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Preface

The 2nd International Conference on Modern Medicine and Global Health (ICMMGH 2024) is an annual conference focusing on research areas including Biomedical Engineering, Pharmaceutical Science, Medicine, and Public Health. It aims to establish a broad and interdisciplinary platform for experts, researchers, and students worldwide to present, exchange, and discuss the latest advance and development in Biomedical Engineering, Pharmaceutical Science, Medicine, and Public Health.

This volume contains the papers of the 2nd International Conference on Modern Medicine and Global Health (ICMMGH 2024). Each of these papers has gained a comprehensive review by the editorial team and professional reviewers. Each paper has been examined and evaluated for its theme, structure, method, content, language, and format.

Cooperating with prestigious universities, ICMMGH 2024 organized three workshops in Kuala Lumpur, Guildford and Wuhan. Professor Mohammed J.K. Bashir chaired the workshop "Assessment and Disinfection of Pathogenic Microorganisms in Water Supply Systems", which was held at Universiti Tunku Abdul Rahman. Dr. Roman Bauer chaired the workshop "Computational Analysis and Modeling for Biomedicine", which was held at University of Surrey. Dr. Shuai Chen chaired the workshop "Dietary Nutrient Bioactive Compounds and Chronic Diseases", which was held at Wuhan University.

Besides these workshops, ICMMGH 2024 also held an online session. Eminent professors from top universities worldwide were invited to deliver keynote speeches in this online session, including Professor Mohammed J.K. Bashir from Universiti Tunku Abdul Rahman, Dr. Omda Omran from University of Central Florida. They have given keynote speeches on related topics of Biomedical Engineering, Pharmaceutical Science, Medicine, and Public Health.

On behalf of the committee, we would like to give sincere gratitude to all authors and speakers who have made their contributions to ICMMGH 2024, editors and reviewers who have guaranteed the quality of papers with their expertise, and the committee members who have devoted themselves to the success of ICMMGH 2024.

Professor Mohammed J.K. Bashir

General Chair of Conference Committee

Workshop

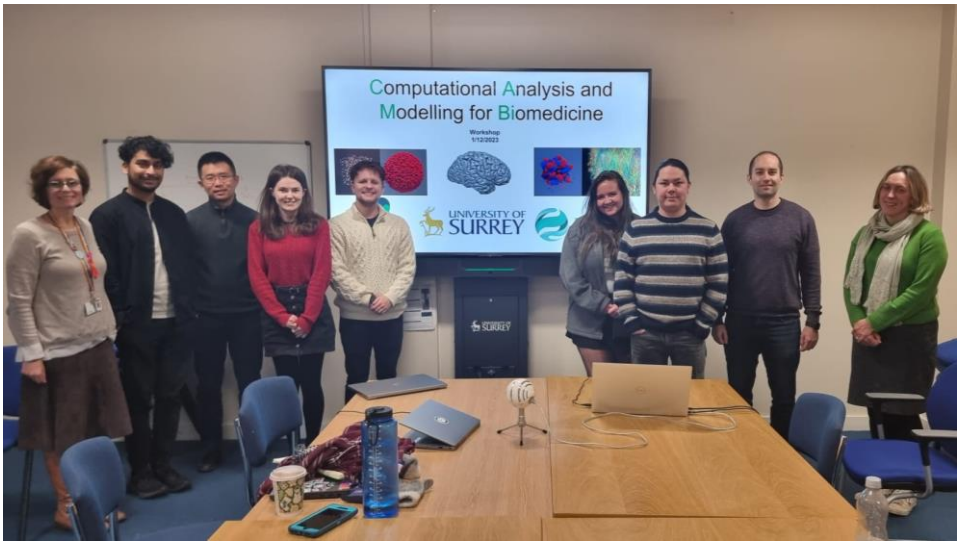
Workshop – Kuala Lumpur: Assessment and Disinfection of Pathogenic Microorganisms in Water Supply Systems

January 5th, 2024 (UTC+8)

Department of Environmental Engineering, Universiti Tunku Abdul Rahman

Workshop Chair: Professor Mohammed J.K. Bashir, Professor in Universiti Tunku Abdul Rahman

Workshop – Guildford: Computational Analysis and Modeling for Biomedicine



December 1st, 2023 (GMT)

Department of Computer Science, University of Surrey

Workshop Chair: Dr. Roman Bauer, Senior Lecturer in University of Surrey

Workshop – Wuhan: Dietary Nutrient Bioactive Compounds and Chronic Diseases

December 2nd, 2023 (UTC+8)

Wuhan University Food Nutrition Research Center, Wuhan University

Workshop Chair: Dr. Shuai Chen, Associate Professor in Wuhan University

The 2nd International Conference on Modern Medicine and Global Health

ICMMGH 2024

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Proceedings of the 2nd International Conference on Modern Medicine and Global Health
DOI: 10.54254/2753-8818/46/20240557

The importance of neuroplasticity and excitability of adult-born neurons in alleviating depression and anxiety-like behaviour in mice

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Abstract. The increase in excitability of newborn adult neurons had been shown to have an antidepressant effect in rodents in previous studies, however whether the same result would apply to mature neurons remains unknown. This paper therefore proposes two experiments that investigate the role of neuroplasticity and mature neuron excitability in reducing depressive behaviour in rodents. Old neurons are known to have reduced neuroplasticity compared to newborn neurons, and the main factor influencing this seems to be the reduced NMDA:AMPA receptor ratio in mature neurons. By knocking out the NR1 gene that codes for the NMDA receptor in newborn neurons, experiment one investigates whether reduced neuroplasticity in the rodent hippocampus would induce anxiety-like behaviour, thus the importance of adult hippocampal neurogenesis (AHN) in maintaining neuroplasticity in the brain and normal affective behaviour. Subsequently the artificially made-mature neurons in the hM4Dq+ tamoxifen inducible Cre-recombinase transgenic mouse line are being activated through injection of tamoxifen followed by CNO, which increases neuronal excitability. This is hypothesised to reduce depressive behaviour to an extent however less effective than activating newborn neurons. These experiments can potentially be valuable in the application of relevant depression treatments in humans, alongside with the maintenance and stimulation of AHN with regards to its anti-depressive effect.

Keywords: Neuroplasticity, Depression, Excitability, Neurogenesis.

1. Introduction

Adult Hippocampal Neurogenesis (AHN) is the process that involves the generation of new neurons from the division of stem and progenitor cells in the hippocampus of adult mammals [1]. Studies have suggested that the level of neurogenesis occurring in the hippocampus could be involved in the development of depression [2]. One reason for this may be due to the greater neuroplasticity and excitability shown by young neurons in comparison to mature neurons [3]. Our investigation examines this idea further by exploring the effect of altering the plasticity and excitability of young and mature neurons on depressive behaviour in mice. The outcome of our investigation may suggest the potential for analogous effects in humans and promote our understanding of how neurogenesis may be significant in human depression.

AHN occurs continuously in specific neurogenic regions located in the subventricular zone (SVZ) of the lateral ventricles and the subgranular zone (SGZ) of the hippocampus throughout the lifespan of

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adult mammals [4], the SGZ being the area that our experiments will focus on. Adult neurogenesis generates granule neurons in the subgranular zone through the asymmetric division of radial glial stem cells [5], whose daughter cells then produce neuroblasts that move into the granule cell layer, where they undergo differentiation into granule neurons [6].

Adult neurogenesis has been shown to occur in many mammal species, including within the primate order, and is notably robust in rodents. Numerous studies have also presented findings supporting the occurrence of adult neurogenesis in the human hippocampus [1]. Although the precise role of adult neurogenesis remains unclear, past experiments that inhibited AHN led to behaviour impairments related to learning and memory, suggesting a role for newborn neurons in cognitive function [7]. Diminished neurogenesis has also been linked to the onset of anxiety and depression, although direct evidence is still needed to support this [2]. Several factors have been demonstrated to impact the rate of neurogenesis in the adult hippocampus. For example, stress often downregulates the process [4], whereas antidepressants such as fluoxetine [8] and stimulation of the amygdala can both upregulate the amount of neurogenesis taking place [9].

Since there is evidence that activating newborn adult neurons can achieve an antidepressant effect [8], our experiments aim to investigate the significance of neural plasticity and excitability in the apparent antidepressant effect of AHN by modifying these features of old and new neurons in mice. Young neurons generally have greater plasticity than old neurons, meaning they have a greater capacity to form Long Term Potentiation. This plasticity is influenced by the NMDA:AMPA receptor ratio, which is higher in young neurons. New neurons also have higher excitability – for example they are less constrained by GABAergic inhibition [3]; whereas old neurons have lower excitability – for example GIRK channels in mature dentate granule neurons lower their resting potential [10].

The prevailing assumption is that immature and plastic newborn neurons exert the most significant influence on behaviour [3]. One possible reason for this may be that new-born neurons, alongside astrocytes, can be incorporated into the pre-existing hippocampal neural circuitry. which has been shown to be necessary for recovery from stress and mood regulation [11].

In our investigation, we will reduce the plasticity of young neurons by reducing the number of NMDA receptors (thereby decreasing the NMDA:AMPA ratio). We will also increase the excitability of new and old neurons using Clozapine -N-Oxide (CNO), which is known to have this effect [8]. The consequence of these changes on depressive behaviour in mice will then be tested to shed further light on the significance of the plasticity and excitability of neurons in the hippocampus.

My hypothesis is that there will be a significant relationship between both plasticity and excitability on depressive behaviour in the mice. More specifically: reducing the plasticity of new neurons (and therefore making them behave more like old neurons) will increase depressive behaviour because mature neurons; increasing the excitability of new and old neurons will alleviate depressive behaviour; and increasing the excitability of new neurons will have a greater antidepressive effect than increasing the excitability of old neurons.

2. Methodology

2.1. Behavioural tests

Below are the behavioural tests that will be carried out to evaluate anxiety and depression-like behaviours of the mice:

2.2. Tail suspension test (TST)

Before the test, groups of mice are kept in a controlled environment with 12:12 light-dark cycles, along with access to food and water. In the test, the mouse's tail will be attached to a clip at the top of a vertical structure, and the mouse will be suspended in a manner that prevents it from touching the floor or any other structure with its paws. The suspension typically proceeds for 6 minutes in a quiet and dimly lit room to minimise external disturbance. Each mouse will be tested individually in succession. The length of immobility time exhibited by the mouse will be recorded with a stopwatch. The immobility period is

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considered to represent a state of behavioural despair, and is therefore used as an indicator of depressive symptoms [8].

2.3. Open field test (OFT)

This test operates on rodents' innate tendency to avoid bright areas and favour darkness. In the OFT, the mouse is transferred from its home cage to a novel and bright arena with enough space for free movement. The arena is divided into central and peripheral units, allowing for the recording of locomotion and rearing behaviour. Due to its photophobicity, the mouse would avoid the bright, open spaces, tending to remain in proximity to the walls. Exploratory or locomotive behaviour in relation to the distance from the walls will be assessed, as well as autonomic activities such as urination and defecation. An automated infrared beam array system is employed to measure locomotion, rearing, and the duration spent in distinct zones within the arena. However, it is worth nothing that this test is vulnerable to various internal and external factors [12].

2.4. Elevated-plus maze (EPM)

The EPM is constructed from black polypropylene and comprises two open arms and two closed arms that are raised 70 cm above the floor. In the center, there is a junction measuring 10 x 10 cm. Mice will be placed individually in the junction and allowed 5 minutes of free exploration. The total number of entries to different alleys will be counted, and the proportion of time spent in the open arms will serve as an indicator of anxiety. Mice with high anxiety would have fear-induced inhibition of open-alley exploration and therefore spend reduced time in the open arms of the maze [13].

2.5. Animals

All experiments will be performed accordance with the Public Health Service Policy on Humane Care and Use of Laboratory Animals, and all procedures are carried out in accordance with EU Directive 2010/63/EU and NIH guidelines on animal care and experimentation [8].

Two- months old naïve C57bl/6 male and female mice (200 – 250g) are housed three per cage and maintained under standard laboratory conditions (12 h light: 12 h dark cycles, 22˚C, relative humidity of 55%, ad libitum access to food and water) [11] for experiment 1 which is the knockout of NR1 gene. Groups of mice (n = 6 per group) are randomly assigned to 4 experimental groups: two of which will have their NR1 gene knocked out with CRISPR-Cas 9, and two control groups. For experiment 2, hM3Dq floxed mice are mated to mice containing tamoxifen-inducible Cre recombinase under the control of the Ascl1 promoter (Ascl1-CreER^{TM}) to produce the transgenic progeny Ascl1-CreER^{TM};R26$^{LSL-hM3Dq}$ (+ hM3Dq) [8]. The progenies (n = 6 per group) are then randomly assigned to the following 4 experimental groups: +hM3Dq without NR1 gene + saline, +hM3Dq + saline, +hM3Dq without NR1 gene + CNO, +hM3Dq + CNO. The mice are genotyped with PCR using genomic DNA and primers, and the Cre and/or DREADD-negative littermates from heterozygote breeding are used as controls [8].

2.6. Experiment 1

To investigate whether the contrast in antidepressant effect of newborn adult neurons and mature neurons is caused by their difference in plasticity, the main factor affecting synaptic plasticity is considered to be the number of NMDA receptors present in the neurons. Previous research has indicated that augmenting the quantity of NMDA receptors may reduce the decline in synaptic plasticity [14], indicating that young neurons have more NMDA receptors and hence more plasticity compared to aged neurons. Therefore, by knocking out the gene that codes for the NMDA receptor on the newborn neurons, we can artificially induce mature neuron properties on the young ones in the hippocampus of the mice groups, then carry out behavioural tests to assess whether there is an increase or decrease in depressive behaviour and anxiety level.

The NMDA receptor complex consists of 4 subunits: two NR1, NR2 and NR3 [14]. By removing any one of these subunits, the functional receptor would not be produced, meaning the newborn neurons

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would have no NMDA receptors and thus behave like old neurons. Therefore to achieve this, we use CRISPR-Cas 9 to knockout the gene that encodes for the NR1 subunit by customising the guide RNA specific to the gene sequence, while the Cas-9 nuclease creates a double-stranded break and the DNA itself disrupts the gene of interest by non-homologous end joining [15].

To determine whether the knockout was successful, after CRISPR-Cas 9 has knocked out the gene we dissect the mouse to obtain the target neurons and lyse them, then Western blot analysis is performed. If the protein band for NMDA receptor isn't present, then a successful knockout of the gene can be determined which will allow us to carry out the formal experiment on the 4 experimental mice groups. 2 groups will be injected with viral vectors with modified genes, and the 2 control groups will be injected with 0.9% saline. Behavioural tests are carried out 24 hours after injection during light hours.

2.7. Result prediction

The prediction is that the two experimental groups would show a greater extent of depressive behaviour compared to the two control groups.

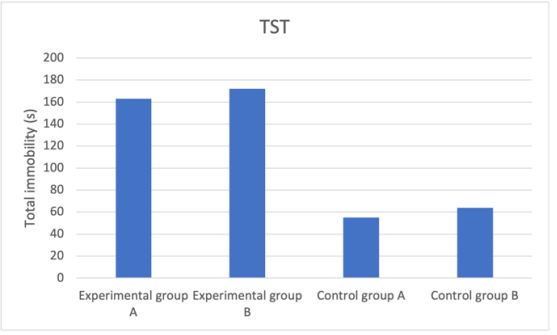

Figure 1a. Decreased neuroplasticity effect on tail suspension test predicted results

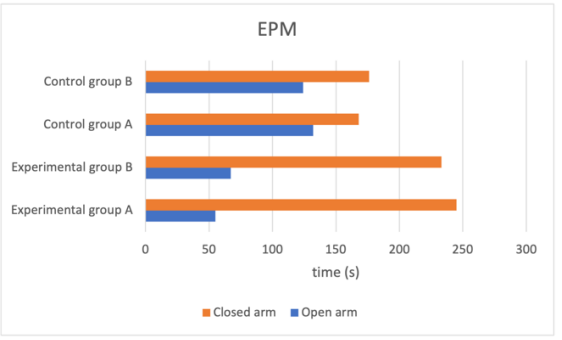

Figure 1b. Decreased neuroplasticity effect on elevated plus maze predicted results

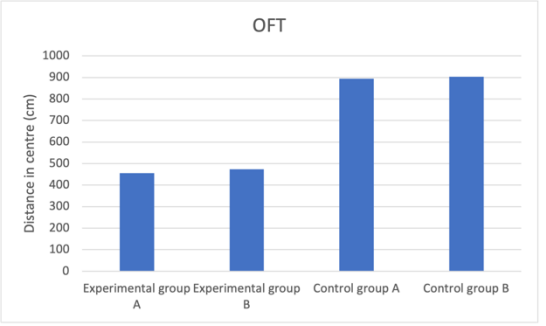

Figure 1c. Decreased neuroplasticity effect on open field test predicted results

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Fig. 1 Reducing number of NMDA receptors in newborn neurons induces increased depressive behaviour and anxiety level. a Mice group with reduced neuroplasticity had longer freeze time, indicating increased anxiety compared to the control group. b Experimental group mice spent much longer in the closed arm with proportion to time spent to open arms compared to control groups, showing higher prevalence of depressive behaviour. c Experimental groups with reduced neuroplasticity exhibited shorter distance in centre, suggesting an inclination to stay closer to the walls, which shows heightened depressive behaviour.

Reduced number of NMDA receptors may result in heightened depressive behaviour due to less Long term potentiation (LTP) will be able to form and therefore the neurons would be less active. This would mean that decrease in neuroplasticity leads to an increase in anxiety and depression, implicating that any difference in antidepressant effect between newborn and mature neurons may also be influenced by their difference in plasticity. When all the new neurons have their NMDA receptor gene knocked out, all the neurons in the mouse's hippocampus would be behaving like mature neurons, which is mimicking the phenomenon that happens when all animals age. Past studies found that neurogenesis declines with age in both humans and animals, meaning less and less newborn neurons will be generated as we age, leaving mainly mature neurons making up the neuronal population, while the prevalence of depression tends to increase with age [9]. This consolidates the idea that newborn neurons in the adult brain are necessary for regulating mood and are essential for the effectiveness of antidepressant treatments [9], and hence also the crucial role of adult neurogenesis in mood regulation.

2.8. Experiment 2

The aim of this experiment is to investigate whether activating neurons with reduced plasticity (i.e. old neurons) would also have an antidepressant effect like newborn neurons. We first cross the hM3Dq floxed mice with mice containing tamoxifen-inducible Cre recombinase to produce the transgenic progeny +hM3Dq mouse line, so upon injection of tamoxifen the Cre protein would remove unwanted genes and lead to the expression of the DREADD protein, which would activate neurons in the dentate gyrus when treated with Clozapine-N-oxide (CNO) [8].

4 experimental +hM4Dq mice groups (n = 6) will be used, 2 of which will have their NR1 gene knocked out with CRISPR-Cas 9, meaning they are artificially made-mature neurons. When the mice are 2 months old, inject 180 mg kg^{-1} tamoxifen intraperitoneally for 5 continuous days, while the control groups receive saline injection in the same time course. 2 hours before the behavioural tests, inject 2mg kg^{-1} CNO to the 2 experimental groups and 0.9% saline to the 2 control groups. CNO injection activates the DREADD tagged neurons and increases their excitability, which leads to increased firing of the neurons and therefore should decrease depression and anxiety in the behavioural tests, the results from which will be statistically analysed and compared.

Statistical analyses are done using SPSS software (SPSS, Chicago, IL, USA) [11]. Once the homogeneity of data is confirmed, statistical analyses are conducted. Behavioural results are assessed using one-way analysis of variance (ANOVA), with F-values and P-values derived from the between-group ANOVA analysis. Differences between groups are determined through Bonferroni's post-hoc multiple comparison test, and the corresponding P-values are presented. In cases where appropriate, a t-test is utilized to evaluate differences between two groups. Statistical significance is considered when $P<0.05$ [11].

2.9. Result prediction

Both experimental groups that had CNO treatment would show lower levels of anxiety and depression compared to the control groups, and the mice group with a complete set of genes (with intact NMDA receptor gene) present greater antidepressant effects upon CNO treatment compared to the mice group with made-old neurons.

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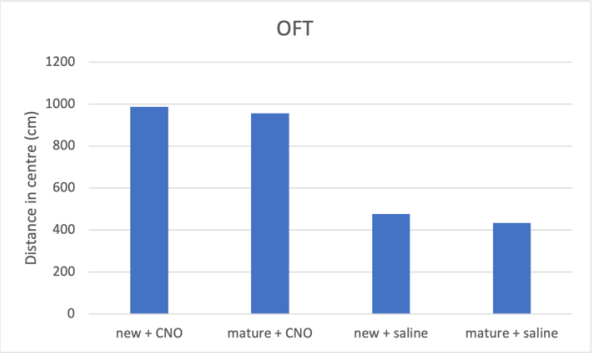

Figure 2a. Increasing neuron excitability on open field test predicted result

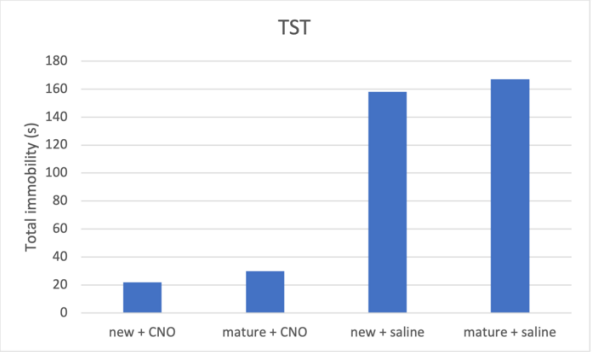

Figure 2b. Increasing neuron excitability on tail suspension test predicted result

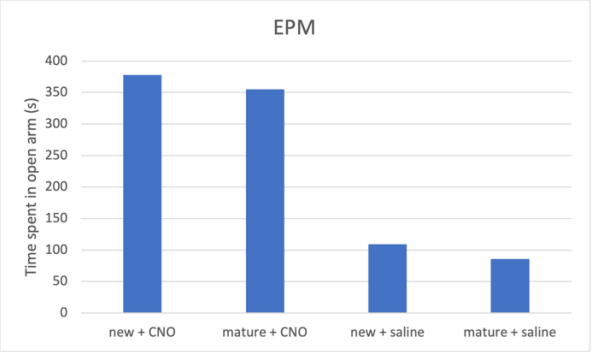

Figure 2c. Increasing neuron excitability on elevated plus maze predicted result

Fig. 2 CNO treatment induces an antidepressant effect, which is more prominent in newborn neurons compared to mature neurons. a Activating both mature and new neurons caused corresponding mice groups to spent longer distance in centre, indicating reduced depressive behaviour. Mice with activated newborn neurons show less anxiety compared to mature neurons. b Both mature and newborn neurons with increased excitability induced decrease in total immobility in mice, showing an antidepressant effect. Mice with activated newborn neurons exhibited even shorted immobility time than mature ones. c Increasing mature and newborn neuron excitability increase time spent in the open arms in elevated plus maze by mice groups, more so in the newborn neuron group.

Therefore I predict that activating mature neurons would also have an antidepressant effect, however not as robust as newborn neurons due to decreased plasticity and lower threshold for LTP. If activating mature neurons result in no positive effects in alleviating depressive behaviour, possible reasons may include a) Aged neurons may have experienced prolonged oxidative stress and inflammatory responses,

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leading to cellular damage and decreased function. b) The activity-enhancing methods used in the experiments may not be appropriate for aged neurons or may lead to abnormal activation of neural circuits causing the opposite effect. c) Older neurons having already integrated into the neural circuit may find it more difficult adapting to new environments or respond to stimuli, affecting their antidepressant effect when activated.

3. Limitations

The main limitation throughout the experiments is that the difference in number of NMDA receptors must not be the only characteristic that distinguishes between mature and newborn adult neurons. However it is not possible to artificially include all the factors that make a neuron old, due to the complexity of natural biological growth and other external influences such as inflammation or infections. Therefore we have included a supplementary experiment that can be carried out to overcome this limitation:

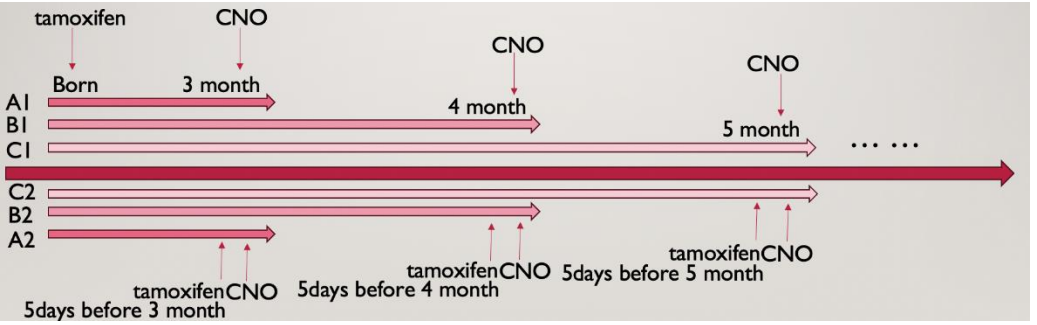

Figure 3. Alternative experiment timeline

As fig 3 shows, this experiment is carried out throughout the course of 6 months, starting from when the mice are 3 months old, inject tamoxifen and apply CNO treatment every month followed by the behavioural tests. This allows the neurons to age naturally and the antidepressant effects during different age stages can be assessed and compared. The groups can be compared horizontally, which is comparing the ability of neurons to regulate depression at different ages; or it can be compared vertically, which compares the ability of old vs. new neurons in alleviating depression and anxiety.

Additional limitations arise from variations in the levels of stress and fear induced by different behavioural tests. These variations can activate distinct subsystems of the neurogenic interactome, potentially introducing interference with the final results [9]. However, since all mice groups undergo the same experiments and tests, the slight impact that this has can be ignored. Furthermore, neuronal morphology is found to vary substantially, even within the same animal [3]. Therefore, the antidepressant effects of newborn and mature neurons may vary from mouse to mouse and might not be sufficient to draw a conclusion for all rodents or even humans.

4. Conclusion

To summarise, our first experiment tested the importance of neuroplasticity in inducing depressive behaviour by knocking out the NR1 gene that encodes for the NMDA receptor, which gave young neurons mature neuron properties, reducing their plasticity. The prediction is that decreased neuroplasticity would lead to increased depressive behaviour, which would highlight the importance of newborn adult hippocampal neurons in mood regulation.

Our second experiment used CNO to increase the excitability of both newborn and artificially made-mature neurons to evaluate which one would have a more significant antidepressant effect. Presumably the newborn neurons would have a greater effect in alleviating anxiety and depression since they have more impact on behaviour, according to Cole, Espinueva et al., 2020, and more plasticity.

Therefore, the distinctive features of newborn neurons and their clinical values can be further studied in application to future depression treatment and in academia.

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A comprehensive literature review of spermidine

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Abstract. This review explores the role of spermidine, a naturally occurring polyamine, in promoting healthy aging and increasing lifespan. Spermidine was initially discovered in human sperm, and it is important in several cellular processes across eukaryotic organisms. We deeply study the historical background of spermidine discovery and its research developments. The paper examines the mechanisms through which spermidine influences lifespan, including its interaction with RNA, stimulation of autophagy, anti-inflammatory effects, and impact on the cell cycle. Each mechanism is discussed with its contribution to cellular health and potential in lifespan extension. Besides, several human diseases that spermidine could reduce the risk of are shown. Moreover, the review highlights food sources of spermidine, especially the Mediterranean diet. Spermidine rich eating pattern is related to improved health outcomes and longevity. Despite the benefits of spermidine, the paper also discusses the potential risks of spermidine intake, indicating that spermidine is related to increased cardiovascular disease risk. In this review, we aim to offer a balanced perspective on how spermidine might help with healthy aging, what we currently know about its effects on the body, and the further research needed to understand what spermidine means for human health.

Keywords: Spermidine, polyamine, RNA

1. Introduction

Spermidine, a natural polyamine first found in human sperm, plays an important role in the life of eukaryotic organisms. Spermidine has a precursor called putrescine. Spermidine is formed by spermidine synthesis, which is two putrescines being combined. As aliphatic hydrocarbons with amino groups at both ends, polyamines like spermidine are fundamental to nearly all living entities [1]. It increases lifespan and health span, and it could be found in our daily diet.

In 1678, Antonie van Leeuwenhoek, the father of microbiology, discovered crystalline substances in human semen. These crystals, later named “spermine,” were found in semen which had been standing for several days rather than in fresh samples. In 1791, Vauquelin identified these crystals as phosphate derivatives. In 1878, Schreiner classified this compound as an organic base. The name “spermine” was officially introduced by Ladenburg and Abel in 1888. As time passed, the medical potential of spermine was recognized. In 1898, von Poehl confirmed spermine’s therapeutic use for various illnesses. The structure of these polyamines, including both spermidine and spermine, was finally deciphered in 1924 by Rosenheim, which concluded the initial chapter of polyamine research [2].

Today, people are still working on exploring spermidine. Knowledge about the lifespan effects of spermidine continue to be studied, expanding the areas of clinical application.

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2. Reasons Why Spermidine Increases Lifespan

There are four main reasons why spermidine increases lifespan, which are spermidine interacting with RNA, spermidine stimulating autophagy, spermidine fighting against inflammation, and spermidine affecting cell cycle.

2.1. RNA

Spermidines are critical for the regular growth and differentiation of cells in prokaryotes and are necessary for eukaryotic life. In eukaryotes, spermidine is important for activating eukaryotic translation initiation factor 5A (eIF5A), a protein which plays a good role in synthesizing eukaryotic proteins. This process depends on post-translational modifications, and the spermidine acts as a substrate for eIF5A's maturation so that it significantly helps with protein synthesis. Under normal physiological pH, polyamines, including spermidine, are positively charged. This allows them to form weak bonds with intracellular molecules that are negatively charged such as nucleic acids, phospholipids, and ATP. Polyamines, including spermidine, most frequent bind with RNA, which affects the structure of mRNA and therefore regulates protein translation. Such kind of interactions are important for a large number of cellular processes such as proliferation, biofilm formation, activity enhancement, and detoxification. Therefore, it is significant to underline that spermidine and related polyamines are important in maintaining the normal functions of organisms [1].

2.2. Autophagy

Autophagy could be seen as a cleanup crew inside human cell. It is a process that helps get rid of damaged or unneeded parts of the cell by breaking them down and recycling them. This process happens in different ways including microautophagy, macroautophagy, and chaperone-mediated autophagy. Macroautophagy is the most common form: the cell's waste is packaged into a structure called an autophagosome, and then fuses with a lysosome or vacuole to break down the contents.

Autophagy keeps cells healthy, but it can increase or decrease based on different factors like aging, stress, or inflammation. For example, as human age, more damaged parts accumulate in cells, and the cells become less efficient at cleaning them up. Autophagy helps fight against aging by removing these damaged parts, therefore autophagy is linked to longer life span and health span.

Spermidine is good at stimulating autophagy in various organisms and cells, from yeast and flies to mice and human cells. There are several ways for it to accomplish it. First, it changes the levels of certain genes (Atg genes) that are involved in autophagy. When these genes are more active, autophagy increases and helps to extend the lifespan. Second, spermidine helps produce more specific molecule (TFEB) that is involved in controlling autophagy. Third, spermidine works by preventing the addition of acetyl groups to proteins involved in autophagy. Spermidine does this by reducing the activity of certain enzymes and the availability of acetyl-CoA, a molecule involved in adding acetyl groups [3].

2.3. Anti-inflammatory

Spermidine can increase lifespan because of its anti-inflammatory properties. Inflammation is like human body's alarm system: it fights against infections, but it can also become harmful if the alarm is always on, which leads to "inflammatory aging". This is a major factor in growing older and developing age-related diseases like heart and kidney disease. At this time, spermidine steps in to help calm this inflammation down. There are several ways for spermidine to reduce the body's inflammatory responses. First, spermidine decreases the levels of certain signals (such as TNF-α) that can cause inflammation, which helps to prevent health issues especially in the heart. It can also stop the accumulation of harmful reactive oxygen species and prevents immune cells from moving to inflammation sites, and therefore together reduce the body's overall inflammatory reaction [3].

Furthermore, spermidine can help convert certain immune cells (macrophages) to a type that does not promote inflammation (M2 macrophages). The M2 macrophages help to control the inflammation. Spermidine accomplishes this by increasing the levels of genes and enzymes that promote this peaceful state. It is especially effective to convert these macrophages during the early life of the macrophages,

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which makes them to be anti-inflammatory at the very beginning. In general, all these actions of spermidine work together to reduce chronic inflammation, which helps delay aging and increase lifespan, making spermidine good at promoting a longer, healthier life [4].

2.4. Cell Cycle

Spermidine could increase lifespan by positively affecting the cell cycle. The cell cycle is a set of checkpoints and stages for a cell to go through so that the cells could grow and divide. Spermidine helps cells go through these checkpoints smoothly, ensuring their healthy growth and division, which leads to healthy human tissues and organs. Studies show that the absence of spermidine can stop cell growth at G1 phase by influencing cell cycle regulators' expression, which indicates the importance of spermidine [3].

There are several methods for spermidine to affect cell cycle. First, spermidine helps promote cells to replicate their DNA and move through the cell cycle, which creates new healthy cells. This is like giving the cells a gentle nudge to keep moving and not get stuck. Additionally, spermidine helps in cell differentiation, promoting cells to become the specific types they need to be, which is important for repairing and maintaining human bodies. Cell differentiation is a process when cells from the same source finally produce cell groups different in morphological structures and functions. Studies shows that spermidine can enhance differentiation in differentiated chondrocytes and in adult stem cells [3].

Moreover, spermidine regulates how cells die. Spermidine can slow down the aging process by avoiding apoptosis. Cell necrosis is the cell death under the induction of extreme serious pathological factors. Studies show that the increase levels of spermidine can suppress cell necrosis, increase the lifespan and improve health in aging yeast [3].

3. Human Diseases in which Spermidine Reduces the Risk

There are five types of human diseases that spermidine can reduce the risk of, and they are all age-related diseases. The first type of disease is metabolic diseases, and the examples are type 2 diabetes mellitus, obesity, and metabolic syndrome. The second type of diseases is musculoskeletal diseases, and the example are Osteoporosis, Sarcopenia, and Osteoarthritis. The third type of disease is immune diseases, and the examples are influenza, cytomegalovirus infection, colitis, and inflammatory bowel disease. The fourth type of disease is cardiovascular disease, and the examples are coronary artery disease, heart failure, and essential hypertension. The fifth type of disease is neurodegenerative diseases, and the examples are Parkinson's disease and Alzheimer's disease [3].

4. Foods Rich in Spermidine

Since spermidine is beneficial for human life span and health span, it is necessary to know what foods are rich in spermidine and spermidine's precursor, putrescine. Fruits and cheese have the highest amount of putrescine, and vegetables and meat products are rich in spermidine.

Products resulting from fermentation processes that involve polyamine-generating bacteria and fungi are rich in spermidine. The content of polyamines in cheese changes between studies. In studied Swedish dairy products, matured cheese has the highest polyamine contents with values of 52.3 and 1.2mg/kg for putrescine and spermidine. Besides, low fat milk has higher putrescine and spermidine, 1.2 and 1.0 mg/kg, than the other types of milk [5].

Spermidine also presents in all unprocessed plant-derived foods, such as fresh green pepper, broccoli, and soybean. The food with the highest contents of spermidine are cereals, legumes and soy derivatives [6].

It is reasonable to indicate that much of the Mediterranean diet contains spermidine rich foods because Mediterranean diet is a plant-based eating plan. The traditional Mediterranean diet is known for its high consumption of plant-based foods, including fruits, vegetables, breads (primarily minimally refined), cereals, potatoes, beans, nuts, and seeds. It emphasizes minimally processed foods that are fresh, seasonal, and locally sourced. Fish and poultry are eaten in low to moderate quantities, while red meat is consumed sparingly [7]. Spermidine increase human life span explains why Mediterranean

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dietary pattern raises life expectancy, decreases the risk of chronic disease, and improves human life qualities and well-being. According to studies, the Mediterranean diet is considered the gold standard of preventive medicine, likely because of its harmonious blend of numerous components with antioxidant and anti-inflammatory qualities, which overwhelm the benefits of any individual nutrient or food item [8].

If people struggle to get enough spermidine in their diet, they can also get it as a spermidine supplement.

In summary, adopting a diet rich in spermidine to increase spermidine levels through human body presents a hopeful approach for encouraging healthy aging.

5. Risk of Spermidine Intake

The intake of spermidine has been linked to potential health risks, especially concerning cardiovascular health according to studies. One thing that needs to be noticed is that the studies' findings are not yet widely understood or confirmed by large community-based studies. Studies have indicated that higher levels of spermidine in the blood may be related to an increased risk of stroke. To be more specific, individuals with raised serum spermidine levels, especially those with levels at or above 205.9 nmol/L, have been found to have a significantly higher risk of having a stroke compared to those with lower levels. This relationship has been found consistently among several studies, which suggests a strong connection between high spermidine levels and stroke risk. Furthermore, the use of statistical models has confirmed that including spermidine levels in risk assessments can improve the accuracy of stroke predictions [9]. Although spermidine is naturally present in the human body and certain foods, these findings highlight the fact that it is important to know the potential risks associated with spermidine intake, especially for those individuals who have high risk of cardiovascular issues. Therefore, it is necessary to consider carefully and make further research before the spermidine consumption to avoid any potential health risks.

6. Conclusion

In conclusion, spermidine, a polyamine discovered in human sperm, has been significant to reach healthy aging and increased lifespan. In this review, we have explored spermidine's several roles in cellular functions, including its interaction with RNA, stimulation of autophagy, anti-inflammatory properties, and influence on the cell cycle. These interactions promote spermidine's ability of prolonging life by maintaining cellular health, preventing premature aging, and fighting against various age-related diseases. We have also discussed how spermidine-rich diets, particularly the Mediterranean diet, can be a natural source of spermidine, which helps preventing chronic illnesses and aging. However, it is also important to balance the benefits with potential risks. It is necessary to know that high spermidine levels have been associated with increased cardiovascular risks like stroke. Therefore, while spermidine could be seen as a helpful supplement to increase life span, further research and a fully understanding are necessary to reduce any potential risks. As we continue to explore spermidine, it is clear that spermidine plays a significant role in the future of health and aging.

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Efficiency of fluoxetine and alprazolam in the treatment of panic disorder

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Abstract. Panic disorder, a mental illness that causes panic attacks as the main symptom, is an important subgroup of anxiety and fear disorders, but it has received little attention and research until recent decades. After it was included in the DSM-II in 1980, researchers began studying drug treatments for the disorder. Among the many drugs, the two most effective are 5-HT reuptake inhibitors and benzodiazepines, and the two most representative drugs are fluoxetine and alprazolam. The purpose of this study was to analyze the effectiveness of fluoxetine and alprazolam in the treatment of panic disorder by studying the structure, synthesis, pharmacology and economic benefits of the two drugs, as well as to analyze the methods of clinical use of the drugs.

Keywords: Fluoxetine, Panic disorder, Alprazolam, Mental illness

1. Introduction

In today's anxia-ridden society, panic disorder, an accessory disorder, also affects many groups. After a period of study and comparison, it was found that the research on drug treatment of this disease was not highly concerned at the social level, and there were few treatment cases and targeted studies. Therefore, the topic of this paper is to explore the effects of SSRIs and two of the most common benzodiazepines, fluoxetine and alprazolam, on panic disorder. It is intended to expand the cognitive scope of the treatment of this disease, and provide effective drug comparative studies for patients with panic disorder, so as to facilitate the reference of drug selection. In addition to in-depth research and analysis of the effects of these two drugs, this study aims to help the progress of drug research for panic disorder and aims to win more social attention for patients with panic disorder, providing support and help for patients from both drug treatment and social attention.

2. Review of Panic Disorder

About 2-3% of people suffer from panic attacks. Seven percent of patients had suicidal thoughts or behaviors [1]. This disease is briefly described in the following paragraphs.

2.1. Symptoms of panic disorder.

The symptoms of panic disorder are varied and are described in detail in the DSM-5. Common symptoms can be heart palpitations, suffocation, body tremors, depersonalization, or fear of death [2].

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According to the textbook “Psychiatry” published by China People’s Medical Publishing House, symptoms above can be summarized into three of the most common clinical symptoms: panic attacks, apprehensive expectation and avoidance behavior

2.1.1. Panic attack. Panic attack refers to when a patient feels sudden fear and tension, accompanied by a strong sense of loss of control and near-death and occurs in the absence of a special terror environment. At the same time, most patients have symptoms of autonomic disorders, including chest tightness, shortness of breath, and tremors. These symptoms usually last about 20-30 minutes, and have not been recorded for more than an hour.

2.1.2. Apprehensive expectation. Apprehensive expectation refers to when patient in the interval after the onset of the disease is still worried about the recurrence of the disease. However, in this condition, the patient’s experience of anxiety is not prominent, but is replaced by weakness, and the symptoms can take hours to days to fully recover.

2.1.3. Avoidance behavior. Avoidance behavior occurs when the patient suffers from constant anxiety and concern over a recurrence of the illness, and consequently avoids places such as school and their workplace. for fear of the unfortunate consequences of the illness. Avoidance behavior does not occur in all patients, only about 60% [1].

2.2. Impact of Panic Disorder

Studies in Europe and the United States have shown that 2-3% of adults develop panic disorder within a 12-month period, and this rate is significantly lower in Latinos, African-Americans and Asian-Americans than in whites and Native Americans. Experimental data in countries in Asia, Africa and Latin America show that the number of cases during the same period is as low as 0.1-0.8%. In all regions, the incidence is mainly during adolescence or post-adolescence adulthood, and the incidence is not high in children or the elderly (less than 0.4% before 14 years of age and less than 0.7% after 67 years of age) [2]. Most of the time panic disorder is not a lifelong disease, only more than 6 months of the course of the disease can become chronic. 30% of patients diagnosed with panic disorder have a good response within a few years and no recurrence, 25% have an intermittent course, 45% have a relatively poor response, and about 7% have suicidal tendencies or behaviors [1].

2.3. History of Panic disorder

Panic disorders were not recognized by the American Psychiatric Association nor included in the DSM-111 until 1980. Before that, patients were often diagnosed as “stressed” or “nervous.” Due to a lack of understanding among health professionals about all anxiety disorders, including panic disorder, few receive effective treatment. Although anxiety disorders have only recently been officially recognized, they have been around throughout human history. The word fear first comes from the name of Pan in ancient Greek mythology. It is said that he can make people who occupy his territory feel a sudden great fear, so people call this feeling “panic” [3]. In the second half of the 19th century, the field of anxiety symptoms began to change gradually. When the word “panic” was first used in psychiatry in 1879, Henry Maudsley, in his book The Physiology and Pathology of the Mind, describes much of the knowledge of insanity and a depressive panic [4]. In 1964, Donald Klein published an article stating that patients with panic attacks do not realize the difference between the disorder and generalized anxiety disorder because chronic anxiety masks the specificity of panic attacks [5]. His observations and descriptions influenced the third edition of the Diagnostic and Statistical Manual of Mental Disorders (DSM-III, 1980), in which the term panic disorder first appeared in an official classification. Over the past 50 years, people have uncovered the mysteries of panic disorder through basic and clinical research [6].

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2.4. Causes of Panic disorder

Panic attacks are easy to cause, and most people have experienced panic or fear at some point in their lives. These brief episodes can be triggered by a variety of factors, such as sudden shock. But when panic attacks turn into panic disorder, the cause of the illness changes. While researchers are still uncertain about the causes, common theories include genetics, CO2 hypersensitivity, and psychosocial factors.

I. genetic

In studying the genetic causes of panic disorder, researchers have chosen two methods: family studies and twin studies. Family studies examined clusters of depressive disorders by comparing phenotypic prevalence between preexisting relatives and control relatives without the disease. The researchers found that when the first degree relative of the first panic disorder was younger than 20 years old, the family risk increased by 17 times. Over the age of 20, the risk increases six-fold. Although this study demonstrates the clustering of the disease in families, the process of genetic and environmental factors is not well distinguished, resulting in significant limitations. Twin studies complement family studies, and twin studies of panic disorder show that the disorder is moderately heritable, and that identical twins are more likely to have it than fraternal twins. Researchers estimate the heritability of the disease to be 30 to 40%. A recent study of 5,000 twins in Virginia showed a heritability of about 0.28 for panic disorder [7].

II. Carbon dioxide hypersensitivity theory

The researchers found that panic attacks were induced when 5 percent CO2 was inhaled in patients with panic disorder, and that intravenous sodium lactate or sodium bicarbonate had the same effect. It is speculated that the hypersensitivity of CO2 receptors located in the locus coeruleus of the brain stem in the patient causes the patient's body to alarm for suffocation when inhaling CO2, leading to panic attacks [1].

III. Social psychological theory

Psychosocial factors suggest that panic disorder is caused by the individual's fear of subconscious impulses affecting real life, or by a conditioned connection formed by a traumatic event in life. But these theories still need scientific confirmation [1].

3. Description of Drugs

3.1. Drugs origin

3.1.1. Fluoxetine. In the 1960s, autopsy studies found that depressed people who were suicidal had reduced serotonin levels, so pharmaceutical companies began investigating serotonin's role in depression. Among them, Eli Lilly has developed a method that effectively and selectively inhibits serotonin reuptake by the serotonin operator, thereby increasing serotonin concentration in the synaptic cleft. The drug, fluoxetine, was demonstrated by several scientists in 1975 to be an antidepressant and has a weak affinity for the norepinephrine functional protein, which is where fluoxetine differs pharmacologically from other drugs. Fluoxetine was approved by the US Food and Drug Administration in December 1987 and launched in 1988 under the brand name Prozac, selling $3.5 billion a month later and earning $2.6 billion that same year. It has since become a world-famous antidepressant [8].

3.1.2. Alprazolam. Alprazolam belongs to a class of drugs called benzodiazepines, which were first discovered by chemist Leo Sternbach in 1955. This class of drugs appeared to be less toxic, less likely to cause dependence than older drugs, and did not have respiratory depression. This led to a skyrocketing demand for this drug in the 1970s, and it topped the list of "most commonly prescribed drugs" [9]. Alprazolam was first released in 1981 by Upjohn Laboratories (now part of Pfizer) as the first drug approved for the treatment of panic disorder. Although there have been concerns in the medical community about the drug's addictive nature and its dangers when used in the elderly, Alprazolam is now the most commonly used benzodiazepine in the United States because of its usefulness [10].

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3.2. Structure and Synthesis

3.2.1. Fluoxetine

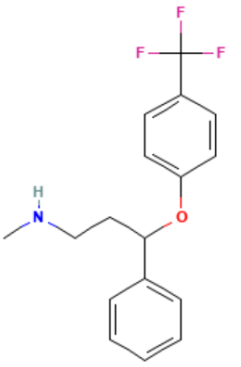

Figure 2. The structure of Fluoxetine

Fluoxetine is an insoluble solid with the structure n-methyl-3-phenyl-3 -[4-(trifluoromethyl) phenoxy] propane-1 amine and the formula C17H18F3NO, an aromatic ether composed of 4-trifluoromethyl phenol, where the hydrogen of the phenol hydroxyl group is replaced by 3-(methylamino) -1-phenylpropyl [11].

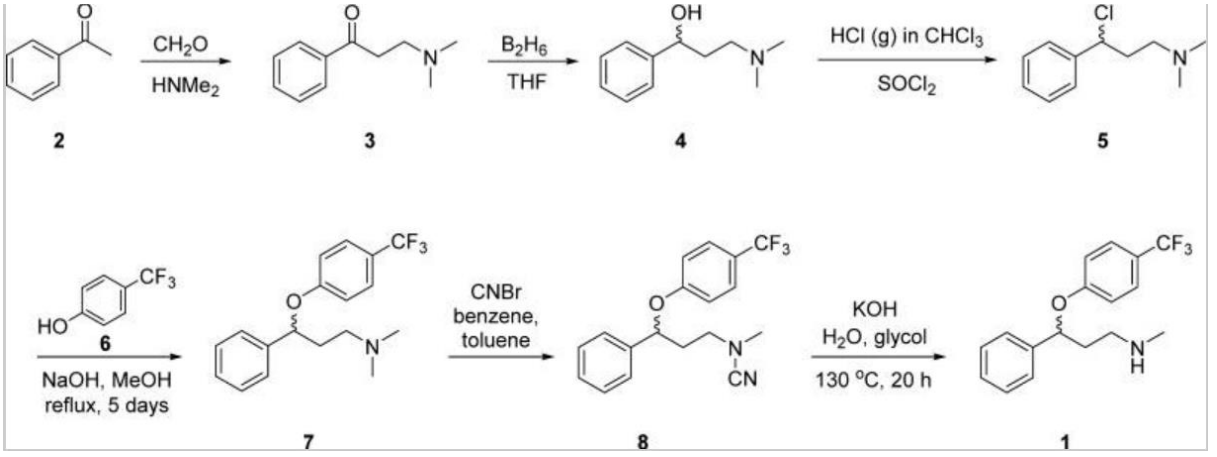

Figure 3. The original synthesis process of fluoxetine

The original synthetic route of fluoxetine (label 1) was reported by Molloy and Schmiegel in 1982 in the United States. Using acetophenone (label 2) as raw material, they obtained β-dimethylaminopropenone oil (label 3) through the Mannich reaction. Dissolve it in tetrahydrofuran and drop it into tetrahydrofuran solution of tetraborane and stir overnight. Then add an equal amount of diborane and stir again overnight. The acid treatment provides the key racemic secondary alcohols (labeled 4). The alcohol is then dissolved in CHCl3 and saturated with anhydrous HCl gas while dropping SO2Cl to maintain reflux for about 5 hours. After the solvent evaporates, the substance labeled 5 in the figure is obtained and collected as crystalline hydrochloride. Then 5 is added to the alkaline solution (label 6) and reflux for 5 days to obtain phenoxyether (label 7). Racemic fluoxetine (label1) is then provided as a free base by an N-cyanide derivative (label 8) and subsequent alkaline hydrolysis [12].

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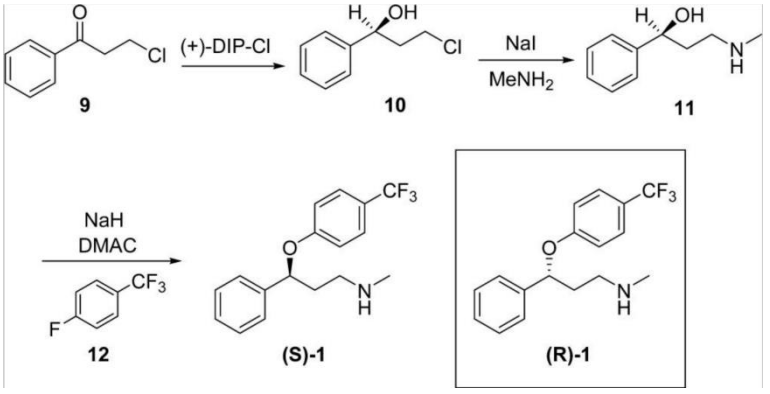

Figure 4. Eli Lily's method of synthesizing fluoxetine

Eli Lilly's method of making fluoxetine is to use the Finkelstein reaction to prepare the corresponding iodide in situ, and then replace it with methylamine to obtain the substance labeled 11 in the figure. The material was deprotonated in DMAC with NaH, then 1-fluoro-4 -(trifluoromethyl) benzene (labeled 12) was added, and (S) -fluoxetine (S)-1 was delivered at an S:R ratio of 96:4. Since only (+)-DIP-Cl is available, this asymmetric synthesis allows only (S) -fluoxetine to be obtained. To obtain (R) -fluoxetine (R)-1, Robertson and colleagues employed a classical resolution technique for the d- and L-mandelates of racemate 1. After conversion to the corresponding (R)-1-(1-naphthyl) ethyl urea, HPLC and NMR confirmed that the S:R enantiomer ratio of (S) -fluoxetine (S)-1 was greater than 99:1, and the S:R enantiomer ratio of (R) -fluoxetine (R)-1 was 1.5:98.5 [12].

3.2.2. Alprazolam

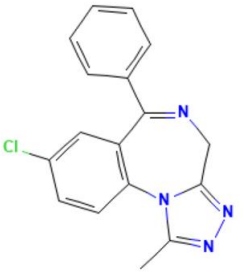

Figure 5. The structure of alprazolam

Alprazolam is a member of the triazolium benzodiazepine class, a 4H-[1,2,4] triazolium [4,3-a][1,4] benzodiazepine class that carries methyl, phenyl, and chlorine substituents at positions 1, 6, and 8, respectively. Alprazolam is only found in people who take the drug [13].

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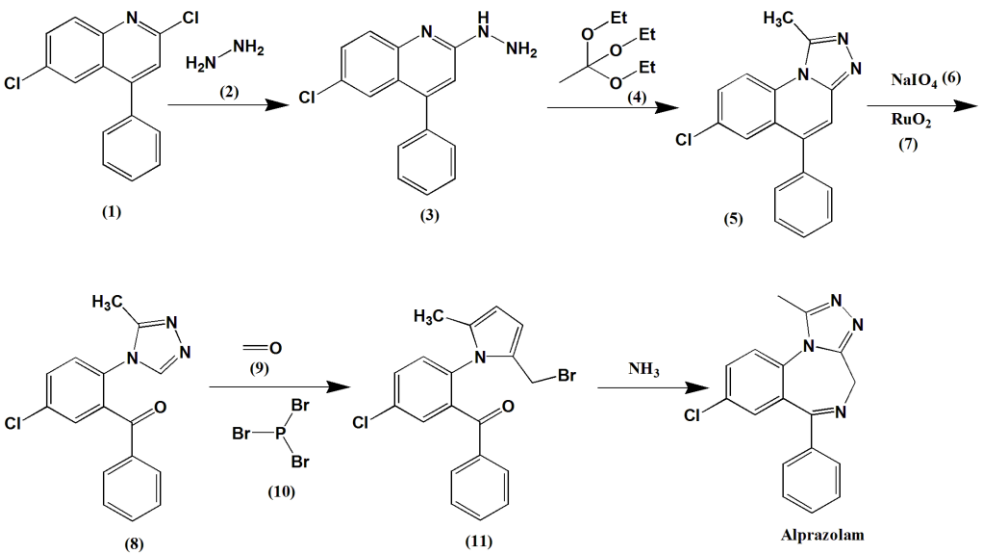

Figure 6. The synthesis of alprazolam

The synthesis of alprazolam is to firstly let 2,6, -dichloro-4-phenylquinoline (label 1) reacts with hydrazine (label 2) to give 6-chloro-2-hydrazine-4-phenylquinoline (label 3). Then, phenylquinoline and triethyl acetate (mark 4) were boiled in xylene to heterocyclic form triazole derivatives (mark 5). 2-[4-(3 '-methyl-1,2, 4-triazole)] -5-chlorobenzophenone (8) is produced by oxidative cleavage of sodium periodate (mark 6) and ruthenium dioxide (mark 7) in an acetone-water system. The hydroxyl group is then replaced by phosphorus tribromide (mark 10) to give 2-[4-(3 '-methyl-5' -bromomethyl-1,2, 4-triazole)] -5-chlorobenzophenone (mark 11). Finally, the bromine atom replaces the amino group with ammonia and spontaneously heterocycles to form alprazolam [14].

3.3. Pharmacology

3.3.1. Fluoxetine. Fluoxetine increases serotonin levels by blocking the reabsorption of the presynaptic end working protein and preventing serotonin from being reabsorbed by the body. It is slightly active on both the 5-HT2A receptor in the cerebral cortex and the 5-HT2C receptor in the limbic system, but has little effect on the reuptake of norepinephrine and other neurotransmitters. When the cytochrome P450 enzyme (CYP2D6) acts on fluoxetine, it stimulates its production of the active metabolite norfluoxetine, and precisely because fluoxetine is metabolized on the CYP2D6 isoenzyme, norfluoxetine has several drug interactions. Equally important is that fluoxetine has a half-life of 2 to 4 days and norfluoxetine has a half-life of 7 to 9 days [15]. In treating panic disorder, the researchers conducted two 12-week randomized, multicenter Phase III trials. Data from the first trial showed that 42% of subjects in the fluoxetine treatment group did not have a recurrence after the trial, while this proportion dropped to 28% in the placebo group. In the second trial, 62% of fluoxetine-treated patients did not have panic attacks at the end of the study, compared with 44% of the placebo group [16]. At the same time, in patients who received 10 or 20mg of fluoxetine or placebo, the researchers found that the 20mg daily dose of fluoxetine had the most significant improvement in panic disorder. And they believe that the drug is well tolerated and that its safety in treating panic disorder is about the same as in treating other conditions [17]. These trials strongly demonstrated the effectiveness of fluoxetine in treating panic disorder.

3.3.2. Alprazolam. Alprazolam belongs to a class of drugs known as benzodiazepines. These drugs bind to the GABA-A receptor. This receptor consists of five subunits, and the GABA-A receptor commonly found in the central nervous system consists of two alpha-1 subunits, two beta-2 subunits, and one gamma-2 subunit. Benzodiazepine binding sites are located between the alpha-1 and gamma-2 subunits.

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A mouse study has shown that alpha-1 subunits mediate the sedative, forgetting, and disordered effects of benzodiazepines, and alpha-2 and alpha-3 subunits mediate the antianxiety and muscle relaxation effects of benzodiazepines. In addition, studies have shown that benzodiazepine-1 receptors affect sedation and anti-anxiety, while benzodiazepine-2 receptors affect muscle relaxation, anti-convulsive activity, memory, and motor coordination. When bound to GABA-A receptors, the main inhibitory neurotransmitter GABA mediates the sedative or inhibitory effects of alprazolam on the human nervous system [18].

One trial suggests that benzodiazepines may have advantages in improving panic symptoms and reducing the number of participants who drop out of treatment. Of the 4,233 participants, 2,124 adults with panic disorder who were treated with benzodiazepines had a lower dropout rate, and the results showed that this class of drugs also had advantages in terms of remission and social function endpoint data [19].

3.3.3. Application. Although both fluoxetine and alprazolam are effective treatments for panic disorder, a combination of the two drugs is most commonly used clinically. In the initial stage, the patient's symptoms will be significantly improved, and the early adverse reactions of fluoxetine can be alleviated. However, after 4-6 weeks, tolerance may occur and there is no obvious advantage, so it is possible to stop alprazolam at this time to avoid long-term addiction to alprazolam [1].

3.4. Side effects

3.4.1. Fluoxetine. Some patients who take fluoxetine will have nausea, vomiting, dizziness, headache, fatigue, insomnia, anorexia and other adverse symptoms. Patients with liver disease, diabetes, and cardiovascular disease should use fluoxetine with caution [20]. Even though these side effects of fluoxetine are severe, they mostly appear immediately and go away over time, and it is best to change the timing of treatment after the side effects have resolved on their own. Data show that most of these side effects are dose-related, so if the side effects affect the patient too much, the dose can be reduced, and if these symptoms persist, the patient should try to switch to another drug [15].

3.4.2. Alprazolam. According to the National Center for Addiction, some of the main side effects of taking alprazolam may include reduced mental alertness, confusion, memory problems, dizziness, muscle weakness, slurred speech, blurred vision, or increased depression. In addition to these symptoms, patients who regularly take alprazolam are more likely to become dependent on the drug, even under the supervision of a healthcare provider. At the same time, patients taking alprazolam develop resistance, so in order to produce better results, they need to ingest more of the drug. In such cases, patients are more likely to become dependent. When patients become dependent on withdrawal, they often experience intense withdrawal within hours of the first withdrawal. Common underlying withdrawal symptoms include anxiety, insomnia, hallucinations and seizures. When these symptoms become severe, life-threatening symptoms such as mania and depression can develop [21].

3.5. Economics

Table 1. Average price of fluoxetine

10mg		
quantity	Per unit	Price
21	$0.87	$18.31
28	$0.67	$18.71
30	$0.40-$0.99	$12.01-$29.84
60	$0.23	$13.75
90	$0.16-0.78	$14.48-$70.53
100	$0.14-0.77	$13.28-$77.32

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Depending on the pharmacy visited by patients, cost of fluoxetine oral capsule 40mg id around $13 for a supply of 30 capsules. The oral solution is around $0.37 per milliliters, $44.76 for 120 milliliters. Image above illustrates the average price of fluoxetine with different quantities from drug.com [22].

Table 2. Average price of alprazolam

0.25mg		
quantity	Per unit	Price
6	$1.78	$10.70
15	$0.83	$12.50
30	$0.52	$15.49
100	$0.29	$29.48
500	$0.22	$109.42
1100	$0.21	$209.33

Depending on the pharmacy visited by patients, the cost of alprazolam oral tablet 0.5 mg is around $11 for a supply of 6 tablets. The oral concentrate is around $3.43 per milliliters and $102.92 for 30 milliliters. Image above illustrates the average price of fluoxetine with different quantities from drug.com [23].

4. Conclusion

It can be concluded that fluoxetine and alprazolam are ideal drugs for the treatment of panic disorder. Fluoxetine inhibits serotonin reuptake and alprazolam inhibits GABA. Although both drugs have negative effects that can sometimes threaten the life of the patient, in general, the two drugs can be effective as long as the patient is monitored timely and the dosage is adjusted. Not only that, the use of two drugs can also play a very good therapeutic effect in the clinical treatment of panic disorder together. Of course, in addition to SSRIs and benzodiazepines, many other antidepressants or anti-anxiety medications such as SNRIs and tricyclic antidepressants are effective for the treatment of panic disorder. Although the mainstream anti-panic disorder drugs on the market are mainly the two mentioned above, with the development of the trend of time, the possibility of more novel and effective drugs in the future is also very large.

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Lipid metabolism and insulin sensitivity

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Abstract. Diabetes mellitus is known to be a serious chronic disease that requires great attention because it is the "cancer that never dies" and affects many people worldwide; its later complications are often numerous and frightening, and some of them may even affect their children as hereditary diseases. The relationship between lipid metabolism and insulin sensitivity in relation to diabetes is therefore even more important in this context. Insulin plays an important role as a key metabolic hormone in regulating glucose intake and maintaining lipid metabolic homeostasis. Insulin resistance interferes with the homeostasis of lipid metabolism while triggering related metabolic diseases such as type 2 diabetes. This review focuses on the relationship as well as interactions between lipid metabolism as well as insulin sensitivity and the specific factors affecting lipid metabolism and insulin sensitivity in terms of excess lipid accumulation, lipid deficiency, altered lipid composition, and specific lipid species. Finally, interventions based on the factors affecting lipid metabolism and insulin are proposed to improve metabolic health and reduce the risk of metabolic diseases such as diabetes.

Keywords: lipid metabolism; insulin sensitivity; insulin resistance; diabetes mellitus.

1. Introduction

Diabetes remains a significant metabolic disease, with the World Health Organization's global diabetes statistics for the year 2021 suggesting that 537 million adults are living with diabetes, increasing to an even higher 643 million by 2030 [1]. As a disease characterized by hyperglycemia due to elevated blood glucose, the cause is due to insufficient insulin secretion to produce sufficient insulin resistance. At the same time, an excessive increase of lipids in the liver, which is the main organ regulating lipid and glucose metabolism, impairs insulin sensitivity and leads to abnormal lipid metabolism [2]. For example, in type 2 diabetes mellitus, the regulation of lipid metabolism is affected by insulin resistance and insufficient insulin secretion. Such abnormalities in lipid metabolism can also lead to the development of other health problems.

In this review, I describe the relationship between lipid metabolism and insulin sensitivity to elucidate the effects of lipid metabolism on insulin sensitivity and provide appropriate interventions. By understanding the effects and interventions, it will be possible to better understand lipid metabolism in diabetic patients and to choose optimal treatments to improve insulin sensitivity.

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2. Overview of lipid metabolism

Lipid metabolism often involves several interdependent or cross-regulated pathways [3]. These pathways include lipid digestion, absorption, transport, storage, lipid catabolism, and biosynthesis [4]. In the digestive system, pancreatic lipase breaks down triglycerides into fatty acids, monoglycerides, and some free small molecules of glycerol [5]. These fat digests are then absorbed into the enterocytes, where they resynthesize triglycerides in the endoplasmic reticulum and are moved and transported around the body in transporters incorporated into celiac particles, relying on those lipoprotein particles that are formed through the transport of cholesterol and triglycerides in combination with proteins [4].

The normalization or lack thereof of lipid metabolism plays an important role in diabetes and insulin sensitivity. Firstly, lipid metabolism helps to maintain energy homeostasis [6], storing a lot of energy and ensuring nutritional homeostasis. However, the accumulation of specific lipid metabolites contributes more to lipid-induced insulin resistance, and insulin sensitivity tends to decrease when intracellular concentrations of lipid metabolites are elevated [7]. As a result, the effect of insulin per unit of glucose is reduced, and the body's ability to break down sugars is correspondingly reduced, with a decrease in the cellular capacity for glucose uptake, leading to an increase in glucose concentrations in the blood and the development of type 2 diabetes mellitus. This abnormality in lipid metabolism is strongly associated with insulin sensitivity and can lead to diabetes and its associated complications.

Therefore, by understanding these concepts of lipid metabolism and insulin sensitivity, it is possible to gain a deeper understanding of the mechanisms of diabetes as well as to find out if there is an increase in insulin sensitivity when there is sufficient insulin secretion, which is a good area of research for improving insulin sensitivity and treating diabetes.

3. Link between lipid metabolism and insulin sensitivity

Insulin resistance is a pathological condition that occurs when insulin signaling is impaired [8] and is also a key factor in the pathogenesis of type 2 diabetes [9]. Lipid accumulation, lipodystrophy due to lipid overaccumulation, lipid deficiency, alterations in lipid composition, and the effects of specific lipid species often leads to dysregulation of lipid metabolism.

3.1. Lipid overaccumulation

Adipose tissue directs lipid metabolism as well as its products [6], Obesity and diabetes are metabolic disorders resulting from excessive lipid accumulation. When lipids in cells and tissues are not absorbed and free fatty acids (FFA) are spilled due to increased metabolic disorders, these FFA interfere with insulin signaling thereby increasing insulin resistance. At the same time, excessive lipid burden can lead to hampered cellular functions [10]. To add insult to injury, excessive accumulation of lipids induces inflammation and produces inflammatory cytokines which impair insulin sensitivity, leading to impaired insulin signaling and systemic insulin resistance [11].

3.2. Excessive lipid deficiency

Conversely, adipose malnutrition can also lead to severe insulin resistance [12]. Because adipose tissue is unable to store excess energy [13], the body's fat metabolism is abnormally low, leading to a decrease in lipid storage, and therefore a greater than usual release of FFA from the plasma, which is abnormally high and interferes to some extent with insulin signaling [9], increasing insulin resistance. However, when the body is over-nourished, leptin, the hormone responsible for regulating appetite, triggers appetite, increases tissue uptake of glucose by activating the sympathetic nerves, protects pancreatic β-cells from lipotoxicity, and increases insulin sensitivity [13].

3.3. Altered lipid composition

Alterations in the iron content, lipocalin content, and resistin content of lipids at various levels may affect insulin sensitivity.

When iron content is increased in adipocytes, iron acts as a strong oxidant that catalyzes cellular reactions and increases the level of oxidative stress [14], damaging pancreatic β-cells and creating

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insulin resistance [6], as lipocalin is affected and inhibited during secretion, which increases abnormalities in lipid metabolism and increases fat accumulation, which reduces insulin sensitivity. sensitivity.

Resistin, a small protein secreted by adipose tissue [6], plays a role in the mechanisms of lipid metabolism and insulin resistance. Elevated resistin is known to trigger insulin resistance in vitro and in vivo [6] and vice versa. However, to date, there is no definitive information to suggest a correlation between resistin and insulin resistance [15].

3.4. Effects of specific lipid species

3.4.1. Diacylglycerol (DAG). Diacylglycerol, a lipid metabolite that serves as a link between lipid metabolism and insulin resistance, is hypothesized to phosphorylate the insulin receptor Thr1160 and inhibit tyrosine kinase activity in the insulin receptor along with activation of protein kinase C (PKCε) in the cell [16], which impedes insulin signaling [17]. In the study of the lipid tract model of hepatic insulin resistance, it can be understood that intrahepatic DAG and hepatic insulin resistance have a clear link, and the increase and accumulation of DAG are accompanied by a corresponding increase in the degree of hepatic insulin resistance so that the two are positively correlated with each other. At the same time, increased DAG reduces insulin sensitivity in humans, and both are strongly associated with type 2 diabetes [16]. These conditions interfere with insulin signaling, thus diminishing insulin action on cells, increasing glucose uptake, and affecting lipid metabolism. However, correct dietary DAG intake can effectively improve insulin sensitivity in type 2 diabetic patients [16]. Similarly, reducing the expression of PKCε can then avoid damaging insulin signaling and also reduce hepatic insulin resistance.

3.4.2. Ceramides. Ceramide, a bioactive lipid molecule that regulates cell signaling [18], may disrupt insulin sensitivity and lead to its decline [19]. It is known that there may be a link between insulin resistance and ceramides [17] and that ceramides may increase in tissues due to an increase in fatty acids [18]. Of course, reduced insulin sensitivity does not necessarily correlate with the accumulation of fatty acids in its entirety. Ceramides affect insulin signaling in several ways, including inhibition of phosphatidylinositol 3-kinase (PI3K) signaling, and blocking activation of the anabolic enzyme Akt/PKB [18,20]. In these ways, glucose uptake becomes abnormal and nutrient storage is compromised [19].

3.4.3. Free fatty acids (FFA). Free fatty acids are known to enhance glucose-induced insulin secretion [21]. When a person is overly obese, levels of circulating FFA derived from lipolysis in adipocytes are elevated [22], and excess FFA leads to the accumulation of lipid intermediates in muscle [10], resulting in disturbed insulin signaling as well as dysregulation of lipid metabolism, which increases the occurrence of insulin resistance. In the presence of this resistance, glucose cannot be taken up efficiently, ultimately leading to elevated blood glucose. At the same time, the body produces more insulin to allow FFA to enter the plasma from the fat cells. The abnormal elevation of FFA in plasma is mainly due to the continuous accumulation of lipid derivatives such as diacylglycerol and ceramides in the cells [10].

By studying the link between lipid metabolism as well as insulin sensitivity, researchers can use these to understand the specific causes as well as mechanisms of insulin resistance. This provides a theoretical foundation for the later provision of relevant and specific measures to improve diabetes mellitus.

4. Factors affecting lipid metabolism and insulin sensitivity and interventions

4.1. Factors Affecting Lipid Metabolism and Insulin Sensitivity.

4.1.1. Dietary structure. Dietary structure has an important influence on insulin sensitivity and people with type 2 diabetes. Red meat consumption is positively associated with the risk of type 2 diabetes [14]. Red meat tends to be heme iron-containing, and according to the above article about increased iron

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content in lipids leading to insulin resistance [6], it is understood that red meat intake is highly correlated with heme iron intake [14]. And there is also a positive correlation between red meat intake and the risk of weight gain, thus high amounts of refined carbohydrates [23], as well as high fat and sugar as the main components leading to lipid accumulation, can lead to disorders of lipid metabolism, while obesity mediates the intake of red meat and the risk of diabetes mellitus [14]. Sodium and nitrite contained within processed meats are also toxic to pancreatic β-cells when converted to nitrosamines and also increase the risk of diabetes [14].

Excessive intake of animal proteins and low intake of plant-based proteins in the human body exacerbates insulin resistance [24], as well as worsens metabolic control in diabetic patients, increasing the incidence of type 2 diabetes mellitus. When animal protein is ingested, this ingredient, together with amino acids, activates glucagon secretion, and when plasma glucagon is persistently elevated, the intake of animal protein may induce insulin resistance and type 2 diabetes; in patients with type 1 diabetes, metabolic control is worsened, and vascular damage may occur [24]. High animal protein intake also impairs the inhibitory effect of insulin on glucose in the liver, thus affecting insulin sensitivity in the long term.

4.1.2. Genetic factors. Genetic factors play a significant role in lipid metabolism and insulin sensitivity in humans. The development of insulin resistance in an individual may also be determined by genetic factors [25]. Abnormalities in insulin structure as well as mutations in insulin receptor substrates can be used to explain genetic variation. These mutations lead to abnormalities in lipid metabolism, which in turn affects insulin signaling pathways and conduction, and ultimately insulin sensitivity. It is clear that while exploring genetic factors, the impact of lifestyle, as well as environmental factors, needs to be taken into account [25].

4.1.3. Living environment. Lifestyle also plays an important role in determining insulin resistance and metabolism [25]. Lithium in the environment accumulates in the liver [26], bone, and muscle through the food chain, and high levels of lithium can impair insulin signaling and interfere with intracellular signaling pathways, thus affecting lipid metabolism and insulin sensitivity. Some pollutants in the environment tend to damage the human body, and even at low levels of exposure, chronic diseases can be suffered from prolonged exposure to highly polluted environments. Some persistent organic pollutants, such as organochlorine pesticides, arsenic, and non-dioxin polychlorinated biphenyls (PCBs), affect human endocrine function. In particular, arsenic impairs pancreatic β-cell function and even alters the expression of genes associated with insulin resistance thereby increasing the risk of type 2 diabetes [27]. Constant exposure to highly polluted environments can also lead to increased oxidative stress and inflammatory responses, affecting the balance of lipid metabolism.

Extreme external temperatures can also affect insulin sensitivity. In cold environments, plasma concentrations of insulin are subsequently reduced, while still allowing for glucose uptake and metabolism. Slightly higher temperatures increase the affinity of the insulin receptor for glucose, which affects the uptake of glucose by cells. In addition, high temperatures lead to the loss of insulin receptors, resulting in reduced insulin binding capacity [27].

In addition to this, for example, seasonal changes, sunlight exposure, and sea level height can affect insulin sensitivity.

4.1.4. Physical activity. When many people become fat due to a lack of exercise, the excessive accumulation of lipids in the body cannot be metabolized in time, which will lead to lipid metabolism disorders and related metabolic diseases. At the same time, lack of exercise will not be able to promote cellular uptake of glucose, resulting in the accumulation of glucose in the plasma and increased insulin resistance. Lack of exercise does not increase the synthesis and stabilization of nitric oxide in the vasculature, which has a vasodilatory effect, improving vascular function and inhibiting insulin delivery and glucose uptake [28].

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4.2. Improving Metabolic Health through Interventions.

4.2.1. Dietary composition. Insulin sensitivity is primarily regulated through the influential factor of dietary structure. Weight loss does not enhance insulin sensitivity, and reducing animal protein intake [24], adjusting dietary heme iron intake, and substituting plant foods can enhance insulin sensitivity. Avoiding diets high in animal protein [24] and limiting saturated fat intake [23], such as consuming nuts, low-fat dairy products, or diets high in grains and fiber instead of animal protein can help to slow the release of glucose and reduce the need for insulin, which can significantly reduce the risk of developing diabetes [14,24]. Vegetable-related dietary fiber can increase insulin sensitivity and improve insulin resistance. Reducing the consumption of red meat, especially processed meat, and eating healthier foods can also reduce diabetes risk and cardiovascular risk.

4.2.2. Increase exercise. Proper aerobic exercise has been shown to be effective in preventing cardiovascular risks [28], and of course, resistance exercise has been shown to be most beneficial for glucose metabolism [29], stimulating the release of factors such as bradykinin and increasing glucose uptake [27]. It improves insulin resistance as well as enhances insulin sensitivity through enhanced signaling after receptor enhancement, promotes regulation of lipid metabolism, reduces the excessive accumulation of lipids in tissues, prevents and manages overweight conditions, and maintains a healthy body. At the same time, exercise has been shown to have a weight loss effect [28] and can have a meaningful impact on blood pressure, metabolism, and quality of life. According to one report, 2 years of aerobic exercise resulted in an increase in plasma HDL cholesterol levels of nearly 3-9% [30], which can reduce the incidence of type 2 diabetes. Exercise reduces hyperactivity of sympathetic nervous activity, favoring insulin resistance and improving autonomic nervous system homeostasis [28].

4.2.3. Managing the living environment. To minimize the impact of environmental factors on insulin resistance, the body needs to reduce exposure to high levels of pollution and chemicals and take care of personal environmental cleanliness; however, it also needs to ensure a certain amount of outdoor exercise in order to ensure adequate sunlight exposure. The body needs to be able to sense temperature extremes and make appropriate choices to maintain normothermia, such as adding or subtracting clothing to minimize the effects of temperature extremes on insulin sensitivity. Medical testing and interventions where appropriate.

5. Conclusion

The discussion in this review shows that there is indeed a strong link between lipid metabolism and insulin sensitivity in many ways. Disturbances in lipid metabolism due to excessive accumulation of lipids, triggering an overabundance of FFA and production of inflammatory cytokines; accumulation of DAG as well as ceramides; all of these interfere with normal insulin signaling and ultimately lead to a decrease in the cellular insulin response.

There is no cure for diabetes, so it is important to prevent it. Finding interventions for lipid metabolism and insulin sensitivity is also an important strategy. Dietary changes, lifestyle modifications, physical activity, and the living environment are some of the ways to improve lipid metabolism and insulin sensitivity and reduce insulin resistance and the risk of metabolic diseases. These in-depth studies help to better explain the molecular mechanisms underlying these relationships and provide a theoretical basis for future research and the development of novel therapeutic strategies.

There are many areas where lipid and insulin metabolism need to be investigated for new therapeutic strategies in the future. First, concerning genetic factors, researchers can provide more personalized, unique lipid interventions based on an individual's genetic profile and metabolic status, taking into account genomics, metabolomics, and proteomics. At the same time, researchers can also actively explore new pharmacological treatments to improve normal insulin transmission by regulating the balance of lipid metabolism.

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Going forward, this review is equally important in all respects. For these metabolic diseases, an understanding of lipid metabolism and insulin sensitivity can help in the diagnosis of these diseases as well as provide the rationale for new therapeutic strategies and the development of preventive measures to minimize the long-term effects of the disease on the patient. For patients with metabolic disorders, this information will enable them to understand the mechanisms of disease and health education, as well as to learn to better manage their health by promoting lifestyle changes. For society, these studies can help advance discovery and exploration in clinical practice, deepen understanding of the medical field, and promote cutting-edge research on related diseases.

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Caprylic acid promotes synaptic plasticity potentially through enhancing leptin/NM2B signaling

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Abstract. Synaptic plasticity is crucial for memory formation and can be impaired in metabolic diseases. Insulin and insulin-like growth factors play important roles in synaptic plasticity, while leptin protects neurons and stimulates growth. Diets can affect synaptic plasticity through macronutrients and appetite signals, highlighting the need for deeper research and interventions for synaptic dysfunction in metabolic diseases. Recent research suggests that while saturated fatty acids (SFA) have been viewed as unhealthy for cognition, they may benefit memory and learning in certain conditions. High levels of SFA were found to improve recollection in young people. In specific, low-level intake of caprylic acid may reduce neuroinflammation and protect against neural degeneration. This anti-neuroinflammatory effect of caprylic acid may be mediated through the JAK2/STAT3 signaling pathway, which is a crucial part in the modulation of synaptic plasticity by leptin/NM2B signaling. Therefore, this work hypothesized that caprylic acid can enhance leptin/NM2B signaling pathway by increasing the expression of JAK2/STAT3 signaling pathway. In this proposal, a 12-week mice experiment is proposed with fEPSP measurements, immunoblotting, and immunoprecipitation to test the long-term potentiation (LTP) and the expression level as well as regions of the signaling molecules. This work may offer another perspective on the complex role of SFA in brain health.

Keywords: Caprylic acid; NR2B phosphorylation; Inflammatory cytokine; Leptin signaling pathway; Saturated fatty acid; Synaptic Plasticity

1. Introduction

Synaptic plasticity is a crucial aspect of the mammalian brain, allowing neural activity from experiences to modify circuit function and influence subsequent thoughts, feelings, and behavior. It involves the activity-dependent modification of synaptic transmission strength, enabling the brain to incorporate transient experiences into long-lasting memory traces, a process vital for memory formation.

There are many epidemiological studies on the relationship between degenerative diseases and metabolic diseases like obesity and diabetes. Animal models using high-fat diets, leptin deficiency, and genetic modifications have shown impaired synaptic function and accelerated cognitive decline associated with metabolic diseases. The mechanisms underlying the impaired synaptic function in metabolic diseases are complex. Increased apoptosis decreased levels of neurotrophic factors and reduced synaptic complexity in specific regions of the hippocampus are consistently observed in models of metabolic disease.

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Hormones involved in metabolic diseases have been proven to influence synaptic function. Insulin resistance, often associated with metabolic diseases, may play a causal role in the impaired long-term potentiation (LTP). Insulin and insulin-like growth factors (IGF1 and IGF2) are crucial for hippocampal synaptic plasticity, adult neurogenesis, memory, and learning. Insulin and IGFs promote synaptic plasticity through various signaling pathways. Leptin, an adipokine involved in appetite regulation, also plays a role in protecting hippocampal neurons and stimulating dendrite growth.

As an upstream factor affecting metabolic diseases, diets can influence the synaptic plasticity of hypothalamic neuronal populations either through direct effect due to macronutrients or via peripheral appetite signals. Indeed, understanding the causal relationship between diet and synaptic function is essential, given the increasing prevalence of metabolic diseases and the potential improvements towards cognition and memory brought by healthy diets. Based on current research, the molecular mechanism of macronutrients in neurons are multifaceted. Thus, further research is needed to gain a deeper understanding of the mechanisms involved and explore interventions to synaptic dysfunction based on these mechanisms.

2. Previous Research

For long time, SFA has been believed as unhealthy and impedes cognition, yet recently increasing research papers point out that SFA benefit memory and learning under specific conditions. A previous research paper observed that, contrary to that worse episodic memory is associated with increasing amount of SFA among older people, young people perform better in the recollection test under high-level SFA [1]. SFA may thus contributes to brain function and development, while the excessive health risks caused by high-level SFA in older people outweigh its benefits. In particular, a diet high in SFA can increase the risk of cardiovascular disease and metabolic disorders, indirectly leading to inflammation [2]. Nevertheless, despite the health risks of SFA, it may protect brain against neural degeneration by reducing neuroinflammation under low-level intake. Lauric acid has recently been reported to have neuroprotection in neuroinflammatory degenerative diseases; caprylic acid can ameliorate rotenone induced inflammation; other SFA such as myristic acid and stearic acid also possess potential anti-inflammatory abilities [3]. In this way, SFA leads to better overall cognitive performance by reducing neuroinflammation.

According to previous research, we speculate that such anti-neuroinflammatory effect of SFA may be achieved through JAK2/STAT3 signaling pathway. Caprylic acid, a SFA found in palm and coconut oil, is involved in inhibiting inflammation through upregulation of the ABCA1/p-JAK2/p-STAT3 pathways [4]. Mice fed with caprylic acid-added diet has proven to have higher mRNA and protein expression levels of JAK2 and STAT3, with declined level of multiple inflammatory factors.

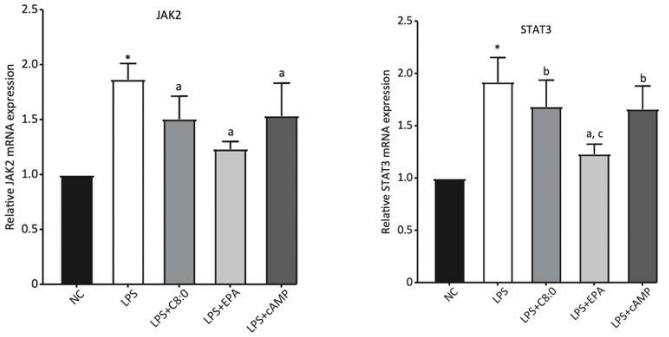

Figure 1. Relative JAK2 and STAT3 mRNA expression levels in C57BL/6 mice fed with different high-fat diets and normal diet (NC). C8:0 refers to 2% caprylic acid feed [5].

In fact, considering previous research on the modulation of synaptic plasticity through leptin signaling, there exist some similarities. Activation of leptin receptors causes following phosphorylation

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of JAK2 and dimerization along with translocation of STAT3, which phosphorylates NM2B, a subunit of NMDA receptor that is crucial to synaptic plasticity, and finally activates NMDA receptor, in turn promoting persistent changes in excitatory synaptic plasticity, usually in form of long-term potentiation (LTP). Within this procedure, JAK2/STAT3 cascade is a crucial component. Since caprylic acid can increase the production of signaling molecules within this cascade, it is reasonable to hypothesize that caprylic acid can enhance leptin signaling by increasing JAK2/STAT3 expression, leading to more NM2B phosphorylation and thus strengthening synaptic function.

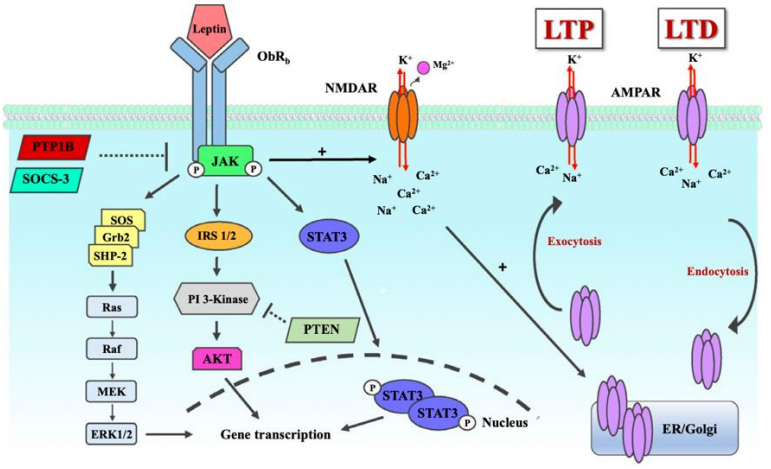

Figure 2. Leptin receptor signaling pathway [6].

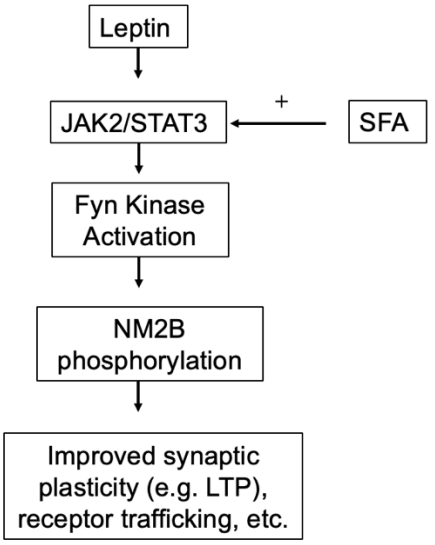

Figure 3. Speculated mechanism of SFA's role in leptin regulation of synaptic function.

3. Methodology

To study the effect of caprylic acid on leptin/NR2B signaling pathway, experimental group and control group are first set. In the experimental group, C57BL/6 mice will be fed with 2% caprylic acid diet, having higher calories than the other group; in the control group, C57BL/6 mice will be fed with standard rodent chow. C57BL/6 mice from both groups will be fed with standard rodent chow for the first 8 weeks after birth and will be randomized into the experimental group and control group afterwards.

Then, mouse types in each group are determined. The widely used C57BL/6 C57BL/6 mice will be applied in this experiment. Male C57BL/6 mice are used because they have relative stable levels of

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hormones, which can affect the result, and they have lower amount of estrogen, which can directly influence the CRE protein.

The C57BL/6 mice will have three types: $LepR^{+}$(C57BL/6 mice with CRE-loxP system and no tamoxifen injection), $LepR^{-}$(C57BL/6 *mice with* $CRE - loxP$ *system and tamoxifen injection*), and WT (Wild -type C57BL/6 mice). The method of constructing CRE-loxP C57BL/6 mice models, as shown in figure 4, will be explained later. By comparing the performance of $LepR^{+}$ and $LepR^{-}$ C57BL/6 mice in experimental and control trials respectively, whether caprylic acid affects synaptic function through enhancing leptin, independently, or in both ways can be known. This will be explained more detailed in the Expected Outcomes. Also, the performance of WT C57BL/6 mice acted as a control group that tests the potential irrelevant variables, if any, brought by CRE-loxP gene editing beside the expression of leptin receptors; In theory, there shouldn't be significant statistical difference between the performance of WT and $LepR^{+}$ C57BL/6 mice ($p<0.05$). Sample size will be determined by power analysis to meet the 3R criteria; in previous research on the effect of caprylic acid on C57BL/6 mice brain, the sample sizes are mostly set between 5~10, so in this experiment, the sample size in each trial is set as 10 C57BL/6 mice. Consequently, there will be six trials and total of 60 C57BL/6 mice.

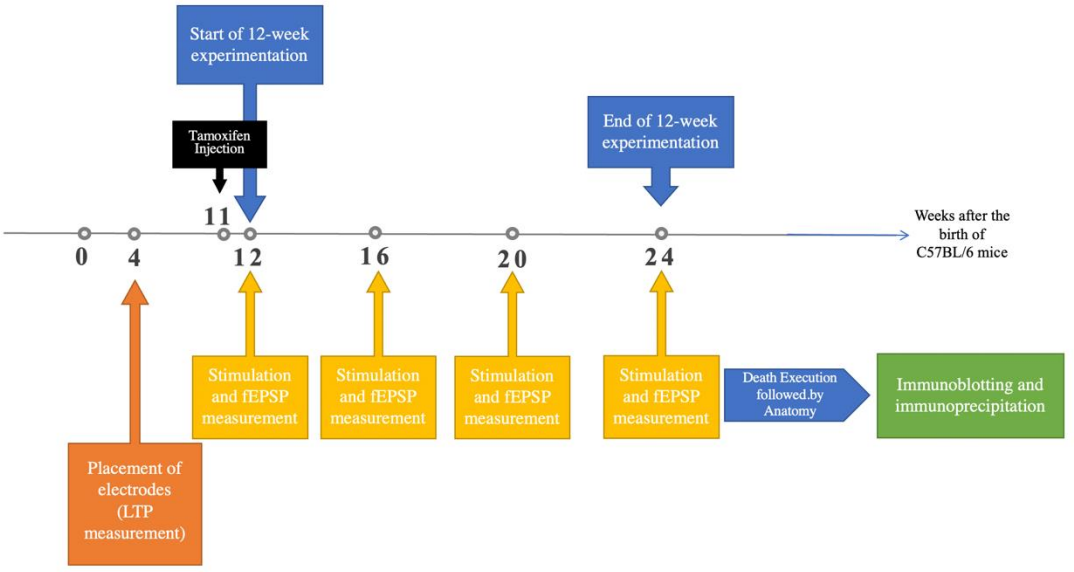

Figure 4. Timeline of the Experiment.

3.1. Transgene Construction and Transgenetic C57BL/6 mice Production

LepRb subtype of leptin receptors is chosen to be controlled through CRE-loxP system. The CRE-loxP system is a genetic tool that uses specific DNA sequences (loxP sites) and the enzyme CRE recombinase to perform DNA-editing. It allows for precise gene insertion, deletion, or rearrangement, enabling controlled and targeted genetic modifications in research. This is because LepRb [7] mRNA is primarily expressed in the dentate gyrus, which is spatially concentrated at the desired position. For promoter, Prox1 gene is chosen: it is known to be highly expressed in the dentate gyrus granule cells, which are a major neuronal population within the dentate gyrus region of the hippocampus. The Prox1 promoter has been widely used to drive gene expression specifically in dentate gyrus granule cells in C57BL/6 mice. Furthermore, with artificial injection of tamoxifen, the time of inhibition of LepRb can be decided precisely. Therefore, CRE-loxP system allows spatiotemporal operations on LepRb gene.

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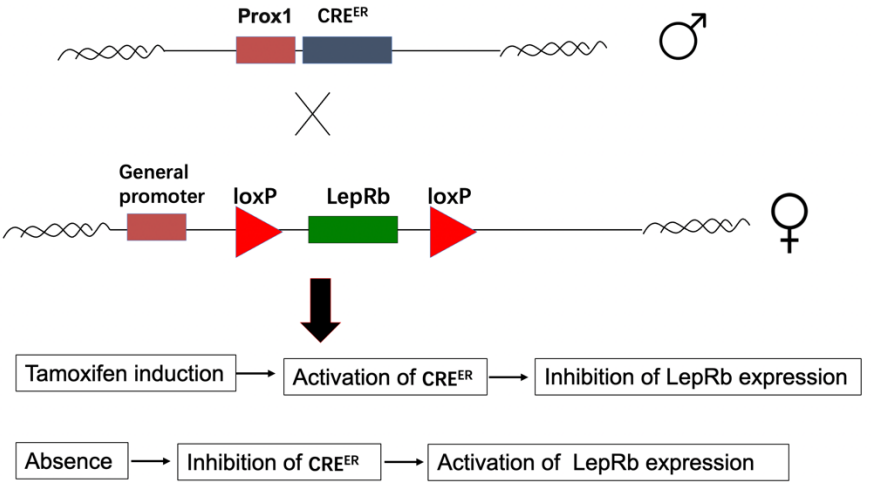

Figure 5. Simple illustration of the preparation of C57BL/6 mice with LepR-specific CRE-loxP system and the control of the LepR expression through tamoxifen.

Tamoxifen will be injected to induce inhibition of LepRb expression. Two methods of injection [8], subcutaneous injection and intraperitoneal injection, will be tested through pretrial. The one that causes less harm to C57BL/6 mice and help to achieve stable inhibition of LepRb expression will be used in the formal experimentation. Tamoxifen injection will be done one week ahead of the experiment, with injection of four consecutive days. Also, before the formal experimentation, patting and mock injection will be done to let C57BL/6 mice get used to surrounding environment and needles.

3.2. Leptin and serum lipid levels

The purpose of this measurement is to prove that the leptin and serum lipid levels of all C57BL/6 mice fell into a narrow range, so that self-produced leptin and serum lipid levels are standardized and thus won't affect the experimentation.

The levels of serum lipid were determined using commercial kits. Blood serum of all C57BL/6 mice is collected first. Serum triglycerides (TG) (Wako,97Osaka, Japan), and HDL-C and LDL-C (Abcam, Cambridge, UK) were measured with commercial kits, and the ratio of HDL-C to LDL-C was subsequently calculated. To be more specific, TG will be measured through GPO-HMMPS, a method for triglyceride measurement. It involves the elimination of free glycerol and the conversion of triglycerides to dihydroxyacetone phosphate and hydrogen peroxide, which react with HMMPS and 4-aminoantipyrine to form a blue pigment for quantification. Also, the ratio of HDL-C to LDL-C will be determined through fluorometric detection method, which utilizes cholesterol oxidase to convert free cholesterol into a reactive compound that reacts with a probe to generate color and fluorescence.

Blood was collected at the end of study after 12 weeks and processed to measure serum leptin level using mouse leptin (no. EZML-82K, Millipore Sigma) ELISA kits, respectively, where leptin in the sample will bind to specific antibodies on a plate and thus enables fluorometric detection for analysis.

For procedural repetition, this measurement will be repeated for three times on each C57BL/6 mice brain slice.

3.3. fEPSP Measurements

The LTP of C57BL/6 mice is measured through fEPSP under live situation, by fixing electrodes in live C57BL/6 mice's brains. By comparing the result between C57BL/6 mice fed with standard rodent chow and 2% caprylic acid, it can be determined whether caprylic acid diet can influence synaptic plasticity in the end.

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Permanent monopolar recording electrode will be fixed for detecting responses of perforant path-dentate gyrus (DG) and hippocampal CA3. The placement of electrodes will be done to C57BL/6 mice with surgery under anesthetized condition between postnatal days 109 and 120. Electrodes will be placed both in the DG and CA3 region. Before the surgery, C57BL/6 mice will familiarize in the environment for at least one week. After the surgery, C57BL/6 mice will be given antibiotics and analgesics. Stimulation of the lateral and medial perforant paths produced monosynaptic responses in the DG and CA3, which were confirmed through decreased field excitatory postsynaptic potential (EPSP) slopes and paired pulse facilitation.

To measure responses, awake and behaving C57BL/6 mice will be subjected to constant current biphasic pulses. Input/output curves were collected to determine the current intensities that evoked field EPSPs at 50% of the maximal response. Response magnitudes were calculated by determining the slope of the field EPSP. C57BL/6 mice's responses will be collected at 0, 4, 8, and 12-weeks after the beginning of the 12-week-experimentation. Each time, after 15 minutes of daily baseline responses, LTP will be induced by delivering five "theta burst" stimulation trains. The responses will be collected for an additional hour after the tetanus.

Data analysis involved normalizing and presenting the percent change in field EPSP slopes relative to baseline responses collected before tetanus. The magnitude of LTP was presented as the percent change in field EPSP slopes compared to baseline responses collected three days before tetanus.

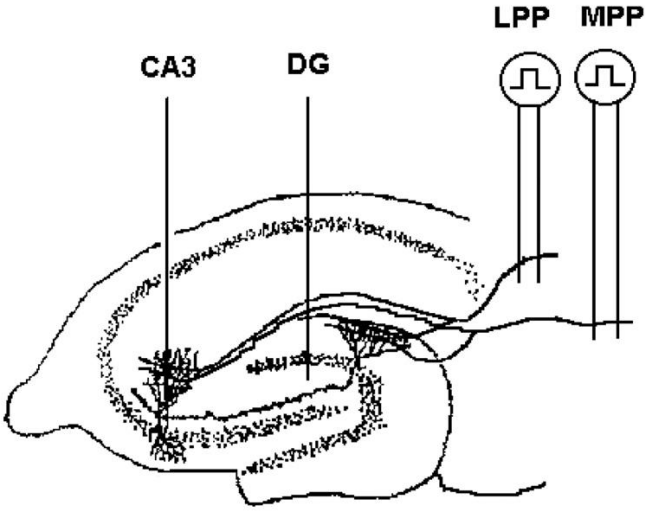

Figure 6. Electrode placement for perforant path for DG and CA3 responses [9].

3.4. Immunoblotting and Immunoprecipitation

Immunoblotting of LepRb, JAK2, and STAT3 are performed for quantifying the expression levels of these signaling molecules in all groups of C57BL/6 mice. Comparing the LepRb expression level between experimental groups and control groups can help to affirm the effectiveness of CRE-lop system in controlling the presence of LepRb ($LepR^{-}$ C57BL/6 mice should not have LepRb expression). Moreover, according to previous research, JAK2 and STAT3 level will increase in C57BL/6 mice fed with additional caprylic acid [10], so the immunoblotting of JAK2 and STAT3 will aid validation, reassuring caprylic acid's role in enhancing leptin/NR2B signaling by increasing the amount of JAK2 and STAT3.

DG granular cell layer of all C57BL/6 mice will be collected and pre-treated. LepRb will be immunoprecipitated by Protein A/G agarose beads (Santa Cruz) pre-incubated with the correct amount of anti-LepRb antibody (1:500; no. ab5593, abcam), overnight at 4 °C [11]. In similar manners, immunoblotting of JAK2 and STAT3 will be done with their specific antibodies. The relative quantity of signaling molecules can bind to enzyme-linked secondary antibodies that can catalyze a colorimetric

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or chemiluminescent reaction, where the fluorescent signal can be quantified with specialized instruments.

For procedural repetition, this measurement will be repeated for three times on each C57BL/6 mice brain slice.

Immunoprecipitation will be done to p-JAK2, p-STAT3, and p-NM2B in DG granular cell layer with C57BL/6 mice brain slices. These assays are designed to test the influence of caprylic acid on specific signaling molecules participating in the leptin/NR2B pathway. With immunoprecipitation, the expression level of the above signaling molecules can be roughly compared between different trials; also, the interaction of these molecules can be studied by finding the overlapping fluorescent dots representing the presence of these molecules after staining. This uncovers the exact molecular mechanism of caprylic acid.

DG granular cell layer of all C57BL/6 mice will be collected. Proteins were separated by SDS–polyacrylamide gel electrophoresis and incubated with monoclonal antibodies to phosphe-JAK2 (1:1,000; no. 3771, Cell Signaling Technology), phospho-STAT3 (1:1,000; no. 9131, Cell Signaling Technology), and phospo-NR2B (1:1,000; no. 5355, Cell Signaling Technology).

For procedural repetition, immunoprecipitation experiment will be repeated for three times on each C57BL/6 mice brain slice.

4. Expected Results

Overall, it is expected that caprylic acid diet can strengthen synaptic plasticity through enhancing leptin/NR2B signaling. Specific expectations are discussed in the following passage: First, comparing the experiment group (caprylic acid diet) and control group, the experimental group should have higher JAK2, STAT3, p-JAK2, and p-STAT3 expression levels, as the leptin/NR2B signaling pathway is enhanced. Second, the experimental group may have greater changes in the fEPSP slope, bearing better LTP. Third, before the experiment, C57BL/6 mice from both groups should have similar serum lipid levels and leptin levels; it is assumed that these variables should be relatively constant in both groups.

5. Discussion

Admittedly, there are limitations in the methodology design, yet they are detailly addressed, as explained in the following passage:

First, the effective doses of tamoxifen injection may not correspond with previous experiment, as this is a novel CRE-loxP system. Therefore, pilot experiment will be done to determine the effective dose of tamoxifen injection. In particular, 0, 1, 10, 100, 200, 300 mg/kg/day will be tested on C57BL/6 mice for four consecutive days, and then the mice will be executed and tested the presence of LepRb with immunoprecipitation to see the effectiveness of tamoxifen in cutting off LepRb gene.

Second, another limitation is due to the self-produced leptin level in mice. Currently, this factor is assumed to be statistically insignificant ($p<0.5$) after controlling the mice' diet and their subspecies. However, since the precise quantitative relationship between leptin and strength of leptin/NR2B signaling pathway is unknown, if the C57BL/6 mice is highly sensitive to leptin compared to caprylic acid, the influence of self-produced leptin level can be the main factor that affects the experiment result. Therefore, before the experiment, the fluctuation in self-produced leptin of all C57BL/6 mice will be assessed through T-test, and the fEPSP measurements may also be taken and analyzed through T-test. Also, if the fluctuation is too large, we may consider inhibiting the self-production of leptin in mice and artificially inject leptins regularly.

Third, the repetition in theta burst stimulation during fEPSP measurement can influence the experiment. Prolonged or excessive stimulation can potentially lead to tissue damage in the neural circuit under study. We will take precautions to ensure that the stimulation parameters used are within safe limits to minimize any potential harm.

Overall, the outcomes can contribute to the research problem by revealing the possible signaling mechanism of caprylic acid in enhancing leptin/NR2B. The experiment can reflect the interaction

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between caprylic acid and important signaling molecules in leptin/NR2B signaling pathway, such as JAK2 and STAT3. In this way, a detailed signaling mechanism of caprylic acid can be known.

6. Contribution To The Field Of Knowledge

Currently, the influence of macromolecules on brain function, in this case synaptic function and adult neurogenesis, lacks research. There can be contradictory relationship between these macromolecules and brain functions, especially for SFA, a formally believed totally unhealthy component that has been recently revealed to have some benefits in brain function. This research will continue this direction and reveal more possible influence of SFA on brain and health.

7. Conclusion

This proposal proposed a research on the effect of caprylic acid in the leptin/NM2B signaling pathway, based on previous results suggesting the common JAK2/STAT3 signaling existed in both caprylic acid's signaling in anti-neuroinflammation and leptin/NM2B signaling. Hypothesizing that the existence of caprylic acid can increase the expression of JAK2/STAT3 signaling molecules, this study designed a detailed 12-week mice experiment, with general control group with standard rodent chow and experiment group with 2% caprylic acid, which focuses on the three following measurements: 1. fEPSP measurement will reveal the difference in LTP. 2. Immunoblotting regarding signaling molecules can reflect the difference in the expression of signaling molecules. 3. Immunoprecipitation suggests the regional expression pattern of these signaling molecules. Altogether, these potential results can give us a clue on the possible signaling mechanism of caprylic acid in enhancing leptin/NR2B. Therefore, this proposed research may offer a new perspective of the role of SFA in learning and cognition.

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Investigating the functional effects of the long-term ketogenic diet on pancreatic islets in epileptic rats

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Abstract. Ketogenic Diet is well studied and applied as a treatment in numerous nervous system diseases, such as epilepsy. However, KD's long term effect on the β cells and the risk of potential insulin resistance are unclear. In this research, we aim to testify whether the usage of KD on patients with epilepsy would increase the risk of diabetes. In this research we firstly verified the cytotoxicity of the islet B cells which might be stimulated by the abundance of fats in the KD. We used a CCK-8 kit to conduct the experiment and obtained a result of higher IC50 values in the keto conditioned cells, which indicates that cells in keto face a higher cytotoxicity than cells in normal condition. Afterwards, we did both vivo and in vitro experiments on the rats. In the in vitro experiment, the islet β cells in a keto-environment showed a higher apoptosis rate. In the in vivo experiment, Enzyme-linked immunosorbent assay (ELISA) was used to detect the insulin level and blood fat in plasma of rats. The result out of ELISA demonstrates a continuously decreasing level of insulin and lower body fat. In addition, oral glucose tolerance test (OGTT) was also used to find the blood sugar in the rats which came out as a result that the glucose metabolism has decreased. Although the experiments exhibit several side effects of KD, it might not be applicable to humans since we only had animal experiment. This research aims to reevaluate the benefits and side effects of application of KD to curing epilepsy.

Keywords: ketogenic diet, diabetes, pancreatic islets, β cells, insulin.

1. Introduction

The Ketogenic Diet (KD) has been employed as a cure for intractable epilepsy and related neurological disorders since the 1920s. KD itself is a dietary regimen characterized by a substantial reduction in carbohydrate intake and a significant increase in fat consumption. In this diet, carbohydrates occupy less

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than 10% of the total calorie content. As documented in the Hippocratic collection, the KD is postulated to act as a remedy that could generate the same advantages of fasting. In this research, we aim to testify whether the usage of KD on patients with epilepsy would increase the risk of diabetes.

The KD is a low carbohydrate-high fat (LC-HF) diet, which causes ketosis, an elevated blood level of ketones that are metabolized by the brain as the main source of energy when glucose is absent. Ketones are fatty acids produced in the liver [1]. While there is no clear definition for the various KD variants, KD can be defined as a diet that induces ketosis. Nevertheless, ketosis can also occur without fat consumption. The KD is characterized by low carbohydrate intake (less than 50g/day) and a relatively high proportion of proteins and fats. The study of the metabolic effects of KD dates to the pioneering work of Cahill and colleagues in the 1960s [2], however, the importance of the diet from a clinical point of perspective became apparent in the early 1920s when it was effectively applied to treat epilepsy [3]. In recent years, interest in a very low carbohydrate ketogenic diet (VLCKD) has increased. There is growing evidence that it can have a positive effect on many diseases. Since the 1970s, VLCKD has been established as a treatment which treated epilepsy effectively. Most recently, more attention has been paid on this particular diet due to the positive impacts it has on several diseases. However, more research is needed to fully fathom this relatively novel idea before applying it to all patients. Therefore, this research revisits the function of KD on curing epilepsy and the potential side-effects it brings as the result.

2. Material and method

2.1. In vitro experiment

2.1.1. Preparing pancreatic β cells.

Material

Pancreatic β cell strand from procell

Insulin high range HTRF test kit from cisbio

2-Deoxyglucose (2DG) Uptake Measurement Kit from FujiFilm

DMEM medium and no glucose DMEM medium from ThermoFisher Scientific.

Method

All pancreatic β cells used in the experiment will be replicate from one cell strand for consistency in DEME medium. Obtain pancreatic β cell strain from procell. Add 10 mL DEME medium without antibiotic for cell replication to a 15cm petri dish, and place cell in it. Add 1mL of cell to petri dish and keep under 37° (normal body temperature). Check and record cell volume under microscope daily. Once the cells reached a consistent multiplying rate, start transferring them out to other 15 cm petri dishes. Repeat the wait and harvest process until 20 petri dishes are filled with the clones of the original strain.

2.1.2. Set two groups of β cells and the variable is the environment. Twenty 15cm petri dishes filled with identical panctratic β cells, labeled as 1 through 20, split into two groups of 10 petri dishes. Group A (dish 1 through 10) is controlling group placed in DMEM medium. Group B (dish 11 through 20) is experimenting group placed in high fat medium. The high fat medium is no glucose DMEM spiked with fatty acid (1g per litre). For 3 weeks, check pancreatitis β cell's population, fludeoxyglucose concentration, and insulin conentration in cell supernatant with 2-Deoxyglucose (2DG) Uptake Measurement Kit and insulin high range HTRF every 6 hours under the microscope, record signs of mitosis as evidence of population growth. Record decrease in fludeoxyglucose and insulin concentration as proof of change in metabolism.

2.1.3. Test the cytotoxicity of the islet βcells. In order to verify the cytotoxicity of the islet βcells which are stimulated by the fats in the ketogenic diet. We used CCK-8 kit as a way to find out the result. CCK-8 use the dye WST-8 inside to test the cytotoxicity. And the dye can be reduced by dehydrogenase in the cell to form formazan which is an orange water-soluble product. Furthermore, there is a correlation between the produced formazan and the count of viable cells. Thus, the viability of cells can be estimated

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using a microplate reader to measure the optical density (OD) of the formazan at 450 nm. The darkness of the color shows correlation with cell proliferation and cytotoxicity. Cell proliferation shows directly proportional to the OD and cytotoxicity shows inversely proportional relationship.

We used 2 main groups which are islet βcells in the keto environment and in normal environment. Each group is provided with 36 wells, and a blank group is set at the same time with 24 wells in the marginal. Add Ham f-12 culture solution to each experimental well for 24 hours. PBS buffer is added to the most marginal wells to reduce the influence caused by evaporation, and then all the cells are cultured in a 5% CO2 cell incubator at 37°C for 12-24 hours. Add about 5000-10000 cells and 100ul to each well (the specific number of cells used in each well depends on the size of cells and the speed of cell proliferation).

Observe that the cells are well adhered to the wall and suck out the culture medium of each well. Normal medium and ketogenic medium spiked with fatty acids (1g per liter) and without glucose were added to the corresponding wells. Then the plate is incubated in a cell incubator with 5% CO2 air at 37°C for 12-48 hours. After that, each well was directly added with 10 μL of CCK-8 solution and the culture plate was incubated with CCK-8 for 1-4 hours in a 37°C 5% CO2 incubator.

2.1.4. Measure the apoptosis rate of islet cells in mice. We used propidium iodide to test whether the ketogenic environment increases the rate of apoptosis in mouse pancreatic islet βcells. Propidium iodide (PI), in collaboration with annexin V, is commonly used to determine whether cells are apoptotic, viable or necrotic, based on differences in plasma membrane permeability and integrity. PI is a widely preferred nuclear stain since it is stable, cost-effective and an excellent indicator of cell viability, as it only stains non-living cells. The capability of PI to penetrate a cell relies on the permeability of the cellular membrane. As long as the plasma membrane remains intact, propidium iodide (PI) does not mark living cells or cells that are in the initial stages of apoptosis. In apoptotic and necrotic cells in the late stage-when the integrity of nuclear membranes and the plasma is decreased-PI may penetrate the cell membrane, access the nucleic acids and emit a red fluorescent signal [4]. Group A cells were cultured in a simulated ketone body environment as might be found in mice, and Group B cells were cultured in a simulated normal mouse. The cells were incubated with Annexin V-FITC. The desired method induced apoptosis. As a positive control, Jurkat cells were treated using 2 μM cisplatin. The cells were collected using centrifugation. Resuspend the cells in 500 μL of Annexin V binding buffer followed by the addition of 5 μL of Annexin V-FITC and 5 μL of propidium iodide. Incubate for 5 minutes at room temperature in the dark.

The FITC signal detector and the phycoerythrin emission signal detector were used to evaluate PI staining and Annexin V-FITC binding assayed by flow cytometry. To examine adherent cells, The cells underwent trypsinization and were thoroughly washed with serum-containing medium before being incubated with Annexin V-FITC. Subsequently, the presence of the cells was detected by fluorescence microscopy.

2.2. In vivo experiment

2.2.1. To make the model of epileptic rats. 30 rats are used to prepare the model of epilepsy and each of them is weighted 200-300g. 5 of them are normal control group and the rest of 25 rats are model preparation group.

On the first day, inject lithium chloride (3mmol/kg body weight) intraperitoneally into the experimental group. In the next day (24 hours later), inject pilocarpine to the same group with 20mg/kg body weight every 30 minutes. If the rats have status epilepticus (reaching level 5 and lasting for more than 1 hour) within 3 injections, the injection of pilocarpine is terminated. If the rats did not have status epilepticus within three injections, they continued to be injected with pilocarpine, and the dose was reduced to 10mg/kg body weight, once every 15min minutes until status epilepticus appeared, then injection was stopped.

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When the status epilepticus appears for more than 60min, the seizure is terminated by intraperitoneal injection of diazepam (10mg/kg body weight). If diazepam cannot be terminated once, 15% ~ 25% of the first dose is injected every 10min until the seizure is basically terminated. After injection of pilocarpine, rats in the model preparation group showed different degrees of activity reduction, tremor, nodding, scratching, "face-washing-like activity", facial convulsion, one-limb clonus, wet dog-like jitter and imbalance, limb tonic clonus accompanied by standing and forelimb clonus, which further developed to level 5, and generalized tonic clonus accompanied by standing and falling, showing a state of epilepsy.

At last, exclude some rats which are died during the experiment and control group, choose the best 20 rats to do the rest experiment.

2.2.2. Set two groups of epileptic rats and the variable is their diet. One group with ketogenic diet and it is considered as group A. The other group takes normal diet, and it is group B. For the group of rats with ketogenic diet. They should take lipid: protein + carbohydrate at a ratio of 4 : 1. The protein should less than 1g/day/kg body weight, and according to the proportion, carbohydrate should less than 1g. Moreover, fibre should be taking every morning and night with 15g.

Each group takes 10 rats and have specific diet for 6 months. And record the frequency of occurrence of epileptic. Use balance to measure the weight of these rats every day. And before weighing them, they should fast for 12-24 hours to reduce the influence of food.

2.2.3. Test insulin and blood fat. The enzyme-linked immunosorbent assay (ELISA) is used to measure insulin and lipid levels in the blood serum or plasma of rats every fortnight. The serum is obtained by drawing blood from the tail of the rats. It coagulates naturally at room temperature for 10-20 minutes. Then the blood is centrifuged and left for about 20 minutes. The supernatant is carefully removed.

10 wells are made with the standard on the enzyme-labelled coated plate. For the 1st and 2nd wells, 100 μL of standard is added followed by 50 μL of diluted standard. The 3rd and 4th wells each receive 100 μL solution from the 1st and 2nd wells, followed by 50 μL standard diluent. Discard 50 μL solution from each of the 3rd and 4th wells, pour 50 μL of this into the 5th and 6th wells and top up with 50 μL standard diluent. Mix thoroughly after each step and then transfer 50 μL of solution from the 5th and 6th wells to the 7th and 8th wells, correspondingly. Then add 50 μL of standard dilution to each 7th and 8t h well. Then add 50 μL of solution from the 7th and 8th wells to the 9th and 10th wells, correspondingly. Then add 50 μL of standard diluent to each of the 9th and 10th wells and discard 50 μL of solution from the ninth and tenth wells after mixing.

Both control wells and sample wells are prepared for the experiment. Add 40 μL of the sample diluent to the sample well, which has a collected supernatant on the enzyme-labelled coating plate. Then, add 10 μL of the collected supernatant for testing purposes. Add the sample to the bottom of the well in the enzyme-labelled plate carefully, while avoiding possible contact with the walls. Afterwards, gently shake and mix the sample.

Incubate the sealed plate for 30 minutes at 37°C. For later use, dilute the concentrated wash fluid 30:1 with distilled water, carefully remove the sealing foil, discard the fluid, spin dry the plate and add wash fluid to each well. Allow to stand for 30 seconds, discard, and repeat five times. Finally blot dry the plate. Add 50 μL of the enzyme-labelled reagent to all wells except the blank wells. Repeat the incubation and washing procedure as described above. 50 μL of Developer A and 50 μL of Developer B are added to each well. Then gently shake, mix and incubate for 15 minutes at 37°C in the dark. Stop the reaction by adding 50 μL Stop Solution to each well.

2.2.4. Test blood sugar and glucose metabolism. Use the OGTT to test the blood sugar and glucose metabolism every 2 weeks. Oral glucose tolerance teat (OGTT) is a method used widely to treat the islet function. Let all of the rats fasting for 12 hours and take the blood sample of these fasting rats before the experiment to measure the baseline levels of insulin and glucose. Give oral glucose solution to rats with 2g/kg and record the administration time as 0 minutes.

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At 30, 60, 90, and 120 minutes after administration, blood samples of rats are collected and measured blood sugar level. Simultaneously measure the insulin levels at each time point. Evaluate rat pancreatic islet function and glycometabolism by analyzing blood glucose curves and change in insulin levels. Above all, all the experiments should be done for 6 months. And after 6 months, these rats are going to die and for pathological analysis.

2.2.5. Detect the apoptotic cells in pancreas of the mice [5]. After killing the epileptic mice, we obtained their pancreas without damaging it. We then performed the TUNEL assay as described below.

TUNEL, the abbreviation for terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling, is commonly used as a staining method to identify the cells that are apoptotic in tissue sections. Microwave heating combined with protein hydrolysis in solutions with extreme pH, resulted in strong staining of 70-80% of cells that are apoptotic and apoptotic bodies on archived tissue blocks with low background. Cells that are in the early stage of apoptosis can be observed because of the increased sensitivity, potentially extending our understanding of apoptotic cells beyond images of atrophic necrosis. Inhibition of endogenous peroxidase was not performed as H2O2 weakens TdT activity [6] and induces DNA breaks [7].

3. Result

3.1. Pancreatic cells had a change in metabolism method.

Cells in Group A are expected to continue to duplicate and producing insulin and fludeoxyglucose at a consistent rate. Certain portion of cells from Group B could show signs of necrosis, consider the possibility of pancreas inflammation after prolong period of keto diet. Cells from Group B should show signs of decreas in both fludeoxyglucose and insulin concentration at similar rates due to less carbonhydrate intake, proving that the pancreatic cells had a change in metabolism method.

3.2. Higher apoptosis rate of islet cells in mice

For analysis of adherent cells, they are grown on a coverslip directly. After incubation, turn over the coverslip onto a microscope slide and observe the cells. Alternatively, the cells can be rinsed with Annexin-V Binding Buffer and treated with 2% formaldehyde before observation. The cells should be observed under a fluorescence microscope with a double filter set for rhodamine and FITC or with separate filters. The plasma membrane of cells that have bound Annexin V-FITC will be green. In cells that have lost their membrane integrity, the nuclei show continuous red PI staining, and a halo of green staining (FITC) appears on the plasma membrane. We monitored the apoptosis rate of the β-islet cells in each group by examining the proportion of red fluorescence at 30 and 60 days after the start of the experiment. The fluorochromes used were PKH-26 and annexin V-FITC.

We found that cells in group A showed redder fluorescence than cells in group B each time we observed the cells. Hence, we came to the conclusion that cells in group A had a higher apoptosis rate compared to the cells in group B, which means islet βcells have a higher apoptosis rate in a keto-environment.

3.3. Lower insulin and lower blood fat

Measure the absorbance (OD value) of each well in sequence, using a wavelength of zero and 450nm of blank air conditioner. Conduct the measurement within 15 minutes after adding the stop liquid. The darkness shows direct relationship with blood fat in the sample. And we found out that the concentration of lipids is decreased. The treated serum or plasma sample reacts with a certain concentration of insulin detection reagent, and then the absorbance of the sample is detected by enzyme-labeled instrument. Draw a standard curve with absorbance as abscissa and insulin concentration as ordinate and calculate the absorbance of the sample to measure the fasting insulin level of rats.

Finally, the result indicates that the level of insulin is continuously decreasing.

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3.4. Lower blood sugar and glucose metabolism

Analyze blood glucose curves and change in insulin levels to evaluate rat pancreatic islet function and glycometabolism.

As a result, we found out that the blood sugar of these rats is decreased and the secretion of insulin has delayed. Also, we discovered that the level of glycosylated hemoglobin (HbA1c) and glycated albumin have decreased. So the function of glucose metabolism has decreased.

3.5. The cell in keto environment has higher cytotoxicity.

The OD value will increase with the increase of time. The OD value can be measured at 0.5h, 1.0h and 2.0h correspondingly, and the OD value can be controlled at about 1.0. Take out the 96-well plate, detect the OD value of each well at the wavelength of 450nm by enzyme-labeled instrument, analyze the processed data and draw the proliferation curve. To measure the semi-inhibition rate, firstly measure the inhibition rate of the two experiment groups. Use the formula inhibition rate. When the inhibition rate reaches 50%, the concentration of the solution is the semi-inhibition rate (IC50). The higher IC50, the stronger cytotoxicity.

Above all, we predicted that the group of cells in keto environment has higher IC50 value which means it has higher cytotoxicity than cells in normal environment.

4. Conclusion

The ketogenic diet is an alternative therapy for intractable epilepsy. It is a strict diet that is high in fat and low in carbohydrates and protein. It is mainly used for children whose seizures have reached the refractory stage. This diet is strict and must be followed for a long time, and it is said to have a high success rate [8-10]. However, long-term ketogenic diets have been shown to impair pancreatic function and can lead to type 2 diabetes [11-12]. For this reason, insulin levels and other body parameters need to be monitored while on a ketogenic diet, and if abnormalities occur, appropriate medication needs to be administered or the ketogenic diet discontinued.

This study provides data suggesting that long-term consumption of a ketogenic diet leads to a decrease in βcells. In this study, we investigated the specific causes of βcell reduction due to long-term ketogenic diet intake, focusing on changes in the energy metabolism pathways of pancreatic islets. Using the number of apoptotic cells as an indicator, in this study, the number of apoptotic cells was compared between lipid metabolism, glucose metabolism as the main energy supply pathway, and glucose metabolism as the main energy supply pathway after the addition of spies. Based on the available information and experimental data, we can expect that at least the shift in the energy metabolism pathway is an important reason for the reduction of βcells. At the same time, the fact that fat metabolism has no functional advantage over glucose metabolism in providing energy means that other cells in the body may also be damaged or even die due to lack of energy supply, with subsequent side effects. The ketogenic diet has become a hot topic of research due to its efficacy in the treatment of type 2 diabetes and a variety of neurological disorders such as Epilepsy and Alzheimer's disease. Many previous studies have shown that the ketogenic diet reduces the number of βcells, with a variety of potential side effects. However, no experiments have been conducted to examine the reasons for this. This study provides ideas for research on ketogenic diets with a focus on energy metabolism. If the problem of inadequate cellular energy supply due to altered energy metabolism pathways can be addressed, many of the side effects will be mitigated. We can expect to utilize the ketogenic diet more effectively and safely for the treatment of type2 diabetes, obesity, and many neurological disorders.

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GLP-1 analogue semaglutide regulates pancreatic beta-cell proliferation via PDX-1 expression control

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Abstract. Semaglutide as an agonist of glucagon-like peptide-1 receptor was shown to potentiate insulin release and suppress food motivation targeting pancreatic islet beta cells and brain region including subfornical organ and hypothalamus, which effectively treats type 2 diabetes and obesity. The investigation of prolonged response with semaglutide on beta cells triggering proliferation and apoptotic resistance is yet to be confirmed. Previous research findings have shown that glucagon-like peptide-1 and liraglutide as agonists for glucagon-like peptide-1 receptors on beta cells result in proliferation through upregulation of PDX-1 transcription factor. Semaglutide is investigated to show whether long-term beta cell survival regulation is achieved through the same downstream signalling pathways. Both in vivo and in vitro methods were designed to show the proliferation effect of the semaglutide in C57BL/6 mice using tissue imaging, cell counting and immunosorbent assay for quantitative analysis of PDX-1 expression. The proliferation effect would broaden the application of semaglutide from insulin augmentation to the sustained benefit of beta cell survival.

Keywords: glucagon-like peptide-1; diabetes; beta-cell; PDX-1

1. Introduction

Semaglutide is a glucagon-like peptide-1 (GLP-1) analogue which acts as an agonist targeting GLP-1 receptors. The GLP-1R were discovered on the pancreatic islet beta cells and within brain in the subfornical organ and hypothalamus [1]. GLP-1, a 30-amino acid peptide produced in the intestinal epithelial endocrine L-cells, is a gut-derived incretin hormone that regulates insulin secretion in a glucose-dependent manner in beta cells. Its action on GLP-1R also results in a prolonged response with activation of genetic transcription involved in proliferation and apoptotic resistance in islet beta cells. Endogenous GLP-1 was shown to induce the proliferation of rat primary islet cells and b-cell lines [2]. And liraglutide, which also acts as an agonist for the GLP-1R, showed increasing proliferation of beta

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cells after alloxan injection during chronic liraglutide treatment [3,4]. This broadens the application of GLP-1 agonists from potentiating insulin secretion to promoting the retention and proliferation of beta cells through the translocation of pancreatic duodenal homeobox 1 (PDX-1) transcription factor to the nucleus [5,6].

In response to the effectiveness of GLP-1 on insulin potentiation and eating behaviour regulation, several GLP-1 receptor agonists are produced to mimic the GLP-1 hormone carrying out these functions. Semaglutide, as one of them, was clinically approved at a dose of 2.4 mg administered as a once-weekly, subcutaneous injection by the UK Medicine and Health Products Regulation Agency and the Food and Drug Administration (FDA) in 2021 [7]. On 28 March 2022, the approval of a 2.0 mg dose of Ozempic for adult type 2 diabetes (T2D) treatment by the FDA was announced by the company Novo Nordisk which developed the semaglutide sold under the name Ozempic® [8].

Being 94% structurally similar to the native GLP-1, semaglutide is designed to be having a longer half-life and higher affinity to the GLP-1 receptor [9]. Its peptide backbone was modified at position eight to substitute alanine with 2-aminoisobutyric acid, with 2-aminoisobutyric acid being resistant to Dipeptidyl Peptidase IV cleavage and a high affinity to GLP-1R [10]. Utilising semaglutide on C75BL/6J mice, a model prone to type 2 diabetes and obesity, effective attenuation on glucose defects and insulin intolerance that were induced by high fat diet was shown with the administration of semaglutide treatment. The reduced blood glucose area under the curve (AUC)and insulin concentration after overnight fasting show a strong effect of semaglutide on attenuating hyperleptinemia and beta-cell glucose sensitivity [11-13]. During clinical trials, the participants with T2D treated with semaglutide significantly improved insulin potentiation thus blood glucose level regulation. It shows profound effects on the insulin concentration, secretion rate and the reduced concentration of plasma glucagon for endpoints from an intravenous glucose tolerance test (IVGTT) [14,15]. Semaglutide has also shown a favourable proinsulin-to-insulin ratio, a lowered proinsulin concentration, and a significant reduction of HbA1c and insulin resistance using homeostatic model assessment (HOMA-insulin resistance) in T2D patients which suggests improved efficiency of beta cell functioning and augmented production of insulin [14,16].

While semaglutide has a profound effect on beta cell activity, like insulin potentiation, GLP-1 and liraglutide have been shown with additional effects resulting in the transcription of genes involved in proliferation. PDX-1, which acts as an insulin transcription factor, is upregulated for the prolonged response on the GLP-1R signalling pathway regulating differentiation and proliferation of beta cells [17,18]. GLP-1 was shown to participate in regulating PDX-1 by increasing its total protein levels and translocating to the nucleus both in vitro and in vivo. Beta-cell regeneration measuring isolated beta cells and beta-cell buds has been significantly stimulated with GLP-1 in vivo [18,19]. In vitro, GLP-1 was also shown to induce a re-distribution of cell cycle to decrease the proliferation rate before the cell differentiation were promoted that would lead towards an endocrine-like phenotype. Thus, islet cells proliferation is promoted which result in an increase of pancreatic beta cell mass [20]. Liraglutide, as a GLP-1R agonist structurally similar to the semaglutide, had also shown increased beta cell survival with the measurement of cell viability in vitro [21].

Structural similarity between semaglutide and GLP-1 is supported by an overall identical crystallisation of the unacylated semaglutide peptide backbone in complex with the GLP-1R to that of native GLP-1 [22]. This discovery leads to functional similarities in affecting GLP-1R downstream signalling pathways with the rapid potentiation of insulin secretion from beta cells. This paper aims to investigate if semaglutide action on the prolonged response of beta cells follows the same signalling pathway, resulting in beta cell proliferation through the translocation of pancreatic duodenal homeobox 1 (PDX-1) transcription factor to the nucleus. Both in vivo and in vitro experiments were designed to investigate PDX-1 expression and beta cell proliferation.

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2. Methods

2.1. Animals, diets, and treatment

Six-week-old male C57BL/6N mice were purchased from The Charles River Laboratories, Chesterford Research Park, Saffron Walden, UK. The group were supported in ventilated cages under regulated temperature and humidity conditions (NexGen system, Allentown Inc., PA, USA) at 20 ± 2 C°, 12 h/12 h dark/light cycle, and free access to food and water. The C57BL6 mice were divided into 5 groups, with no less than 20 mice in each group. They are Healthy C57BL6 test mice (without any drug treatment), PDX-1 gene normal diabetic C57BL6 test mice (Drug-treated), PDX-1 gene-deficient diabetic C57BL6 test mice (Drug-treated), PDX-1 gene normal diabetic C57BL6 test mice were added with inhibitors (Drug-treated), PDX-1 gene normal diabetic C57BL6 test mice (without drug treatment). The healthy C57BL6 test mice (without any drug treatment) were acclimated for 1 week, followed by a low-fat diet (LFD, 10kcal% from fat, D12450J, Research Diets, New Brunswick, NJ, USA). Besides, the PDX-1 gene normal diabetic C57BL6 test mice (Drug-treated), PDX-1 gene-deficient diabetic C57BL6 test mice (Drug-treated), PDX-1 gene normal diabetic C57BL6 test mice were supplied with inhibitors (Drug-treated), and PDX-1 gene normal diabetic C57BL6 test mice (without drug treatment) were acclimated for 1 week, followed by a high-fat diet (HFD, D12492 with 60kcal% from fat, Research Diets) feeding for 10 weeks.

Semaglutide was injected into three groups. These three groups are PDX-1 gene normal diabetic C57BL6 test mice (Drug-treated), PDX-1 gene-deficient diabetic C57BL6 test mice (Drug-treated), PDX-1 gene normal diabetic C57BL6 test mice were supplied with inhibitors (Drug-treated). Semaglutide (diluted in sterile 0.9% NaCl) was subcutaneously administered at 40 mg/kg once every three days. The duration of treatment was 12 weeks.

2.2. Semaglutide effect under the influence of T2D, rapamycin and PDX-1 deficiency

In the first set of experiments, to verify whether semaglutide can treat diabetic mice, two groups of C57BL6 mice (Drug-treated PDX-1 gene normal diabetic C57BL6 test mice and PDX-1 gene normal diabetic C57BL6 test mice without drug treatment) were used to compare the body weight, blood glucose level, insulin and C-peptide of the two groups of mice. Drug-treated PDX-1 gene normal diabetic C57BL6 test mice were subcutaneously treated with semaglutide using the illustrated method. The duration of treatment was 12 weeks. PDX-1 gene normal diabetic C57BL6 test mice were reared normally for 12 weeks without drug treatment. AST, 24h meal stimulation test and GGIT were used to measure glucose, insulin and C-peptide every three days. Then record the data. After 12 weeks, compare the data of the two groups.

In the second set of experiments, the control variable was using inhibitors. Drug-treated PDX-1 gene normal diabetic C57BL6 test mice were injected intravenously with rapamycin targeting the mTOR pathway in beta cell proliferation signalling [23,24]. Treatment was given every 7 days for 3 weeks with 0.5 mg/kg/day. AST, 24h meal stimulation test and GGIT were used to measure glucose, insulin and C-peptide every three days. Then record the data. After 3 weeks, compare this data with the Drug-treated PDX-1 gene normal diabetic C57BL6 test mice, which we had measured in the first set of experiments. In the third set of experiments, the control variable was whether the islet PDX-1 gene was normal. Twenty islet PDX-1 gene-deficient C57BL6 mice were purchased, which were the Drug-treated PDX-1 gene-deficient diabetic C57BL6 test mice mentioned above. Semaglutide with 40 mg/kg dosage was administered subcutaneously every three days, and the data were recorded (blood glucose levels, insulin and C-peptide in the Drug-treated PDX-1 gene-deficient diabetic C57BL6 test mice). The duration of treatment was 12 weeks. Compare this data with the data of Drug-treated PDX-1 gene normal diabetic C57BL6 test mice, which we had measured in the first set of experiments.

2.3. Arginine stimulation test (AST)

For the arginine stimulation test, the following protocol is administrated. An intravenous glucose injection with 150 mg/kg is applied to reach hyperglycaemia with a blood concentration of 16 mmol/l,

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followed by a 5g arginine intravenous injection after 2 hours. The blood samples were drawn frequently within 35 minutes of arginine administration to analyse insulin, C-peptide, glucose and glucagon concentration [25].

2.4. 24h meal stimulation test

Three standard meals were served at 0h, 5h and 10h, with a high-protein meal at the 10h. The test is temporally separated from the arginine stimulation test. The glucose metabolism at fasting and postprandial status were evaluated over this 24h period achieved by the extraction of blood samples which also measured insulin, C-peptide and glucagon concentrations [15].

2.5. Graded glucose infusion test (GGIT)

A graded glucose infusion test was performed to assess beta cell responsiveness. Carried out in both groups with T2D and healthy mice, the intravenous glucose infusion was determined to achieve sequential plasma glucose concentration in a 3-hour period. The concentration is separated by 3 mmol/l reaching 17 from 5 mmol/l, with a 45 minutes interval between each target. Blood samples were constantly drawn to monitor the concentration of the compound illustrated above.

2.6. Evaluation of endotrophin

The present study evaluated its effects on phase I and II insulin secretion, beta cell function and indices of glycaemic control. Endostatin is a peptide derived from collagen VI associated with obesity-induced insulin resistance. It is produced in adipose tissue and is thought to contribute to insulin resistance. Thus, the beneficial effects of semaglutide on glucose metabolism and weight loss may also impact endotrophin levels. We will measure endotropins in serum collected from diabetic ZDF rats by using rodent PRO-C6 ELISA. PRO-C6 ELISA is a specific laboratory technique used to measure the concentration of PRO-C6 (collagen type VI pro-peptide) in biological samples. It utilises antibody and enzyme reactions to detect and quantify specific proteins or molecules in biological fluids. Thus, ELISA is used to detect specific molecules such as hormones, proteins, or antibodies related to beta cell or insulin secretion, and the marker quantifies endotropins in the serum of PC patients. ELISA was performed for the determination of endotrophin levels.

The abundance of endotrophin in pancreatic islets was determined by enzyme-linked immunosorbent assay (ELISA Kit) to detect the PRO-C6 in PC mouse serum. The eyes of the mice were poked using a syringe. The blood was removed, put in, and centrifuged for 10 minutes to separate the blood from the serum. The serum was taken as the required sample solution in the EISLA kit. All the reagents and samples were placed at room temperature, and protein standards and extracted mouse serum were added to the respective wells. Incubate at room temperature. Pour out the solution, wash with washing solution and blot with a clean paper towel. Add 100 ul 1x Biotinylated Detection Antibody to each well. Shake gently at room temperature. Add the prepared HRP-Streptavidin Concentrate. Incubate at room temperature. Add TMB and measure wavelength every minute. Calculating the results of each set of experiments can be analysed by plotting using SIgmaPlot software.

2.7. Islet isolation

Perform collagenase perfusion to expand the pancreas. Inject type V collagenase (Sigma), fetal bovine serum, and Hank's balanced salt solution into the pancreas to cause it to swell. The pancreas tissue was then taken out and placed in a test tube, then incubated in a water bath at 37.5°C for 15 minutes for collagenase digestion and vigorously shaken by hand for 15 seconds. Filter through a nylon cell strainer. The islets were returned by hand, fix them in formalin, embedded in paraffin, and prepare slides. Evaluate the mouse pancreatic tissue with HE staining, fluorescence microscopy for insulin expression, KI-67 (proliferation level), TUNEL staining, and GLP-1R antibody staining.

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2.8. Detection of PDX-1 expression

PDX-1 is essential for the growth of endocrine and exocrine compartments and controls the transcription of glucose-regulated insulin genes, which are major metabolic regulators of β-cell function. In vivo, gene ablation experiments with PDX-1 and BETA2 in mice have identified their critical roles in pancreatic development [26]. The PDX-1 can be examined in insulin by using the Rat PDX1 (Sandwich ELISA) ELISA Kit - LS-F20295 for the Quantitative detection of Rat PDX1 in samples of Plasma and Serum. All the samples for the experiment were taken from islets in the pancreas of mice, which were chopped up in a homogeniser using and homogenised into a slurry before being pipetted into each well. Add 100μl of homogenised islet tissue and incubate for 2 hours at 37°C. Add 100 μl of Detection Reagent A working solution to each well and stir to ensure that the two are well mixed. Aspirate the liquid from each well and wash with buffer using an automatic washer. Blot up the remaining wash buffer with clean absorbent paper. Incubate each well with Detection Reagent B Working Solution. Rinse again with Wash Buffer. Add TMB substrate solution to each well and monitor periodically to document optimal colour development. Finally, add the termination solution to each well. The blue colour will immediately change to yellow. The value is determined using the reader.

2.9. Cell culture

A certain number of beta cells are divided into three groups: 1. beta cells with semaglutide (10-100ug/mL) 2. Beta cells with PDX-1 mutation and semaglutide (10-100ug/mL) 3. beta cells without semaglutide (control group). The standard curve is first established. In the second step, the number of beta cells in the pre-made cell suspension was counted with a cell counting board, and then the cells were inoculated. The medium is then diluted proportionally to a cell concentration gradient. After inoculation, the cells were cultured for 2-4 hours, 10 μL CCK-8 reagent was placed into the medium every 100 μL, and the OD value within a certain period of time was determined, and the standard curve was drawn (cell number was horizontal coordinate, OD value was vertical coordinate). According to this standard curve, the number of cells in the sample can be determined. CCK-8 (Cell Counting Kit-8) is a colourimetric detection Kit based on WST-8, widely used in cell proliferation and cytotoxicity studies. CCK-8 solution can be added directly to the cell sample without additional ingredients. The kit was used to evaluate how beta cell proliferates with the effect of semaglutide.

2.10. Cell activity detection

The cell suspension (100 μL/ well) with the above three groups of beta cells pre-cultured in the incubator for 24 hours were inoculated into the 96-well plate. Then 10 μL CCK-8 solution was added to each well. After completion, the culture plate is placed in the incubator and cultured for 1-4 hours. The absorbance at 450 nm was then measured with enzyme labelling.

2.11. Cell proliferation - toxicity detection

Different concentrations of the drug to be tested were added to the culture plate (the culture plate should be incubated in the incubator), and 10 μL CCK-8 solution was added to each well. The culture plate was placed in the incubator (culture for 1-4 hours). At the same time, the absorbance at 450 nm was measured with enzyme labelling. After collecting all the required data, the survival and inhibition rates of beta cells were calculated using the following formulas: (As-Ab) /(Ac-Ab) ×100% and (Ac-As)/(Ac-Ab)]. ×100%. (As: Experimental hole absorbance. Ac: Control hole absorbance. Ab: Blank hole absorbance)

2.12. Measurement of cellular insulin secretion

The INS ELISA kit is a solid-phase sandwich enzyme-linked immunosorbent assay (ELISA). Samples containing beta cells with known secreted insulin concentration and samples containing beta cells with unknown secreted insulin concentration are added to the microporous enzyme label plate for detection. First, the substance to be tested is incubated with biotin-labelled antibodies. After washing, avidin-labeled HRP was added. After incubation washing, the unbound enzyme binding is removed, and the enzyme binding is acted on to produce colour.

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3. Result

3.1. Predicted analysis of the in vivo treatment stage

Semaglutide has both transient and prolonged effects on beta cells, so to explore the effect of semaglutide on beta cell proliferation, we decided to use the control variable method to conduct experiments.

Rapamycin acts as an immunosuppressant, has been shown with an anti-proliferative ability, and was chosen to inhibit beta-cell proliferation [24,27]. It inhibits the mammalian target of rapamycin (mTOR) kinase, which mainly controls cell growth and proliferation and the sensing of nutrients as well as hormonal signals, including insulin released from pancreatic beta cells [28]. Since beta cells in the control group were inhibited in proliferation, the effect of semaglutide on beta cell proliferation was suppressed, and thus the effects on beta cell proliferation were controlled.

The effect of semaglutide on beta cell proliferation could be observed by comparing the experimental group with the control group. However, in order to further investigate the effect of semaglutide on beta-cell proliferation, three comparative experiments were designed. The control variables were semaglutide use, rapamycin cell proliferation inhibitor use and PDX-1 gene.

3.2. Arginine stimulation test (AST) 24h meal stimulation test and Graded glucose infusion

Three test methods were used to measure the blood glucose level, insulin and C-peptide of C57BL6 test mice. For the 24 h meal stimulation test, the level of glucose and glucose that were indicated by AUC responding to semaglutide during fasting, postprandial state and overall state were predicted to show a significant reduction. (AUC[0-24H].) The arginine stimulate test was predicted to show that the semaglutide treatment would result in a increased maximal insulin capacity. During the graded glucose infusion, significant increases of insulin secretion rate would be recorded with semaglutide treatment, reaching level of that in control group.

3.3. Predicted analysis of in vivo end stage

The semaglutide hypothesis, related to islet regulatory processes, suggests that progressive hyperglycemia caused by type 2 diabetes results in defective insulin gene expression and suboptimal insulin levels. The primary basis for this hypothesis is the studies carried out in b-cell lines, and our study examined for the first time whether treatment of C57BL6 animals with semaglutide also proliferates and reduces apoptosis of beta cells and prevents the loss of β cell mass. Over the course of the study, nutrient levels were significantly elevated in rats treated with the drug, reflecting the progression of the disease. Treatment with semaglutide all attenuated this increase, and at the end of the study, endothelial nutrient levels were significantly lower than the drug, respectively. Serum endotrophin levels in drug-treated rats increased over time, consistent with endotrophins predicting disease progression in diabetic patients. semaglutide reduced endotrophin levels, respectively.

3.4. Predicted analysis of in vitro stage

Based on the results of the experiment, we could conclude whether semaglutide can promote proliferation and insulin secretion in beta cells.

The first experiment shows that the beta cells with semaglutide increased significantly compared to the other two groups of cells. The specific proliferation of beta cells varies at different concentrations. The lower the concentration, the less cell proliferation; The higher the concentration, the more cells multiply.

In the second experiment, we got the result that beta cells with semaglutide secreted more insulin than the other two groups of beta cells. The higher the concentration of semaglutide the beta cell contains, the more insulin it secretes. Conversely, the lower the concentration, the less is secreted. Although different concentrations of insulin secreted will be different, overall, more than the other two groups.

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4. Conclusion

To present a comprehensive investigation, the experiments were designed to assess both in vivo and in vitro effects of semaglutide on beta cell proliferation under different conditions. With previous supporting research, the experiment could lead to a promising result, indicating beta cell proliferation by semaglutide due to its stimulation of the GLP-1R. The PDX-1 activity was predicted to be an essential step for triggering beta cell proliferation. Overall, the experiment setup could be used to measure beta cell activity based on insulin, C-peptide and glucagon concentration. The glucose concentration, both in fasting and postprandial status, could also be assessed. ELISA technique and staining method allow the beta cell proliferation in the tissue to be measured. While in vitro stage, cell counting and imaging could be used for cell proliferation measurement. The essence of PDX-1 could be compared by assessing PDX-1 deficiency on beta cell activities. ELISA is also used for the detection of PDX-1 activities. Although the relevant research supports the prediction, the result still needs to be supported by the administration of the methods.

In conclusion, beta-cell proliferation is significantly augmented with the semaglutide application both in vivo and in vitro when PDX-1 activities are sustained. The increased activity of beta cells is consistent with previous studies [5]. The insulin responses in both the first- and second-phase significantly increased during the arginine stimulation test and graded glucose infusion test with the diabetic group treated with the semaglutide vs. without. The fasting and postprandial glucose and glucagon levels were reduced in the 24h meal stimulation test in the diabetic group with semaglutide injection vs. without. The pancreatic tissue showed an enlargement and an increased proliferation level and insulin expression after islet isolation. In vitro, a significant increase in the survival and inhibition rate is observed in the cell plates with semaglutide and wildtype PDX-1 compared to the group without semaglutide and wildtype PDX-1, and the group with semaglutide but mutated PDX-1. However, the enlargement and increased proliferation level were predicted only to be observed in the diabetic group. The two groups with the injection of rapamycin or PDX-1 deficiency showed no enlargement and significant differences in proliferation level with semaglutide injection vs. without, supporting the hypothesis that PDX-1 expression is essential for semaglutide to augment beta cell proliferation. If the enlargement and increased proliferation level were still observed in these two groups, alternative potential signalling pathways should be tested [21, 23]. Semaglutide was shown to be an effective drug in treating type 2 diabetes, with the additional role of augmenting beta cell proliferation apart from beta cell activity and responsiveness. The result indicated the potential long-term benefit of semaglutide treatment for diabetes.

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Gender difference in coronary artery disease

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Abstract. Background: Coronary artery disease (CAD) continues to exert a substantial impact on global health, being the main factor causing morbidity and mortality in North America and Europe. Men and women have different incidences of CAD and risk factors, which has been widely reported across populations. In light of a notable scarcity of studies examining the variation in CAD risk among different subgroups. We investigated how cholesterol, glucose, blood pressure, and age affect CAD risk in different sex and smoking groups. **Method**: Framingham Heart Study, a longest-running cardiovascular epidemiological investigation used furnishing valuable insights into CAD risk. Investigating the relationship between these characteristics and the occurrence of CHD involved using logistic regression. **Result**: For blood pressure variable among smoking group, there were significant increase in male group (β=0.0255, P<0.001) compared with non-smoking group (β=0.0164 in male, β=0.0162 among female group, P<0.001) and reduction among female group (β=0.0089, P<0.001). Within the female group, the age variable exhibited a notable reduction in smoking group (β=0.0460, P=0.0020) compared with the non-smoking group (β=0.0829, P<0.001). After adding the interactions between smoking and blood pressure, smoking and age. In the male group, both smoking and the interaction become insignificant, whereas in the female group the interaction is significant. **Conclusion**: Our study suggested that high level of glucose, total cholesterol and blood pressure increased the risk of CAD. By dividing into subgroup, we discovered that the combined presence of smoking and increased blood pressure, and of smoking and age could potentially exert a more adverse effect on pressure wave reflection in women compared to men.

Keywords: cardiovascular diseases, coronary disease, gender, smoking

1. Introduction

Coronary artery disease (CAD) continues to exert a substantial impact on global health, being the main factor causing adverse health outcomes and fatalities in Europe. Its complex nature, characterized by risk factors like hypertension, smoking, cholesterol profile (total cholesterol, LDL(low-density lipoprotein) cholesterol, HDL(high-density lipoprotein) cholesterol), and glucose level, underscores the challenge of detection [1]. These risk factors have significantly contributed to our understanding of CAD vulnerability. Population studies have facilitated predictive models that forecast CAD occurrence by integrating more factors.

Differences in CAD incidence and risk factors between genders have been extensively documented among various populations [2, 3]. Recent findings have highlighted the identification of novel cardiovascular disease risk factors specific to women. However, our comprehension of sex-specific risks remains limited, and the strategies for averting and handling stroke and cardiovascular risk elements are

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fundamentally indistinguishable for both sexes. This is the case despite an increasing body of evidence showcasing noteworthy sex-based variations in the occurrence of conventional CAD risk factors, as well as in how these factors influence CAD outcomes.

Another aspect related to gender is that the smoking habits of males and females are different. Across the general population, the frequency of smoking is greater among males compared to females, despite the fact that the recent reduction in the prevalence of smoking has been more conspicuous among men in comparison to women [4]. Nonetheless, smoking cigarettes undoubtedly stands as the most significant contributor to abrupt cardiac death among younger women, whose smoking prevalence is currently rising, as smoking is responsible for more mortality from CAD and stroke than any other medical conditions [5, 6].

In light of a notable scarcity of studies examining the variation in CAD risk among different subgroups, which potentially leading to missed opportunities for integrating subgroup-specific considerations into the design of CAD prevention strategies, we investigated how cholesterol, glucose, blood pressure, and age affect CAD risk in different sex and smoking groups.

2. Method

2.1. Data source

Our analysis drew on research information sourced from the Framingham Heart Study, a longest-running heart and circulatory epidemiological investigation initiated in 1948. National Institutes of Health and overseen by Boston University supported this study which encompasses three generations of well-characterized individuals of White ethnicity and two additional cohorts representing diverse racial and ethnic backgrounds. These cohorts are richly characterized, with extensive longitudinal monitoring, furnishing valuable insights into cardiovascular and noncardiovascular aspects of human physiology across the lifespan. Furthermore, they aid in the identification of significant factors contributing to the risk of cardiovascular disease.

The Institutional Review Board from the Boston Medical Center gave its approval to the study protocol after receiving the written informed consent of all participants.

2.2. Characteristics

There is a total of 4238 residents in this ongoing study. The extracted variables encompass both general information and personal history. In terms of general information, these variables include gender, age, and body mass index. Personal history, on the other hand, covers factors such as smoking, alcohol consumption, as well as other lifestyle and health-related behaviors.

Participants in the heart research had a variety of evaluations during each visit, including physical exams, anthropometry measurements, measurements of blood pressure, and phlebotomy to evaluate factors associated with vascular risk. Participants were asked to place their left arm against the sphygmomanometer, which had a mercury column, and a cuff that was the right size. The examination blood pressure was determined using the average of two measures taken by doctors. Standardized enzymatic techniques were used to measure serum total cholesterol levels. Self-reporting was used to establish smoking status. Higher fasting glucose readings, specifically 126 mg/dL for the offspring cohort and 140 mg/dL for the original cohort, were recognized as signals of diabetes. Additionally, the administration of insulin or oral hypoglycemic drugs to regulate blood sugar levels was also considered indicative of the condition. During the heart investigation, the examining physician relied on self-report to identify whether antihypertensive drug use was present.

The occurrence of CAD episodes and mortality were continuously tracked for all research participants. As indicated by the findings of the Framingham Heart Study, CAD encompasses a range of cerebrovascular incidents including ischemic stroke, hemorrhagic stroke, and transient ischemic attack. Additionally, it involves various events linked to coronary heart disease (CHD) such as fatal coronary outcomes, myocardial infarction, instances of coronary insufficiency, and episodes of angina. Additionally, heart failure and peripheral artery disease (intermittent claudication) were included. A

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thorough strategy, including medical histories, clinic-based physical examinations, hospitalization records, and correspondence with personal physicians, was used to collect information concerning CAD episodes during follow-up. A team of three skilled investigators carefully evaluated any possible new incidents after carefully going through all pertinent medical information. A neurologist from the heart research team analyzed the majority of participants who were thought to be stroke suspects, and cases of cerebrovascular events were reviewed by a separate review committee involving a neurologist.

2.3. Statistical analysis

Logistic regression was applied to examine the association between cholesterol, glucose, blood pressure, age and occurrence of CHD. All models were first adjusted for known determinants of CHD risk as shown in Table 1. For the final model selection, we used a subset of different gender and smoking situation subjects data from FHS to derive the logistic model with five-fold cross-validation, recall value and roc_auc score. We added interactions term between blood pressure, hypertension, glucose and diabetes and evaluated whether they improved the model performance. R software, version 4.3.0 (R Foundation for Statistical Computing, Vienna, Austria), was used for all analyses.

3. Result

A total of 4238 participants were subject to continuous monitoring for the occurrence of CAD events and mortality. Among smokers, with a sensitivity of 0.75, a specificity of 0.64 and an ROC-AUC of 0.74, the model demonstrated its performance. In the nonsmoking group, the model attained a sensitivity of 0.7, specificity of 0.73, and an ROC-AUC of 0.79.

Table 1. Clinical characteristics in study subjects in male and female

	Male(n=1819)	Female(n=2419)
Current smoker(n)		
No	713	1431
Yes	1106	988
Prevalent stroke(n)		
No	1809	2404
Yes	10	15
Prevalent hypertension(n)		
No	1209	1673
Yes	570	746
Diabetes(n)		
No	1767	2362
Yes	52	57
	Male(n=1819)	Female(n=2419)
Age	49±9	50±9
Total cholesterol	233±42	239±46
Systolic blood pressure	131±19	133±24
BMI	26.2±3.4	25.5±4.5
Glucose	82±24	82±22

Table 1 shows the distribution of characteristics of the study population. More than half of the population were women. Non-smoking rates among women (59.16%) are significantly higher than among men (39.20%).

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Table 2. Results of logistic regression analysis to assess the relationships of risk factors and occurrence of CAD in four groups classified by gender and smoking

	Male			Female		
	β	95% CI	P	β	95% CI	P
Smoking						
Total cholesterol	0.0025	-0.001,0.0060	0.1636	0.0016	-0.0032,0.0063	0.5119
Glucose	0.0078	0.0012,0.0146	0.0215	0.0063	-0.0014,0.0138	0.0948
Blood pressure	0.0255	0.0176,0.0336	<0.001	0.0089	-0.0007,0.0182	0.0652
Age	0.0595	0.0400,0.0792	<0.001	0.0460	0.0168,0.0753	0.0020
nonsmoking						
Total cholesterol	0.0043	-0.001,0.0096	0.1084	0.0002	-0.0035,0.0038	0.9218
Glucose	0.0093	0.0031,0.0162	0.0048	0.0068	0.0011,0.0125	0.0188
Blood pressure	0.0164	0.0068,0.0261	<0.001	0.0162	0.0098,0.0227	<0.001
Age	0.0617	0.0365,0.0877	<0.001	0.0829	0.0594,0.1071	<0.001

Table 2 presents the associations between total cholesterol, glucose, blood pressure, age and occurrence in different subjects. For blood pressure variable among smoking group, there were significant increase in male group (β=0.0255, P<0.001) compared with non-smoking group (β=0.0164 in male group, β=0.0162 among female group, P<0.001<0.001) and reduction among female group (β=0.0089, P<0.001). In the female group, the age variable exhibited a considerable reduction in smoking group (β=0.0460, P=0.0020) compared with the non-smoking group (β=0.0829, P<0.001).

Table 3. Results of logistic regression model to assess the effect of interaction between smoking and blood pressure, as well as smoking and age on risk of CAD in male and female

	Male		Female	
	β	P	β	P
Total cholesterol	0.0031	0.0357	0.0006	0.6845
Current smoker	0.5301	<0.001	0.2277	0.1029
Glucose	0.0085	<0.001	0.0065	0.0048
Blood pressure	0.0216	<0.001	0.0130	0.0652
Age	0.0612	<0.001	0.0666	0.0020
Interaction with blood pressure				
interaction	0.0087	0.1647	0.0119	0.0246
Total cholesterol	0.0030	0.0430	0.0006	0.6602
Current smoker	0.6672	0.4437	1.9034	0.0118
glucose	0.0085	<0.001	0.0065	0.0042
Blood pressure	0.0163	<0.001	0.0168	<0.001
Age	0.0607	<0.001	0.0684	<0.001
Interaction with age				
interaction	0.0016	0.9207	-0.0443	0.00877
Total cholesterol	0.0031	0.0339	0.0007	0.6398
Current smoker	0.4366	0.6164	2.5655	0.00467
Glucose	0.0085	<0.001	0.0065	0.0046
Blood pressure	0.0218	<0.001	0.0138	<0.001
Age	0.0598	<0.001	0.085	<0.001

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Table 3 shows how these four variables related to the occurrence of CAD after adding the interactions between smoking and blood pressure, smoking and age. In the male group, both smoking and the interaction become insignificant, whereas in the female group the interaction is significant.

4. Discussion

Our study suggested that high level of glucose, total cholesterol and blood pressure increased the risk of CAD. Smoking, blood pressure and age were identified as important factors contributing to risk of CAD. A potential disparity based on gender might exist in how these factors influence the level of risk. These factors might collaboratively amplify risk increase in females. In contrast, among males, these factors might independently raise incidence without interacting. Consequently, the combined presence of smoking and elevated blood pressure, and of smoking and age could potentially exert a more adverse effect on pressure wave reflection in women compared to men.

Previous research has demonstrated that smoking has a substantially more negative relative effect on CAD in women. Although men typically had a higher incidence of myocardial infarction, women who smoke heavily exhibited a greater frequency than men who had never engaged in smoking [7]. The mechanism of smoking is probably involving alterations to platelet function and clotting factors, as well as the acceleration of atherosclerosis and stimulation of the sympathetic nervous system. But the effect of smoking in females can be different. It is stated that women exhibited a higher propensity for plaque erosion than males were from the Burke study, and they also experienced less luminal narrowing and plaque calcification [8]. In our study, synergistic effect of smoking and blood pressure, smoking and age in women also suggest a different mechanism of smoking on female.

An increasing body of research indicates that women are less advantaged than men in numerous areas. According to epidemiological data, no variation is evident in the rate of CAD progression upon reaching menopause [9, 10]. There is no indication of a rise in the mortality rate due to CAD among women aged 45 to 55 years, nor is there evidence of a convergence of rates between women and men. Consequently, women might possess an unjustified sense of optimism concerning the level of protection against CAD attributed to estrogen, and this can cause people to overestimate their risk for cardiovascular disease. Although anecdotal evidence suggests that despite the regular emphasis on adopting a healthy lifestyle in women's periodicals and other platforms, many women may retain uncertainties about various factors related to the development of CAD [11]. further studies will be required for this potential lack of advocacy among women and the underlying mechanism.

While the current study uses reliable, standardized CAD incidence criteria and a sizable community-based sample under ongoing surveillance, it's important to acknowledge various limitations inherent to this study. Although medications for hypertension affect CAD risk, the present study did not examine the influence of these medications. A further study to examine the ethnical impact on the CAD risk factors is also proposed. Considering the predominantly white composition of the Framingham sample, it becomes essential to assess the applicability of the CVD risk function in different sample populations. It's worth noting that other risk functions from Framingham have demonstrated their applicability in diverse contexts [12].

5. Conclusion

In conclusion, our study suggested that high level of glucose, total cholesterol and blood pressure increased the risk of CAD. By dividing into subgroup, our findings revealed that combined presence of smoking and increased blood pressure, as well as smoking and age could potentially exert a more adverse effect on pressure wave reflection in women in contrast to men. It suggests a different mechanism of increase in risk. Subsequent investigations are necessary to explore the underlying mechanisms of these findings.

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EVs as biomarkers of cardiovascular disease

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Abstract. Exosomes are an emerging research direction in biology, and their current definition is: "Exosomes are small vesicles (30-150nm) containing complex RNA and proteins, and nowadays, they are specifically dish-like vesicles with diameters of 40-100nm." Many cells can produce exosome under physiological or physiological conditions. The mechanism is formed by the deposition of polyvesicles after binding to the extracellular membrane. Therefore, it can be seen from this definition that exosomes are a general term for a large class of vesicle-wrapped biological contents, which are physiological substances used by organisms to maintain normal physiological activities. Therefore, exosomes have a very rich classification, subclasses, and subsets.

Keywords: Exosomes, disease, cell.

1. Introduction

Extracellular vesicles are nanometer-sized, highly heterogeneous spherical solid bilayer proteolipid vesicles that are released into the extracellular space by virtually all different kinds of cells, including all different kinds of cells [1]. Extracellular bodies are a kind of substances with physiological functions, such as proteins, lipids, nucleic acids and so on. they are excreted in large quantities through their own physiological activities and enter into different organisms [2]. Extracellular vesicles consist of heterogeneous vesicles with different biological origins, compositions, and biological properties, including exosomes, microvesicles, and apoptotic cells [3,4]. Cells rely on extracellular vesicles to interact with the extracellular environment, and secretion of extracellular vesicles plays an important role in intercellular communication [5]. Extracellular bodies play an important role in regulating immune response (such as inherent and adaptive), angiogenesis, blood coagulation and miRNAs transmission [2,4]. In addition, EV is also involved in the pathogenesis of many diseases, such as tumorigenesis, growth and progression, and tumor invasion and metastasis is the key link of tumor treatment [1].

The contents of extracellular vesicles are expected to discover liquid biomarkers for prostate, kidney, and bladder cancers [6]. In addition, EV is also a medium that can help maintain the stability of the intra-articular environment [7]. Among them, EV microRNAs may be important in maintaining cardiovascular homeostasis and can coordinate the maintenance of cardiovascular homeostasis [8]. Cardiovascular diseases, including coronary heart disease, stroke, hypertension and peripheral artery disease, are not only the major economic burden on human health and society, but also the largest cause of cardiovascular disease morbidity and mortality worldwide [9]. EVS has a certain guiding significance for the occurrence and development of heart disease, and for clinical diagnosis, treatment and monitoring of heart disease [10](see Figure 1).

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Figure 1. The Cardiovascular Health and Disease.

2. EV mirna as a CVD biomarker

MiRNAs, which is specifically expressed in intestinal epithelial cells, can be used as a prognostic and diagnostic marker in many diseases [11]. In 2007, Jan L€otvall and other studies showed that mutant RNA such as miRNAs and mRNAs can be mediated by the host in different hosts. In 2010, it was reported that transitive miRNAs could function in receiving cells [1]. Ev-mirna may be a predictor or indicator of early CVD detection. Deddens et al. noted that EV released into the circulation from a damaged heart contained mirna and proposed that EV mirna could be used as an early biomarker of cardiac injury. EV miRNAs can be used as an early diagnostic index of acute heart failure. Matsumoto et al. identified three p53-responsive mirna, which predicted the development of HF one year after acute myocardial infarction, by screening extensively for mirna in serum EVs. In addition, non-coding rna (ncRNA), especially circular rna (circRNA), which is carried in large quantities by EVs, has been recognized as a potential biomarker [11].

EV mirna has also been associated with cardiovascular risk factors (i.e., exposure to particulate matter, diabetes, dyslipidemia, obesity, MetS). Alterations in EV miRNA from particulate matter exposure to air pollution have been associated with elevated blood pressure and coagulation status, and have been shown to increase the risk of cardiovascular disease, as well as cardiovascular morbidity and mortality. It has been demonstrated that the transfer of miR-320 from cardiomyocytes to endothelial cells via EV has an inhibitory effect on myocardial angiogenesis. Down-regulated levels of both miRNAs in plasma EVs of patients with familial hypercholesterolemia (FH) and elevated levels of miR-130a resulted in decreased coronary atherosclerosis in patients with CAD, suggesting its use as a potential biomarker for CAD. Excessive obesity is another crucial risk factor for CVD, and EV mirna has been proposed as an early biomarker for predicting CVD events in obese patients. After coronary artery bypass grafting (CABG), EVs expresses a large amount of EVs miRNAs in circulation, which is closely related to coronary artery intima injury and can be used as a marker of ischemia-reperfusion injury in patients with coronary artery disease [12].

2.1. EV proteins as CVD prognostic biomarkers

The expression of virus (EV) is affected by various physiological and pathological factors. The protein profile may have changed in the disease's early stages, making the content a potential early biomarker. EV proteins are considered as prognostic biomarkers for cardiovascular events. It has been shown that increased circulating CD31/membrane link protein 5-positive EV has an independent predictive effect

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on cardiovascular risk in patients with stable coronary atherosclerosis and that its release of high levels of EVs correlates with higher mortality rates and higher need for hemodialysis due to CVD [13].

2.2. EVs lipids as CVD prognostic biomarkers

The amount of lipids in EVs may be associated with atherosclerosis once these lipid accumulations are associated with toll-like receptor-mediated macrophage foam cell formation and apoptosis, leading to atherosclerosis. EV is rich in arachidonic acid, which can be secreted by activated platelets and stimulate the production of thrombin, thus accelerating the formation of thrombus [13].

2.3. EV counting as a biomarker of CVD

EV quantification has potential applications as a biomarker for diagnosis and therapeutic monitoring. About 70% of EV in normal people's blood is secreted by platelets. Therefore, the EV in circulation is dominated by activated platelets, namely p-EV [11]. It has been shown that platelet-derived EV (p-EV) counts are associated with CVD. Under conditions of platelet-derived EV (p-EV) activation, such as myocardial infarction, there is an increased release of circulating EV in the plasma. In patients with ACS, the EV value increases, resulting in an ischemic pressure response. The number of EV also increased significantly in STEMI patients. In addition, EV increases rapidly in circulation after pathological stimulation. Deddens et al. found that the results show that this method can detect plasma vehicles quickly. Previous work found that EV increased within 1 hour after MI. The number of EVs may be an essential indicator for differentiating the severity of heart failure. The increase in the number of EVs derived from circulating endothelial cells is related to cardiac insufficiency, cardiac insufficiency, and so on. The concentration of EVS in patients with cardiac insufficiency was also higher than that in healthy people [13].

3. Conclusion

The cardioprotective effect of EV, especially the expression of miRNAs in cardiomyocytes, makes it a potential target for cardiomyocytes. EV proteins and lipids have been recognized as prognostic biomarkers of cardiovascular events. Quantification of EVs has potential applications as a biomarker for diagnosis and therapeutic monitoring. Exogenous or exogenous EV in blood and blood of EV has been proved to improve cardiac function and improve cardiac function, but its role in clinical practice remains to be further explored [11].

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Potential regulatory mechanisms for NKCC1 and KCC2 that induce temporal lobe epilepsy

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Abstract. The specific cause for mesial temporal lobe epilepsy at a molecular level has remained unknown for decades. Much research has proposed possible mechanisms that would induce mesial temporal lobe epilepsy. In this article, we briefly summarize some of the present theories on the cause of this disease and then introduce our theory on NKCC1 upregulation in hippocampal pyramidal cells as a potential cause of epilepsy. We specifically discussed the WNK pathway that regulates NKCC1 and KCC2 as a potential cause of epilepsy and explored another possible mechanism that overrides WNK and would potentially induce mesial temporal lobe epilepsy. Eventually, we discussed future expectations on medication according to our theory.

Keywords: NKCC1, KCC2, Temporal Lobe Epilepsy, WNK pathway, regulatory mechanism.

1. Introduction

As a prevailing type of Temporal Lobe Epilepsy, Mesial Temporal Lobe Epilepsy happens mostly in the middle part of the temporal lobe, which involves the hippocampus and surrounding areas. Temporal Lobe epilepsy can be detected through MRI, where one can sometimes observe hippocampal sclerosis. It is also a widespread neuronal dysfunction in the central nervous system [1]. Additionally, several medications have already appeared to be efficient in treating mesial temporal lobe epilepsy, which includes Carbamazepine, Gabapentin, Lamotrigine, Topiramate, and Oxcarbazepine [2].

NKCC1, encoded by Slc12a2 and thought to be the cost of 30% of epilepsies, is a protein that is commonly found in neurons in the central nervous system, peripheral nervous system, and some glial cells and is responsible for the uptake of chloride ions from the extracellular matrix. NKCC1 predominates in the immature neuron, while KCC2 is kept in the minority due to some age-dependent factor, causing the chloride concentration higher within the cell, making the neuron excitatory. However, as the neuron matures, KCC2 upgrades and NKCC1 degrades, making the chloride concentration within and without cell alters, turning the neuron to be inhibitory [3].

Although not yet determined, the cause at a molecular level for mesial temporal lobe epilepsy had long been a prevailing topic, in this article, we focused on NKCC1 as a potential cause for this disease and try to set up a corresponding theoretical framework that justifies NKCC1 and its associated mechanism as a potential cause for mesial temporal lobe epilepsy, and will provide expectations for medicine accordingly.

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2. Current Understanding Of The Potential Causes For Mesial Temporal Lobe Epilepsy

Taking up about 80% of all epilepsy, the cause of mesial temporal lobe epilepsy (MTLE) at a micro level remains unknown, it is often observed many macro or general causes would induce mesial temporal lobe epilepsy, which may include physical traumas in the brain area, genetic inheritances, and Neurocysticercosis according to a recently published paper [4]. Therefore, throughout the decades, researchers have hypothesized and testified several possible causes.

2.1. Reelin loss

There are varied explanations for the possible causes of MTLE, one of them that had been convincing in the decades is the theory about the loss of Reelin, a secreted neurodevelopmental glycoprotein, has long been thought to be associated with the formation of MTLE. In a paper in 2006[4], they proposed that loss of Reelin in the epileptic adult hippocampus induced aberrant integration of newborn Granule Cell Dispersions (GDC) [4]. Then later in a paper published in 2022, [5], they provided insight into the effect of Reelin, GDCs on mesial temporal lobe epilepsy, whereas they suspected that loss of Reelin affected GDCs, which is commonly found in epileptic brains [5]. This suspicion is then being supported by research in 2023 [6]. In this research, they found that loss of Reelin induced a decrease in its downstream target, disabled 1 (Dab1), which inhibits cofilin to avoid aberrant neuronal migration. Moreover, they found in the patient a significant overexpression of Cofilin, indicating the pathway they introduced to be quite plausible [6].

2.2. miRNA-induced epilepsy.

miRNA, as to Reelin, has been suspected for an extended period to be one of the causes of epilepsy. In a paper published in 2011 [7]. They first demonstrated a miRNA alternation after seizures [7]. Later, as more studies about miRNA's role in Temporal Lobe Epilepsy, the idea that miRNA affected some pathological responses in the brain area eventually led to epilepsy [8]. In a study in 2016 [9], they suggested that a decrease of generalized miR-146a-mediated leads to a decrease of complement factor H and is likely to induce temporal lobe epilepsy in the rat model [9]. Moreover, other studies suggested that miRNA is associated with neural function and plasticity that may also contribute as a factor for temporal lobe epilepsy [8]. For instance, in research in 2013 [10], the research group demonstrated that miRNA expression alternation induced functional changes in the dentate gyrus and thus had a large impact on causing temporal lobe epilepsy [10].

2.3. Blood-brain barrier (BBB) leakage induces temporal lobe epilepsy.

Unlike Reelin losses and miRNA dysregulation, Blood-Brain Barrier disruption is a rather new and minor area of consideration on what induced temporal lobe epilepsy. However, immune cells' transmigration or invasion into the brain parenchyma can also induce epilepsy [11]. This had been supported by a paper published in 2008 [12]. They demonstrated a pathogenetic link between leukocyte-vascular interaction, BBB damage, and the formation of seizures [12].

However, in a recently published paper in *2022*[11], the research group applied functional magnetic resonance imaging (fMRI) to 90 subjects with Temporal Lobe Epilepsy (TLE) and ran a statistical experiment to test the patients' functional connectivity in their brain areas. They demonstrated that the memory network of the TLE patients had experienced changes inside the mesial temporal lobe and frontal lobe when compared to the control group, and a higher level of disease burden is associated with weaker connectivity with the inter-mesial temporal lobe and intra-mesial temporal lobe, which support verbal and visual memory [11]. These new findings inspired a new possible mechanism for explaining the fundamental cause: the role of hippocampal pyramidal cells in causing mesial temporal lobe epilepsy.

3. Nkcc1 Upregulation In The Hippocampal Cells Induced Mesial Temporal Lobe Epilepsy

Hippocampal pyramidal cells are learned to be associated with spatial learning [13]. Thus, functional disability of hippocampal pyramidal cells would cause alternations in spatial learning and furthermore affects episodic memory. Moreover, hippocampal pyramidal cells contain NKCC1 and KCC2 proteins

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[14], which are recently found to be key proteins in inducing epilepsy [15]. Therefore, we hypothesized such following mechanism: upregulation of NKCC1 associated with downregulation of KCC2 [16] induced intracellular chloride ions accumulation, thus, alternating the charge within the neurons turning it from inhibitory to excitatory [15], causing hyperexcitement, which damaged the original function of hippocampal pyramidal cells, causes epilepsies, and disrupts episodic memory. In some cases, a high level of intracellular chloride ions would become toxic for the neurons, therefore inducing more than 50% of cells in the hippocampus to go through apoptosis and inflammation, which on a macro scale would display as hippocampal sclerosis [17]. Additionally, this hypothesized mechanism follows the pattern of neural maturation. Since NKCC1 must experience a downregulation while KCC2 has to experience an upregulation as the neuron matures, any mutation or functional disability during this process of transforming would result in an outbalanced NKCC1 and KCC2 ratio, which makes the maturing stage of the neurons having the most threats for experiencing hyperexcitement. This provides a plausible explanation for why it is common for the mesial temporal lobe to display epilepsy since it is one of the areas that mature first in the brain [18].

3.1. WNK-SPAK/OSR1 function in regulating NKCC1 and KCC2 and as possible cause for epilepsy

Several recently published papers have extended our previous hypothesis and have adjusted our focus on the whole mechanism. In these two papers [19] [20], WNK had been demonstrated as both chloride concentration gated, and cell volume gated (Figure), whereas the cell is exposed to a hypertonic Cl environment, the cell would go through hyperosmotic.

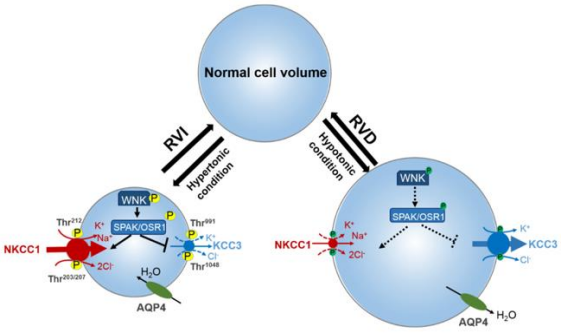

Figure 1. Roles of WNK, NKCC1, and KCC2 on regulating cell volume and intracellular ion concentration [20].

Thus, activating the WNK-SPAK/OSR1, and upregulating NKCC1 to bring in Cl to restore cell volume. Moreover, the WNK pathway had been found to regulate NKCC1 and KCC2 at the same time, and when WNK is activated, it upregulates NKCC1 as well as downregulates KCC2[19] [20]. And as research indicated that WNK has an autophosphorylation mechanism [21], it remained unclear how WNK function in immature neurons. According to existing researchers, in immature neurons where intracellular chloride is much higher than in mature neurons, the WNK pathway should be inhibited, and NKCC1 should be downregulated while KCC2 should be upregulated, but NKCC1 still prevails in immature neurons. There must present an unknown factor that regulates WNK and he co-transporters. Though some mechanisms had been found to furthermore regulate WNK, NKCC1, and KCC2 (Figure 2), more are yet to be discovered, since these factors, when malfunctioning or mutated, have the potential to disrupt the expression of WNK or directly disrupt the co-transporters, thus inducing hyperexcitement and causing epilepsy. Therefore, it is critical to identify as many as mechanisms to avoid many unknown causes of epilepsies as possible.

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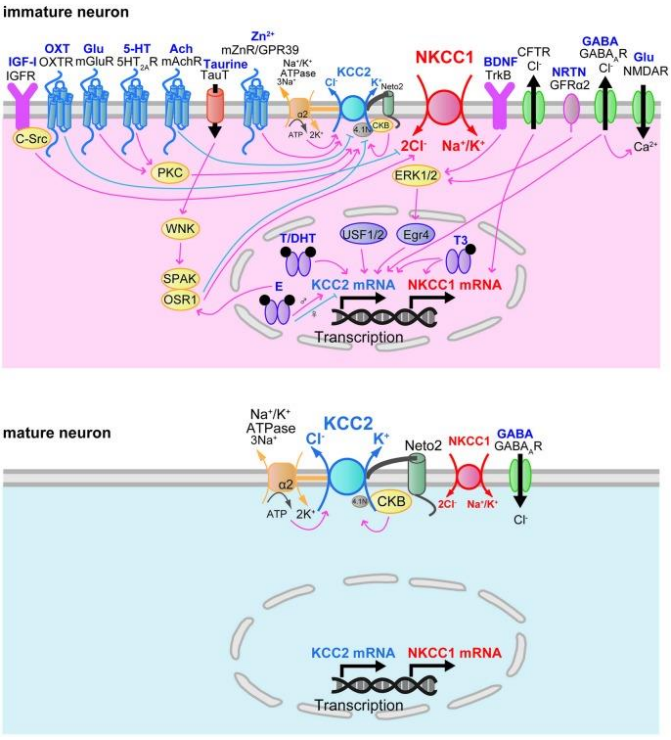

Figure 2. Currently known proteins for regulating WNK, NKCC1, and KCC2[18].

3.2. Possible mechanisms other than the WNK pathway in regulating NKCC1 and KCC2.

We further categorized the mechanisms that regulate WNK, NKCC1, and KCC2 into three categories: an unknown mechanism that regulates WNK, an unknown mechanism that regulates KCC2/NKCC1 along with WNK, different regulation patterns of WNK in immature and mature neurons (Figure3). And all three these categories do not stand against each other, they can be stand-alone, dependent on each other, and even coexist.

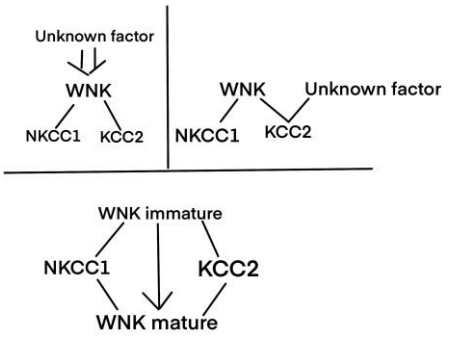

Figure 3. Three possible categories of mechanisms regulate WNK, NKCC1, and KCC2.

For the first categories, the unknown factor regulates WNK and furthermore regulates the co-transporters, has examples of Kelch-like 3(KLHL3) and Cullin-Ring ubiquitin 3 (CUL3) [22], which both would inhibit WNK kinase expression. KLHL3 BTB binds the C-terminus of L-WNK1 or WNK4, and Ring-box protein 1 of CUL3 binds to KLHL3 in the BTB domain, using enzymes that would degrade the two WNKs, and thus is inhibited [22]. Other than KLHLU3 and CUL3, Taurine happens to be another natural-occurring inhibitor or WNK family [18]. Another type of WNK regulator is SGK1, but SGK1 and WNK are in bilateral regulation (specifically for WNK1 and WNK4), whereas SGK1

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phosphorylates WNK at S1169 and S1196[22], while the WNK family's N termini displayed effectiveness to strong activation of SGK1 [23].

In the second category that WNK and an unknown factor both regulate the co-transporters, a paper published in 2017 provided an example that when spinal alpha-7 nicotinic acetylcholine receptor (nAChR) is activated, it upregulates KCC2 in rats [24], whereas brain-derived neurotrophic factor/tyrosine receptor kinase B expressions are reduced by nAChR that leads to an upregulation of KCC2. Moreover, in a paper published in 2019, during Hypoxic-ischemic encephalopathy (HIE), hypoxia-inducible factor-1α (HIF-1α) upregulates NKCC1 and nuclear factor of activated T cells 5 (NFAT5) that down-regulates NKCC1 [25]. This mechanism serves another typical type of multiple factors regulation on the cotransporters.

The last category of the mechanism, the difference in functions in mature and immature central nervous systems, has a limited number of studies compared to the other two categories. However, there is still insight into this category. In a paper published in 2021, *WNKs are potassium-sensitive kinases* [26], proposed that potassium is a chloride-independent WNK inhibitor. Moreover, the paper demonstrated a potassium secretion increase when bathed in a hypotonic environment, following a WNK-, Fray-, NKCC- dependent manner [26]. This provides insight into a possible mechanism of WNK in an immature state of neurons, where we can hypothesize that WNK in immature neurons is more potassium-gated than chloride-gated.

4. Envisioned Treatment (Medication)

For mesial temporal lobe epilepsy induced by NKCC1 overexpression, we do not believe medications should be inhibiting NKCC1 itself, even though it's the direct cause of the disease. In most cases, the upregulation of NKCC1 often comes with the downregulation of KCC2 [19] [20], which makes the cell vulnerable to hypertonic environments and increases the percentage of apoptosis in such an environment. Thus, if a medication inhibits NKCC1 protein, it may release the symptom for a limited period, but it potentially decreases the cell's ability to respond to hypotonic environments as well, which would eventually proliferate the threat on cell apoptosis. Under such saying, we think the medication should be targeting the WNK pathway or other mechanism that regulates the co-transporters, wherein such means, we could restore the organism's ability for maintaining homeostasis, and thus self-cure epilepsy.

5. Conclusion

Through the analysis of the current understanding of mesial temporal lobe epilepsy, this article introduced several possible causes of mesial temporal lobe epilepsy and focused on one cause to analyze its current stage and propose future development. The article also includes envisioning medications for the specific cause of the disease previously described. The causes of epilepsy are diverse and largely varied, and the process of uncovering the causes at a molecular level could be time-taking and burdening. However, finding the cause of a disease at a molecular level means the discovery of potential targets, and potential biomarkers, which leads to a higher cure rate and early detection. Under such a saying, it has always been critical to seek the potential cause of every disease.

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Comparing Doxycycline and Azithromycin in treating cholera

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Abstract. Cholera is a famous bacterial infection that was found in the Indian Ganges Delata during the 19th century. People who were infected suffered a lot from the symptoms like diarrhea and dehydration, which impacted people's living standards. Doxycycline and Azithromycin are the most common drug treatments used for cholera patients. Doxycycline can inhibit the synthesis of bacterial protein by combining with the 30s ribosome and shorten the duration of this disease. Doxycycline is used over a wide range of age groups. Azithromycin can combine with the 50s ribosome to inhibit the synthesis of protein. However, using antibiotics over the given time period will cause the bacteria to grow resistant to them. This essay will discuss the two antibiotics that are used nowadays for Cholera treatment.

Keywords: Cholera, Doxycycline, Azithromycin

1. Overview of disease

1.1. Introduction

Cholera is a global disease that has nearly 4.0 million cases per year and with high mortality. It was first discovered in the 19th century in the Indian Ganges Delata and became a serious public health problem worldwide. The main symptoms of it are diarrhea and dehydration. For developing countries, Cholera may have a higher death rate, because the fewer medical resources. Therefore, effective medical treatments are important for decreasing the severity of Cholera. There are two antibiotics, Doxycycline and Azithromycin, that contribute a lot in treating Cholera.

This essay will be focused on comparing Doxycycline and Azithromycin in treating cholera. First provide the background information on Cholera, including history, symptoms, and influences. After that, introduce two antibiotic synthesis processes, pharmacology, and mode of delivery. Finally, discuss the two drugs' side effects, and drug resistance. The comprehensive research presented in this paper can broaden the understanding of Cholera. Contribute to minimizing the effect of Cholera.

1.2. Introduction of Cholera

The infection known as cholera is caused by *Vibrio cholera* bacteria that is spread through the water and food containing the bacterium *Vibrio cholerae*. Toxin-producing *V. cholerae* causes the disease. It's a highly motile, comma-shaped gram-negative bacterium with many serogroups, including pathogenic and non-pathogenic strains. It exists in the aquatic environment and infects the small intestine. The epidemics caused by cholera are mainly attributed to the two phenotypic variants, monophyletic 'classical' strains and 'El Tor' of *Vibrio cholerae* [1].

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1.3. Symptoms of cholera

The most common symptom of infection is watery diarrhea. The symptoms will show after people ingest the food or water containing the bacterium. However, people won't easily recognize that they are infected because of these two factors. The first is that diarrhea is a common symptom of many illnesses, making it difficult to identify the illness they have. The second is that cholera typically has an incubation period of around 1-2 weeks until the symptoms appear. The vibrio cholera bacterium will spread when the water containing it is digested [2]. The two toxigenic serogroups, *Inaba and Ogawa,* are responsible for the two serotypes, *classical and El Tor*, as well as the two biotypes.

Due to *V. Cholerae*'s motility and adherence characteristics, *V. Cholerae* could colonize efficiently the intestinal wall. However, *Vibrio Cholerae* requires a high infectious dose to produce the cholera toxin. After excretion, which transmission occurs through the contaminated water with the 24-hour-hyper-infectious phase of the bacteria, the two toxin factors will play an important role in the infection: the toxin-coregulated pilus and cholera toxin. They play a crucial role in the host's gut mucosa layer being colonized and the passage of the gastro endothelium wall that results in watery diarrhea [3].

At the same time, Biofilm formation ensures the facilitating colonization in human and aquatic reservoirs of the bacteria.

1.4. Impact and number of mortalities of cholera

Cholera is a disease that has been discovered for a long history. Even though Cholera has impacted 120 countries, it is a disease that took place in developing countries that lack medical resources. Around the time of the fourth era of B.C., Greek Hippocrates; Sushruta Samhita, from India in the fifth century B.C.; and Aretaeus, who is a Cappadocian, are writing in the first century A.D., all reference cases of cholera-like disorders [4].

One of the earliest thorough descriptions of the spread of cholera was given by Portuguese historian Gaspar Correa, author of Legendary India. He addressed a pandemic that plagued both nations in the Ganges Delta in the early spring of 1543. The illness is referred to regionally as "moryxy." It developed quickly, leading to a high death rate that the patient would die within 8 hours of the symptom developing. This outbreak marks the origin of cholera. Then in the 19th century, the first and second cholera pandemics spread from India to Asia, Europe, and the Americas. The third was the deadliest pandemic, which was around 1852–1859 years and impacted multiple continents. (*Cholera*, 2017). Nevertheless, the improvement of public health and infrastructure have helped control pandemics, but cholera is still a significant problem in developing nations, like Africa that outbreak the current seventh pandemic that began in 1961 [5].

Around 1.3 to 4.0 million cases of the present Cholera epidemic in countries that are developing, and there are global-related death cases of cholera that are around 21,000 and 143,000 [6].

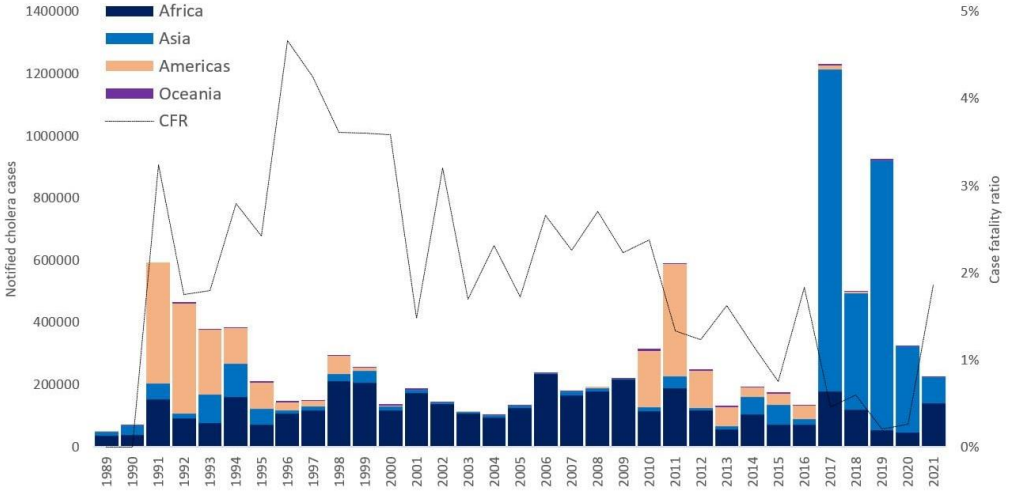

Figure 1. Global cholera cases in 1989-2021 [6]

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Figure 1 demonstrates that cholera epidemics nowadays are primarily found in Asian and African regions. In those areas, people don't have insurance as in nations like America and other countries with high living standards and medical care, which causes a lack of infrastructure and water quality regulation. With the shortages the resources, people are unable to safeguard themselves from the Vibrio cholera. As a result, this area has the highest incidence of cholera and the highest number of cases.

1.5. Significance in China and other countries in the world of cholera

The nations with the greatest cholera risk are Bangladesh, India, Nigeria, China, Ethiopia, and Nigeria (Ali et al., 2015). China has to be taken into account in estimations of worldwide trends of the spread of cholera since it served as both a source and a sink during the seventh pandemic of V. cholerae. Each outbreak in China could be considered the consequence of a different introduction of these bacteria from another country. As a result, China is considered a **"**source**"** or possibly **"**amplified**"** the epidemic's spread to other regions [7].

2. Treatment of cholera

Doxycycline and azithromycin are two drugs that are used to treat cholera nowadays.

2.1. Doxycycline

Doxycycline is synthesized and formed. The effective invention of penicillin during the Second World War, which encouraged others to study antibiotics, may have been the point of departure. As Robert Woodward claimed that these two medicines maintained the same four aromatic rings, scientists have found two broad-spectrum antibiotics. They are tetracyclines as a result. Then, Charlie Stephens' modifications to chemicals resulted in the generation and manufacture of the antibiotic doxycycline, which the FDA approved for use in 1967 [8].

Following the discovery of the first tetracycline group in the 1940s, doxycycline was synthesized as an antibiotic from the soil-dwelling bacteria Streptomyces aureofaciens [9].

2.1.1. Synthesis of doxycycline. Doxycycline, which is among the largest number of partially synthesized tetracycline derivatives. They stand apart from tetracycline by their positioning of a single hydroxyl group that is present.

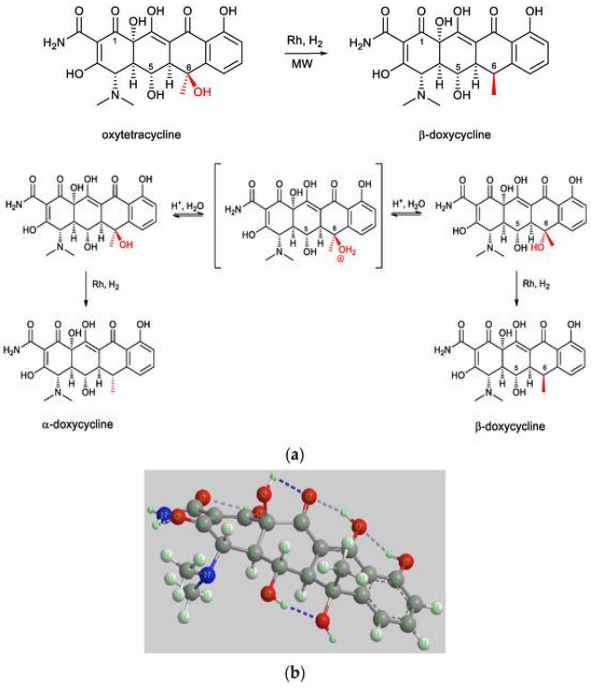

Figure 2. The synthesis of Doxycycline [10]

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Traditionally, doxycycline can be considered as an outcome - a transfer of the hydroxyl group from C6 to C5. Methacycline serves as an intermediary in the modern industrial manufacture of doxycycline. The methods that were used during the synthesis of Doxycycline were conventional and MW-Assisted Methods, Complexation of Oxytetracycline with Cyclodextrins, and Subsequent Hydrogenolysis Reaction. Doxycycline could possibly, in fact, produce two epimers during synthesis: the α and the β forms. The α epimer is the most often used form of doxycycline these days, and it has pharmacological activity. The β epimer would be preferred while using the heterogeneous at the time when it may reach the site of the methyl substituent [10].

2.1.2. Nomenclature of Doxycycline. Doxycycline is a tetracycline in which the 5beta-hydrogen is replaced by a hydroxy group, with molecular formula of C22H24N2O8, and a molar mass of 444.4g/mol. Doxycycline's IUPAC name is (4S,4aR,5S,5aR,6R,12aR)-4-(dimethylamino)-1,5,10,11,12a-pentahydroxy-6-methyl-3,12-dioxo-4a,5,5a,6-tetrahydro-4H-tetracene-2-carboxamide. Vibramycin is the trade name of Doxycycline [11].

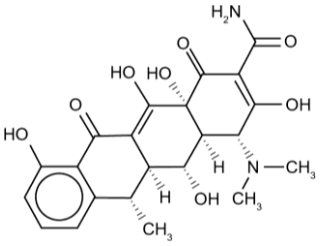

Figure 3. Structure of Doxycycline [12]

2.1.3. Doxycycline's chemical and physical characteristics

Table1. Chemical and physical properties of doxycycline [11].

Drug name	Chemical and Physical properties
Doxycycline	MW: 444.4 g/mol Character: Yellow crystal in rtp. Melting point: 201°C Solubilities: Partially dissociated in ether and chloroform, alcohol, readily soluble in diluted acids, alkali hydroxide, and in water. pKa: 3.09

2.1.4. Drug pharmacology of doxycycline. Doxycycline prevents the synthesis of V. cholerae proteins by irreversibly interacting with the 30S ribosomal subunit. Therefore, it inhibits aminoacyl-tRNA by interacting with the bacterial ribosome [13]. By attaching to the 70S ribosomes, it also affects the synthesis of mitochondrial proteins and functions as an antibacterial agent as a result. In the later stages of the malaria cell cycle, doxycycline combines with apicoplast subunits of ribosomal proteins from Plasmodium falciparum to hinder the synthesis of fatty acids and the generation of heme. Both a pH-sensitive active transport system in the inner cytoplasmic membrane and pores that are hydrophilic in the outer cell membrane which enables doxycycline to enter cells. It additionally reduces angiogenesis and apoptosis, facilitates gum fibroblast attachment, and aids in wound healing, among other things. Specific metalloproteinases, a group of proteolytic enzymes made by inflammatory cells, get similarly affected by doxycycline. Doxycycline's potential for usage in anti-inflammatory and anti-tumor treatments is therefore suggested [12].

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2.2. Azithromycin

Compare to erythromycin, Azithromycin is more easily taken up and has fewer side effects. Azithromycin is produced by a series of process that involves oximation, reduction rearrangement and so on, from erythromycin A. It also has bacteriostatic activity that affect both to the Gram-positive bacteria and Gram-negative bacteria, which include *B. pertussis* and *Legionella* [14]. The reason Azithromycin is a more effective antibacterial is that it is less likely to separate from the ribosome of the gram-negative bacteria [15].

2.2.1. Synthesis of Azithromycin: A sophisticated chain of chemical reactions results in the semi-synthetic form of the 15-membered macrolide antibiotic azithromycin, which is created from erythromycin. Azithromycin is created through an oximation, Beckmann rearrangement, reduction, and N-methylation. In comparison to other antibiotics in its class, azithromycin has higher stability, oral absorption, a longer half-life, and a broader activity. This subsequently improves its antimicrobial properties.

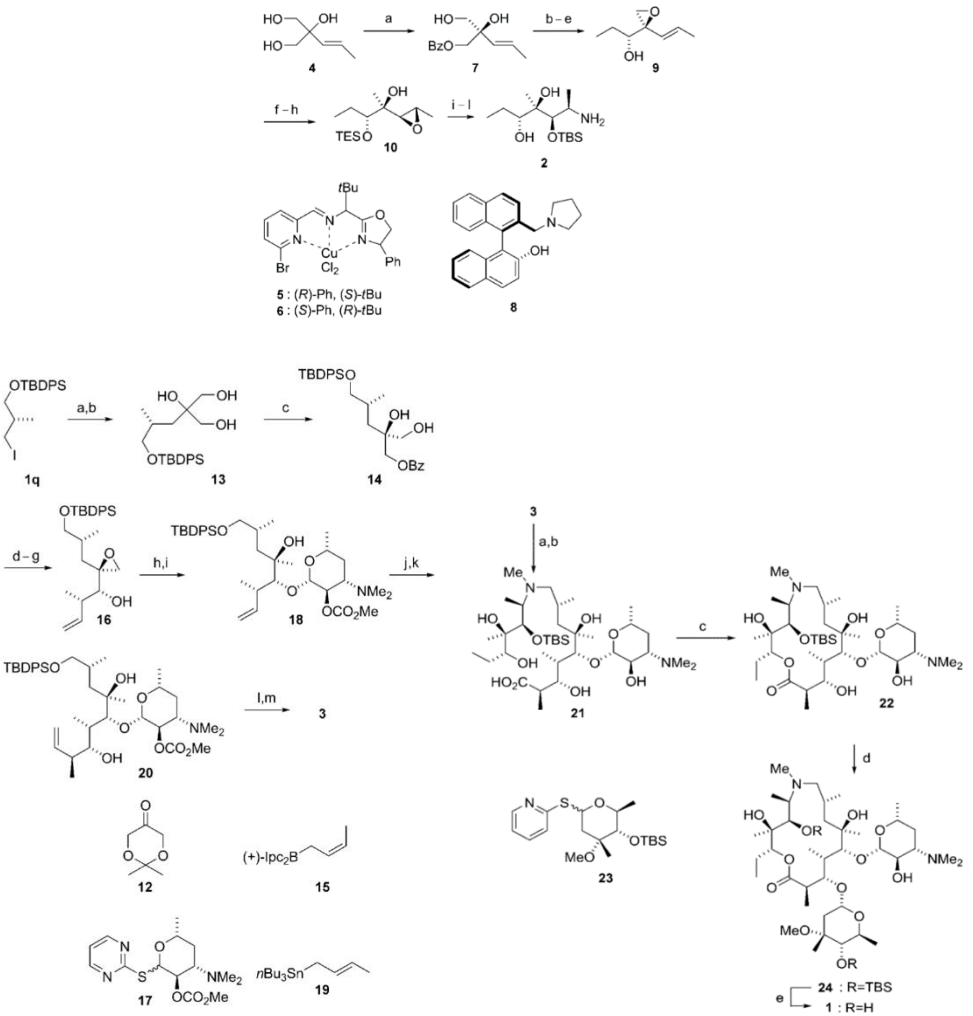

Figure 4. The synthesis of Azithromycin [16].

The synthesis of aminol chain 2 on the west side and hydroxycarboxylic acid chain 3 on the east side is initiated by severing segment 1 at the lactone linkage and C9-N9a bond. The timing of glycosylation stages for efficient macrolactonization, and methods like regioselective epoxide openings, asymmetric ethyl addition, and enantioselective desymmetrization have been used. A triol must first be

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desymmetrized in order to create mono benzoate 7, which is subsequently transformed into epoxy benzoate. The epoxy compounds created R and diastereomeric S alcohols that are required. The western amine segment 2 is created after additional adjustments. Segment 3 is built using a desymmetrization process for a quaternary carbon core and stereogenic centers that are made by crotylation reactions. Then the eastern carboxylic acid chain 3 is created via a number of procedures.

Completion reaction of the synthesis of segments 2 and 3 will take place when they are coupled, by macrolactonization of the carboxylic acid, and a subsequent glycosylation process. Segment 3's main hydroxy group undergoes oxidation and is joined to segment 2 by reductive amination. The 15-membered lactone 22 is produced by macrocyclization, and b-anomer 24 is required by further glycosylation. Therefore, the final product azithromycin, is obtained after purification [16].

2.2.2. Nomenclature of Azithromycin. Azithromycin is a macrolide, which is a natural compound, and is a member of the azalide subclass of macrolides. It consists of a ring with a 15-membered and a nitrogen methyl-substituted group that stands in for of a carbonyl group at position 9a on the aglycone ring, which contributes to simpler the evasion of metabolism. It shares a structural association with erythromycin [14]. The IUPAC name of it is (2R,3S,4R,5R,8R,10R,11R,12S,13S,14R)-11-[(2S,3R,4S,6R)-4-(dimethylamino)-3-hydroxy-6-methyloxan-2-yl]oxy-2-ethyl-3,4,10-trihydroxy-13-[(2R,4R,5S,6S)-5-hydroxy-4-methoxy-4,6-dimethyloxan-2-yl]oxy-3,5,6,8,10,12,14-heptamethyl-1-oxa-6-azacyclopentadecan-15-one [17].

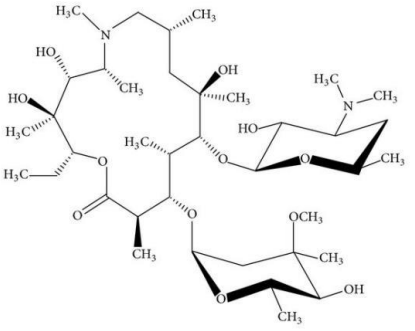

Figure 5. Structure of azithromycin [18].

2.2.3. Chemical and physical properties of azithromycin

Table 2. Chemical and physical properties of azithromycin [17].

Drug name	Chemical and Physical properties
Azithromycin	MW: 749.0 g/mol Character: Amorphous solid Melting point: around 113 to 115 °C Solubility: fully dissociated in ethanol and DSMO, and partially dissociated in water pKa = 8.74

2.2.4. Drug pharmacology of azithromycin. Similar to the azalide subclass of macrolides, azithromycin attaches to the 23S location of 50S bacterial ribosomal subunits. It limits the synthesis of bacterial proteins by obstructing the ribosome's capability to transport aminoacyl-tRNA and the protein-forming process. Azithromycin functions largely as an antibacterial agent, which implies that it inhibits protein

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synthesis instead than aggressively eradicating germs, in contrast to other macrolides and other macrolide antibiotics. In addition, azithromycin may have bactericidal effects, especially at higher doses when used to treat streptococci and H. influenzae. Azithromycin has antibacterial properties and also demonstrates antiviral characteristics in vitro.

In terms of pharmacokinetics, azithromycin could enter quickly into the tissues from the bloodstream, then they could cross the cellular membranes more easily, and fight successfully off intracellular infections. Azithromycin blocks the 50S ribosome found in the apicoplast, which is the non-bacterial organism that has a protein-synthesis mechanism similar to bacteria and is essential for metabolic processes, using apicomplexan parasites including species like; Babesia, Toxoplasma, and Plasmodium to inhibit the protein synthesis [15].

3. Mode of delivery

3.1. Treatment for cholera

The primary treatment for cholera involves oral or intravenous hydration. The patients have extreme symptoms and the special situation will use antibiotics at the same time. the antibiotics would be used based on the patient's tolerance of oral medication and in accordance with regional patterns of antibiotic susceptibility. In the majority of situations, Doxycycline is first recommended to both adults and children; however, if doxycycline resistance is established, alternatives such as azithromycin and ciprofloxacin are available [19].

Azithromycin, which is available in oral and intravenous forms, is typically given once daily for 3 to 5 days. It effectively penetrates tissues undergoes hepatic metabolism, and allows a shorter treatment duration compared to other antimicrobials. The strong tissue penetration, extended half-life, and suitability for patients with renal disease without dosage adjustment make azithromycin a valuable choice in treatment. Its formulations include tablets, packets, suspensions, intravenous solutions, and ophthalmic solutions for bacterial conjunctivitis [15].

For a long time currently, tetracycline has been the first-choice medication used for cholera. Presently, oral tetracycline is the most effective antibiotic medication for treating both cholera patients and carriers, but it must be taken in multiple doses over a period of two or three days. In order to effectively treat this condition, replacement fluid therapy is required. It shortens the time that vibrio excretion lasts and reduces the amount of fluid lost [20]. Doxycycline has been suggested as the treatment of choice for adults, including pregnant women, and children since it has been shown to be similar to treatment with a single 300-mg dose of tetracycline [19].

3.2. Side effects and resistance to doxycycline and azithromycin

3.2.1. Resistance and Side Effects of Doxycycline. The majority of doxycycline resistance is caused by genes like "tet" and "otr," which are always formed by plasmids and transposons. Ribosomal protection proteins and efflux proteins are the main mechanisms of how resistance develops. Since 1953, both Gram-negative and Gram-positive bacteria have shown an increase in resistance to the antibiotic reagent like doxycycline. H. pylori and N. gonorrhoeae are rarely chromosomal mutations but they clinically significant have impact on the resistance of doxycycline [12]. The side effect that is less frequent take place include diarrhea, irritation of the vagina, and being uncomfortable during sexual activity. Severe adverse effects that unusually appear are digestive disorders, visual abnormalities, dermatological issues, etc [21].

3.2.2. Resistance and Side Effects of Azithromycin. Azithromycin increased its resistance by being frequently abused. The 23S rRNA target is mostly modified by methylation, which causes crossover resistance with other antimicrobial agents as a result it makes the bacteria have resistance [22]. Azithromycin is generally regarded as its security, however, 15% of people may have dizzy spells, headaches, and gastrointestinal problems those symptoms. There have been reports concerning

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consequences such as severe cardiac points by rotations and QT prolongation. At the same time an uncommon symptom, liver injury is another issue. Stevens-Johnson syndrome and anaphylaxis are relatively uncommon but they are potentially fatal adverse effects [14]. Nevertheless, the therapeutic efficacy of these medications is constrained by the rise in drug resistance, but at the same time, those adverse effects which vary from moderate to severe need necessitate consideration of treatment [15].

4. Conclusion

To summarize, doxycycline and azithromycin all act as bacterial inhibitors to prevent protein synthesis. cholera treatment comprises hydration and antibiotics such as doxycycline or azithromycin, chosen based on local susceptibility when needed. During outbreaks, it's crucial to monitor antibiotic resistance. Antibiotics should be used alongside aggressive hydration. Azithromycin's characteristics make it an important option for treating cholera (Antibiotic Treatment, 2022). Since cholera possesses a long history of occurrence and treatment with doxycycline and azithromycin, as a result, it makes the V. cholerae bacteria develop the bacterial resistance to those antibacterial agents. Therefore, people are still improving doxycycline and azithromycin continuously by modification. The affordable price, of doxycycline and azithromycin, compared to drugs like fluorouracil and docetaxel, which gives the underprivileged people a chance to treat this illness and aid in the reduction of cholera cases and its effects. Although azithromycin and doxycycline can be produced through chemical synthesis, the biological method is much more effective than the chemical method. Therefore, doxycycline and azithromycin are primarily produced using biological methods. The potential that these two agents have on chemical synthesis and the reasonable price make doxycycline and azithromycin play an important role in treating cholera.

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Stimuli-responsive drug delivery system for breast cancer treatment

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Abstract. In the annals of cancer treatment within the domain of nanomedicine, the utilization of stimulus-response drug delivery systems has emerged as a focal point of considerable significance. While a multitude of diverse methodologies are presently employed for the treatment of tumors, the preeminent and most efficacious approach, particularly in the context of intricate scenarios, pertains to the targeted administration of therapeutic and diagnostic nanoparticles into the tumor milieu. These nanoparticles exhibit the inherent potential to acclimatize to the nuanced microenvironment of the tumor, thereby surmounting the challenges posed by tumor heterogeneity. The stimuli-responsive attributes inherent to nanoscale particles can broadly be delineated into two distinct categories: endogenous and exogenous, with a minority manifesting characteristics of both classifications. This scientific review provides an overview of the discourse surrounding exogenous drug delivery systems, which leverage ultrasound and magnetic fields as responsive stimuli, alongside endogenous drug delivery systems employing pH variations and enzymatic cues as triggering mechanisms. The findings of this review emphasize the significance of stimuli-responsive drug delivery systems as a promising strategy to enhance the specificity and efficacy within the sphere of breast cancer treatment. Finally, the review concludes with a discussion on the future directions and potential clinical applications of these systems.

Keywords: Breast Cancer, Stimuli-responsive, Smart Delivery Systems, Drug Delivery.

1. Introduction

Breast cancer is a prevalent and debilitating disease that strikes millions of individuals worldwide. The World Health Organization reported an incidence of more than 2.3 million cases each year [1]. Despite the modern advancements made in medical research and treatments of breast cancer, the overall mortality and morbidity are still high and remain a significant public health concern. Recent treatment

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strategies include several options depending on the severity of the cancer: chemotherapy, radiation therapy and surgical resection. Among all, chemotherapy is the most frequently used systemic treatment due to its capacity to suppress the proliferation of cancer cells [2]. Nevertheless, having the therapeutic agents utilized in chemotherapy to selectively target cancer cells without influencing healthy cells pose a great challenge. This has undesirable consequences such as hair loss, fatigue, appetite loss, nausea and vomiting, and is associated with a high risk of damage to various organs [3].

With the development of nanoscale drug delivery, controllable and specified therapeutic releases based on stimuli-responsive biomaterials have been exploited to surmount the constraints mentioned above. The stimuli-based drug delivery system involves the materials that possess the ability to respond to particular triggers, enabling the controlled release of pharmaceutical substances at precise anatomical sites within the organism. In particular, approaches involving drug release with exogenous stimulation (including magnetic field, ultrasound, and light) and endogenous stimulation (including pH and enzyme) have attracted considerable attention recently. This review discusses the above-mentioned stimuli in responsive drug delivery for breast cancer treatment. Furthermore, this study provides insight into the current challenges and future directions.

2. Exogenous Stimuli-Responsive Drug Delivery System

2.1. Ultrasound-responsive Drug Delivery Systems(URDDS)

2.1.1. Mechanisms of URDDS

Ultrasound-responsive drug delivery systems possess diverse applications in the sector of cancer treatment as a result of their capacity to selectively release medical payloads at tumor sites in response to externally transmitted ultrasounds. The propagation of ultrasound waves in the body triggers physical phenomenon such as pressure variation, acoustic streaming, hyperthermia and cavitation [4]. Hence, these physical alterations could be utilized as stimuli for ultrasound-mediated drug release. The characteristics of URDDS hold great potential for enhancing targeting precision, facilitating deep penetration into tumor tissues, and mitigating adverse effects by modulating the frequency of ultrasound within a defined range [5].

2.1.2. Ultrasound-responsive Nanocarrier

Nanocarriers refer to encapsulating particles characterized by nanoscale dimensions that are specifically engineered to facilitate the transportation and delivery of therapeutic agents to targeted sites within the body. Due to their diminutive dimensions, a solitary cell is capable of up taking one or more nanocarriers for the purpose of transporting the drug payload [4].

In particular, the delivery of therapeutic agents using nanocarriers that respond to ultrasound for cancer treatment is mainly accomplished through the aid of micro/nanobubbles. These bubbles are comprised of a gas core encased by a stabilizing shell of nanocarriers. Additionally, the major constituents consist of the cargo (holds gene, drug etc.) and surface modification ligands, which is visually demonstrated by Figure 1.

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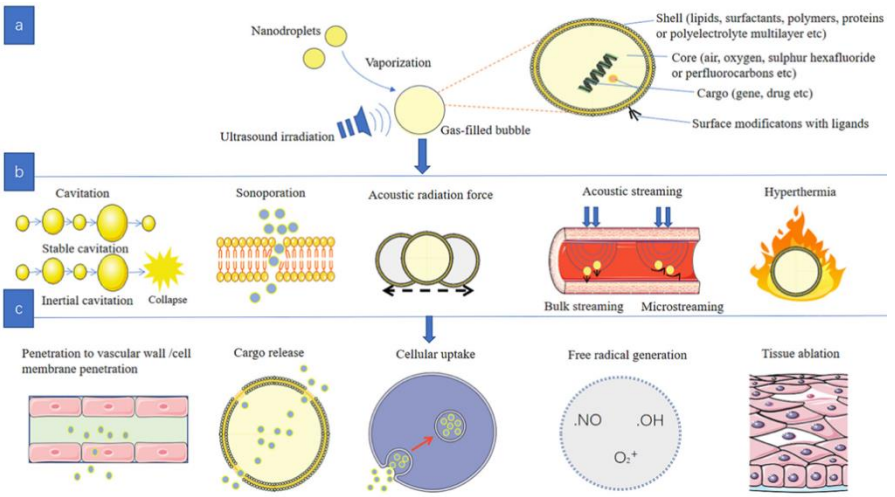

Figure 1. (a): The provided diagram illustrates the structural composition of the bubble. (b): The therapeutic mechanisms underlying the use of bubble-assisted ultrasound. (c): Biological consequences of bubble-assisted ultrasonography [6].

They possess the capability to enhance the targeting of therapeutic medicines due to their distinctive interaction with ultrasound. The utilization of targeted ultrasound stimulation elicits a sequence of dynamic phenomena, including expansion, contraction, oscillation, and forceful collapse of injected micro/nanobubbles, this phenomenon is known as cavitation [7]. The presence of gas within drug delivery vehicles facilitates the generation of acoustic activity, hence reducing the cavitation threshold. This heightened sensitivity to ultrasound enables local activation, release, and distribution of drugs by the carriers [8].

2.1.3. Ultrasound-responsive Nano-particles for Breast Cancer Treatment

This section will analyze the application of ultrasound-responsive micro and nanoparticles of various types in preliminary studies with regard to breast cancer treatments. The preliminary research findings have been succinctly summarized in Table 1.

Table 1. (Preclinical studies of ultrasound-responsive nanoparticles for treatment of breast cancer)

Nanoparticle	**Therapeutic**	**Effect**	**Reference**
Microbubble (RDG-MBs)	PTX	The ultrasonic trigger leads to enhanced drug accumulation in tumors targeted for (TNBC)	[9]
Liposome	Calcein (Model Drug)	Increase albumin uptake in tumor cells	[10]
Polymeric micelle	Paclitaxel	The application of ultrasonic stimuli has been shown to enhance the uptake of drugs and inhibit cellular proliferation in breast cancer tumours.	[11]

[9] devised a novel approach involving the creation of a dual-modal RGD-lipid microbubble (RDG-MBs) that encapsulates the chemotherapeutic agent PTX. This technique is further enhanced by applying the technique of ultrasonic targeted microbubble destruction (UTMD). Additionally, the integration of sulfur hexafluoride is employed to enhance the resolution and sensitivity of ultrasound images. The findings of this study indicate that the RDG-MBs combined with UTMD exhibited enhanced

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internalization by Triple-negative breast cancer (TNBC) cells, leading to a notable enhancement of the inhibitory effect on TNBC cells in an in vitro setting.

Using a different approach, [10] investigated the efficacy of pegylated liposomes conjugated to human serum albumin as an approach of delivering the therapeutic agent calcein to breast cancer cells, employing another novel methodology. Liposomes are lipid-based nanocarriers characterized by a spherical shape and composed of amphipathic layers. They have been extensively investigated due to their advantageous attributes, including low toxicity, high efficacy, and biodegradability [12]. According to the research conducted by [10], it was observed that the uptake of calcein by two breast cancer cell lines, namely MDA-MB-231 and MCF-7, exhibited a statistically significant increase subsequent to exposure to ultrasound. The authors also emphasize the utilization of targeted liposomal formulation in conjunction with ultrasound triggers as a means to achieve a safer, more effective, and site-specific targeting URDDS.

In comparison with liposomes, micelles are characterized by their smaller size as nanocarriers. This characteristic enables micelles to accumulate pharmaceuticals more effectively at the desired location due to the higher permeability and retention effect. To provide further clarification, micelles are composed of surfactants wherein the hydrophilic tails are oriented towards the exterior and the hydrophobic heads are oriented towards the interior. This structural arrangement possesses resemblance to certain characteristics observed in biological transport systems and enables micelles to properly protect insoluble hydrophobic drugs [13]. A preliminary investigation conducted by [11] implements polymeric micelles containing encapsulated paclitaxel for the treatment of a human breast cancer cell line. The experimental findings indicate that their usage of encapsulated paclitaxel in URSDDS resulted in an important improvement of drug uptake, surpassing a 20-fold increase. Furthermore, the stimuli of ultrasonic signals led to a substantial inhibition of cellular proliferation, with a reduction of nearly 90%. The research conducted by [11] provides clarification on the notable cytotoxic effects caused by micellar-encapsulated paclitaxel, specifically when exposed to ultrasound. This finding holds promise for minimizing systemic toxicity while boosting the potential of targeting tumors.

2.2. Magnetic-responsive Drug Delivery Systems

2.2.1. Mechanism of Magnetic-responsive Nano-particles

As a typical external stimulus, magnetic stimulation-responsive nanoparticles are commonly used in magnetic resonance imaging (MRI) and have the ability to penetrate human tissues [14]. The interaction between clusters of magnetic nanoparticles is mainly due to dipole-dipole interaction, which makes magnetic anisotropy, structure, and particle size the main factors affecting their performance. Because of they are extremely small in size (with an average diameter ranging from 1-100nm) and solvent affinity, magnetic nanoparticles can form suspensions in water [15]. However, the properties of high permeability of magnetic-responsive nanoparticles originate from their characteristics of super-paramagnetism.

Unlike bulk magnetic materials, nanoscale particles made from the same material only form a single magnetic domain due to their small size, which means that magnetic ordering does not occur within a certain region [16]. Similarly, in cases where magnetic reversal is required, bulk magnetic materials require a higher Curie temperature (such as iron, which requires a temperature of 770 degrees Celsius), while nanoscale magnetic particles only require room temperature [17]. This also demonstrates the existence of super-paramagnetism, which makes magnetic responsive nanoparticles highly sensitive to external magnetic fields, thus completely different from macroscopic magnets. In an experimental report co-authored by Ji Ma and Kezheng Chen, they discovered a unique phenomenon of super-paramagnetism in submillimeter-sized single crystal magnetite that differs from any known ferromagnetic behavior [18]. Figure 2(a) presents the XRD spectrum of the Fe3O4 porous single crystals (PSCS) before and after calcination, which was obtained using a powder X-ray diffractometer. The figure reveals several sharp and intense peaks that are closely related to the cubic structure of Fe3O4. The slope in Figure 2(b) demonstrates a lattice strain value (Δd/d) of -1.21×10-2 in the Fe3O4 PSCS,

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which is approximately two orders of magnitude larger than other structures of Fe3O4. Due to the effect of super-paramagnetism, the strength of the super-exchange interaction between individual iron ions is significantly influenced.

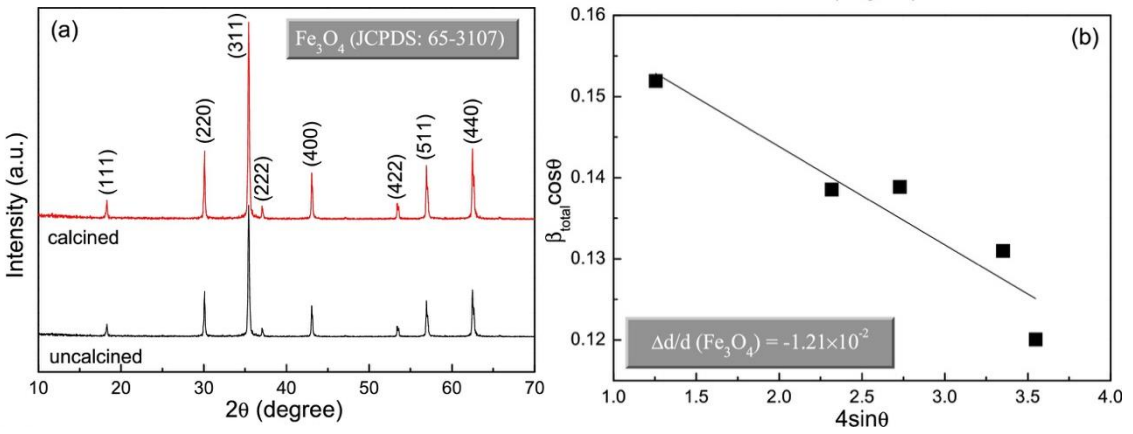

Figure 2. (a) Fe3O4 PSCS before/ after calcination shown by XRD patterns; (b) Linear adaptation of the XRD peak of uncalcinated Fe3O4 PSCS was done using a Williamson-Hall relationship [19].

2.2.2. Application of Magnetic-responsive Nano-particles in Breast Cancer Treatment

As previously mentioned, medicine provides various treatment options for breast cancer. To be more specific, breast cancer is classified into invasive and rare types, with HR+ and HER2- being the most common. Biopsy and imaging examinations are crucial for diagnosis and treatment. Approximately 80% of patients are eligible for surgical treatment, while conservative treatment is available for histologically rare types [20]. The primary goal for drug delivery to breast cancer tissues is to maximize treatment effectiveness while minimizing drug delivery to non-target sites. Magnetic stimulation-responsive nanoparticles can help achieve this objective. Due to the lack of rejection response from biological tissues towards magnetic fields, the magnetic nanoparticles can interact with an external magnetic field to deliver drugs maximally to specific human tissues [21].

The mechanism of superparamagnetism of magnetic-resposive nanoparticles lays the foundation for two of their major clinical uses in breast cancer treatment: #1 the magnetic hyperthermia drug releasing mechanism driven by superparamagnetic nanoparticles (SPMNPS) and #2 drug targeting mechanism guides by magnetic field [22]. Due to the utilization of these biotechnologies, magnetic stimulation-responsive nanoparticles (MNPS) have made significant advances in cancer imaging and treatment.

Magnetic hyperthermia is a widely used clinical approach for treating tumor lesions [23]. It involves using MNPs to generate targeted nanoscale heating effects on tumors by applying an alternating magnetic field (AMF), which results in localized induction heating. This method offers advantages such as strong penetration, minimal human immune response, and precise targeting since they are small in size to reach most of the human body tissues [24]. To achieve the desired effect, it is important for MNPs to have a high specific absorption rate (SAR) while maintaining a small volume to minimize side effects during clinical treatment [25].

$$SAR = \frac{c\Delta T}{\Delta t} \quad (1)$$

The specific absorption rate (SAR) can be calculated in the equation (1) above, where Δt records the change in time while ΔT records the change in temperature and c stands for the specific heat capacity [26].

Although there are many types of magnetic responsive nanoparticles, currently the most suitable MNPs for magnetic hyperthermia are primarily composed of iron oxide nanoparticles. By utilizing ion oxide nanoparticles that accumulate selectively within breast tumor cells, this innovative approach allows for precise targeting. When subjected to an alternating magnetic field, these nanoparticles

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generate localized heat, effectively damaging or eliminating cancer cells while minimizing harm to surrounding healthy tissue [27]. In research leads by [25], he and his team studied both the advantages and disadvantages of spherical and cubic iron oxide particles in terms of heat transfer, and concluded that cubic particles have a higher heating efficiency compared to spherical nanoparticles. Figure (2) shows a comparison of the SAR data for two nanoparticles of the same size (20nm) but different shapes: spherical and cubic. Under the same experimental conditions, the data shows that the SAR value of the cubic nanoparticles is nearly 20% higher than that of the spherical nanoparticles [25].

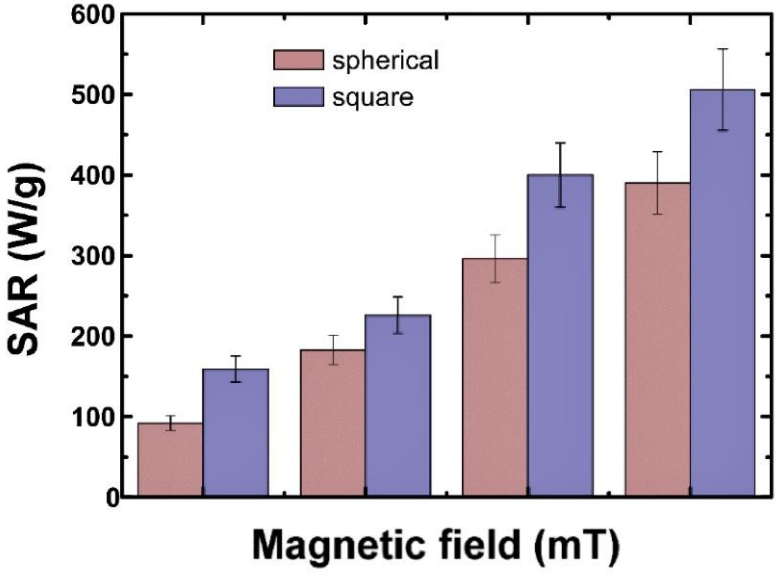

Figure 3. Comparison of SAR data between two types of iron oxide nanoparticles with similar composition but different shapes [25].

According to research, it has been demonstrated that cubic-shaped iron oxide nanoparticles exhibit superior magnetic heating efficiency, making them a significant advancement in the field of magnetic hyperthermia for cancer treatment [28]. These highly targeted and thermally conductive iron oxide nanoparticles can specifically heat tumor tissue through magnetic hyperthermia, effectively killing breast cancer cells and preventing the spread of cancer cells from the source [27].

3. Endogenous Stimuli-Responsive Drug Delivery System

In addition to exogenous stimuli, endogenous stimuli are more often targeted by researchers targeting drug delivery systems. To date, researchers have successfully devised numerous nano drug carriers that facilitate prolonged release of drugs. These nanoparticles release drugs in response to various endogenous stimuli, such as changes in pH, enzymes, PDA, ROS, temperature or two or more combinations of them.

3.1. PH-responsive Drug Delivery System

3.1.1. Mechanism of pH-responsive Drug Delivery System

pH-responsive nanoparticles are currently one of the most popular study domains. This is mainly owing to the fact that the pH of the environment near each tissue in the human body changes, for example, the pH of the stomach is approximately 1, but the pH of surrounding tissues closer to the stomach increases as they proceed away from the stomach [29]. In normal human blood vessels, the pH is usually around 7.2 to 7.4, but around tumor cells, the pH appears weakly acidic (with a pH of around 5.5 to 6.0). Based on the characteristics of this difference between the normal cells and the pathological cells, researchers

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have designed many nano-carriers such as liposomes, high molecular polymers, hydrogels, etc. They can be hydrolyzed or swelled in a slightly acidic environment to achieve the targeted drug release effect.

3.1.2. pH-responsive Drug Delivery System for triple- negative breast cancer

As one of the most difficult types of breast cancer to cure, triple negative breast cancer has always been a difficulty that drug developers are trying to overcome. However, researchers have created a novel course of treatment using pH-responsive targeted drug delivery systems. As early as [30] proposed using PCNDXR to treat triple- negative breast cancer, confirming the feasibility of using the PCN platform as a pH- responsive carrier [30]. Since then, the potential of pH-responsive drug delivery systems in the management of triple-negative breast cancer has been extensively studied by researchers. Furthermore, [31] designed an Artemisin (ART) dimer. This dimer can be carried on liposomes and hydrolyze under acidic conditions to release drugs. And the effect of this drug on triple negative breast cancer has been confirmed through experiments [31]. Chaudhari D and colleagues designed a pH sensitive liposome loaded with paclitaxel (PTX) and validated its heterogeneous release in acidic and medium environments, opening up an effective and safe formula [32]. [33] used pH responsive bonds to connect Doxorubicin (Dox) and aminoglutethimide (AGM), creating a family of pH responsive poly-L-glutamic acid (PGA) composite conjugates. The utilization of pH responsive bonds was then used to complete a significant amount of drug production.

In addition, scientists are also attempting to improve drugs that target other cancers to meet the specificity of triple negative breast cancer. In 2016, there was an attempt to apply DNA damaging platinum-based compounds, which had been proven to have specific effects on cancer cells, to the treatment of TNBC [34]. In 2019, [35] used pH responsive degradable Zeolitic iminazole frameworks (ZIFs) loaded with DOX as a nanodrug delivery system and successfully managed to survive 80% of experimental mice after 40 days [33]. What is more, pH responsiveness can easily combine with other stimuli to form multi-responsive molecules. In a 2017 work, [36] and his research team synthesized a poly (AA-b-NIPAAm) copolymer (PAA-b-PNIPAAm). This copolymer is responsive to stimuli including temperature, pH, and enzymes. In 2021, a new type of targeted nanoultrasound contrast-enhanced nanobubbles was prepared, which can not only consciously accumulate in acidic tumor cells, but also be further treated by introducing photodynamic therapy through ultrasound contrast agents [37].

The above methods have been effective in killing cancer cells, but in clinical practice, their safety and efficiency still need to be confirmed.

3.2. Enzyme-responsive Drug Delivery System

3.2.1. Mechanism of Enzyme-responsive

Enzymes are another factor of great concern. Enzymatic-responsive materials are usually very beneficial, mainly because enzymes play a central role in cellular regulation and activity. The secretion of enzymes has precise spatial and temporal control, and the structural characteristics of enzymes make them highly specific to certain substrates. Therefore, in terms of accuracy, enzyme-responsive drug delivery system is more advantageous compared to other endogenous stimuli. Enzymatic reactive nano-carriers can protect drugs from degradation during transportation and selectively release drugs within tumors. Research has identified that compared to normal cells, multiple enzymes secrete more and have higher concentrations in tumor cells and tumor related cells, such as endothelial cells and macrophages.

3.2.2. Enzyme-responsive Drug Delivery System for Triple- negative Breast Cancer (TNBC)

TNBC lack the expression of estrogen receptor (ER), progesterone receptor (PR) and human epidermal growth factor receptor 2 (Her 2) [38]so it is difficult to achieve the same targeted treatment as other breast cancers. But scientists still find some specific enzymes that can serve as target sites. Renoux B and colleagues prepared a method for selectively releasing the potential monomethylauristatin E in the tumor microenvironment of TNBC, targeting the extracellular vesicles of tumors β-Glucuronidase is a drug delivery system that responds to drug release. The therapeutic efficacy of this intervention has been

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demonstrated in experimental studies with mice [37]. [39] synthesized and characterized an enzyme responsive peptide (MSN-AP-FA-PEP-S-Sn) through physical and chemical techniques. In repeated experiments, this silica-based nanoparticle showed targeted diagnostic ability, which can effectively inhibit tumor growth and reduce liver and kidney toxicity. [40] also utilized higher concentrations of β-Glucuronidase prepared tubulin destabilizer prodrug 17a. This prodrug exhibits high selectivity towards cancer cells pretreated with β-glucuronidase and can promote the specific release of highly efficient but systemic toxic tubulin destabilizer. In mouse experiments, it has also been confirmed that this prodrug can maintain high efficacy of tubulin destabilizer without causing significant damage to organs.

4. Challenges and Future Directions

Overall, despite the substantial preclinical evidence supporting the potential of stimuli-responsive drug delivery systems for targeted modulation of therapeutic effects, there remains a paucity of clinical trials, particularly in the context of triple-negative breast cancer, a therapeutic domain that currently faces limited treatment alternatives. It is imperative to ensure the translation of in-vitro and in-vivo methodologies into clinical research, with a primary focus on the preservation of safety and adherence to ethical considerations.

4.1. Challenges and Future Directions of USRDDS

One of the primary concerns of the USRDDS pertains to the lowered drug encapsulation capacities presented by ultrasonic sensitive nanoparticles. More precisely, it has been observed that materials with higher loading performance tend to possess comparatively lower levels of responsiveness. Therefore, it is necessary to employ higher frequencies of ultrasound for the purpose of releasing drugs from said materials. It is important to note that this process may potentially result in the disturbance of adjacent tissues. The development of nanoparticles with high drug encapsulation capacities and the ability to be triggered by low ultrasonic energies might yield significant advantages. Moreover, it is imperative for ultrasound responsive nanoparticles to focus on the application of degradable materials in order to ensure both efficacy and the mitigation of any detrimental effects.

4.2. Challenges and Future Directions of Magnetic-responsive Nanoparticles

Magnetic hyperthermia could offer a minimally invasive and targeted therapeutic option, complementing traditional treatments like surgery, chemotherapy, and radiation. While further research and clinical trials are necessary to fully establish its efficacy and safety, magnetic hyperthermia represents a compelling avenue for advancing the treatment landscape for breast cancer patients.

4.3. Challenges and Future Directions of Endogenous Stimulus Responsive DDS

The advantage of endogenous stimulation over exogenous stimulation is that its stimulating factors are spontaneously generated by cancer cells and do not require additional human assistance, making it a less energy consuming and more convenient approach. Nevertheless, the intricate and dynamic internal microenvironment of the human body contributes to variations in physiological conditions between individuals, hence posing additional challenges for the targeted therapy of cancer cells by endogenous stimulus response drug delivery systems. The pH-responsive DDS mentioned above requires nano-carriers to be able to unload drugs in slightly acidic environments near cancer tissue, while also protecting drugs in neutral blood and strongly acidic digestive tract environments. This puts forward strict requirements for researchers of biomaterials. To develop enzyme-responsive drug delivery systems (DDS), it is imperative to conduct preliminary investigations on enzymes that exhibit a substantial concentration disparity between normal and cancer cells throughout cellular processes. Subsequently, efforts should be directed towards identifying responsive molecules for these enzymes and establishing their connection to nanocarriers. This is not only a high demand for material researchers, but also a high demand for tumor researchers. What is more, the endogenous stimuli in the body vary depending on the type of cancer and individual, and not all drugs can be used as a single carrier. This is

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the reason why most endogenous stimulation drug delivery systems are currently difficult to use in clinical practice, and it is a problem that scientific researchers need to solve.

5. Conclusion

The above content describes in detail the principle and current situation of two exogenous stimuli (ultrasound, magnetic field) and two endogenous stimuli (acid-base, enzyme) as drug response targets in targeted drug delivery systems for breast cancer treatment. This review provides a brief insight into the contributions made by scientists in these areas over the past decade. Although people have come a long way in developing new responsive nano-drug delivery systems. Yet, the findings in most laboratories are still far from clinical use. It is anticipated that this analysis will facilitate a rapid comprehension of the subject matter for readers, while also encouraging further investment by researchers to support the advancement and practical application of these studies.

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Factors influencing the survival of cervical cancer

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Abstract. More studies on the effect of socioeconomic (SES) factors on the survival of cervical cancer appeared while lacking comprehensive studies on both SES factors and traditional survival-influencing factors. Our study included baseline factors, SES factors, tumor features, and therapy. It thoroughly analyzed their influences and interactions on the survival of the cervical cancer population in the US. A total of 28471 cases from the Surveillance, Epidemiology, and End Results (SEER) database were adapted in our study, using the Kaplan-Meier method and log-rank test as univariate analysis and the Cox proportional hazard model as multivariate analysis. Surgery, marriage, younger age, and higher income were found to have improving effects on survival, and chemotherapy and radiotherapy did the same after adjusting baseline and SES factors. Hispanics have the best survival, while Blacks have the worst. Survival in metropolitan areas decreased as the population increased, while the opposite appeared in nonmetropolitan areas.

Keywords: Cervical cancer, survival analysis, SEER.

1. Introduction

Cervical cancer is a significant and pervasive malignancy afflicting women worldwide. According to the International Agency for Research on Cancer's latest report, the incidence and mortality rate in 2020 is 13.3% and 7.3% among women affected by cervical cancer, positioning it as the fourth leading cause of female mortality due to cancer [1]. Cervical cancer is prevalent in middle- to low-income countries, where limited access to screening and vaccines compounds its prevalence [2]. Non-genetic factors influencing cervical cancer, such as human papillomavirus (HPV) infection, smoking, C. trachomatis infection, and oral contraceptive use, have garnered attention [3]. Within all non-genetic factors, human papillomavirus infection is the predominant cause of most cervical cancer cases, with HPV 16/18 contributing 71. The most common histopathological type of cervical cancer is squamous cell carcinoma, comprising over 80% of cases, followed by adenocarcinoma, and squamous cell carcinoma is the predominant histopathological type in cervical cancer [4]. The clinical promise of alternating chemoradiotherapy for high-risk cervical cancer patients, including those with advanced nodal disease, is underscored by its effective outcomes and manageable toxicity [1].

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Besides, social factors, including race and ethnicity, socioeconomic status, access to health care, insurance status, and others, have been studied for their impacts on the incidence and prognosis of cervical cancer [3, 5, 6]. A study on area-specific economic factors found that Medicare coverage and healthcare expenditure significantly influence the surgery coverage of cervical cancer in the U.S., thus leading to differences in the incidence and mortality among different districts [7]. Black and Hispanic or Latina women were said to have a higher probability than White women of developing cervical cancer and dying from it [8]. Black women were also found to have the highest incidence rate of cervical squamous cell carcinoma and the highest mortality in adenocarcinoma, compared with the higher risk of adenocarcinoma in Hispanic and White women [9]. Patients with lower education, low annual household income, and window/divorce status were more likely to delay seeking medical care, leading to later stages of diagnosis [10]. A study from Brazil found fertility to be positively related to mortality [11]. Socioeconomic status (SES) is a measurement of the overall social standing of an individual, including social factors such as income, education, and occupation; lower SES was associated with poorer outcomes in cancers [12]. Disparities in cervical cancer survival related to health insurance coverage and SES were discovered [5]. Individuals with lower economic status and living in regions with less healthcare expenditure were less likely to access HPV vaccines, causing higher exposure to cervical cancer [13]. Lower insurance status also caused less access to outcome-improving therapies such as brachytherapy [14]. Briefly, social factors have a confounding influence on different aspects of the whole procedure of cervical cancer, including screening, diagnosis, treatment, and prognosis.

In recent years, increasing studies have attempted to include the analysis of lacking social datasets [15]. A study from 2006 on the importance of SES for survival after cervical cancer is diagnosed and a combined conceptual model of social factors(combining SES indicator, healthcare provider factors, individual factors, and shorter cervical cancer survival time) is used in the study to comprehensively analyze the impact of social factors [10]. Our study referred to the conceptual model but used more recent data (until 2020). However, studies like this often completely shifted their focus to the aspect of social or socioeconomic factors and overlooked the importance of keeping the original approach to analyzing cervical cancer. This trend of focusing on one group of factors with similar characteristics or employing the simple standard analysis of cervical cancer fails to contribute to a new nuanced understanding of survival rates of cervical cancer among women on a national basis.

This article takes a comprehensive approach to observing the relationships between cervical cancer and various determinants of different risk levels. The study's principal objective is to conduct a survival analysis to determine how a combination of factors could influence the risks of cervical cancer and women's health. Specifically, the scope of the data analyzed encompasses a wide range of potential social factors, epidemiological factors, and tumor characteristics identified. Our findings will provide a basis for additional considerations for various determinants leading to a better understanding of women's health.

2. Methodology

2.1. Study population and data selection

Data from the Surveillance, Epidemiology, and End Results (SEER) registries were used for cervical cancer analysis. SEER compiles and releases data on cancer occurrence and survival rates obtained from cancer registries among the U.S. population. The datasets are gathered for each instance of cancer reported across 22 geographical regions in the U.S. (SEER). The database selected for this study is the Incidence – SEER Research Data from 17 registries (SEER 17) in the years ranging from 2000 to 2020. According to the 2020 census, SEER 17 covers approximately 26.5% of the U.S. population [16].

To ensure the specificity and significance of the results, only cases signifying “Cervix Uteri” according to the site recode ICD-O-3 with complete records of survival months, cause of death classifications, and follow-up details were included. The cases diagnostic confirmation is only limited to positive histology, and cases reported without autopsy or death certificate were excluded. After

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excluding every case with missing records in each covariate and under 15 in age, 28471 cases were included in our research.

2.2. Cervical cancer survival outcome

The main survival outcome of this study is the survival class data that combines both SEER 17 cause-specific death classifications and survival months. Cause-specific death classifications represent cases' survival status, with dying from cervical cancer as positive results and other as negative. Individuals with negative cause-specific death classifications were treated as censored data points. Survival months are calculated as those measured after cancer diagnosis until death or last follow-up up to December in 2020 [17].

2.3. Covariates

Median household income and rural-urban continuum code were chosen as SES factors available in the SEER database. The county attribute variables are computed utilizing the 5-year data files of the American Community Survey (ACS). Detailed technical documentation for the ACS files during this period is available for access through the United States Census Bureau [18].

Other factors relevant to the influence of cervical cancer survival analysis are grouped into three categories based on characteristics: the therapy group, the tumor feature group, and the baseline factors group. The therapy group includes radiation recode, chemotherapy recode, and surgery recode. Utilization of each therapy was identified as yes or no. The tumor feature group consists of the histology recode of the tumor (adenocarcinoma(ADC), squamous cell carcinoma(SCC), epithelial, other types), primary site (Endocervix, Exocervix, Overlapping lesion of cervix uteri, Cervix uteri), combined summary stage (Distant, In situ, Localized, Regional, Unknown/unstaged), grade recode (Grade I/II/III/IV), and record number record(1, 2, equal or more than 3). The baseline group contains age code, race, and origin recode (Hispanic, American Indian/Alaska Native, Asian or Pacific Islander, Black, White), and marital status at diagnosis (Divorced, Married including common law, Separated, Single never married, Widowed).

For each covariate treated as a categorical variable, groups with the most numbers were chosen as reference groups while studying, like White for race, married including common law for marital status, ADC for histology, and Cervix uteri for primary site, except for Counties in metropolitan areas ge 1 million pop for continuum code, Grade I for grade, and no for each therapy.

2.4. Statistical analysis:

Univariate analysis was conducted by using the Kaplan-Meier method to estimate the survival condition and assess the impact on survival of each covariate. Kaplan-Meier curves were generated to show the changes in survival over time, and 1-, 3- and 5-year survival rates were calculated. The log-rank test was used to compare the differences between distant groups in each covariate. Cox regression model was also used to identify the influence of each covariate on survival.

Covariates statistically significant in the univariate cox regression model were included in multivariate analysis. The Cox proportional hazard model was employed to analyze the net impact on survival of all covariates included separately, and adjusting different factors to find the relationship between each factor: adjusted the baseline (age, marital status at diagnosis, race) individually and respectively with socioeconomic (median household income, rural-urban continuum code), histology recode, grade and stage, tumor feature, therapy; adjusted both baseline and socioeconomic respectively with histology, grade and feature, tumor features, and therapies; and we have adjusted all factors into one Cox proportional hazard model to examine the net relationship of all factors. The hazard ratio (HR) was calculated in every Cox model. All the statistical computations mentioned above were executed using R statistical software (version 3.6.1, http://www.R-project.org/). A less than 0.05 in p-value was denoted as statistically significant in this study.

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3. Results

Table 1. Counts and percentages of each group of the 28471 cervical cancer patients included from the SEER 17 database from 2000 to 2020.

Characteristic	N=28,471
Year of diagnosis	2,011.0 (2,007.0, 2,014.0)
Age recode with <1 year olds and 90+	16 (<0.1%)
15-19 years	
20-24 years	230 (0.8%)
25-29 years	1,181 (4.1%)
30-34 years	2,501 (8.8%)
35-39 years	3,392 (12%)
40-44 years	3,842 (13%)
45-49 years	3,667 (13%)
50-54 years	3,299 (12%)
55-59 years	2,791 (9.8%)
60-64 years	2,347 (8.2%)
65-69 years	1,796 (6.3%)
70-74 years	1,261 (4.4%)
75-79 years	940 (3.3%)
80-84 years	645 (2.3%)
85-89 years	383 (1.3%)
90+ years	180 (0.6%)
Race and Origin Recode NHW, NHB, NHAIAN, NHAPI, Hispanic	
Hispanic All Races	6,652 (23%)
Non-Hispanic Asian or Pacific Islander	210 (0.7%)
Non-Hispanic Asian or Pacific Islander	2,665 (9.4%)
Non-Hispanic Black	3,639 (13%)
Non-Hispanic White	15,305 (54%)
Marital Status at Diagnosis	
Divorced	3,556 (12%)
Married Including Common Law	12,807 (45%)
Separated	645 (2.3%)
Single Never Married	8,499 (30%)
Unmarried or Domestic Partner	113 (0.4%)
Widowed	2,851 (10%)
Median Household Income Inflation adj to 2021	
< $35,000	335 (1.2%)
$35,000-$39,000	565 (2.0%)
$40,000-$44,999	986 (3.5%)
$45,000-$49,999	1,591 (5.6%)
$50,000-$54,999	1,638 (5.8%)
$55,000-$59,999	2,469 (8.7%)
$60,000-$64,999	2,815 (9.9%)
$65,000-$69,999	5,724 (20%)
$70,000-$74,999	2,970 (10%)
$74,999+	9,358 (33%)
Rural-Urban Continuum Code	
Counties in metropolitan areas ge 1 million pop	17,186 (60%)

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Table 1. (continued).

Counties in metropolitan areas of 250,000 to 1 million pop	5,783 (20%)
Counties in metropolitan areas of It 250 thousand pop	2,170 (7.6%)
Nonmetropolitan counties adjacent to metropolitan area	1,886 (6.6%)
Nonmetropolitan counties not adjacent to a metropolitan area	1,446 (5.1%)
Behavior Recode for Analysis	
Malignant	28,471 (100%)
Histology Recode – Broad Groupings	
SCC	18,777 (66%)
ADC	6,602 (23%)
Epithelial	1,945 (6.8%)
Other Subtype	1,147 (4.0%)
Primary Site – Labeled	
C53.9-Cervix Uter	21,480 (75%)
C53.0-Endocervix	5,847 (21%)
C53.1-Exocervix	582 (2.0%)
C53.8-Overlapping lesion of cervix uteri	562 (2.0%)
Combined Summary Stage 2004+	
Distant	3,948 (14%)
Localized	12,874 (45%)
Regional	11,184 (39%)
Unknown/Unstaged	465 (1.6%)
Grade Recode thru 2017	
Well differentiated; Grade I	11,976 (42%)
Moderately differentiated; Grade II	11,432 (40%)
Poorly differentiated; Grade III	1,032 (3.6%)
Undifferentiated; anaplastic; Grade IV	4,031 (14%)
Reason No Cancer-Directed Surgery	16,889 (59%)
Radiation Recode	16,785 (59%)
Chemotherapy Recode yes, no/unk	
No/Unknown	14,039 (49%)
Yes	14,432 (51%)
Record Number Recode	
>=3	193 (0.7%)
1	26,744 (94%)
2	1,534 (5.4%)
SEER Cause-Specific Death Classification	
Alive or Dead of other Cause	19,493 (68%)
Dead Attributable to this Cancer dx	8,978 (32%)
Survival Months	61 (21, 119)
Patient ID	34,901,095 (22,091,334, 49,730,636)
[1]Median (IQR); n (%)	

Our sample size includes 28,471 cases, excluding subjects with missing information.

Table 1 shows the distribution of each covariate in our data. The data contains age, race, marital status, income, rural-urban continuum code, histology recode, cancer stage, primary sites, grade recode, record number, surgery, radiation recode, and chemotherapy recode. The categories that include the most cases of the groups are age ranging from 40 to 44 years (13%), non-Hispanic white population (54%), married including common law (45%), earning of $75,000+ (33%), counties in metropolitan areas per 1 million population (60%), SCC histology recode (66%), Cervix uteri primary site (75%),

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localized summary stage (45%), grade III recode (40%), reason no cancer-directed surgery (59%), record number 1 (94%), alive or death of other cause (68%). Among most covariates, we have very equivalent distributions among different groups.

Table 2. 1-,3-,5-year overall survival rate of the 28471 cervical cancer patients included from the SEER 17 database in percentage calculated by Kaplan-Meier method and the 95% confidence interval.

Year	Survival rate(95%CI)
1-year	0.87(0.87, 0.88)
3-year	0.74(0.73, 0.74)
5-year	0.69(0.69, 0.70)

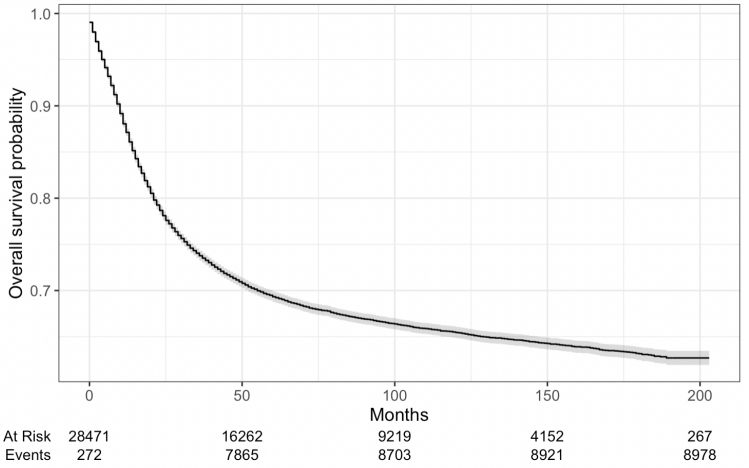

Figure 1. Overall 200-month Kaplan-Meier survival curve of the 28471 cervical cancer patients included from the SEER 17 database from 2000 to 2020.

We have calculated the survival rates for 1-year, 3-year, and 5-year shown in Table 2. We would like to note our 5-year survival rate as it is the common timeframe for survival rates. Figure 1 is the Kaplan-Meier curve for the overall survival results.

Table 3. Univariable and Multivariable Hazard Ratio Analyses for Associations Between Age, Race, Marital Status, Income, Continuum Code, Tumor Feature, and All.

Covariates	Unadjusted model HR(95% CI)	p-value	Adjusted model race, marital status, income, continuum code HR(95% CI)	p-value	Adjusted model race, marital status, income, continuum code, tumor feature HR(95% CI)	p-value	Adjusted model all HR(95% CI)	p-value
Age	1.33(1.31, 1.35)	<0.01	1.33(1.31, 1.35)	<0.01	1.16(1.14, 1.18)	<0.01	1.11(1.09, 1.12)	<0.01

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Table 4. Univariable and Multivariable Hazard Ratio Analyses for Associations Between Race, Age, Marital Status, Income, Continuum Code, and All.

Covariates	Unadjusted model HR(95% CI)	p-value	Adjusted model race, marital, income, continuum code HR(95% CI)	p-value	Adjusted model race, marital, income, continuum code, tumor feature HR(95% CI)	p-value	Adjusted model all HR(95% CI)	p-value
White	ref	ref	ref	ref	ref	ref	ref	ref
American Indian/Alaska Native	1.11(0.88, 1.40)	0.4	1.16(0.92, 1.47)	0.2	1.14(0.90, 1.44)	0.28	1.15(0.91, 1.45)	0.25
Asian or Pacific Islander	0.94(0.87, 1.02)	0.12	0.92(0.85, 0.99)	<0.05	0.96(0.89, 1.04)	0.28	0.93(0.86, 0.95)	0.05
Black	1.47(1.38, 1.55)	<0.01	1.31(1.24, 1.39)	<0.01	1.26(1.19, 1.34)	<0.01	1.12(1.06, 1.20)	<0.01
Hispanic	0.91(0.86, 0.96)	<0.01	0.93(0.88, 0.99)	<0.01	0.94(0.89, 1.00)	<0.01	0.89(0.85, 0.95)	<0.01

Table 5. Univariable and Multivariable Hazard Ratio Analyses for Associations Between Marital Status, Age, Race, Income, Continuum Code, and All.

Covariates	Unadjusted model HR(95% CI)	p-value	Adjusted model race, marital, income, continuum code HR(95% CI)	p-value	Adjusted model race, marital, income, continuum code, tumor feature HR(95% CI)	p-value	Adjusted model all HR(95% CI)	p-value
Married including common law	ref	ref	ref	ref	ref	ref	ref	ref
Divorced	1.45(1.36, 1.54)	<0.01	1.28(1.20, 1.37)	<0.01	1.28(1.20, 1.37)	<0.01	1.13(1.06, 1.21)	<0.01
Separated	1.39(1.21, 1.59)	<0.01	1.38(1.20, 1.58)	<0.01	1.37(1.19, 1.57)	<0.01	1.18(1.03, 1.36)	<0.05
Single never married	1.38(1.31, 1.45)	<0.01	1.44(1.37, 1.52)	<0.01	1.44(1.37, 1.51)	<0.01	1.22(1.16, 1.28)	<0.01
Widowed	2.20(2.06, 2.34)	<0.01	1.25(1.17, 1.35)	<0.01	1.24(1.16, 1.33)	<0.01	1.20(1.12, 1.29)	<0.01

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Table 6. Univariable and Multivariable Hazard Ratio Analyses for Associations Between Therapy, Age, Race, Marital Status, Income, Continuum Code, and All.

Covariates	Unadjusted model HR(95% CI)	p-value	Adjusted model age, race, marital status HR(95% CI)	p-value	Adjusted model age, race, income, continuum code HR(95% CI)	p-value	Adjusted model all HR(95% CI)	p-value
Surgery(1 to 0)	0.21(0.20, 0.22)	<0.01	0.25(0.23, 0.26)	<0.01	0.25(0.23, 0.26)	<0.01	0.42(0.40. 0.44)	<0.01
Radiotherapy(1 to 0)	2.21(2.10, 2.31)	<0.01	0.80(0.75, 0.86)	<0.01	0.80(0.75, 0.85)	<0.01	0.81(0.76, 0.86)	<0.01
Chemotherapy(1 to 0)	2.27(2.17, 2.37)	<0.01	1.24(1.16, 1.32)	<0.01	1.24(1.17, 1.32)	<0.01	0.68(0.64, 0.72)	<0.01

Table 7. Univariable and Multivariable Hazard Ratio Analyses for Associations Between Income, Age, Race, Marital Status, Continuum Code, Histology, and All.

Covariate	Unadjusted model HR(95% CI)	p-value	Adjusted model age, race, marital status HR(95% CI)	p-value	Adjusted model age, race, marital status, code, histology HR(95% CI)	p-value	Adjusted model all HR(95% CI)	p-value
Income	0.92(0.91, 0.94)	<0.01	0.94(0.92, 0.95)	<0.01	0.92(0.90, 0.94)	<0.01	0.95(0.93, 0.97)	<0.01

Table 8. Univariable and Multivariable Hazard Ratio Analyses for Associations Between Continuum Code, Age, Race, Marital Status, Income, and All.

Rural Urban Continuum Code	Unadjusted model HR(95% CI)	p-value	Adjusted model age, race, marital HR(95% CI)	p-value	Adjusted model age, race, marital, income HR(95% CI)	p-value	Adjusted model all HR(95% CI)	p-value
Counties in metropolitan areas ge 1 million pop	ref	ref	ref	ref	ref	ref	ref	ref
Counties in metropolitan areas of 250,000 to 1 million pop	1.00(0.95, 1.06)	<0.01	1.02(0.97, 1.08)	<0.01	0.97(0.92, 1.03)	<0.01	0.99(0.93, 1.04)	<0.01
Counties in metropolitan areas of It 250 thousand pop	1.03(0.95, 1.11)	<0.01	1.04(0.85, 0.99)	<0.01	0.91(0.84, 1.00)	<0.01	0.90(0.82, 0.98)	<0.01

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Table 8. (continued).

Nonmetropolitan counties adjacent to metropolitan area	1.12(1.03, 1.21)	<0.01	1.10(1.01, 1.19)	<0.01	0.91(0.82, 1.00)	<0.01	0.86(0.78, 0.95)	<0.01
Nonmetropolitan counties not adjacent to a metropolitan area	1.14(1.04,1.25)	<0.01	1.15(1.95,1.26)	<0.01	0.92(0.82, 1.03)	<0.01	0.98(0.88, 1.09)	<0.01

Table 9. Univariable and Multivariable Hazard Ratio Analyses for Associations Between Record Number Recode, Age, Race, Marital Status, Histology, Primary Site Recode, Stage Recode, Continuum Code, Grade, and All.

Covariate	**Unadjusted model HR(95% CI)**	**p-value**	**Adjusted model age, race, marital status, histology, site, stage, code, grade, records HR(95% CI)**	**p-value**	**Adjusted model all HR(95% CI)**	**p-value**
Record Number Recode	1.28(1.20, 1.37)	<0.01	1.11(1.03, 1.19)	<0.01	1.11(1.03, 1.19)	<0.01

Table 10. Univariable and Multivariable Hazard Ratio Analyses for Associations Between Primary Site, Age, Race, Marital Status, Histology, Primary Site, Stage Recode, Grade, Record Number Recode, and All.

Primary Site	**Unadjusted model HR(95% CI)**	**p-value**	**Adjusted model age, race, marital statis, histology, site, stage, grade, records HR(95% CI)**	**p-value**	**Adjusted model race, marital, income, continuum code, tumor feature HR(95% CI)**	**p-value**	**Adjusted model all HR(95% CI)**	**p-value**
C53.9-Cervix Uter	ref	ref	ref	ref	ref	ref	ref	ref
C53.0-Endocervix	0.61(0.58,0.65)	<0.01	0.77(0.72, 1.01)	<0.01	0.78(0.73, 0.83)	<0.01	1.13(1.06, 1.21)	<0.01
C53.1-Exocervix	0.67(0.56,0.79)	<0.01	0.86(0.73,1.01)	<0.01	0.86(0.73,1.01)	<0.01	0.88(0.75,1.04)	<0.05
C53.8-Overlapping lesion of cervix uteri	0.87(0.75,1.01)	0.073	0.95(0.82,1.11)	0.53	0.95(0.82,1.11)	0.55	1.06(0.91,1.24)	0.43

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Table 11. Univariable and Multivariable Hazard Ratio Analyses for Associations Between Histology, Age, Race, Marital Status, Primary Site, Stage, and All.

Histology	Unadjusted model HR(95% CI)	p-value	Adjusted model age, marital statis, histology HR(95% CI)	p-value	Adjusted model, age, race, marital status, histology, site, stage HR(95% CI)	p-value	Adjusted model all HR(95% CI)	p-value
ADC	ref	ref	ref	ref	ref	ref	ref	ref
SCC	0.69(0.66,0.73)	<0.01	0.79(0.75,0.83)	<0.01	1.18(1.11,1.26)	<0.01	1.26(1.18,1.34)	<0.01
Epithelia	1.63(1.52,1.75)	<0.01	1.76(1.64,1.89)	<0.01	1.35(1.25,1.45)	<0.01	1.41(1.31,1.52)	<0.01
Other Subtypes	1.23(1.12,1.35)	<0.01	1.27(1.15,1.40)	<0.01	1.46(1.32,1.61)	<0.01	1.88(1.70,2.08)	<0.01

Table 12. Univariable and Multivariable Hazard Ratio Analyses for Associations Between Grade Recode, Age, Martial Status, Stage Recode, Histology, Primary Site, Record Number, and All.

Grade Recode Thru 2017	Unadjusted model HR(95% CI)	p-value	Adjusted model age, marital statis, grade, stage HR(95% CI)	p-value	Adjusted model, age, marital status, grade, stage, histology, stie, records HR(95% CI)	p-value	Adjusted model all HR(95% CI)	p-value
Well differentiated; Grade I	ref	ref	ref	ref	ref	ref	ref	ref
Moderately differentiated; Grade II	2.32(2.11,2.54)	<0.01	14.65(13.67,15.69)	<0.01	1.43(1.3,1.58)	<0.01	1.48(1.34,1.63)	<0.01
Poorly differentiated; Grade III	4.12(3.76,4.51)	<0.01	4.18(3.92,4.45)	<0.01	1.93(1.75,2.12)	<0.01	1.97(1.79,2.17)	<0.01
Undifferentiated; anaplastic; Grade IV	5.46(4.83,6.18)	<0.01	5.68(4.90,6.59)	<0.01	1.99(1.76,2.26)	<0.01	2.06(1.81,2.34)	<0.01

Age had a significant effect on survival under either adjustment ($p<0.01$); the older the worse the prognosis (Table 3).

Across all adjustments, Hispanics had the best survival, and HR(<1) is almost unaffected by various adjustments. Black consistently had the worst survival, but HR(>1) gradually decreased with the increase of adjustment. After adjusting for age and marital status, Asian or Pacific Islander was significantly better than White, but this advantage disappeared after further adjustment for SES (Table 4).

Survival of different marital status are significantly different ($p<0.01$). Among them, the survival status of married, including common law, was significantly better than others under all adjustments. Without any adjustment, the survival of the divorced group was significantly worse than that of the other

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groups (HR=2.20) but was similar to that of the other non-married groups after adjusting for age. There was no significant change in the separated and divorced group after age, race, and SES were adjusted. HR decreased in all groups after balancing tumor features and therapy (Table 5).

All therapy factors significantly affected survival ($p<0.01$). Patients who underwent surgery had significantly better survival than those who did not (HR=0.21), but HR(<1) increased slightly after further adjusting the tumor feature. Before any adjustment, radiotherapy had a significant negative effect on survival (HR=2.21). However, it changed to a positive effect after balancing baseline (HR=0.80), and there was no significant difference after further balancing SES and tumor features. Chemotherapy had a significant negative effect on survival before any adjustment (HR=2.27) and after balancing baseline and SES (HR=1.24), but the effect became positive after balancing tumor features (HR=0.68)(table 6).

According to Table 7, after adjustments, high income's positive influence on survival decreases. After additionally adjusting code and histology, high income's advantage in survival then increased.

Table 8 shows no notable distinctions in the survival rates of Counties in metropolitan areas of 1 million population and Counties in metropolitan areas of 250,000 to 1 million population under, regardless of any adjustments made. Incorporating income data and implementing additional modifications result in improved survival rates for counties in metropolitan areas with a population of 250 thousand. (<0.05). Nonmetropolitan counties adjacent to a metropolitan area have an advantage in survival over Counties in metropolitan areas of 1 million population, except when income and adjustments(age, race, marital status) are considered.

According to Table 9, after adjustments, the negative impact of the record number recode decreases since it is a sequential number of individuals' submissions within SEER.

According to Table 10, there are no statistical differences examined for the C53.8-Overlapping lesion of cervix uteri ($p>0.01$), while the others do in all scenarios ($p<0.01$). C53.9-Cervix uteri has the greatest negative impact on survival (HR<1 for all other factors). The negative impact of the primary site increases while adjusting more factors, but there are no significant differences as more factors are adjusted.

According to Table 11, the Histology Recode significantly impacts survival in all adjustment scenarios (<0.001), while acc has the most negligible negative impact on survival under all scenarios. Epithelia has the worst survival before adjusting more factors, but its survival improves after adjustments. The survival of the subtype gets worse compared to the adc while adjusting more factors. The survival overall decreases as more factors are considered for histology to recode.

Table 12 shows that the grade recode significantly influences survival in all adjustment scenarios ($p<0.01$), and Moderately differentiated; Grade II, Poorly differentiated; Grade III and Undifferentiated; anaplastic; Grade IV have a significantly more significant impact than Well differentiated; Grade I. Grade 4 has the most significant negative impact on survival before adjustment, and grade 2 has an extremely large negative impact on survival (HR=14.56) while adjusting to age, marital status, and stage.

4. Discussion

Age was a significant factor affecting the survival of cervical cancer patients, and survival decreased with increasing age. This is similar to past research by Beavis, and found that Black women's incidence rises as their age increases [19]. Cohen et al. observed age-specific incidence rate differences in their study of racial and pathological differences in the US cervical cancer population using the SEER database, whose incidence was exceptionally high among people above 65-year-old, presumably due to a lack of screening during the past decade [9]. The effect of age on survival is likely to be achieved by influencing other factors, such as older people are more likely to have an advanced stage at diagnosis. For example, in our study, HR changed from 1.33 to 1.16 after adjusting tumor features; and young people are less likely to have their independent insurance [20].

There has always been a great difference in the incidence and mortality of cervical cancer among different races, with one of the largest Black-White mortality gap [21]. A significant racial difference was also observed in our study, with Hispanics having the best survival (HR=0.91) and Blacks having

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the worst (HR=1.47) compared to Whites. This is consistent with previous studies [13, 20]. Cohen et al. found that Hispanics had the highest 5-year survival but also a high incidence, speculating that nativity contributed to the difference in survival among Hispanics [9]. Gomez et al. found that foreign-born Hispanics had a survival advantage over the US-born in the study of the Hispanic cervical cancer population, which may be related to cultural preference [22]. Black people are consistently reported to have the highest mortality rates [8, 23], but a relatively lower incidence rate at present [9]. The reason for the high mortality rate of Black people may be related to insurance-related disparities and insufficient follow-up of abnormal pap test [23, 24], and possibly access to treatment (like Brachytherapy) [14]. In fact, in our study, as every additional part of adjustment is added, Black's HR will produce a certain change. Cohen et. al found in their study that [9] API had the lowest mortality rates. However, interestingly in our study, a similar trend only appeared when balancing the baseline factors, and it disappeared after balancing the SES factors, inferring that it is mainly influenced by SES factors. Observation of changes in HR also suggests that SES is an important factor affecting the disparities of mortality in different races.

We found that people who married in common law had the best survival. This is consistent with previous studies, suggesting that marriage is a factor that promotes the prognosis of cervical cancer [25]. Many studies have shown that unmarried patients are often at later stages when diagnosed [26], which might be related to the higher acceptance of screening in married group [27]. Married patients also have higher treatment compliance, are more willing to accept radical treatment, such as surgery, and are more likely to receive chemotherapy than unmarried patients [25]. The survival advantage in married groups may also come from the emotional and economic support from their children and spouses [28]. In our study, widowed women have the worst living conditions (HR = 2.20), but after the adjustment of age and race, no significant difference was left; this trend also appeared in the divorced group, suggesting the survival disadvantage in these two groups might be mainly related to age. Some studies also proposed that marital status may be affected by cultural background and social status of different races, but more than present evidence is needed to support this conclusion [26]. It is worth mentioning that there was no significant difference in survival between each group before and after adjustment for SES factors, but there was a significant decline after adjustment for tumor feature and therapy, suggesting that the survival advantage of marriage may be more attributed to the latter than to economic status.

In general, it is shown that higher income levels correlate to better survival, even when other variables like continuum code and histology are considered. Previous research, such as the one on socioeconomic status and survival following cervical cancer, has also examined this trend. Rising income level enables higher levels of education, which may explain the variations in survival among cervical cancer patients [6].

Socioeconomic factors such as income and continuum code play significant roles in the survival of patients. Patients in nonmetropolitan areas may experience poorer survival rates initially, but the tendency is reversed with further adjustments, most likely due to the influence of income. Other studies regarding rural and urban areas' effects on cervical cancer make contrasting conclusions. Research on rural–urban and racial/ethnic disparities in invasive cervical cancer incidence in the United States stated that rural counties had a higher incidence of cervical cancer than urban counties at every stage. The comparisons between cervical cancer frequency and age-adjusted incidence are made. However, the amount of adjustment factors put into consideration is limited. Lulu Yu and the team utilized the comparisons between cervical cancer frequency and age-adjusted incidence [29]. Our approach differs as much more comprehensive factors, such as age, marital status, tumor features, therapy, income, and histology, are adjusted to test whether they influence the continuum code's effects on survival. Areas with lower populations and higher income levels may have greater access to healthcare services and treatments. Patients with higher salaries may also be able to afford better treatments, leading to better survival rates. The affordability of healthcare may be a problem in areas with higher populations and lower average income [30].

Nonmetropolitan areas might have poorer survival rates with the possibility of fewer people having access to Medicare, which prevents screening [31]. Because of this, most cases are at an advanced stage

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at the time of diagnosis. After adjusting for tumor features, this disadvantage disappears [32]. This differs from the studies that only included age for adjustments since they will not detect other insights from multiple factors. Metropolitan locations are more likely to have advanced medical facilities and specialists, leading to rapid diagnosis [33]. This could result in more cases being detected at earlier stages, allowing for more effective treatment and improved survival rates. The advantage is evident when tumor characteristics are taken into account for adjustment [34]. Lower SES often correlates with reduced access to public healthcare, including proper cervical cancer screening and timely treatment, leading to increasing HR values after tumor features are adjusted to the Cox regression [15]. Age is associated with both the marital status of females and the affordability of individuals for their treatments, leading to an increasing HR after adjusting age to tumor features [35]. The significance of tumor feature factors (histology recode, primary site, grade recode) was indicated by an increasing HR value after adjusting to SES. The increases in HR values also suggest a significant association between considered histology type and survival month of cervical cancer. The increasing HR value implies that the histology type has an independent and significant influence on the survival month. Biologically, the histological type of the tumor determines how the cancer behaves and responds to different types of treatments [36]. Our research shows that the advanced histology types of epithelia and squamous cell neoplasms may be more resistant to considered treatments like radiation therapy and surgery, causing failure and reduced survival rates [37]. These histologic types are relatively more challenging to detect in an early stage since they are less responsive to screening tests such as Pap smears or do not produce obvious symptoms [38].

In our study, survival was significantly affected by implementing every treatment, including surgery, radiotherapy, and chemotherapy. Surgery was the most significant contributor to survival in multivariate analysis (HR=0.42), consistent with the previous study [19]. For cervical cancer at its early stages, radical surgery is the most recommended treatment, and there are always great benefits for stage I and II, so early detection is a very important [39]. As surgery can only be performed at the early stage, the specific effect of surgery on prognosis will be interfered with by the stage at diagnosis; in our study, the promoting effect of surgery decreases after balancing tumor features. Although there was no significant change in the effect of surgery before and after adjusting baseline factors in our study, some studies have shown that there are differences in the performance of surgery by race like blacks are more likely to receive radiotherapy than surgery [8], and the difference in survival between races before adjusting Hysterectomy is vastly underestimated [19].

Interestingly, chemotherapy and radiotherapy were found to hurt survival without any adjustment. After balancing the baseline factors, radiotherapy changed to have a positive effect, and after further balancing tumor feature, chemotherapy changed to a positive effect. This suggests that both chemotherapy and radiotherapy actually had a positive effect on survival, and radiotherapy is mainly affected by baseline factor, while chemotherapy is mainly affected by tumor feature. Care should be taken to balance these confounding factors while studying the effects of therapy on cervical cancer. Although we found no influence of SES factors on the effect of the therapy, studies have shown that Medicare coverage, health care expenditure, and insurance greatly impact surgery coverage [7, 23]. Advanced-stage concurrent chemotherapy with radiotherapy is the best treatment [37], but in low-middle-income areas the capacity of radiotherapy is limited, leading to the decline of radiotherapy utilization rate and the deterioration of prognosis of cervical cancer patients in these areas [7].

There are several limitations in our study. We had some missing data among many of our covariate groups; some cases have been removed during the final model analysis process, which may lead to bias in the results. Further covariates such as education level and poverty were not included in this model due to lack of access. Furthermore, the changes in the record pattern of the SEER database from 2000 to 2020 might cause differences in results, such as the “unmarried or domestic partner” group in marital status, which refers to other single conditions such as homosexual partners, was only started to be recorded after 2010, so we included it into the “single never married” group in the final analysis. Insurance is also a factor frequently mentioned to influence the survival of cervical cancer [5, 8], but a database containing records of individual insurance conditions additional to other essential basic factors

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(like covariates studied in this study) is hard to found. Further study on a more comprehensive scale is expected.

5. Conclusion

Overall, we examined the relationships between baseline factors, SES factors, tumor features, and therapy and their impact on the survival month in cervical cancer with a large and representative sample size of 28,471 subjects. The distribution of covariates (age, race, marital status, income, rural-urban continuum code, histology recode, cancer stage, primary site, grade recode, record number, surgery, radiation recode, and chemotherapy recode) was assessed within different groupings. Age emerged as a significant factor impacting survival, with older individuals experiencing worse prognosis. The influence of race on survival months was pronounced, with Hispanics showing the best overall survival rate and Black individuals facing the worst rate. Patients who underwent surgery indicated a notably higher survival rate than those who did not. Examining covariates like rural-urban continuum code, record number recode, primary site, and histology recode relationships with the survival month. This study provides valuable insights into the complicated nature of cervical cancer survival, displaying the significance of considering more comprehensive factors. Further cohort studies are expected to verify the results.

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Applications of machine learning to electronic health record data in liver-related disease

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Abstract. Electronic Health Records (EHRs) has gained its increasing significance in modern healthcare as its promising prospects in the application of machine learning. The accumulation of vast clinical data holds potential for repurposing in clinical research such as prediction, diagnosis and prognosis. However, the collection and preparation of EHR data present challenges, primarily due to the inherent incompleteness of the data and the associated privacy and security concerns. To address the issue, text-mining tools based on domain-specific lexicons, data sharing and multiple de-identification methods have been suggested. In terms of methodologies, various machine learning models and algorithms used in EHR data analysis are analyzed, including logistic regression, decision trees, random forests, and natural language processing, each with its unique application scenarios in the healthcare domain. Liver related diseases, including HAV, HBV, HCV and especially Liver Cancer, has affected hundreds of millions of people around the world. The incidence and mortality rates for these diseases are still rising continually. With recent advancements of Machine Learning techniques, such as the attention mechanism and BERT-based embedding, which have shown exceptional results in EHR analysis when applying to liver diseases. While EHRs offer a treasure trove of data for clinical research, the challenges associated with their collection, processing, and analysis cannot be ignored. It underscores the need for robust methodologies and tools to harness the full potential of EHRs while ensuring data integrity and patient privacy. In this paper, we will gather and review the existing application in the realm of liver-related diseases.

Keyword: EHR, machine learning, deep learning, text-mining, embedding, NLP, liver-related disease

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1. Introduction

Liver cirrhosis, the end-stage consequence of various chronic liver diseases, is characterized by the accumulation of fibrillar collagen-rich extracellular matrix[1]. This condition signifies a critical juncture wherein liver function becomes severely compromised, leading to a cascade of potential complications. Ascites and bleeding varices stand out as common and life-threatening consequences of cirrhosis.

The liver, a vital organ with multifaceted functions, plays a pivotal role in metabolism, detoxification, and protein synthesis. Its well-being is crucial for overall health, but a range of liver diseases pose significant global health challenges. Concurrently, the integration of machine learning in healthcare offers promising avenues for improving clinical decision-making and early disease detection.

Hepatitis A virus (HAV) globally causes hepatitis through oral-fecal transmission, leading to liver inflammation and damage. Symptoms range from mild to severe, with untreated cases potentially resulting in cirrhosis, cancer, and death.[2]. Chronic HBV infection affects 350 million globally, causing 1 million annual deaths from liver disease. Immune response often leads to cirrhosis, liver failure, or HCC in 40%. Factors like age, gender, immune/viral status, and external influences impact progression.[3]. HCV infection, a global pandemic with 170 million cases, is 5x more common than HIV-1[4]. Mainly transmitted through blood (needles, drug use), it's widespread, especially in specific populations. Most HCV-infected develop chronic disease, a major reason for liver transplants. [4].

Liver cancer (Hepatocellular Carcinoma or HCC) is a global, aggressive malignancy from liver cells, often linked to pre-existing liver conditions like cirrhosis. It ranks fourth in cancer-related deaths worldwide. By 2025, over 10,000 people are projected to be diagnosed annually [5], with rising incidence and mortality rates, posing significant health and societal challenges.

Fatty liver disease, a metabolic condition with a global prevalence, is marked by the abnormal buildup of fat within liver cells. This condition encompasses two main types: non-alcoholic fatty liver disease (NAFLD) and alcoholic fatty liver disease (AFLD).

With the flourishing development of machine learning, implementations of machine learning models to EHR data have gained increasing significance in modern health care. For their potential to seek latent insights in massive amounts of medical data, machines are enabled to improve clinical decision-making and detect diseases at an early stage. On the flipside, machine learning techniques could also handle unstructured data such as the medical history and the medical prescription of individuals which could also assist the clinical decision-making.

2. EHRs and machine learning

2.1. EHR

EHRs is a digital way to collect and store patients' longitudinal healthcare data, including Medical History, Medications, Test Results, Allergies, Clinical Notes, etc. The primary purpose of EHRs is to provide more accurate and higher-quality healthcare. These data are maintained by healthcare providers [5], such as hospitals and clinics, and collected during routine delivery of patients' health care. Nowadays, EHRs are omnipresent in healthcare, virtually replacing traditional health records. Its widespread use led to the accumulation of substantial clinical data, which is considered can be effectively repurposed for clinical research [6].

2.2. Data Preprocessing

Collection and preparation of EHR data are essential steps prior to employing machine learning, given the data's inherent incompleteness and the significant privacy and security considerations involved. Hence, specific methods are necessary to alleviate their impacts on the machine learning process and potential ethical concern.

Incompleteness stands as a primary challenge to the EHR data collection, arising from a variety of underlying factors. Researchers has discovered several different methods to make improvements. To reduce the amount of unavailable, inaccessible or incomputable data within a data warehouse, Botsis et al. [7] suggest using a text-mining tool, which requires a source- and domain-specific lexicon to extract

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valuable data. In their research, they also noticed that data fragmentation is very likely to happen on individuals with terminal illnesses. This occurs due to the possibility of these patients being referred to different institutions for more effective treatments [7]. Thus, establishing a health information exchange network across healthcare entities, such as Health Information Exchange (HIE), is recommended to mitigate this problem [7]. Additionally, implementing an industrial standing for EHRs, including using clinical registries with predefined format and defining "standard content" for data, is also recognized as effective ways to enhance data completeness and quality [5], [7].

Automatic de-identification is an important data preparation procedure to solve privacy and security concerns of EHR data before being used in secondary research purposes. It involves the process of eliminating sensitive personal information while retaining the clinical data at the same time. Nowadays, named entity recognition (NER) is considered as the most common method for de-identification [8]. A long time before the adoption of NER, researchers have evaluated different de-identification systems at that time to find the best one. They have found some effective techniques that encompass machine learning methodologies, such as CRF, Decision Trees, Maximum Entropy models, and SVM [9]. These are coupled with dictionaries and occasionally regular expressions. However, these researchers claimed that many systems perform well on the specific documents, but they should be tested on a wider variety of documents to assess their generalizability [9]. Then, a system based on LSTM-CRF model, with both a label prediction bidirectional LSTM layer and a CRF layer, was introduced. This model exhibited a slight performance improvement over CRF-based methods [8]. Still, it requires fine-tuning to achieve best performance. In recent years, Ahmed et al. [10] invented a new model that combines the self-attention mechanism and stacked Recurrent Neural Network. They claimed that this system not only maintains computational efficiency but also surpasses the performance of state-of-the-art models. However, like above, it is hard to say how good is the general performance of this model without testing on multiple heterogeneous documents.

2.3. Algorithm and model

For the analysis of structured data such as body mass index, laboratory results, and heart rate, supervised learning is generally applied which let the machine learn the features from labeled training data to make predictions or decisions. The frequently used models and algorithms in the field of EHR data analysis encompasses [6]:

1) Logistic regression: a classification algorithm that is usually used to predict the probability of a binary output and it is effective in coping with the linearly separable data. Its application scenarios lie in risk assessment and diagnostic assistance.

2) Decision tree: a tree-like algorithm that outputs decisions based on the condition of the branches which is generally used for classification and regression. It is often implemented for diagnostic accuracy improvement.

3) Random forest: an algorithm that combines various decision trees to generate accurate decision while each decision tree is trained on a portion of the original input data and makes its own decision. The final prediction is based on a majority vote (for classification) or an average (for regression) of predictions from all the trees.

4) Support vector machine: a classification algorithm that maps the input data to a hyperplane that distinguishes them linearly. It is commonly applied to enhance clinical calculations and improve surgical outcomes.

In terms of the unstructured data, which is mostly text-based including pathology or radiology inspection reports, admission or discharge details, and progress notes, natural language processing (NLP) is more commonly applied to effectively analyze and extract crucial information from them. Within the NLP algorithm, it first tokenizes the text into small units and then analyzes the structure of the text linguistically by recognizing the grammar regulations after which the machine could parse the text semantically. In the case of EHR data, the NLP algorithm is commonly applied to [8]:

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1) Medical Text Classification and prediction: Enabling the machine to categorize clinical notes into different sections or types, such as patient pathology history, diagnosis, and treatment. Besides, segmenting the sentences and phrases semantically from the text and identifying specific medical terminology like drug names, disease names, and patient identifiers.

2) Bidirectional Encoder Representations from Transformers (BERT) -based Embeddings [8]: a powerful pre-trained language model that contextualizes word representations in the text, which capture the meaning of words based on the surrounding words in a sentence or text. It is shown to be effective in capturing the complex semantics and domain-specific language present in medical texts.

3) Information Extraction: Extracting relevant and imperative information such as radiology inspection results, medications, and symptoms from unstructured clinical narratives.

4) Generation: Providing feedback and comment based on the clinical text generation and medical language translation that parses the diagnoses and predictions generated from other supervised machine learning models.

2.4. Validation and Evaluation

Validating machine learning models applied to EHR data is crucial to ensure that the models are accurate, reliable, robust, and generalizable. EHR data is complex and heterogeneous, presenting unique challenges for model development and validation. General performance of a certain model could be measured by its confusion matrix which summarizes the performance of a classification model. It encompasses metrics such as false negatives (FN), true negatives (TN), false positives (FP), and true positives (TP), which can help assess model accuracy and errors. Other specific and more detailed validation methods that could be applied to the machine learning models mentioned previously are listed below:

1) Cross-Validation: Dividing the dataset into several subsets to train and validate the model iteratively, which provides an estimation of model performance with more robustness

2) External Validation: Testing the model on an independent dataset that was not used during training to assess generalization to new, unseen data.

3) Temporal Validation: Evaluating the model's performance over time, using historical data for training and testing on future data points to assess predictive capabilities.

Validation procedures for machine learning models should be carefully designed that is catered to address the specific challenges of healthcare data, including privacy concerns, data quality issues, and potential biases. The purpose is to develop models that are accurate, safe, and ethically sound for use in real-world clinical scenarios.

3. Applications on liver disease management

3.1. Utilizing Supervised Learning on Structured and Unstructured Data

The 11th leading cause of death worldwide is liver disease, particularly cirrhosis, which is a significant health problem. It accounts for 2.1% of global deaths and impacts 5.2016% of disability-adjusted life years in a two-year span. Each year, 320,000 deaths occur due to Chronic Liver Disease (CLD), with a gender distribution of two-thirds male and one-third female. One of the primary challenges is early detection, given its almost imperceptible initial symptoms. Today's healthcare system produces a deluge of patient data, posing challenges for clinicians. However, artificial intelligence, particularly supervised machine learning methods like Support Vector Machines (SVM), Decision Trees (DT), and Random Forests (RF), holds promise in aiding early diagnosis. These methods learn from labeled data and predict outcomes for new, unlabeled data. Applied to liver diseases, they can foresee disease progression and treatment outcomes by analyzing patient records and biomarkers. Such models equip doctors with tools to make precise diagnostic and treatment choices, enhancing patient outcomes and survival.

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Incorporating these techniques is crucial for innovating liver disease management and could save numerous lives.

Firstly, machine learning techniques have demonstrated their effectiveness in tracking and predicting liver disease development, in terms of disease progression. Researchers have analyzed different liver disease datasets using various machine learning algorithms to evaluate the analysis performance with different parameters and optimization techniques. This study underscores the significance of machine learning optimization in adjusting hyperparameters to minimize cost functions[11]. Concurrently, the study by Mostafa et al. utilized various machine learning algorithms to analyze patients' clinical data to more accurately predict the progression of the disease[12]. The application of this technique can not only assist doctors in diagnosing diseases earlier but also provide more personalized treatment recommendations for patients. For liver disease diagnosis, a mixed approach using the Adaptive Neuro Fuzzy Inference System (ANFIS) and Particle Swarm Optimization (PSO) has been suggested by some experts. This intelligent diagnostic method combines inference systems and optimization processes, aiming to adjust ANFIS hyperparameters based on the dataset[12].

Next, when it comes to death rates, we have shown that computer programs for learning can guess right about the chance of dying after getting a new liver. Deep learning models used by Li et al. try to predict risk factors after liver transplantation, providing doctors with a more accurate tool to assess the risks and benefits of transplantation. The application of this technology can help doctors better select suitable donors for patients, thereby increasing the success rate of transplantation[13].

Lastly, machine learning techniques have been effectively used to identify valuable clinical features in radiology reports, resulting in more precise forecasts about a patient's future health condition. The study by Liu et al.[14] Used machine learning models and natural language processing methods to examine radiology reports, giving doctors important data about patient outcomes. [14]. Some researchers have proposed a way based on a deep neural network model for predicting Non-Alcoholic Fatty Liver Disease (NAFLD) using multimodal inputs, including metadata and facial images. This method outperforms techniques that use only metadata[15].

3.2. Classification

3.2.1. Medical text classification

The process of categorizing or classifying clinical narratives into predefined classes has gained significant attention due to its potential to enhance healthcare decision-making. Gao et al. [16] have provided a comparative analysis on a bunch of methods of clinical text classification techniques, discussing limitations and breakthroughs related to unstructured narratives and emphasizing the role of self-attention mechanism and long document splitting. Building on this they dive into deep learning methods, particularly CNNs, and hierarchical self-attention network (HiSAN), showcasing their prowess in capturing intricate patterns from clinical texts. Notably, the introduction of BERT [16] has revolutionized medical text classification, as researchers fine-tune this transformer-based model on medical text datasets to achieve state-of-the-art results in multiple clinical classification tasks.

3.2.2. Segmentation

Efficiently segmenting clinical narratives into meaningful sections is pivotal for extracting relevant information and improving data organization. Ganesan and Subotin [17] had an overview on the previous clinical text segmentation methods, highlighting the complexity of unstructured data and the challenges in model generalization. They propose a Logistic regression model that is competent to segment the top-hierarchical sections of clinical narratives and meanwhile could remain its segmentation accuracy to unseen dataset. Recognizing the lack of labeled data for unsupervised segmentation Tepper et al. [18] introduce a heuristics-free machine learning approach for the rapid adaptation to unlabeled data, specifically, they applied maximum entropy model with beam search and Gaussian prior smoothing.

As healthcare data continues to grow, the integration of advanced NLP techniques offers a promising avenue for extracting valuable insights and improving patient care through accurate classification and

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effective segmentation of clinical narratives. The field remains dynamic, with ongoing research and developments shaping the future of NLP in healthcare.

3.3. BERT-based Embeddings

In NLP, embedding denotes a numerical representation of a word, phrase, or text within a vector space. These representations have found applications in modeling the biomedical text semantics, tracking the patients' trajectory, and a multitude of other assignments [8]. By utilizing a masked language model that can predict words concealed within a contextual order at random, BERT is asserted to possess superior word embedding capabilities compared to their predecessors [8], [19]. However, pretraining is required due to its poor generalizability. Hence, many different versions of pretrained BERTs have emerged. For instance, BioBERT for biochemical domain is pretrained on biomedical research papers, while ClinicalBERT, EhrBERT and MS-BERT for EHR domain are trained on clinical notes [8]. They all have better performance in their respective areas.

BERT has already been widely implemented in solving liver-related problems. Liu et al. [19] utialized BERT-based deep learning to figure out the evidence related to liver cancer diagnosis. This research is based on Chinese Radiology Reports, and Zhang et al. [20] provides a fine-tuning BERT for Chinese clinical documents. Furthermore, Liu et al. [19] introduce a BERT-BiLSTM-CRF model in their research, where this model is composed of the fine-tuned BERT language model for word embedding and BiLSTM-CRF technique for pattern extraction. While extracting features of APHE (hyperintense enhancement in the arterial phase) and PDPH (hypointense in the portal and delayed phases), two important diagnosis evidence for hepatocellular carcinoma, these researchers compared three distinct models: CRF, BiLSTM-CRF, and BERT-BiLSTM-CRF. The result turns out that, no matter in report level or character level, the recognition result of BERT-BiLSTM-CRF outperformed both other two models significantly. Overall, Liu et al. [19] acknowledge the exceptional performance of the BERT-based machine learning model in extracting radiological features from Chinese radiology reports, and demonstrate that both APHE and PDPH stood out as the two most crucial features for diagnosing liver cancer.

3.4. Information Extraction

Information Extraction (IE) involves the automated identification of crucial information within unstructured natural language text [9]. In this section, our primary emphasis will be on presenting an overview of its applications within EHRs.

The focus of the relationship extraction task is to identify and capture specific types of relationships between different entities from the text. In a medical context, a precise understanding of the relationships between various medical entities is essential to a comprehensive understanding of a patient's medical records. The early relationship extraction problem was often viewed as a multi-label classification task, where categories represented labels with a particular type of relationship. However, as time goes by, more complex feature modeling methods are gradually introduced into deep relation extraction systems to improve the modeling effect of entity relationships.

Convolutional neural networks (CNNs) are one of the methods used to solve the problem of relation extraction. In clinical discharge summary, standard CNN combined with word-level features is applied to extract the relationship between entities [21]. Another investigation integrated Convolutional Neural Networks (CNNs) with Recurrent Neural Networks (RNNs) to extract biomedical relationships from both linear and dependency graph representations of candidate sentences [26]. This hybrid CNN-RNN model was subjected to benchmarking across various protein-protein and drug-drug interaction corpora, showcasing the collaborative efficacy of CNNs and RNNs in relation extraction tasks [9]. In addition, some studies employ RNNs to label entities of interest one by one and infer relationships between them. In one study, by comparing SVMs, RNNs, and rule induction systems, it was found that in adverse drug event (ADE) detection tasks, the RNN model performed best in relation extraction [22]. Similarly, in the task of intra-sentence relationship extraction, researchers adopt a similar BiLSTM-CRF model [23]

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and use the Transformer-based method to capture the long-distance dependencies of inter-sentence relationships [24].

3.5. Generation

Research in the terms of Natural Language Generation (NLG) of generating EHR has become increasingly important. [25]. An encoder-decoder model enables the creation of synthetic main complaints based on factors such as age group, gender, and discharge diagnosis in EHRs. This method is effective at creating realistic chief complaint text and also keeps a lot of the original epidemiological data intact. A unique benefit of this approach is that the synthetic complaints don't contain any personally identifiable information (PII), which provides a way to de-identify text in EHRs. When integrated with technologies such as Generative Adversarial Networks (GANs), the method can even be expanded to generate fully synthetic EHRs. This facilitates better sharing of data between healthcare providers and researchers, and also enhances the optimization of healthcare-related machine learning models. Particularly in cases involving diseases such as cirrhosis, hepatitis, liver cancer, and fatty liver disease, this technology may contribute to a more profound understanding and analysis of the prevalence trends and characteristics of these conditions. Consequently, it could provide valuable insights to support clinical decision-making processes[26].

4. Discussion

4.1. Current ML-related limitations

4.1.1. Data limitation

The main part of the EHR data is often unstructured and could be incomplete or inconsistent. It may contain errors due to various reasons such as typos, missing information, not recorded regularly. Apart from that, the EHR data would only be approachable with the permission of both the patients as well as the medical clinics. Besides, the EHR data collected from different hospitals, even different doctors could be varied in structure and content, which may pose greater challenges for the machine to parse the data.

4.1.2. Model performance

These issues mentioned can drastically affect the performance of machine learning models, which includes the failure for models to converge or reduced prediction and classification accuracy. Especially for the unstructured data, its chaotic form and disorder will hamper the deep learning model from extracting the pattern from the training data as the lack of context or clear structure can make it challenging to disambiguate word meanings or infer relationships between words [27] thus the irrelevant noise could further confuse the model and even mislead it to biased state, in addition, many NLP tasks involve understanding long-range dependencies and relationships within text based on the proper sequence of the data, and failure of capturing these long-term dependencies could lead to suboptimal performance.

4.2. Ethical and Privacy Considerations

Despite the implementation of data de-identification and data privacy protection laws in the context of EHR data collection, concerns about the potential breach of personal privacy continue to trouble many patients. First of all, automatic de-identification is process where its performance larges depends on the source of documents and clinical institutions. It can lead to poor performance if the model is not fine-tuned. Also, with the development of re-identification techniques, the security of de-identification can become compromised. Secondly, for some institutions that are lack of data security awareness, unauthorized individuals may access and abuse these data. They are vulnerable to hacking and data breaches if these servers are not well maintained. Even doctors or employees with access to EHR systems might abuse their privileges by selling patients' private information to illegal organizations.

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Additionally, the health information exchange network and the secondary utilization of EHRs mentioned earlier aim to enhance data accuracy and utility. However, both approaches involve the sharing of data among institutions, consequently increasing the risk of data exposure. All these privacy and security concerns will create challenges in obtaining consents of patients sharing their EHR data to clinical institutions. Consequently, this will diminish the comprehensiveness and value of EHR data.

4.3. Future Research Trends and Prospects

Machine Learning for Privacy: As privacy concerns about EHR data rise, future research should focus on building improved privacy-preserving machine learning algorithms. Improving data de-identification methods, investigating safe multi-party computation, and creating federated learning systems that enable collaborative analysis without revealing raw data are all part of this effort. Researchers may build comprehensive ethical frameworks and protocols for EHR data collection, sharing, and analysis to address the ethical challenges connected with EHR data utilization. These frameworks should take into account patient consent, data ownership, and data management. Interoperability and Data Standardization: enhancing data quality and accessibility requires enhancing interoperability across different EHR systems and standardized data formats. Future study might concentrate on the development of universal standards for EHR data interchange in order to improve data integration and analysis. Deep Learning Advances: Advances in deep learning approaches, such as self-attention mechanisms and transformer models like BERT, will almost certainly play a large role in extracting important insights from unstructured clinical narratives. Further research should be conducted to determine how these models might be fine-tuned for specific healthcare purposes. Initiatives for Collaborative Research: Collaboration is vital among healthcare organizations, researchers, and regulatory agencies. Collaborative research projects and data-sharing networks can help to expedite development in the area while also addressing data privacy and ethical concerns.

5. Conclusion

In this comprehensive review, the paper delves into the potential of machine learning (ML) techniques, especially in the domain of liver disease, using Electronic Health Records (EHRs). It examines the challenges and opportunities that EHRs present for clinical research, such as data incompleteness and privacy concerns. The study extensively reviews various ML methods for EHR data analysis tailored to liver disease, spotlighting recent advancements like self-attention mechanisms and stacked Recurrent Neural Networks. Furthermore, it underscores the importance of robust methodologies to extract relevant insights for liver disease while maintaining data integrity and patient confidentiality. Although this review advocates the establishment of Health Information Exchange Networks (HIE) and the implementation of industrial standards as potential solutions, it acknowledges that the domain has not yet fully tackled data privacy and ethical challenges. Notably, the BERT-BiLSTM-CRF model, as highlighted, excels in medical diagnostics, suggesting that its application in liver disease diagnostics could be a valuable research focus. While this study marks an essential stepping stone in EHR-based research for liver disease using advanced ML techniques, it also flags the critical need for addressing data privacy and ethics in subsequent studies. Overall, this research offers both theoretical and practical contributions, with a pronounced emphasis on liver disease diagnostics and treatment enhancements using ML algorithms.

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Advances in medications and treatments for reducing memory loss in Alzheimer's disease

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Abstract. Alzheimer's disease (ad) is a disease in which brain cells deteriorate, leading to dementia and cognitive decline. It is mainly caused by cholinergic functional problems and the accumulation of amyloid plaques in the brain. Age, genetics, head damage, vascular disease, infection and environmental factors can also cause the disease. Currently, only two drugs have been approved for the treatment of Alzheimer's disease, but they only target symptoms and cannot cure or prevent the disease. Ongoing research aims to better understand Alzheimer's disease and develop treatments that can slow or change its progress in response to mechanisms such as beta-amyloid plaques, inflammation and cholinergic dysfunction with abnormal tau metabolism. The focus of this review is on current drug therapy and the causes of Alzheimer's disease, including those that change the disease, as a partner, or use natural compounds.

Keywords: Alzheimer's disease(ad), Beta-amyloid plaques, Inflammation, Cholinergic dysfunction.

1. Introduction

Alzheimer's disease is a progressive degenerative brain disorder that primarily affects memory, thinking, and behavior. It is one of the leading causes of dementia and affects millions of individuals worldwide. Over the years, significant advancements have been made in understanding the disease and developing drugs and treatments to reduce memory loss in Alzheimer's. Slowing memory decline is important because it can positively impact an individual's quality of life, functional abilities, mental well-being, relationships and social engagement, rehabilitation and recovery, healthcare costs, and research advancements. It helps individuals maintain cognitive abilities, independence, and overall well-being. Studying methods to slow memory decline is crucial in addressing the challenges associated with cognitive impairment [1-4].

2. Alzheimer's Disease and Memory Loss

Alzheimer's disease (AD) is a neurodegenerative disease characterized by progressive memory loss and cognitive decline. This is the most common form of dementia. Alzheimer's disease is characterized by

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the formation of plaques and tangles in the brain. Plaques are clumps of an abnormal protein called beta-amyloid that pile up between nerve cells, while tangles are twisted fibers of another protein called tau that pile up inside cells. These plaques and tangles disrupt communication between neurons, resulting in the loss of brain cells and impaired function of various brain regions. The progression of Alzheimer's disease is divided into mild cognitive impairment (MCI), mild AD, moderate AD, and severe AD. In the early stages, individuals may experience memory loss, and as the condition progresses, the memory impairment becomes more severe, affecting the ability to recall recent events, recognize familiar faces or objects, and engage in conversation.

The progressive nature of memory loss in AD has significant implications for daily living. In the early stages, individuals may require reminders for appointments or assistance with organizing daily tasks. As memory decline worsens, more support is needed to ensure medication adherence, personal hygiene, and maintaining a safe living environment. Ultimately, patients may struggle to recognize their loved ones, remember their own identity, and perform basic self-care activities. The impact extends beyond memory loss, affecting other cognitive functions such as language, problem-solving, and judgment. Individuals may become disoriented, have difficulty following directions, and experience personality and behavioral changes. AD can also lead to challenges in social interactions and withdrawal from previously enjoyed activities.

The causes and mechanisms of memory decline in Alzheimer's disease are unknown, but various factors are thought to contribute to its development. Genetic factors play a role, with certain mutations in genes such as APP, PSEN1 and PSEN2 increasing the risk of developing AD. However, most cases are sporadic and may involve a combination of genetic, environmental and lifestyle factors. In Alzheimer's disease, the accumulation of beta-amyloid plaques and tangles of tau protein disrupts the normal function of neurons. Beta-amyloid is thought to disrupt synaptic communication between neurons, leading to memory and cognitive impairments. Tau tangles, on the other hand, contribute to the destruction of microtubules, which are basic structures for cell transport and support. In addition, neuroinflammation, oxidative stress, and dysfunction of neurotransmitters such as acetylcholine are thought to play a role in memory decline in Alzheimer's disease. These mechanisms lead to progressive degeneration of brain cells and an overall decline in memory function in AD patients [5-6] (Figure 1).

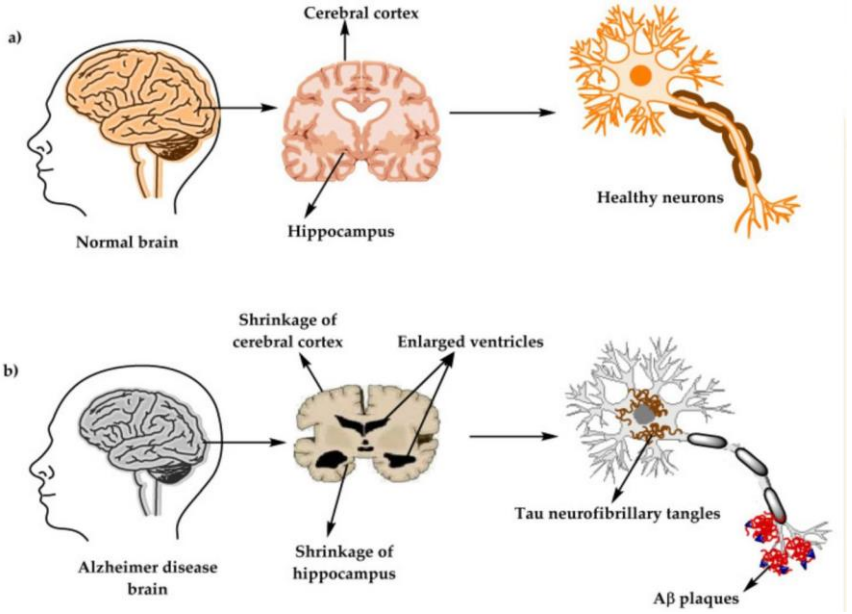

Figure 1. Differences between normal brain and Alzheimer disease brain

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3. Current Approaches to Memory Loss Management

Current approaches to memory loss management include cholinesterase inhibitors and NMDA receptor antagonists

3.1. Cholinesterase Inhibitors

Cholinesterase inhibitors are a class of drugs that block the activity of acetylcholinesterase. This enzyme is responsible for breaking down the neurotransmitter acetylcholine in the nervous system. By inhibiting this enzyme, cholinesterase inhibitors increase the level of acetylcholine in the brain, which improves cognitive function, memory, and overall brain activity. These drugs are often used to treat Alzheimer's disease because the disease is characterized by a deficiency of acetylcholine in the brain.

3.2. NMDA Receptor Antagonists

NMDA receptor antagonists are a class of drugs that block the activity of N-methyl-D-aspartate (NMDA) receptors. The NMDA receptor is a glutamate receptor involved in synaptic plasticity, learning and memory formation. By blocking NMDA receptors, these antagonists regulate levels of glutamate, an excitatory neurotransmitter, in the brain. NMDA receptor antagonists have been studied for potential therapeutic use in a variety of diseases, including Alzheimer's disease, depression, and neuropathic pain [7-8] (Figure 2).

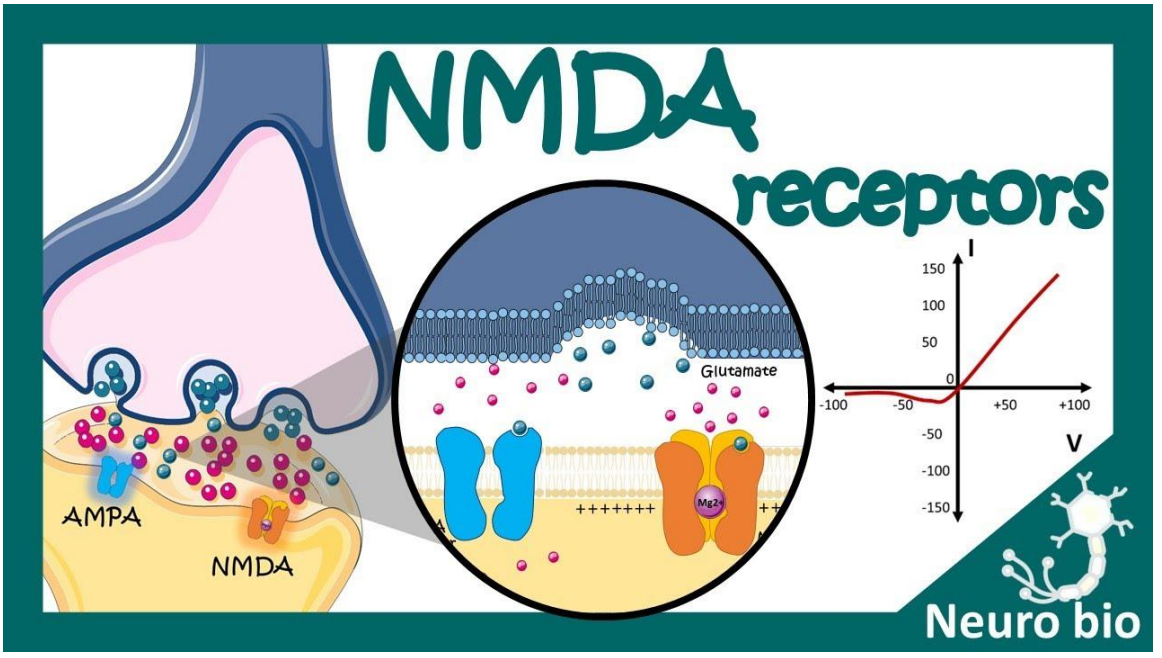

Figure 2. NMDA receptors

4. Emerging Therapies and Experimental Treatments

There are several emerging and experimental treatments for Alzheimer's disease that are being explored.

1. Immunotherapy: This approach involves developing drugs that target and remove abnormal proteins, such as beta-amyloid, that build up in the brains of people with Alzheimer's disease. These drugs help stimulate the body's immune system to clear these proteins from the brain.

2. Gene therapy: Scientists are exploring the use of gene therapy to potentially modify or replace defective genes linked to Alzheimer's disease. This approach aims to restore the normal function of the affected genes and slow or stop the progression of the disease.

3. Stem cell therapy: Stem cells have the potential to develop into different cell types, and scientists are investigating using them to replace damaged brain cells in Alzheimer's patients. Stem cell therapy may help restore cognitive function and slow the progression of the disease.

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4. Tau targeted therapy: Tau is another protein that accumulates in the brains of Alzheimer's patients. Researchers are developing drugs that target tau tangles to prevent their accumulation and subsequent damage to brain cells.

5. Reduce inflammation: Chronic inflammation in the brain has been linked to the development of Alzheimer's disease. Researchers are studying drugs and interventions that can reduce inflammation to potentially slow the disease.

6. Cognitive training: Cognitive training programs involve engaging individuals in mental exercises and activities to improve cognitive abilities. Research suggests that these interventions may help slow the cognitive decline associated with Alzheimer's disease.

7. Lifestyle changes: Certain lifestyle changes, such as regular physical exercise and maintaining a healthy diet. These have been linked to a reduced risk of Alzheimer's disease. These approaches are being further explored for their potential therapeutic benefits.ll experimental and require further research and testing to determine their safety and effectiveness [9].

5. Multimodal Approaches and Personalized Medicine

Multimodal interventions in memory loss management involve combining pharmacological (medication-based) and non-pharmacological strategies to address cognitive deficits comprehensively. For Alzheimer's disease, pharmacological approaches include cholinesterase inhibitors that raise acetylcholine levels, and NMDA receptor antagonists like memantine regulating glutamate. Non-pharmacological strategies encompass cognitive stimulation, physical exercise, nutritious diets, social engagement, sleep optimization, stress management, cognitive rehabilitation, and environmental modifications. These approaches counteract the multifaceted causes of memory loss.

The synergy between pharmacological and non-pharmacological methods enhances outcomes. Medications provide a cognitive base, while lifestyle changes bolster brain health. This blend minimizes medication side effects and improves the overall quality of life. However, intervention efficacy varies based on individual health and causative factors. Consulting healthcare professionals is vital for personalized, evidence-based plans. Overall, these combined strategies offer a holistic approach to memory loss management, addressing its multifaceted nature and yielding potential benefits for cognitive function and well-being.

Personalized medicine, or precision medicine, tailors medical care to an individual's genetic makeup, environment, and lifestyle for effective treatments. It's especially relevant in genetics, oncology, and neurology, where individual variations impact disease susceptibility, progression, and treatment response. In memory loss and cognitive impairment contexts:

1. Genetic Profiling: Genetic testing identifies gene variants linked to memory disorders, aiding early detection and personalized prevention strategies.

2. Biomarker Assessment: Biomarkers like amyloid and tau levels gauge disease progression, shaping personalized treatment plans.

3. Pharmacogenomics: Genetic analysis predicts drug responses, reducing adverse reactions and guiding medication choices.

4. Cognitive Profiling: Identifying cognitive strengths and weaknesses customizes rehabilitation programs.

5. Lifestyle Factors: Tailoring interventions to a person's lifestyle, environment, and preferences maximizes efficacy.

6. Data Integration: AI and data analysis combine genetic, medical, and lifestyle data to offer personalized treatment recommendations.

7. Longitudinal Monitoring: Ongoing assessment adjusts treatments based on individual responses.

8. Ethical Considerations: Protecting sensitive data and ensuring informed consent are paramount.

Despite its potential, challenges include standardization, regulations, evidence for efficacy, and accessibility to advanced diagnostics. As these obstacles are addressed, personalized medicine promises targeted and effective interventions for memory loss and cognitive decline.

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Numerous studies have explored synergistic effects from combining treatments to enhance outcomes, including memory loss and cognitive impairment. Some notable studies are:

1. FINGER Study: This study, published in Alzheimer's & Dementia (2015), investigated a multimodal intervention for cognitive decline risk. Combining dietary guidance, exercise, cognitive training, and risk management, it positively impacted cognitive performance in older adults.

2. MAPT Study: Published in the Journal of Alzheimer's Disease (2018), this trial examined antioxidant supplementation, exercise, and cognitive training in mild cognitive impairment. Results indicated that combined interventions improved cognitive outcomes.

3. SNIFF Study: The Alzheimer's & Dementia-published SNIFF study (2011) explored intranasal insulin with cognitive training for mild cognitive impairment. Findings revealed improved memory and cognition with the combination.

4. MIND Diet and Physical Activity Study: This Diabetologia-published study (2016) focused on type 2 diabetes patients, combining the MIND diet and supervised exercise. Positive cognitive effects were observed, indicating a synergistic diet-exercise impact.

5. MAP Study: Published in JAMA (2015), this study evaluated omega-3 fatty acids and lutein/zeaxanthin supplementation in older adults with mild cognitive impairment. The combined supplementation correlated with improved cognitive performance.

These studies showcase the potential benefits of combining interventions like dietary changes, cognitive training, exercise, and supplementation to enhance cognitive outcomes. However, individual responses vary, necessitating further research to validate these approaches. Consulting healthcare professionals before implementing interventions is essential for safety and tailored strategies [10-11].

6. Future Directions and Implications

Reducing memory loss in Alzheimer's disease offers far-reaching benefits. Enhanced well-being, prolonged independence, and improved quality of life result from preserved memory function. Addressing memory deficits fosters functional autonomy, alleviates caregiver burden, and fosters meaningful communication. Mood, social engagement, and overall health benefit, impacting individuals and caregivers positively. Contributions to research and clinical trials arise from improved cognitive abilities, fostering treatment development. Societally, lowered healthcare costs and enhanced productivity stem from reduced cognitive decline. By combining pharmacological, cognitive, lifestyle, and caregiver approaches, memory loss reduction not only benefits individuals but also shapes disease progression and societal outcomes.

Continued research and collaboration are vital in addressing memory loss and cognitive impairment. Research deepens our understanding of the causes and mechanisms of neurodegenerative conditions, guiding interventions. Collaborative efforts facilitate innovative approaches and novel therapies. Optimizing treatments, especially personalized ones, is achieved by collecting diverse patient data. Rigorous research ensures safe and effective interventions. Timing and complexity are addressed through interdisciplinary collaboration, accelerating progress and encouraging innovation. This global impact fosters solutions benefiting diverse populations. Moreover, research educates professionals, caregivers, and the public, reducing stigma and promoting early diagnosis. Ultimately, ongoing research and collaboration drive the development of interventions that enhance the lives of those affected by memory loss and cognitive impairment, offering hope for the future [12-15].

7. Conclusion

Alzheimer's disease (AD) is a worldwide health concern that has led to the revision of diagnostic criteria. The current focus of AD treatment is mainly on managing symptoms rather than altering the disease's progression. Medications such as cholinesterase inhibitors and memantine can improve memory and alertness but do not stop the disease from advancing. Lifestyle changes, including diet and exercise, have shown potential in improving brain health and reducing the risk of AD. However, many drugs being tested in clinical trials targeting the pathological features of AD have not been successful. Researchers are also exploring other potential therapies, such as chaperones and natural extracts from

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Chinese medicine. Early initiation of treatment and the use of biomarkers to monitor disease progression are vital for effectively managing AD. The diagnostic process has expanded to include the early stages of the disease and mild cognitive impairment, with additional diagnostic tests available. Nevertheless, current treatment options for AD focus on providing support and alleviating symptoms. Lifestyle modifications are recommended as the initial intervention for all patients. More research and the development of effective strategies for early detection and treatment of AD are urgently needed.

In conclusion, it is crucial to continue advancing our understanding of memory loss mechanisms and treatment strategies. Memory loss significantly impacts individuals and their loved ones, and its prevalence is increasing as our population ages. By expanding our knowledge, we can develop better treatments and interventions. Currently, options for memory loss management are limited, particularly in severe cases like Alzheimer's disease. Early detection and intervention are important, but without a better understanding, it is challenging to develop targeted therapies. Memory loss is complex, and unraveling its intricacies may help identify new targets for treatment. This knowledge can also aid in developing preventive strategies and have broader implications for other neurological conditions. By dedicating resources to this field, we can improve diagnosis, treatment, and prevention of memory loss disorders.

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Can BDNF manipulate the overshoot-and-decline effect of adult neurogenesis during recovery from sleep deprivation?

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Abstract. Sleep deprivation reduces an organism's ability to perform adult neurogenesis, the generation of new neurons in adulthood. Recently, researchers discovered that recovery in sleep deprivation causes the rate of adult neurogenesis to overshoot the normal rate, and then decline back to normal. This research focuses on brain-derived neurotrophic factor, and whether or not manipulating it and its related pathways could extend the overshoot effect to enhance adult neurogenesis in the long term. The conclusion that is expected from this research is that BDNF will be able to prolong the overshoot of adult neurogenesis that is induced by sleep deprivation. The work seeks to provide more control over the overshoot-and-decline effect of adult neurogenesis during recovery from sleep deprivation, opening new pathways to treatments involving the manipulation of adult neurogenesis.

Keywords: Brain-derived neurotrophic factor (BDNF), tyrosine receptor kinase B (TrkB), BDNF-TrkB pathway, Bcl2, adult neurogenesis, sleep deprivation

1. Background

I would be very happy to be able to research about brain-derived neurotrophic factor (BDNF) and its possible effect on the overshoot-and-decline effect of adult neurogenesis during recovery from sleep deprivation, as discovered by Mirescu et al. Sleep deprivation affects many people around the world. I want to research in order to understand not only the direct effects of sleep deprivation on adult neurogenesis but also how we can harness the effects to help those who struggle with neurological ailments. My research aims to find ways to manipulate the adult neurogenesis overshoot, and I believe that our findings can suggest possible treatments for decreased adult neurogenesis in seniors or victims of neurodegenerative diseases.

In a 2006 PNAS paper published by Mirescu et al., the researchers found that while sleep deprivation did result in decreased adult neurogenesis through the upregulation of glucocorticoids, adult neurogenesis levels exhibited a rebound during the recovery period unrelated to glucocorticoid levels before returning to control levels [1]. Glucocorticoids were already known to have a negative impact on adult neurogenesis by suppressing proliferation of radial stem cells and reducing neuroblast numbers [2]. Sleep deprivation increases stress, which is a manifestation of increased glucocorticoid levels [3]. The BDNF-TrkB pathway also plays a vital role in adult neurogenesis; not only does BDNF promote cell proliferation, but its downstream product Bcl2 also discourages apoptosis [4].

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2. Literature review

Existing research shows that the rebound in adult neurogenesis after one week of recovery from sleep deprivation is not affected by glucocorticoid levels, and that adult neurogenesis levels decrease to normal after three weeks [1]. I find the rebound effect particularly compelling to the discussion of how adult neurogenesis can be enhanced to normal levels in patients with neurodegenerative diseases. The fact that the adult neurogenesis overshoot-and-decline effect does not rely on glucocorticoid levels being lowered as Mirescu and company first imagined could lead to a more consistent treatment method.

I also read that sleep deprivation decreases BDNF levels, which made me curious about the impact that manipulating BDNF levels could have on sleep deprivation [5]. I asked myself if I would be able to dampen or even reverse the negative effects of sleep deprivation on adult neurogenesis by promoting BDNF. My interest in sleep deprivation was immediately piqued by the literature I read.

The BDNF-TrkB pathway is a MAP kinase pathway that not only promotes cell proliferation but also discourages cell apoptosis through the promotion of Bcl2 [6]. BDNF binds to TrkB receptors, which are tyrosine kinase receptors. This activates Erk5 and promotes the downstream production of Bcl2, which encourages senescent cell survival [7]. Bcl2's role in cell survival means that it could be used to sustain the overshoot effect in recovery from sleep deprivation by discouraging apoptosis of new neurons.

Tracking new neurons in a rat's brain is usually done by injecting BrdU into the brain of a mouse, and then using an inverted fluorescence microscope after the rat has been perfused and its brain sectioned. Stem cells, which are indicators of new neurons forming, cycle slowly and retain their BrdU labels well, reducing the need to constantly reinject BrdU [8].

3. Research approach

I predict that my experiment will show that BDNF and Bcl2 can manipulate the overshoot-and-decline effect of adult neurogenesis. My experiment will involve the detection of new neurons through inverted fluorescence microscopy and BrdU indicator. However, BrdU has toxic effects on cells, so groups expected to yield a normal level of new neurons may yield slightly less [9].

In Mirescu's experiment, he sleep-deprived rats for 72 hours, injected their brains with BrdU indicator, then allowed them to recover for 2 hours, 1 week, or 3 weeks before perfusing them and counting new neurons with the BrdU [1].

My first experiment will involve four groups of Sprague-Dawley rats; cage control (CC), small-platform sleep deprivation, cage control with TrkB inhibited during the initial 72-hour period, and small-platform sleep deprivation with TrkB inhibited during the recovery period. All groups will have sub-groups divided into 2-hour recovery to check adult neurogenesis levels right after sleep deprivation, as well as 1-week and 3-week recovery. This will allow me to check if the overshoot phase of sleep deprivation recovery is caused by BDNF. Small-platform sleep deprivation prevents REM sleep in rats by ensuring that they will fall into a pool of water and be forced to climb back up if they fall into REM sleep [10]. TrkB inhibition will be achieved by carefully administering Larotrectinib, a breakthrough cancer treatment that inhibits tyrosine receptor kinases [11]. The main advantage to my experiment method is how definitive the results can be; if cage control with TrkB inhibited during the first 72 hours has no effect versus cage control, I will know that BDNF does not play a role in the overshoot-and-decline effect. If small-platform sleep deprivation with TrkB inhibited during the recovery period does nothing, I will also know that BDNF does not play a role in the overshoot-and-decline effect. A drawback is that I will have to control the dosage of larotrectinib very carefully in the group that receives TrkB inhibition in the initial 72-hour period to make sure that the effects of the larotrectinib do not bleed into the recovery period.

My second experiment focuses on Bcl2 and its possible role in affecting the overshoot pattern by reducing apoptosis of new neurons. It will involve three groups of rats; cage control, small platform, and small platform with Bcl2 promoted during the recovery period. All groups will have sub-groups divided into 2-hour recovery to check adult neurogenesis levels right after sleep deprivation, as well as 1-week and 3-week recovery. Simply promoting Bcl2 through increasing BDNF levels will also induce

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cell proliferation, inflating cell count without knowing the true effect of the reduced apoptosis alone [12]. Promoting Bcl2 alone can be done through acridone derivative A22 [13]. Finding the effect of Bcl2 on the decline phase of sleep deprivation will allow me to learn how to extend the overshoot in adult neurogenesis. The main advantage is that the negative result of the experiment is already known; if promoting Bcl2 has no effect, the level of new neurons will simply return to control levels as shown in Mirescu's experiment [1].

4. Methodology

The first step is to collect at least 105 Sprague-Dawley rats. Every sub-group will require 5 rats averaged for maximum accuracy, and there are 7 total experimental groups each containing 3 sub-groups for the different recovery times. The small-platform sleep deprivation test is inexpensive and very feasible; it requires an upside-down flowerpot for the mouse to stand on inside a bucket of water.

4.1. Animal Treatment and Ethical Methods

The experiment shall be performed to cause the least amount of pain to the rats. BrdU will be injected into the rats' brains, and after a recovery period of 2 hours, 1 week, or 3 weeks, the rats will be perfused with 4.0% paraformaldehyde in 0.1M phosphate buffer to ensure that they die without pain [1]. Their dentate gyruses will be sectioned into 40 micrometer thick slices for surveying with an inverted fluorescence microscope [14].

4.2. Inverted Fluorescence Microscopy

After using BrdU to mark the new neurons in the rat hippocampus, the slides of dentate gyrus will be processed just as they were in Mirescu's sleep deprivation experiment [1]. Then, I will count the BrdU-labeled cells at 1000x zoom using a light microscope and multiply the count by 24 to estimate the count of BrdU-labeled cells per brain.

4.3. Interpretation of Results

The number of BrdU-labeled cells will indicate the number of new neurons during the recovery time after sleep deprivation. A decreased count of BrdU-labeled cells for the 1-week recovery subgroup when the BDNF-TrkB pathway is inhibited would indicate that BDNF has a significant impact on the overshoot effect of recovery from sleep deprivation, because without it the overshoot effect would not happen. An increased count of BrdU-labeled cells for the 3-week recovery subgroup when Bcl2 is promoted would indicate that Bcl2 plays a vital role in the regulation of apoptosis of new neurons in the decline stage of sleep deprivation recovery.

Bar graphs with each bar corresponding to each recovery time will be used to intuitively visualize the change in BrdU-labeled new neuron count. As depicted in Mirescu's experiment, the BrdU-labeled cell count was lowest after 2 hours of recovery, spiked during 1 week of recovery, and then returned to normal after 3 weeks of recovery. Any deviation from this control will suggest that BDNF and Bcl2 have a significant impact on the overshoot-and-decline effect of recovery from sleep deprivation.

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5. Expected results

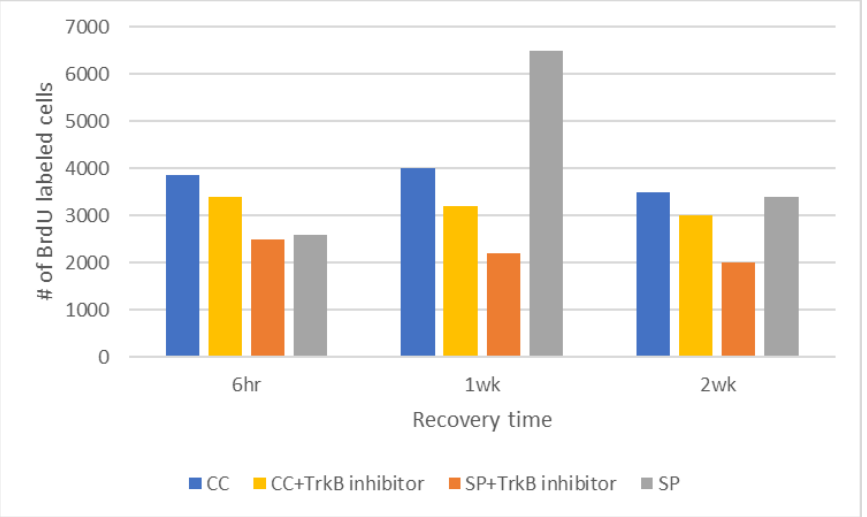

Figure 1. Expected raw data from the first experiment [15]

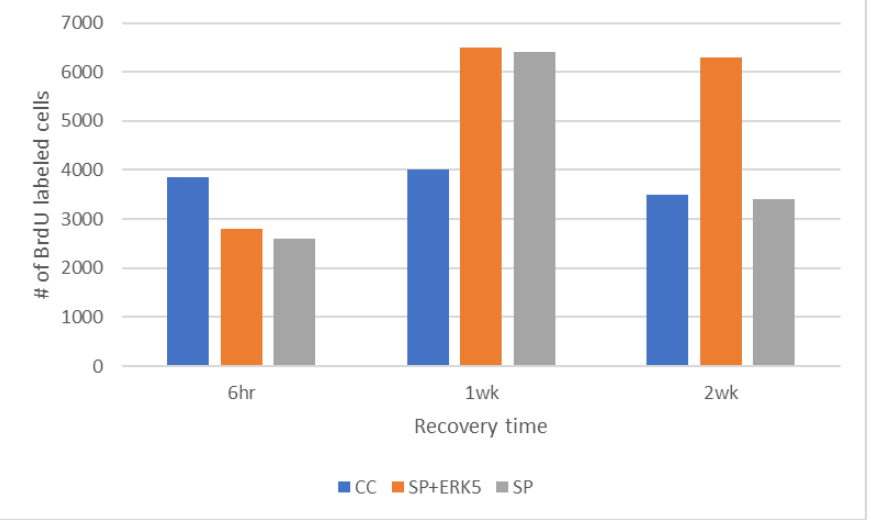

Figure 2. Expected raw data from the second experiment [15]

I expect that BDNF and Bcl2 will affect the overshoot-and-decline effect of adult neurogenesis in sleep deprived rats. BDNF is known to induce growth for neurons, as well as cancer cells and a wide range of other cells [12]. Bcl2 is a main factor in regulation of apoptosis, which most likely drives the decline process of new neurons. Even if the decline in new neurons is not totally mitigated due to other factors in apoptosis, the addition of Bcl2 will at least slow the decline of new neuron count. The possibilities that my research can lead to are vast; BDNF is already being investigated as a therapeutic agent in Parkinson's disease. The notion that sleep deprivation could induce BDNF as shown in the overshoot phase could lead to potential treatment that does not require external stimulation of BDNF, especially since a 2020 experiment by Palasz et al showed that external dosage of BDNF did not relieve symptoms of Parkinson's disease [16]. Parkinson's disease and other neurodegenerative diseases are often long-term and require sustained treatment, so a short burst in BDNF will not be adequate. Assuming that my hypothesis is correct, Bcl2 will be vital in maintaining elevated levels of new neurons.

BDNF has also been found by Björkholm and Monteggia to be an important bridge between antidepressant drugs and the resulting neuroplastic changes that alleviate symptoms of depression [17].

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A drug like acridone derivative A22 after recovery from sleep deprivation could provide a sustained alternative treatment for depression.

6. Conclusion

I genuinely believe that my proposal to research about BDNF and its possible effect on the overshoot-and-decline effect of adult neurogenesis during recovery from sleep deprivation can bring about a novel understanding of sleep deprivation's role in neurological ailment. Being able to manipulate BDNF would allow us to at least slow the symptoms of a neurological ailment, since BDNF is known to be important to at least Parkinson's disease and depression. The ability to control Bcl2 levels to allow for sustained treatment would help us develop a drug that could provide long-term aid to those who suffer from neurological ailments. For the depressed and for the neurologically diseased, I urge you to consider my research for funding.

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A comprehensive review of antimicrobial peptides: Mechanisms, classifications and designations

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Abstract. Antimicrobial peptides (AMPs) are a group of alkaline poly-peptide compounds that have antibacterial activity and are produced by insects. This research examines three key aspects of antimicrobial peptides (AMPs): their mechanism of action, categorization, and designation. Antimicrobial peptides have a wide range of antimicrobial action, with a significant number of them being naturally occurring peptides. Consequently, they are increasingly being recognized as promising candidates for therapeutic applications. Antimicrobial peptides can be categorized into many categories based on their structural characteristics and origin. The field of computer-assisted peptide design integrates computer-aided design technology with the process of drug creation, so leveraging its inherent benefits and offering a rational and efficient theoretical framework for the design of peptides. The identification of novel classes of antibiotics is a promising approach for addressing the issue of antibiotic resistance. Antimicrobial peptides possess significant potential for application within the pharmaceutical sector due to their notable antibacterial efficacy, extensive range of antibacterial activity, diverse structural characteristics, and extensive array of options. Moreover, these peptides have a reduced likelihood of inducing resistance mutations in target strains. Currently, a number of poly-peptide antibiotics are being subjected to preclinical feasibility investigations. One of these antibiotics, magainins, has progressed to the phase III clinical trial stage.

Keywords: antimicrobial peptides, computer-assisted peptide, resistance, bacteria, Microorganisms-Derived Antimicrobial Peptides

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1. Introduction

Antimicrobial peptides (AMPs) are a widely distributed group of short peptides that play a crucial function in the innate immune systems of various animals. The inhibitory effects of the substance are wide-ranging, encompassing bacteria, fungi, parasites, and viruses [1].

The urgency of the situation is emphasized in the 2019 Antibiotic Resistance Report published by the Centers for Disease Control and Prevention (CDC) in the United States. The report provides detailed information on the occurrence of approximately 2.8 million occurrences of illnesses that are resistant to antibiotics, leading to a total of 35,000 deaths. The occurrence of bacterial diseases that are resistant to drugs not only poses a threat to the health of both humans and animals, but also imposes a substantial economic burden [2].

It is worth mentioning that the presence of multi drug-resistant (MDR) bacteria, including Methicillin-resistant Staphylococcus aureus, vancomycin-resistant Enterococcus, Multi-drug resistant Pseudomonas aeruginosa, and Multi-drug resistant Stenotrophomonas maltophilia, intensifies the level of urgency surrounding this issue.

They are readily accessible and consistently utilized within the medical field. Positron emission tomography (PET) probes that utilize peptide targeting typically include of tiny peptides that exhibit a notable affinity and selectivity towards various cellular and tissue targets. The primary benefits associated with these probes encompass their cost-effectiveness compared to conventional antibody-based PET tracers, as well as their efficient chemical modification procedure that allows for radio-labeling with a wide range of radionuclides. These characteristics render them particularly appealing for clinical applications. Request for reconsideration. Presently, in line with the development of drug design, the utilization of computational procedures in the sector is progressively expanding, albeit not yet considered a conventional tool in radio-pharmaceutical design [3].

This revolutionary paradigm introduces a new direction to the study of therapeutic peptides, which includes a range of immunomodulatory functions, non-membrane mechanisms of action, peptide analogues, and formulation approaches to overcome protease degradation [4]. This review aims to provide a comprehensive understanding of the mechanisms underlying antimicrobial peptides, shedding light on this emerging paradigm. The technique presents intrinsic hurdles that are formidable, but it also holds the potential to develop more effective and cost-efficient broad-spectrum peptides, which could significantly impact the future of antimicrobial medicines.

2. Mechanism of antimicrobial peptides

2.1. Mechanism of peptide bond formation

The fundamental concept underlying peptide synthesis is the chemical condensation reaction between the carboxyl and amino groups of amino acids, resulting in the formation of peptide bonds. This chemical process is commonly denoted as a peptide bond-forming reaction or a peptide bond synthesis reaction. The process of peptide bond formation involves the creation of an amide bond between a carboxyl group and an amino group. Peptide bonds has the capability to undergo disassembly and reassembly via uncomplicated hydrolysis and transfer processes [5].

2.2. Mechanisms of antibiotic resistance

2.2.1. Causes of Antibiotic resistance

Microbial resistance to antibiotics is manifested by changes in antibiotic permeability, changes in target molecules, production of inactivating enzymes, and efflux of antibiotics from the cytoplasm. Bacteria and other microorganisms use all of these mechanisms to evade the toxic effects of antibiotics [6].

Antibacterial drug penetration barrier

In order to exert their antibacterial effect, antibiotics must penetrate the interior of bacteria and successfully reach the target spot. Alterations in the permeability of the bacterial cell wall and/or outer membrane can establish a protective barrier that hinders the penetration of antibacterial agents into the

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bacterial core, hence impeding their interaction with the intended target site. This phenomenon significantly compromises the efficacy of antibacterial treatments.

The outer membrane of Gram-negative bacteria is a multifaceted organelle that facilitates the uptake of nutrients while simultaneously serving as a safeguard and barrier. According to new findings from Enterobacteriaceae, it has been observed that the permeability of the outer membrane undergoes dynamic changes during bacterial development, which subsequently impacts the degree to which medications can enter the membrane [7].

Alteration of drug targets

Natural or acquired changes in antimicrobial drug targets that prevent drug binding are a common resistance mechanism. When the DNA or protein target of the drug is mutated or modified, the affinity between the drug and the target is reduced, and the inhibitory activity of antibacterial drugs is significantly weakened, leading to drug resistance. Alterations at target sites are often caused by spontaneous mutations in bacterial genes on chromosomes. Since the interaction of an antibiotic with a target molecule is usually very specific, small changes in the target molecule may have important effects on the binding of the antibiotic [8].

Production of inactivating enzymes

Many drug-resistant bacteria can produce inactivating or inactivating enzymes, hydrolyzing inactivating enzymes. Antibiotics can be modified by inactivating or modifying enzymes. These enzymes are located in transferable elements such as plasmids, transposons, and bacterial chromosomes, and spread between different bacteria, which are destructive to antimicrobial drugs and lead to drug inactivation. Bacteria often (develop resistance to antibiotics after exposure to them. Natural resistance is usually chromosomally mediated, whereas acquired resistance may result from chromosomal mutations or acquisition of resistance-encoding genes from external sources such as plasmids and transposons etc. Required and natural resistance are clinically important and can lead to treatment failure. However, acquired resistance is a major way of bacteria to be inherited [9].

Drug efflux

Drug efflux is a significant mechanism contributing to drug resistance in Gram-negative bacteria. These cellular mechanisms actively transport solutes out of the cell. Microbes employ efflux pumps as a means to manage their internal milieu by eliminating various detrimental chemicals, such as antimicrobials, metabolites, and quorum-sensing signaling molecules [10].

3. Classification of AMPs

The diversity of natural AMPs brings a lot of difficulties in their classification. AMPs are classified according to Source, Amino acid-rich species and Structure. (Figure 1). In the part of source, we will start form these aspects; Insect, mammalian, Aquatic and Microorganisms. In the part of Aminoacid-rich species, we will start from these aspects; Glycine, Proline and Histidine. Finally, in the part of structure, we will start from these aspects; Linear extension, α-helix and β-helix.

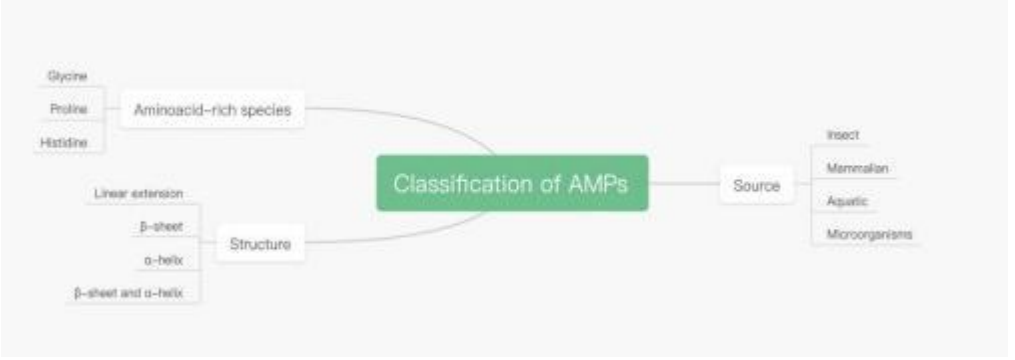

Figure 1. Classification of AMPs

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3.1. Categorization of Antimicrobial Peptides (AMPs) According to their Origins

According to the statistical data provided by APD3, the sources of antimicrobial peptides (AMPs) can be categorized into three main groups: insect, mammalian, and microbes. Additionally, AMPs have the ability to attract marine organisms that inhabit the ocean. Through extensive research, it has been discovered that certain antibacterial peptides possess potent antimicrobial properties against fungi, protozoa, viruses, and cancer cells. Consequently, numerous researchers have been inclined to classify these bioactive peptides as "peptide antibiotics."

3.2. Antimicrobial Peptides Derived from Insects

In instances where insects are exposed to pathogens or foreign chemicals, the hemolymph of these insects will generate a substantial quantity of antimicrobial proteins or antimicrobial peptides. The synthesis of these peptides mostly occurs within the fat bodies and subsequent secretion into the hemolymph. This process serves as a very efficient defense mechanism, capable of rapidly neutralizing or eradicating invasive germs. One example is the family of antimicrobial peptides known as Cecropin, which is widely recognized in insects. These peptides have been identified in several insect species, including guppies, silkworms, bees, and fruit flies. Cecropin A has demonstrated efficacy in combating several inflammatory conditions and malignancies [11]. It is important to note that there is significant variation in the quantity of antimicrobial peptides (AMPs) among different species. For instance, invasive Harlequin ladybirds (Harmonia axyridis) and Black flies (Hermetia illucens) possess as many as 50 AMPs, whereas the pea aphid (Acyrthosiphon pisum) does not possess any AMPs [12]. Jellyin, a peptide derived from royal jelly, exhibits robust biological activity, and demonstrates favorable effects against many bacteria and fungi. Additionally, its conjugated derivative, moonacid, has been found to possess inhibitory properties against Leishmania [13].

3.3. Antimicrobial Peptides in Mammals

Mammalian antimicrobial peptides have been identified in various species, including humans, sheep, cattle, and other vertebrates. The initial discovery of the mammalian antimicrobial peptide Cecropin P1 occurred in 1989, when it was isolated from the small intestine of pigs. The defensins present in the human body are a diverse group of antimicrobial peptides. They can be classified into three categories based on variations in their amino acid spatial structure and secretion site. These categories include human α-defensin (humandα-defensin), human β-defensin (human β-defensin), and human β-defensin (human β-defensin). Human θ-defensin (HβD) has been discovered.

There exist over 35 distinct types of human defensin, among which 10 defensin variants hold significant importance. Antimicrobial peptides can be categorized into many categories based on their structural characteristics, with Cathelicidin and Defensin being the predominant classifications. These entities can be categorized into five distinct classes: 1. Single chain α-helices lacking cysteine residues, or peptides consisting of two α-helices connected by a random coil region. 2. Antimicrobial peptides that exhibit a high abundance of specific amino acid residues, excluding cysteine residues. 3. Polypeptides with antibacterial properties characterized by the presence of a single disulfide bond. 4. Antimicrobial peptides possessing two or more disulfide bonds and adopting a β-folded conformation. 5. Peptides displaying antimicrobial activity derived from larger peptides with established functional roles. The earliest isolated Cecropins and Magainins derived from Xenopus are classified as the initial group of antimicrobial peptides, commonly known as Cecropin antimicrobial peptides. Currently, there is a greater level of depth in the study conducted on antimicrobial peptides. Furthermore, adenosine monophosphate (AMP) present in human breast milk serves a crucial function in the process of breastfeeding by mitigating the incidence of illness and death among infants who are nursed [14]. It is worth noting that Casein201, a peptide derived from beta-casein 201-220 amino acids, is present in varying quantities in colostrum from preterm and full-term humans [15]. Dairy products provide as a significant dietary source of AMP, which is generated through the process of enzymatic hydrolysis of milk. Multiple antimicrobial peptides (AMPs) have been discovered in various proteins such as alpha lactalbumin, beta-lactoglobulin, lactoferrin, and casein. Among these, lactoferrin B (LfcinB) is the most

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prominent peptide, as documented in previous research [16]. Furthermore, it is worth investigating the potential application of antimicrobial peptides derived from dairy products for the preservation of dairy products.

3.4. Microorganisms-Derived Antimicrobial Peptides

Bacterial antimicrobial peptides, commonly referred to as bacteriocins, encompass cationic peptides and neutral peptides, which are capable of being excreted by both gram-positive and gram-negative bacteria. Antimicrobial peptides can be derived from microorganisms such as bacteria and fungus. Notable examples of these peptides are nisin and gramicidin, which are obtained from Lactococcus lactis, Bacillus subtilis, and Bacillus brevis [17]. Bacteria have been revealed to possess four distinct classifications of antimicrobial peptides, including Bacitracin, Gramicidin S, Polymyxin E, and Nisin. Currently, the APD database comprises a total of 119 bacterial proteins. Among these proteins, nisin, a short peptide consisting of 3 to 4 amino acid residues, is produced by Lactococcus. Notably, nisin exhibits acid resistance and remains stable even in low pH environments, such as the stomach. Furthermore, it possesses the ability to inhibit gram-positive bacteria, including Clostridium and Listeria. Bacitracin and mersacidin, which are generated by certain strains of Bacillus spp., exhibit a significant inhibitory impact on methicillin-resistant Staphylococcus aureus (MRSA), a highly drug-resistant bacterium. Intraperitoneal dosing has been observed to effectively eliminate the presence of MRSA germs in various organs of mice, including the blood, lung, liver, kidney, and spleen. Furthermore, this method of delivery does not appear to induce any discernible damage to the organs of the animals.

3.5. Classification of AMPs Based on Amino acid-rich species

3.5.1. Proline

Proline is a representative example of a non-polar amino acid. PrAMPs exhibit distinct behavior compared to other AMPs. Specifically, they gain entry into the bacterial cytoplasm by utilizing the inner membrane transporter SbmA, as opposed to exerting antibacterial effects by membrane disruption [18]. The molecular formula of the compound is C5H9NO2.

It solid formula is (Figure 2).

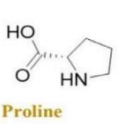

Figure 2. Proline

3.5.2. Histidine

Histidine is a prevalent amino acid with basic properties, and antimicrobial peptides (AMPs) that are rich in histidine demonstrate favorable activity in terms of membrane permeability. Moreover, it has been observed that HV2 exerts an inhibitory effect on bacterial motility, which is contingent upon its concentration. Additionally, HV2 demonstrates a robust anti-inflammatory action by suppressing the synthesis of tumor necrosis factor α (TNF-α) [19]. The molecular formula of the compound is C6H9N3O2. Its solid formula is (Figure 3).

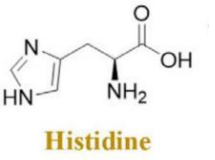

Figure 3. Histidine

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3.5.3. Glycine

In the field of biology, the R group of glycine is commonly categorized as a non-polar amino acid. Glycine-rich antimicrobial peptides (AMPs), such as attacins and diptericins, are found abundantly in various natural environments [20]. Its chemical formula is C2H5NO2. Its solid formula is (Figure 4).

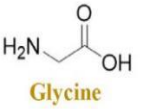

Figure 4. Glycine

3.6. Categorization of Antimicrobial Peptides (AMPs) According to Structural Characteristics

Antimicrobial peptides can be classified into four distinct categories according to their structural characteristics, namely linear α-helical peptides, β-sheet peptides, linear extension structure, and peptides exhibiting both α-helix and β-sheet conformations (Figure 5) [21]. Each individual possesses their own unique function, and hence, both individuals fulfill crucial roles.

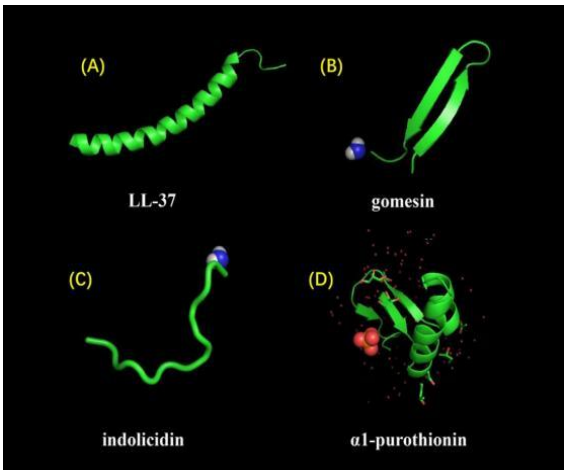

Figure 5. Displays several architectures of antimicrobial peptides (AMPs). LL-37 exhibits a characteristic α-helical structure, as reported in the literature (DOI: 10.2210/pdb2K6O/pdb). Gomesin is a peptide characterized by a β-sheet conformation and its stability is attributed to the presence of disulfide bonds (DOI: 10.2210/pdb1KFP/pdb). Indolicidin is an antimicrobial peptide (AMP) that possesses a linear extended structure rather than a well-defined three-dimensional conformation (DOI: 10.2210/pdb1G89/pdb). The protein α1-purothionin has a conformation that includes both alpha-helix and beta-sheet structures. The extension direction is shown by arrows (DOI: 10.2210/pdb2plh/pdb).

4. Designation

There are plenty of different approaches of designation, which is used to design antimicrobial peptides. Moreover, there are multiple types of Antimicrobial peptides (AMPs). AMPs have following questions with its property so it has a large number of methods on designation. Firstly, different function is an important aspect that should be taken into consideration of designing an antimicrobial peptides. In addition, costs of production and limited techniques narrow the possibility of manufacture. Another vital reason is that they have different stability in different conditions like temperature.

4.1. The Methodology of Template-Based Design

By comparing structurally comparable portions of natural antimicrobial peptides (AMPs) and identifying conservative patterns based on various types of residues (e.g., charged, polar, hydrophobic, etc) [22]. The propensity for helix formation, cationic properties, amphiphilicity, and overall

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hydrophobicity can be systematically altered by means of modification and other parameters. For example, cecropin, magainin, protegrin, and lactoferrin have been employed as templates for antimicrobial peptides (AMPs) [23]. The majority of antimicrobial peptides (AMPs) exhibit a cationic nature, resulting in reduced bactericidal efficacy in high salt conditions. This is attributed to the effective binding of cationic ions with the bacterial membrane [24]. The antimicrobial activity of a chimeric peptide H4, which is a combination of hBD3 and hBD4, was found to be more potent against bacteria like Enterococcus faecalis and S. aureus. Furthermore, this antibacterial activity was observed even in high salt circumstances [25]. Furthermore, the antibacterial activity of hBD3 was enhanced against various bacterial species, including E. coli and Enterococcus faecium, when the N-terminal region was truncated by three amino acids. This improvement was particularly notable under high salt conditions [26]. The antibacterial action of natural θ-defensins in rhesus macaques has been observed to be effective against both bacteria and fungi, even at low concentrations. As an illustration, it was observed that θ-defensin-1 (RTD-1) exhibited a three-fold increase in its bactericidal activity in comparison to the open-chain analog. Notably, this enhanced activity was not influenced by salt concentration [27]. In order to augment the antibacterial efficacy of antimicrobial peptides (AMPs) and mitigate their toxicity, the implementation of dimeric structures can be considered. However, the destabilizing effects on the membrane are diminished following the production of dimers [28].

4.2. Computer-assisted peptide

Computer-assisted peptide design (CAPD) pertains to the utilization of computer technology in the field of protein engineering. It involves the processing, prediction, and evaluation of various protein transformation schemes based on known protein sequence, molecular conformation, structure, and relationship data. CAPD aims to make optimal choices in protein design, with a particular focus on the development of software tools for protein engineering research. Computer design encompasses various methodologies, such as statistical modeling, quantitative structure-activity relationships research [29], neural networks [30], deep learning [31], word embedding [32], and machine learning. The backdrop of the research study

The field of quantitative structure-activity relationships (QSAR) has given rise to two distinct methodologies: a prediction method that relies on the therapeutic properties of AMPs.

In this study, we propose the utilization of an index for the identification of novel potential antimicrobial peptides (AMPs) from the expressed sequence tag (EST) database. Our approach is based on the concepts of highly conserved signal peptide subclasses that are associated with AMPs, as previously described [33]. The utilization of two active compounds to create chimeras is a well accepted practice in the field of computer-assisted drug discovery. This approach is employed by the software programs TOPAS [34] and BREED [35]. Nevertheless, this methodology may not always be suitable for peptides. The addition of acyl moieties is a significant approach for augmenting AMP activity, as these moieties can furnish the requisite hydrophobic regions for the formation of short peptides [36, 37, 38].

In relation to the present body of study on peptide molecules, it is evident that they universally possess a shared characteristic, namely, the ability to modify and enhance naturally occurring molecules. Nevertheless, over the course of conducting research, certain variables may arise that are outside the researcher's control, such as spatial mutations. Computer-aided design (CAD) obviates the need for exhaustive evaluation of each option, resulting in significant time, cost, and labor savings. One of the primary constraints in current computer-directed approaches to forecasting amp function is the requirement for standardized and dependable biological data as an input to facilitate an efficient design process. The field of computational methods has undergone significant advancements, leading to the emergence of training computers and improved analysis techniques. Additionally, several types of Synthetic Aperture Radar (SAR) strategies have been established. These strategies have the potential to be integrated with diverse structures that are rooted in natural sequences. The production of peptides can be achieved with a high degree of precision and can be enhanced through the process of evolving templates in order to generate active peptides [39].

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4.3. The general process of rational drug design

Rational drug design or structure-based drug design is based on the understanding of the molecular pathophysiology of the disease process, according to the molecular structure of the target, and reference to the chemical structure of the effector to design drug molecules for the disease, so as to guide the design to rationalization. The drugs designed by this method are often highly active, specific and have low side effects, so it is called rational drug design.Rational drug design is inseparable from computer, so it can also be called computer-aided drug design.In theory, computer-aided drug design avoids a certain degree of blindness in previous research, greatly speeds up the development of new drugs, and saves the human, material and financial resources for the development of new drugs. Janssen, a Belgian firm, for example, has increased the success rate of computer-aided drug design on a large scale from one in 10,000 to one in 3,000.

4.4. Computer aided drug design methods

4.4.1. Indirect drug design

3D structure search based on pharmacophore.

Pharmacophore model method.

It was found early that 2-deoxy-2, 3-dideoxyd-n-acetylneuraminic acid had an inhibitory effect on sialase bacteria, but the effect was not good in animal models.According to the crystal structure of sialase and its interaction with the enzyme inhibitor Neu5Ac2en, it was found that there were negative electric groups near the action site of the enzyme structure. If the inhibitor 4-carboxyl group was replaced by an amino group, the binding effect would be enhanced, because the amino group formed a salt bridge with the carboxyl side chain of the enzyme Glu119. If the 4-carboxyl group is replaced by guanidine, it can interact with Glu119 and Glu227, showing a stronger affinity.

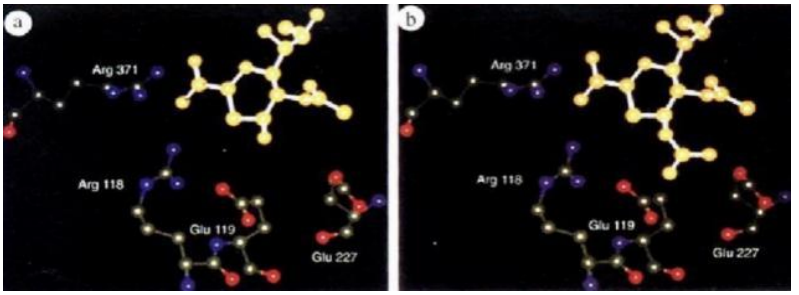

Figure 6. (a)2-deoxy-2, 3-dideoxyd-n-acetylneuraminic acid; (b)4-carboxyl group

4.4.2. Designing antimicrobial peptides

As antibiotic-resistant antibiotics gradually lose their effect, it is urgent to find new antimicrobial methods. Antimicrobial peptides are natural antimicrobials that protect the host organism from invasion, but their direct antimicrobial activity is moderate.[40]

4.5. Computer-assisted design of cyclic peptides and peptidomimetics

4.5.1. Cyclic peptidomimetics

The process of designing and synthesizing tiny molecules with conformational restrictions. The development and production of compact cyclic peptides with limited conformational flexibility is a compelling strategy to capture the essential molecular recognition components found in linear or bigger peptides and proteins. This approach aims to transfer these components to molecules that exhibit enhanced affinity, selectivity, stability, and bioavailability.

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5. Conclusion

Antimicrobial peptides have emerged as a prominent area of research worldwide, with several crucial challenges in their design and application that require immediate attention. There are multiple restrictions that provide obstacles to the development and implementation of amplifier applications. The development of prospective antimicrobial peptides (AMPs) can be enhanced by multidisciplinary interactions including several fields such as biology, materials science, chemistry, bioinformatics, molecular informatics, and pharmacy. In order to enhance comprehension of the correlation between amp and different goals, it is advisable to move beyond unilateral conductive experimental research. This approach will enhance the experimental design, resulting in a more robust and rigorous demonstration of systematic and scientific principles. The process of applying antimicrobial peptides (AMPs) in clinical settings, starting from laboratory research through eventual implementation in medical practice, is expected to be a protracted and challenging endeavor. This research posits that as our understanding of the structural and functional interplay of polypeptide antimicrobials becomes more refined and their stability continues to increase, there will be additional advancements in their clinical use and the emergence of novel breakthroughs in the healthcare domain.

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The overview of Clarithromycin treatment for Helicobacter pylori infection

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Abstract. *Helicobacter pylori* (*H. pylori*) is a Gram-negative bacillus that causes infections worldwide. *Helicobacter* pylori's drug resistance and infectivity are vital factors causing Helicobacter pylori infection. The virulence factors of *Helicobacter pylori* include acid escape virulence factors and epithelial cell colonizing factors, which can directly or indirectly cause chronic gastritis or gastric ulcer. As the resistance of *Helicobacter pylori* increases, a combination of multiple antibiotics is now the recommended therapy, and clarithromycin is one of them. The production process of clarithromycin and its effectiveness are essential points of discussion.

Keywords: Bacterial infection, *Helicobacter pylori*, Virulence factors, Clarithromycin

1. Introduction

Helicobacter pylori is a bacterial infection with a high prevalence that is extensively dispersed worldwide, impacting around 50% of the global population. Even though around 90% of individuals infected with *H. pylori* do not exhibit symptoms, this infection poses significant health concerns and has become progressively more challenging to manage due to the rise in antibiotic resistance in recent times [1]. Research has indicated that the occurrence of *H. pylori*-positive status exhibits variability based on various parameters, including age, geographical location, living conditions, and socioeconomic position. The primary mode of *H. pylori* transmission appears to be through oral-oral transfer [2]. Although *H. pylori* infection is highly prevalent, only a certain group of patients infected with this bacterium suffer severe gastroduodenal pathology. The aetiology of *H. pylori* infection and its subsequent illness manifestation are thought to be regulated by a multifaceted interplay of several variables., including the host, environment, and virulence factors of the bacteria. *H. pylori* has successfully acclimated to the challenging conditions of the human stomach by acquiring a repertoire of virulence genes. These genes facilitate the bacterium's ability to withstand the environment characterized by acidity, navigate to the gastric epithelium, and establish adhesion to gastric epithelial cells. The presence of these virulence factors facilitates the effective establishment of colonization inside the gastric mucosa and maintains a continuous infection *of H. pylori*. This, in turn, induces chronic inflammation and results in tissue damage, potentially culminating in the formation of peptic ulcers or even gastric cancer [3]. The most effective treatment options now available for *H. pylori* infection involve the use of a proton pump inhibitor (PPI) and ranitidine bismuth citrate in combination with two other antibiotics, known as triple

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treatments. Alternatively, a quadruple therapy approach can be employed, which includes the use of bismuth, tetracycline, metronidazole, and PPI. Clarithromycin is a highly effective antibacterial agent in combating *Helicobacter pylori* infections. The compound in question is a macrolide that exhibits acid stability and possesses a wide range of antibacterial properties. It is efficiently absorbed by the body and has a broad distribution throughout various tissue. Additionally, it is associated with few adverse effects. The minimum inhibitory concentration (MIC50) of Clarithromycin against *H. pylori* is rather low, the efficacy of the treatment is augmented through the suppression of acid. When the PPI or ranitidine bismuth citrate is coupled with amoxicillin or metronidazole, it is possible to achieve eradication rates above 95% for susceptible organisms [4, 5].

2. Pathogen virulence factors

H. pylori are successfully acclimated to the hostile conditions in the human stomach by acquiring a repertoire of virulent genes. These genes facilitate the bacterium's ability to endure the acidic environment, navigate towards the gastric epithelium, and establish adhesion to gastric epithelial cells. The presence of these virulence factors facilitates the effective establishment of colonization inside the stomach mucosa and supports the maintenance of a prolonged *H. pylori* infection. This, in turn, induces chronic inflammation and results in tissue damage, potentially culminating in the formation of peptic ulcers or even gastric cancer. A multitude of research has been conducted to examine the frequency and significance of purported *Helicobacter pylori* virulence genes in the development of disease. Although numerous virulence factors with diverse activities have been found, the connections with diseases seem to be less apparent, particularly when considering different study groups [3].

Acid escape virulence factors**:**

Urease:

1. The synthesis of ammonia (NH3) and carbon dioxide (CO2) has the ability to neutralize stomach acidity.

2. The process of angiogenesis has been identified as a contributing factor in the progression of stomach cancer.

3. The progression of malignancies is enhanced through the PI3K-AKT-mTOR pathway activation.

Bacterial shape:

The helical structure of bacteria has the potential to facilitate their entry into the mucous layer, so providing a protective advantage to the bacteria.

Flagella:

1. Motility facilitates bacterial locomotion away from acidic environments.

2. Flagellin activation exhibits increased motility, hence providing protection for the bacteria.

Epithelial cells colonizing factors:

BabA:

It is capable of binding to the epithelial cell receptor, Leb, hence facilitating attachment of bacterial and colonization. Additionally, it has been observed to enhance the translocation of CagA and produce double strand breaks in host cells.

SabA:

It is a bacterial adhesin that specifically interacts with the sialyl-Lex antigen, facilitating the attachment and colonisation of bacteria.

The OipA:

It facilitates the attachment of bacteria to the gastric epithelium, resulting in potential harm to the mucosal layer. This process triggers the release of interleukin (IL)-8 and leads to the death of host cells.

The HopQ:

It facilitates the attachment of bacteria to the stomach epithelium, hence impeding the functioning of immune cells.

Epithelial cells pathogenicity factors:

CagPAI:

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The genetic material under consideration encompasses a type IV secretion system (T4SS) which serves the purpose of aiding the transportation of CagA and peptidoglycan.CagT:

It functions as a critical component within the T4S system, facilitating the translocation process of CagA.

CagY:

It is capable of binding with integrin, thereby exerting influence over the immune response, facilitating bacterial persistence, and inducing modifications in the functions of the Type IV secretion system, also called T4SS.

Cagζ:

It is involved in the functioning of the Type IV Secretion System (T4SS) and has a role in facilitating the transport of CagA.

CagL:

It functions as a central protein within the Type IV secretion system (T4SS) and interacts with integrin, facilitating the CagA translocation and triggering the production of interleukin-8 (IL-8).

CagA:

It induces cellular proliferation by the process of tyrosine phosphorylation. This phosphorylation event also leads to the upregulation of IL-8 production and cell elongation. Additionally, CagA is involved in the downregulation of heat shock protein VacA:

1. It induces vacuolization in epithelial cells, leading to cellular necrosis or apoptosis.

2. The autophagy activation and subsequent rise in cellular death is augmented by endoplasmic reticulum stress.

HtrA:

It functions as a protease, responsible for the degradation of misfolded proteins. Additionally, the facilitation of CagA dissemination is of utmost importance. Moreover, it cleaves tight junction proteins, namely occludin, claudin-8, and E-cadherin.

Outer membrane vesicles:

The internalization of outer membrane vesicles, whether clathrin-dependent or independent, serves as a protective mechanism for pathogens against the detrimental impact of reactive oxygen species. Additionally, this process hinders normal cellular activities while also stimulating dendritic cell functions.

γ-glutamyl transpeptidase:

1. It is involved in transpeptidation and amino acid production, which have been found to promote cell death and suppress cellular growth and cell cycle arrest.

2. The vacuolation of epithelial cells, which leads to the death of these cells.

2. Clarithromycin

2.1. History

The discovery of Clarithromycin can be attributed to the efforts of researchers employed at Taisho Pharmaceutical, a prominent Japanese pharmaceutical business, in the year 1980. The development of the product was driven by the objective of creating a variant of the antibiotic erythromycin that would not undergo acid instability within the gastrointestinal tract, hence mitigating adverse effects such as nausea and stomachache. The pharmaceutical company Taisho initiated the process of seeking patent protection for their medicine in approximately 1980. Following this, they brought a proprietary variant of the drug, named Clarith, to the Japanese market in 1991. In the year 1985, Taisho entered a partnership with the American corporation Abbott Laboratories to acquire the international rights for their product. Additionally, Abbott Laboratories successfully obtained permission from the *Food and Drug Administration* for Biaxin in October 1991. The pharmaceutical product transitioned to a generic form in Europe in the year 2004, followed by its generic availability in the United States during the middle of 2005 [6].

3. Process of traditional synthesis

Comprehensive Elucidation of the Innovation

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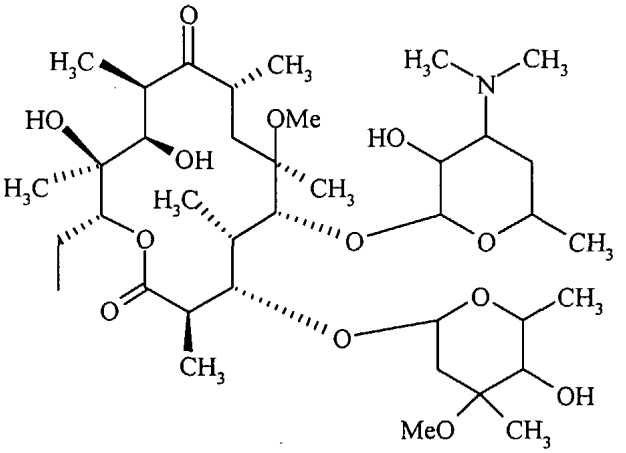
A comprehensive investigation is currently being conducted to explore the large-scale manufacture and high purity of 6-O-Methyl-erythromycin, also called Clarithromycin, a highly effective inhibitor for gram-positive bacteria. Therefore, there was a regulated methylation of the 6-hydroxyl group of erythromycins, which had been protected at the 2' and 4" positions. The current invention pertains to the manufacturing process of achieving an enhanced degree of purity for Clarithromycin (Formula I) [6].

Formula (I)

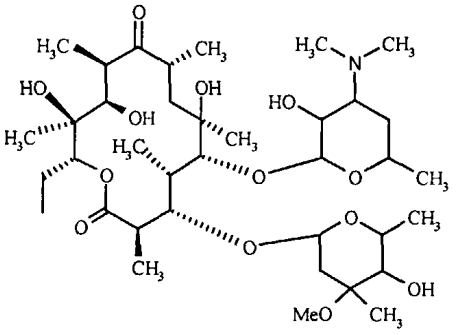


The initial iteration of Clarithromycin produced from this methodology exhibits a concentration of 6,11-O-dimethyl erythromycin-A, a possible byproduct, which is below 1.0%. The current innovation focuses on the industrial process of Clarithromycin, specifically aiming to achieve a controlled amount of side products. The finished product has been subjected to HPLC examination, which has led to the characterization of a total of eight contaminants. In accordance with a specific embodiment of the current invention, the synthesis of Clarithromycin Form II is achieved by the implementation of the subsequent reaction scheme:

(a) The reaction between Erythromycin-A and hydroxylamine hydrochloride, resulting in the formation of Erythromycin-A-9-Oxime.

The hydroxylamine hydrochloride compound is subjected to a reaction with caustic flakes in an aqueous solution of isopropyl alcohol within the range of 10°C to 20°C. This reaction results in the formation of the hydroxylamine base, which is dissolved in the solution. Subsequently, Erythromycin A is added to the solution [6].

The formula of NhLOH HCI isopropyl Alcohol



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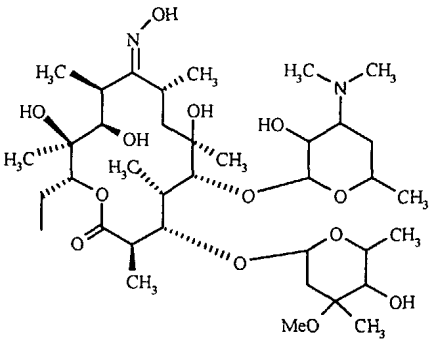
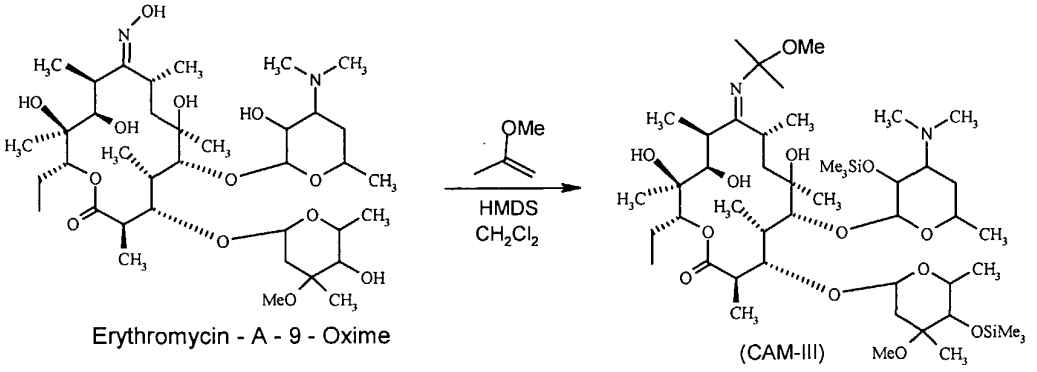


The formula of Erythromycin - A Erythromycin - A - 9 - Oxime

The pH of the reaction mixture is carefully regulated to a range from 6.5 to 7.0 with a gradual and precise supplement of glacial acetic acid. Erythromycin-A is introduced into the agitated reaction mixture, which is thereafter stirred for an additional duration of 28 hours at a temperature of 55°C. Following the conclusion of the reaction, the mixture is subjected to neutralization by the addition of aqueous ammonia and water. The resulting mixture is then subjected to continuous stirring for a duration of one hour. To achieve a precipitate of Erythromycin-A-9-Oxime with a yield ranging from 85% to 90%, an increased quantity of water is introduced into the reaction mixture [6].

(b) The objective of this study is to investigate the oxime protection, T, with the 4"-OH functional groups of Erythromycin-A-9-Oxime. The compound known as Erythromycin-A-9-Oxime, which was acquired through the previously mentioned process, is subjected to a reaction involving 2-methoxy propene and pyridine hydrochloride from dichloromethane. The mixture is subjected to stirring for a duration of 6 hours within a temperature range of 8°C to 120°C, after which hexamethyldisilazane (HMDS) is introduced [6].



The stirring process is maintained for approximately 15 hours at a temperature consistent with the surrounding environment. Following the comprehensive protection of the oxime and 2' & 4" -OH functionalities of Erythromycin - A-9-oxime, the fully protected compound is entirely protected through conventional work-up and filtration methods. CAM-III is obtained through the process of vacuum drying, resulting in a yield of above 90%. The melting point of the substance under consideration ranges from 125 degrees Celsius to 1270 degrees Celsius [6].

(c) The 6-OH group of CAM-III is subjected to methylation, followed by deprotection of the oxime and the 2' and 4'-OH functions.

To achieve this, 2',4"-O-Bis(trimethylsilyl)-erythromycin-A-9- [O- (l-methoxymethyl ethyl) oxime] is reacted with methyl iodide in the presence of potassium hydroxide powder in a suitable solvent system. This reaction results in the formation of 2',4"-disilylated-Clarithromycin-9-methoxypropyl oxime (CAM-IV). Upon further hydrolysis, CAM-IV yields Clarithromycin-9-Oxime, as illustrated below:

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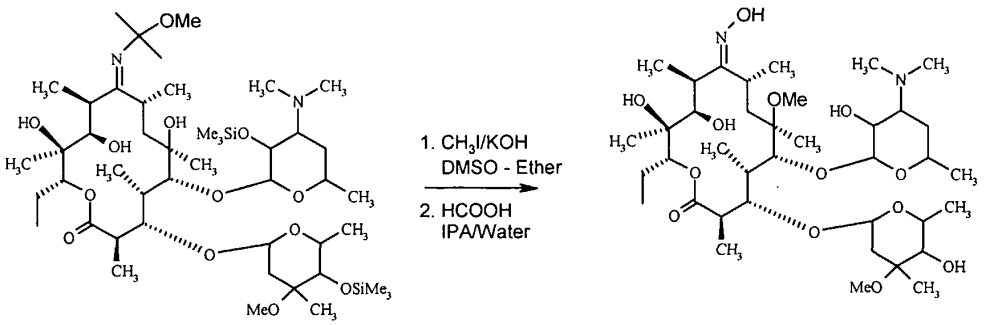


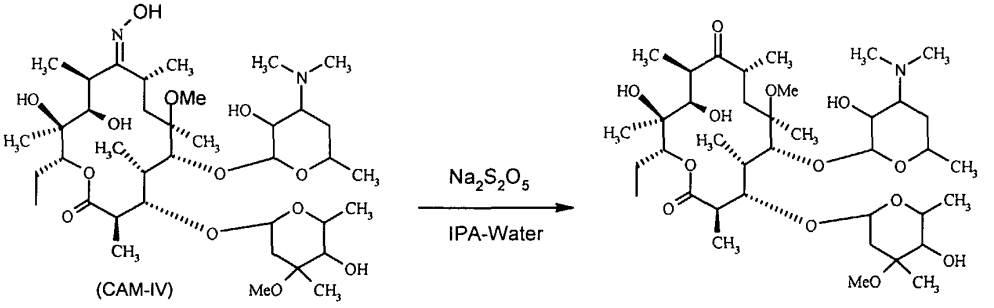
The term "computer-aided manufacturing" (CAM) pertains to the utilization of computer technology for manufacturing purposes. The chemical being examined is clarithromycin-9-oxime. One of the suitable solvent systems for this reaction includes a combination of DMSO and diethyl ether, among other potential options. The ideal proportion of diethyl ether in DMSO falls within the range of around 40% to 60%, with a preferred value of around 50%. The reaction is typically performed within the temperature range spanning between 10°C and 600°C, with a preferred range of approximately 15°C to 25°C. The duration of reactions often falls within a range of 40 minutes to 2 hours, with a favored interval of 80 to 100 minutes [6].

The precise ratio of methyl iodide to potassium hydroxide powder is of utmost importance, as any variation from the appropriate proportion can lead to the generation of unwanted byproducts. Based on thorough examination, it has been shown that the combination of methyl iodide (1.75 moles) and potassium hydroxide (1.20 moles) results in the formation of a resultant substance containing regulated impurities. This substance can be subjected to subsequent purification processes, such as one or two re-crystallizations. An advantage of employing the methylation approach in the current study using DMSO-Diethyl ether is the capacity to isolate the ether layer subsequent to halting the reaction mixture with a 40% dimethylamine solution. The ether layer is comprised solely of the methylated product, while the DMSO layer contains impurities. The DMSO layer is subsequently employed for the retrieval of DMSO, which functions as the solvent. In the second stage of the synthesis, the methylated product, referred to as CAM-IV, undergoes a reaction with 98% formic acid in a volume ratio of 1:1 between isopropyl alcohol and water. The aforementioned reaction is conducted over a period of 30 minutes, within the temperature range observed is between 25°C and 35°C. The intended result of this chemical reaction is the synthesis of Clarithromycin-9-Oxime [6].

(d) The process of converting to Clarithromycin from Clarithromycin-9-Oxime is examined in this study.

The production process of Clarithromycin involves the conversion of Clarithromycin-9-Oxime through a reaction with sodium metabisulphite in a solution consisting of Isopropyl alcohol and water in equal proportions (1:1, v/v). This reaction is carried out at the temperature of 80°C for a period of 6 to 8 hours [6].

Formula (I)



Clarithromycin-9-Oxime Clarithromycin

2.3 Mechanism of actions

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Macrolides exert their mechanism of action by the binding process to ribosomes, specifically targeting the peptidyl transferase loop located in domain V of the 23S ribosomal RNA. This interaction effectively hinders the translocation of aminoacyl transfer-RNA, consequently impeding the subsequent process of protein synthesis. Clarithromycin exhibits a wide spectrum of antimicrobial action, comparable to that of erythromycin. It effectively inhibits a diverse array of microorganisms encompassing both Gram-positive and Gram-negative bacteria, atypical pathogens, anaerobic organisms. Nevertheless, the development of resistance to erythromycin typically indicates a concurrent resistance to clarithromycin. It is worth mentioning that clarithromycin exhibits activity, ranging from active to moderately active, against Campylobacter species. However, it demonstrates greater activity against H. pylori compared to erythromycin, azithromycin, and roxithromycin [6].

2.4 Side effect and precautions

The most documented side effects of clarithromycin included nausea with 3.8%, diarrhea with 3.0%, stomach discomfort with 1.9%, and headache with 1.7% [6]. Taste disturbance is a frequently observed phenomenon that has a correlation with dosage. For instance, when administered concurrently with omeprazole, about two-thirds of patients reported the occurrence of a metallic taste sensation, which subsequently vanished entirely within a few days following the completion of the treatment [7]. Occasional adverse effects such as cholestasis, jaundice, hepatitis, and Steven Johnson syndrome have also been documented.

4. Overview and conclusion

Clarithromycin, a macrolide antibiotic that is developed from erythromycin, serves as the foundation on the treatment of H. pylori infection due to its favorable attributes, including a low minimum inhibitory concentration (MIC), effective absorption into the mucosal lining, and minimal susceptibility to the acidic environment. The variability about the prevalence of clarithromycin resistance in H. pylori, which is the major determinant of the effectiveness of empirically prescribed conventional H. pylori eradication therapy, exhibits significant regional and international differences. The prevalence is contingent upon the selection pressure exerted by the specific group of antibiotics within a particular geographical region [8].

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An in-depth review of pneumonia and the combination therapy of pneumonia with Levofloxacin and Cefixime

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Abstract. Pneumonia is one of the most common diseases in the world. When we go to the hospital, we can see and hear many patients suffering from pneumonia. A large number of people die from pneumonia every year all over the world. With the development of medical industry and the occurrence and invention of many drugs, pneumonia can be dealt with by using the drugs to kill the pathogens. However, how to use a more effective and economic-friendly approach to deal with pneumonia is still worth discussing. Now, there is a more effective way-combining two kinds of antibiotics to deal with pneumonia. In this essay, I will give an introduction of pneumonia, including its history, its symptoms, its impact, etc. One of the main pathogenic bacteria of pneumonia, Streptococcus pneumoniae, will also be discussed. Two approaches with some important disadvantages that people use to deal with pneumonia will be briefly discussed. Then, I will elaborate Levofloxacin and Cefixime, and how people combine Levofloxacin and Cefixime to deal with pneumonia.

Keywords: Pneumonia, Levofloxacin, Cefixime

1. Introduction

Pneumonia is one of the most common diseases in the world. Many people are suffering from pneumonia. What's worse, a large amount of people has died because of pneumonia. It's important for people to find the method of dealing with pneumonia, saving patients' lives. Fortunately, with the development of medical industry, people have found or invented many antibiotics to kill the pathogens of pneumonia, helping people to recover from pneumonia. However, the pathogens of pneumonia have already generated the resistance against some antibiotics, making these antibiotics become less effective when they are used to kill the pathogens [1]. In addition, although some antibiotics are not strongly resisted by the pathogens, some of them are too expensive, leading to the heavy financial burden for patients and governments. Therefore, how to treat pneumonia with a both effective and economic-friendly way is worth discussing. In this essay, I'll discuss the therapy of dealing with pneumonia by the combination of Levofloxacin and Cefixime. Levofloxacin and Cefixime are both effective and economic-friendly antibiotics. Additionally, they are absolutely two different kinds of antibiotics, which means the combination therapy can deal with the resistance against antibiotics. Therefore, the combination therapy will be a both effective and economic-friendly way to deal with pneumonia, which can benefit the whole world.

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2. Pneumonia

2.1. The Definition and Classification of Pneumonia

Pneumonia refers to inflammation of the terminal airways, alveoli, and pulmonary interstitium. It can be caused by pathogens, physical and chemical factors, immune damage, allergies, and drugs. Pneumonia caused by pathogenic microorganisms is the most common. There are three classification methods for pneumonia caused by pathogens. First of all, according to the pathogen types, pneumonia can be divided into bacterial pneumonia, viral pneumonia, atypical pathogen pneumonia, pulmonary fungal disease, and pulmonary parasitic disease. Secondly, according to the patient's environment while being infected, pneumonia can be divided into community acquired pneumonia (CAP) and hospital acquired pneumonia (HAP). CAP refers to pneumonia contracted outside of the hospital, including pneumonia that develops during the incubation period after admission due to pathogen infections with a clear incubation period. HAP refers to pneumonia that occurs in the hospital after 48 hours or more of admission, including pneumonia that occurs within 48 hours after being infected in the hospital and discharged from the hospital. Thirdly, according to the anatomical site where pneumonia occurs in the lungs, pneumonia can be divided into lobar pneumonia, lobular pneumonia, and interstitial pneumonia. In clinical situation, doctors always make the diagnosis based on the chest radiograph [2].

2.2. History of Pneumonia

The initial records of pneumonia could be traced back to Hippocratic era. He, like other ancient medical experts, classified diseases with chest pain symptoms as Perioneumonia, and this term had been used for over 2000 years. Hippocratic used blood letting and hot compress to treat patients with Perioneumonia. In the 17th century, Sydenham, a famous Dutch clinical physician, believed that Perioneumonia was the same disease as Pleurisy, but the scope of Perioneumonia's invasion of lung parenchyma was more extensive. Sydenham reported a case of Perioneumonia Notha, and his student Huxham invented a formula specifically designed to treat it, which was actually a type of alcohol containing cinchona. In 18 century, the famous British clinical physician Boerhaave described two types of peripulmonary inflammation in his book "Proverbs". One occurs at the end of the pulmonary artery, and the other occurs in the bronchus. This may be the earliest distinction between lobar pneumonia and lobular pneumonia. Italian anatomist Morgagni dissected and observed the bodies of pneumonia patients after death, studying pneumonia pathologically. What's more, Baillie first described the liver like changes in the lungs of pneumonia patients. The Western medicine percussion method was invented in the 18th century. Auenbrugger used percussion method in 1761 to analyze the percussion sounds of normal lungs and those of pneumonia patients. The invention of percussion method greatly helped doctors diagnose chest diseases. In 19 century, French doctor La ë Nnec invented the Stethoscope. The appearance of Stethoscope makes it possible for doctors to find chest diseases, such as pneumonia, in time. In 1819, La ë Nec published the classic work "On Auscultation", in which he meticulously described many common auscultation sounds in chest diseases. La ë NEC also noticed that the crepitus heard during chest auscultation is an important indicator for determining the initial stage of pneumonia and a sign of the dissipating stage of pneumonia. Since the invention of percussion and auscultation methods, people's understanding of pneumonia has become increasingly profound. Most pathogenic bacteria were discovered in the last 30 years of the 19th century, allowing doctors to make etiological diagnosis of pneumonia. In 1895, German physicist Roentgen invented X-ray, which was quickly applied in medicine, especially in the diagnosis of pneumonia. In 1920, Ramsay reported Pneumonilis based on X-ray examination results. In 1938, Reimann further introduced this type of pneumonia, and in 1945, Dingle reported that the pathogen of Pneumonilis may be a virus. The invention of antibiotics in the 20th century created miracles for the treatment of bacterial diseases. In 1928, A. Fleming discovered penicillin; in 1935, G. Domagk invented the sulfa drug Prontosil; in 1944, S. A. Waksmann invented Streptomycin; in 1947, he discovered Chloramphenicol; in 1948, he discovered Chlortetracycline; since then, tetracycline, Oxytetracycline

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and other antibiotics have been used in clinical practice. Bacterial pneumonia has been beaten by the antibiotics repeatedly [3].

2.3. The Symptoms of Pneumonia

Acute onset, chills, fever. Cough, phlegm, mostly purulent phlegm, occasionally with phlegm and blood. Typically, Streptococcus pneumoniae pneumonia has rust colored sputum, caused by the release of hemoglobin from red blood cells in a fibrous exudate. Mycoplasma pneumoniae pneumonia and viral pneumonia have low sputum volume, mostly white sputum. Phlegm from pulmonary fungal disease is white and thick, with filamentous symptoms, making it difficult to cough up. Physical examination may show signs of lung consolidation or the smell of moist rales. Severe infections can manifest as circulatory failure, respiratory failure, or multiple organ dysfunction [4].

2.4. The Impact and Mortality of Pneumonia

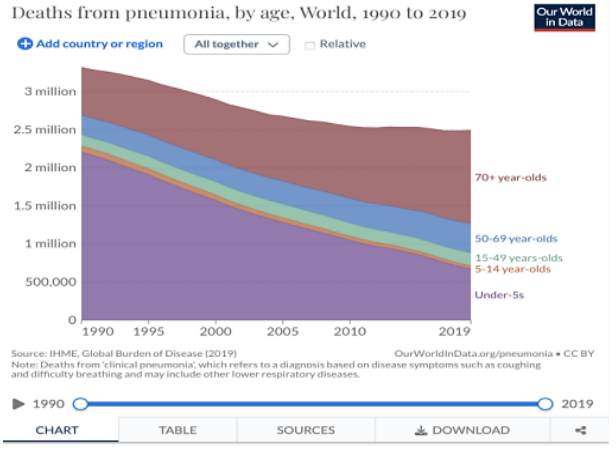

Figure 1. Deaths from pneumonia, by age, from 1990 to 2019 [5].

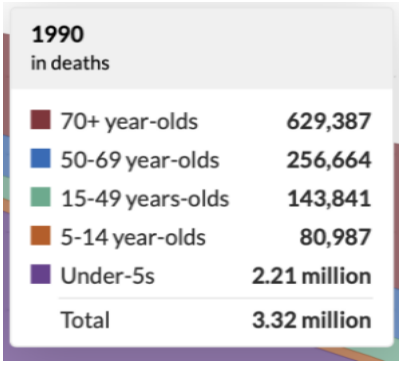

Figure 2. Deaths from pneumonia, by age, in1990 [5].

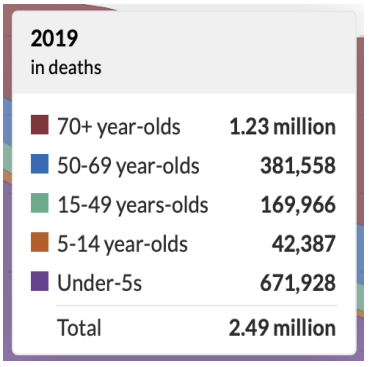

Figure 3. Deaths from pneumonia, by age, in 2019 [5].

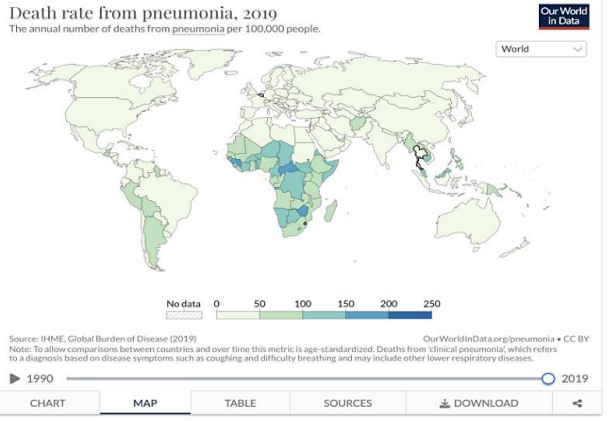

Figure 4. Death rate from pneumonia, 2019 [5].

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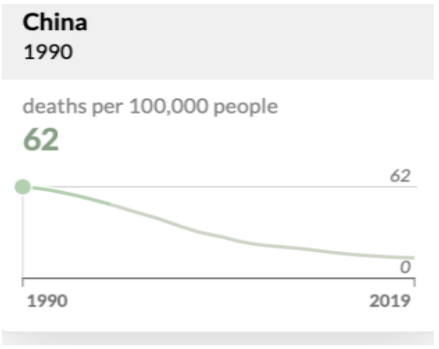

Figure 5. Deaths from pneumonia in China in 1990 [5].

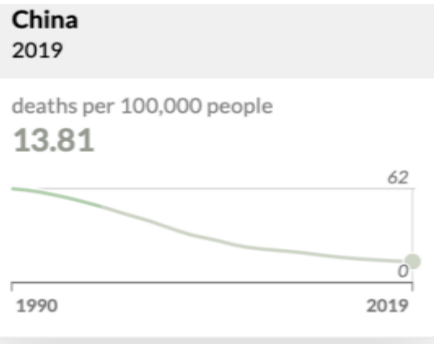

Figure 6. Deaths from pneumonia in China in 2019 [5].

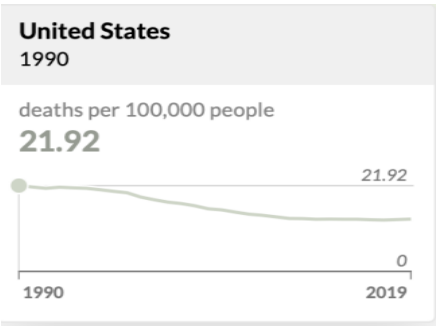

Figure 7. Deaths from pneumonia in the U. S. in 1990 [5].

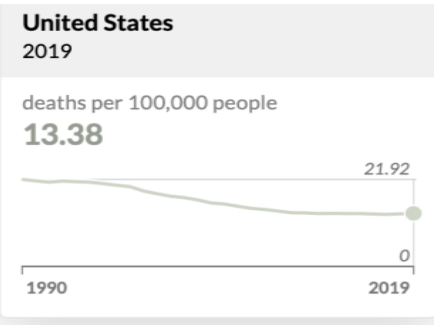

Figure 8. Deaths from pneumonia in the U. S. in 2019 [5].

Pneumonia is one of the most common and influential diseases in the world. As shown in Figure 1 and Figure 2, in 1990, 3.32 million people died because of pneumonia in 1990. In 2019, the deaths number from pneumonia have been reduced. As shown in Figure 3 and Figure 1, in 2019, 2. 49 million people died because of pneumonia [13].

In China, as shown in figure 5 and figure 6, the deaths from pneumonia per 100000 people were 62 people in 1990 and 13. 81 people in 2019. In the United States, as shown in Figure 7 and Figure 8, the deaths from pneumonia per 100000 people were 21. 92 people in 1990 and 13. 38 people in 2019 [14].

The death rate of pneumonia differs from region to region. According to figure 4, the death rates from pneumonia in developing countries are highest in the world, especially for the Sub-Sahara Africa, the South-East Asia, and some countries in South America. And the death rates of pneumonia in developed countries are relatively low [15]. This difference is related to the different developing level of medical industry and different economic strength in different countries.

The number of the deaths and death rate from pneumonia have been reduced, but the problem of dealing with pneumonia is still severe and challenging to people. And how to deal with pneumonia with some both economic-friendly and effective approaches is also worth discussing.

3. Streptococcus pneumoniae-- one of the pathogenic bacteria of pneumonia

3.1. Introduction of Streptococcus pneumoniae and how they infect the lung

Streptococcus pneumoniae, also known as pneumococcus, often reside in the nasopharyngeal cavity of normal individuals. Most Streptococcus pneumoniae are not pathogenic or have weak pathogenicity, with only a few having pathogenicity. They are the main pathogens of bacterial pneumonia [16]. In developing countries, 5 million children under the age of 5 die each year from pneumonia caused by Streptococcus pneumoniae. In recent years, 40% to 50% of community acquisition pneumonia in the UK has been caused by Streptococcus pneumoniae [6]. Streptococcus pneumoniae are Gram positive

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bacteria and anaerobic bacteria with a spearhead like shape, mostly arranged in pairs, with opposite wide ends and outward pointed tips. The bacterial cell is surrounded by a transparent ring, as shown in figure 8. It can also appear as a single or short chain in sputum, pus, and lung tissue lesions. No flagella or spores. capsule can be formed in the body or serum-containing culture medium, and they require special staining to be visible [7]. Streptococcus pneumoniae's pathogenicity are mainly due to the invasiveness of its capsule containing high molecular weight polysaccharides on tissues, and the bacterial exudate spreads through the Kohn pores to the surrounding alveoli, affecting lung segments or lobes (hence also known as lobar pneumonia). Streptococcus pneumoniae does not produce toxins and generally does not cause lung tissue necrosis or other damage to lung structures [8].

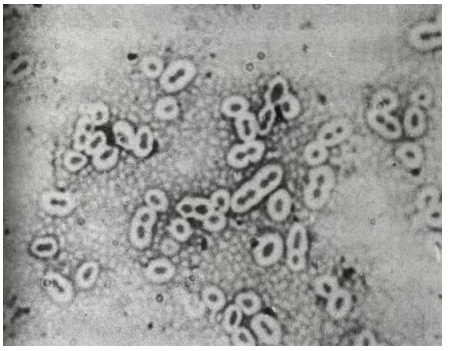

Figure 9. Streptococcus pneumoniae capsule,capsule stain x1500 [9].

3.2. Image of The Infected Lung and The Normal Lung

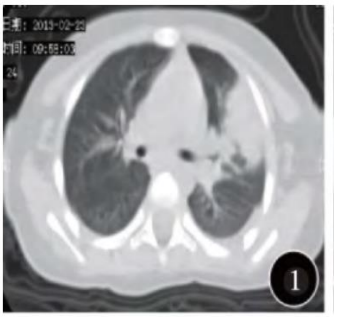

Figure 10. Imaging of Streptococcus pneumoniae pneumonia [8].

The consolidation lesion starts from the periphery of the left upper lobe of the lung, adjacent to the pleura, and spreads towards the center; there is a small amount of pleural effusion on the left side [10].

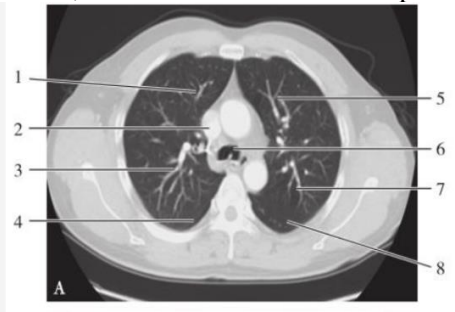

Figure 11. Imaging of the normal lung [10].

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4. Drugs used to treat pneumonia

4.1. Brief introduction of other approaches with some crucial disadvantages that people use to deal with pneumonia

Vancomycin:

People use Vancomycin to deal with pneumonia. Vancomycin is a kind of glycopeptide antibiotic, which is extremely good at killing Gram positive and Gram negative bacteria, including the Streptococcus pneumoniae [11]. And the Streptococcus pneumoniae almost hasn't generated the resistance against Vancomycin. Zhao Xiaoji and others did a research about the rate of resistance against antibiotics generated by streptococcus pneumoniae. They collect specimens from 75201 patients treated at The Third Hospital of Mianyang from January 2017 to December 2020, and a total of 596 strains of Streptococcus pneumoniae were isolated. After conducting bacterial identification, drug sensitivity tests, and statistical analysis of the data, as shown in Table 1, they found that the drug resistance rate of Streptococcus pneumoniae against Vancomycin is 0% in both children group and adult group [12]. However, it rarely metabolizes in human bodies. More than 90% Vancomycin need to excrete through the filtration process of glomerulus. Therefore, in addition to other common side effects, it has a relatively higher Nephrotoxicity. Its adverse effects rate is also relatively high [13]. And its price is also relatively high. I did a survey in Qilu Hospital of Shandong University. I found that the price of Wenkexin Vancomycin Hydrochloride for injection is 103. 99 yuan, and the price of Laikexin Vancomycin Hydrochloride for injection is 78. 00 yuan. And the price of Levofloxacin for injection is 30. 81 yuan; the price of Levofloxacin for oral administration is 24. 00 yuan. And the Cefixime for oral administration is 3. 79 yuan. Obviously, Vancomycin has a relatively high price than Cefixime and Levofloxacin, which means it is not very economic-friendly.

Erythromycin:

People use Erythromycin to deal with pneumonia. Erythromycin is a kind of macrolides antibiotic, which can be used to kill the pathogenic bacteria of pneumonia, including the streptococcus pneumoniae. Its side effects are rare and mild [14]. However, the Streptococcus pneumoniae has generated a strong resistance against Erythromycin, which means its efficiency of killing Streptococcus Pneumoniae is largely reduced. As I mentioned before, Zhao Xiaoji and others did a research about the drug resistance generated by Streptococcus pneumoniae. As shown in Table 1, we can see than the drug resistance rate of Streptococcus pneumoniae against Erythromycin is 94. 7% in the children group; the drug resistance rate of Streptococcus pneumonia against Erythromycin is 98. 6% in the adult group. Therefore, Erythromycin is not a effective way for people to deal with pneumonia [15].

Table 1. Comparison of drug resistance rate of Streptococcus pneumoniae isolated from children group and adult group [strains(%)]. [16].

Antibiotics	Children Group (n=378)	Adults Group (n=218)	The value of x^2	The value of P
Penicillin	31(8. 2)	35(16. 1)	8. 66	0. 00
Amoxicillin	73(19. 3)	58(26. 6)	4. 29	0. 04
Ceftriaxone	21(5. 6)	34(15. 6)	16. 64	0. 00
Cefotaxime	23(6. 1)	36(16. 5)	16. 86	0. 00
Tetracycline	342(90. 5)	206(94. 5)	3. 02	0. 08
Erythromycin	358(94. 7)	215(98. 6)	5. 71	0. 02
Linazolamide	0	0	——	——
Levofloxacin	4(1. 1)	28(13. 2)	37. 80	0. 00
Vancomycin	0	0	——	——

Vancomycin is extremely effective, but it is not very economic-friendly and has many severe adverse effects. Erythromycin is relatively mild and safe, but it is not effective while being used to kill

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Streptococcus pneumoniae. Therefore, there must be other better antibiotics which can be used to kill Streptococcus pneumoniae and deal with pneumonia.

4.2. The Combination Therapy with Levofloxacin and Cefixime to Deal with Pneumonia

4.2.1. Levofloxacin

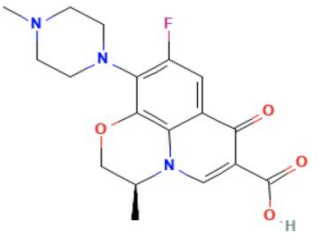

Figure 12. The picture shows the structure of Levofloxacin [16].

(1) Basic Information:

Levofloxacin is a quinolone antibiotic, and it is also the broad-spectrum antibiotic. Its molecular formula is C18H20FN3O4;its IUPAC name is (2S)-7-fluoro-2-methyl-6-(4-methylpiperazin-1-yl)-10-oxo-4-oxa-1-azatricyclo[7. 3. 1. 05, 13]. trideca-5(13), 6, 8, 11-tetraene-11-carboxylic acid. Its molecular weight is 361. 4g/mol. It is freely soluble in glacial acetic acid and chloroform;it is sparingly soluble in water [17]. Its Basic pKa is 8. 12 and its Acidic pKa is 6. 1 [17].

(2) The Mechanism of Action:

As the left-handed isomer of Ofloxacin, Levofloxacin has a good effect on gram-positive bacteria, including Streptococcus pneumoniae. Quinolones can block DNA replication and interfere with the distribution of replicated DNA to offspring cells by inhibiting DNA gyrase and Topoisomerase Ⅳ of bacteria, thus achieving rapid sterilization [18].

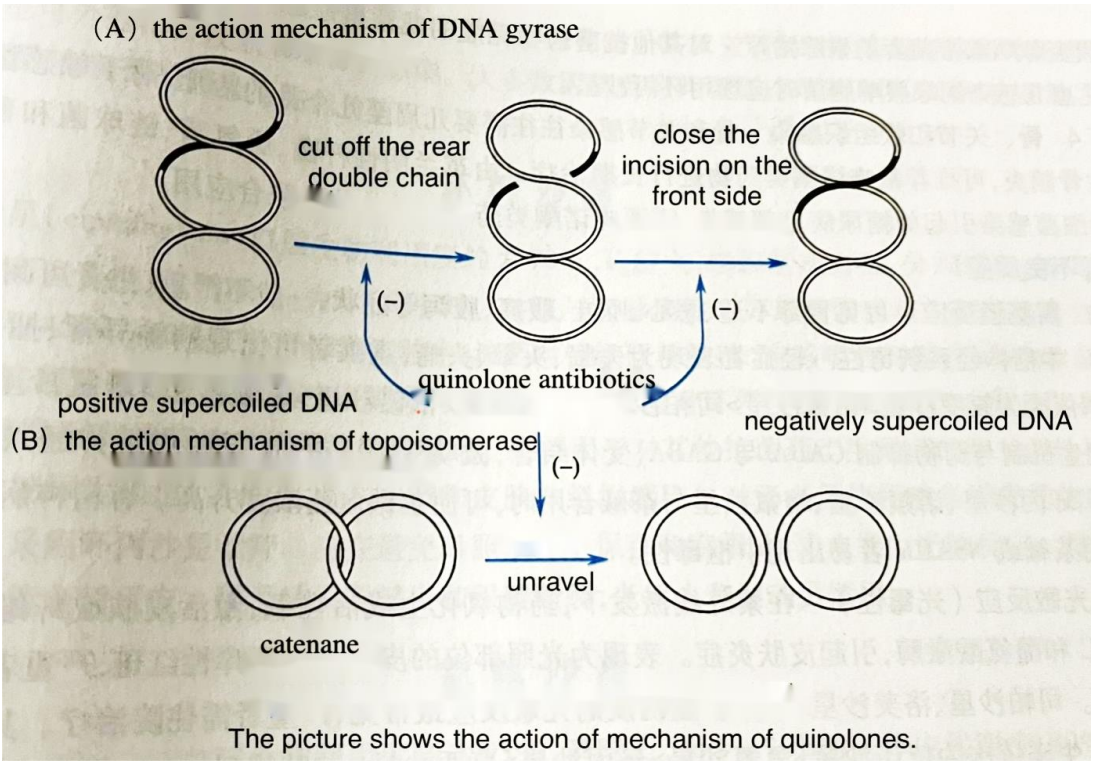

Figure 13. The mechanism of Quinolone antibiotics [19].

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(3) Usage, Dosage, and Some of the Precautions

2007 CAP Diagnosis and Treatment Guidelines in the United States pointed out that when Levofloxacin is used to deal with pneumonia, doctors should maintain the plasma concentration above the mutant prevention concentration as long as possible, ensuring the success of treatment and preventing the occurrence of drug-resistant mutants [20]. In China, Levofloxacin can be used by diluting it into 0. 9% sodium chloride injection or 5% glucose injection for intravenous drip. The adults receive 0. 3g-0. 6g Levofloxacin per day, and it should be given once or twice per day by injection. Depending on the type and symptoms of infection, the amount should be modified appropriately. Moreover, it can also be used for oral administration in China. When it is used to deal with community acquired pneumonia, people should be given 0. 4g per day and twice a day; or people can be given 0. 5g per day and once a day; the treatment period is 7days [21]. Like other quinolone antibiotics, Levofloxacin can't be given to children and pregnant women.

(4) The Efficiency: It's very good at dealing with the pathogenic bacteria of pneumonia, including Streptococcus pneumoniae. Additionally, the resistance against Levofloxacin generated by Streptococcus Pneumoniae is not very high and serious. As shown in Figure 12, according to the research done by Zhao Xiaoji and others, the drug resistance rate of Streptococcus pneumoniae against Levofloxacin is 1. 1% in children group;the drug resistance rate of Streptococcus pneumoniae against Levofloxacin is 13. 2% in adult group [22]. According to the survey I did in Qilu Hospital of Shandong University, the price of Levofloxacin for injection is 30. 81 yuan. And the price of Levofloxacin for oral administration is 24. 00 yuan. It's economic-friendly. Tang Jinwen did a research at Daze Health Center, Xinhui District, Jiangmen City, Guangdong Province. He divided 100 patients who had community acquired pneumonia in half into two groups. In addition to some basic therapies, the patients in the experimental group were treated with Levofloxacin. And in the control group, the patients were treated with conventional therapy. The patients in the experimental group were given Levofloxacin for injection 500mg per time and once per day. When the patients in the experimental group got better, the therapy changed into Levofloxacin with oral administration. After conducting the therapies and collecting the data, Tang Jinwen found that the total effective rate of the experimental group (Levofloxacin Treatment) was 98%, and the adverse effects rate of the experimental group(Levofloxacin Treatment)was only 1. 9%. In the control group (Conventional Treatment), the total effective rate was 86%, and the adverse effects rate was 13. 4% [38]. Therefore, even if we use Levofloxacin alone to deal with pneumonia, it will be pretty effective and safe.

(5) Synthesis: This is a relatively new way to synthesize Levofloxacin-- "One Pot" Synthesis. It's invented by Zhao Feichao, etc. It is more environmental-friendly than traditional approaches;it has low waste and high yield [23].

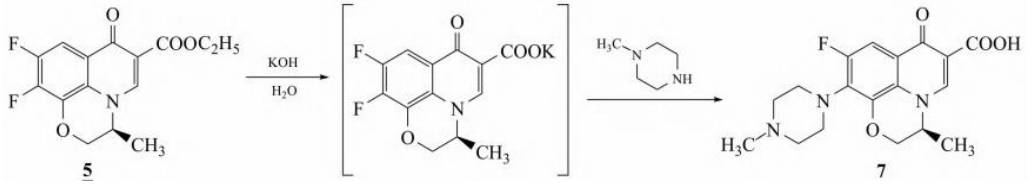

Figure 14. The picture shows the "One Pot" Synthesis of Levofloxacin [40].

The Specific Process of Synthesis:

Add 20. 0g **5**, 4. 0g of potassium hydroxide, and 120mL of water to a 500mL three necked bottle, raise the temperature to 60 ℃, and keep it warm until the spots of levofloxacin cyclic ester disappear after TLC detection. Add 105. 0 g of N-methylpiperazine. Continue to raise the temperature to 100 ℃, keep it warm for 9 hours, and then concentrate until dry. Add 100g of water, adjust the pH to 5. 0 with hydrochloric acid, then adjust to neutral with sodium hydroxide, extract with dichloromethane, and concentrate until dry. Add 100 g of anhydrous ethanol to dissolve at high temperature, and decolorize with an appropriate amount of activated carbon. Slowly cool down, crystallize, filter, and dry to obtain 20. 3 g of light yellow solid-Levofloxacin, with a yield of 87. 0% [24].

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4.2.2. Cefixime

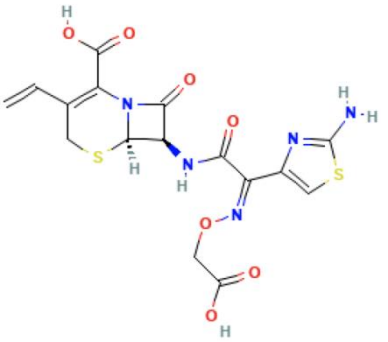

Figure 15. The picture shows the structure of Cefixime [25].

(1) Basic Information:

Cefixime is the Cephalosporin and β Lactam antibiotic, which can be used to kill Streptococcus pneumoniae, etc. Cephalosporin are a kind of antibiotics obtained from natural Cephalosporin C through Semisynthesis [25]. Its molecular formula is C16H15N5O7S2. Its IUPAC name is (6R, 7R)-7-[[(2Z)-2-(2-amino-1, 3-thiazol-4-yl)-2-(carboxymethoxyimino)acetyl]. amino]. -3-ethenyl-8-oxo-5-thia-1-azabicyclo[4. 2. 0]. oct-2-ene-2-carboxylic acid. Its molecular weight is 453. 5 g/mol. This drug was launched in Japan in 1987 and in China in 1994 [25].

(2) The Mechanism of Action

Its antibacterial mechanism is mainly to inhibit the synthesis of mucopeptides in the cell wall of bacteria, thus preventing the cross linking of mucopeptide chains, so that bacteria cannot form a tough wall. In addition, there are special protein molecules that combine with penicillin or Cephalosporin on the cell membrane of bacteria, which is the role of β Lactam antibiotics, namely penicillin binding protein (PBP). Most penicillin or Cephalosporin antibiotics mainly combine with PBP3 and PBP1 to form filamentous and globular bodies, which then cause bacteria to deform and shrink, and gradually dissolve and die [26].

(3) Usage and Dosage

It's generally Oral administration to deal with pneumonia. For children who weight more than 30kg, they should be given 0. 1g Cefixime per time, twice a day. For children who weight less than 30kg, they should be given 1. 5-3mg per kilogram per time, twice a day. The amount should be modified according to age, weight, and disease circumstance. For the adults, they should be given 0. 1g Cefixime per time, twice a day. The amount should be modified according to the disease circumstance [27].

(4) The Efficiency:

The third generation Cephalosporin(including Cefixime) has strong tissue penetration and is widely distributed in the body. It can reach effective antibacterial concentration in tissue, body cavity and body fluid, and is basically non-toxic to the kidney [28]. Ma Xiaoli did a research in Maternal and Child Health Hospital of Chuanhui, Zhoukou, Henan. She divided 140 patients who had light to moderate community acquired pneumonia in half into two groups. Patients in the control group were treated with Amoxicillin for oral administration, and patients in the experimental group were treated with Cefixime for oral administration. After conducting the research and collecting data, Ma Xiaoli found that the total effective rate in the control group was 72. 86%, and the total effective rate in the experimental group was 90. 00%. And the duration of the recovery and treating process was shorter in the experimental group than which in the control group. The adverse effects rate in the experimental group was 4. 28%, and the adverse effects rate in the control group was 14. 28%. All in all, we can see Cefixime is good at dealing with pneumonia for its efficiency and mildness [29]. Although as the third generation Cephalosporin, which is not so good at killing Gram positive bacteria as the first and second generation Cephalosporin, Cefixime can still be used to kill Streptococcus pneumoniae [30].

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(5) The Synthesis:

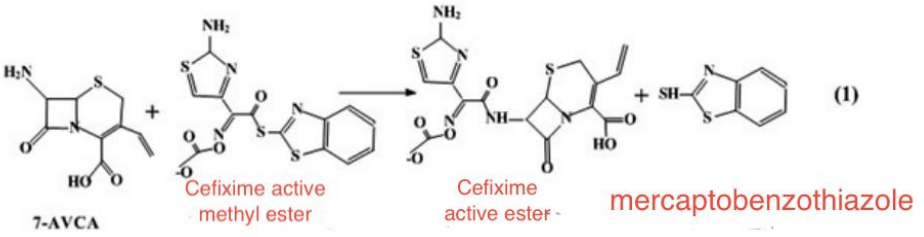

Figure 16. The first step of the synthesis of Cefixime [31].

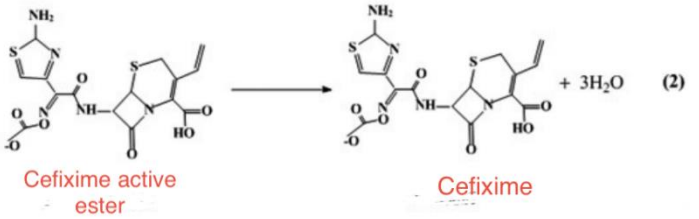

Figure 17. The second step of the synthesis of Cefixime [32].

This approaches replaces traditional tetrahydrofuran, acetone, and other solvents with Cefixime active methyl ester, 7-amino-3-vinyl-cephalanic acid, and ethyl acetate, and undergoes acylation reaction to obtain Cefixime ester. Then, Cefixime is obtained through hydrolysis reaction. The solution is crystallized, filtered, and dried to obtain Cefixime finished product. This method not only reduces the types of solvents used, but also facilitates the recovery and reuse of solvents, which is beneficial for removing reaction by-products such as mercaptobenzothiazole [33].

4.2.3. The combination with Levofloxacin and Cefixime to deal with pneumonia

(1) The Advantages of Combination Therapy

Levofloxacin and Cefixime are two different antibiotics. Levofloxacin is a Quinolone antibiotic, which can block DNA replication and interfere with the distribution of replicated DNA to offspring cells by inhibiting DNA gyrase and Topoisomerase Ⅳ of bacteria, thus achieving rapid sterilization [34]. However, Cefixime is a Cephalosporin antibiotic and has a completely different mechanism. Its antibacterial mechanism is mainly to inhibit the synthesis of mucopeptides in the cell wall of bacteria, thus preventing the cross linking of mucopeptide chains, so that bacteria cannot form a tough wall. In addition, there are special protein molecules that combine with penicillin or Cephalosporin on the cell membrane of bacteria, which is the role of β Lactam antibiotics, namely penicillin binding protein (PBP). Most penicillin or Cephalosporin antibiotics mainly combine with PBP3 and PBP1 to form filamentous and globular bodies, which then cause bacteria to deform and shrink, and gradually dissolve and die [34]. These two antibiotics are both broad-spectrum antibiotics, and both of them can be used to kill Streptococcus pneumoniae, which is one of the main pathogenic bacteria of pneumonia. When they are used alone, their efficiency and safety are both pretty good [34]. [35].

Now, I want to introduce a word-Cross Resistance. Cross Resistance refers to tolerance (as of a bacterium) to a usually toxic substance (such as an antibiotic) that is acquired not as a result of direct exposure but by exposure to a related substance [36]. According to Zhao Xiaoji and other people's research, Streptococcus pneumonia has generated resistance against both Quinolone antibiotics and Cephalosporin antibiotics [37]. If we just use one antibiotic or a single type of antibiotic, the Streptococcus pneumoniae may generate resistance not only against one specific antibiotic but also against one entire type of antibiotic. The problem of cross resistance will get more serious. And the efficiency of the antibiotics will be largely reduced. The combination of Levofloxacin and Cefixime

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can resolve this problem well. They have completely different mechanisms and target points. And they are not the same kind. If they can be used together to kill Streptococcus pneumoniae and deal with pneumonia, the problem of Cross Resistance can be avoided, and they can work together to kill Streptococcus pneumoniae from two targets, which is more comprehensively and effectively. According to the survey I did in Qilu Hospital of Shandong University, the price of Levofloxacin for injection is 30. 81 yuan; the price of Levofloxacin for oral administration is 24. 00 yuan. And the Cefixime for oral administration is 3. 79 yuan. They are both economic-friendly. After calculating, we can see that the combination therapy with Levofloxacin and Cefixime is also economic-friendly.

(2) Research Review

Zhang Rongrong did a research in The People's Hospital of Liuyang. 96 patients with chronic pneumonia treated at The People's Hospital of Liuyang from October 2019 to October 2020 were selected as the study subjects, and they were randomly divided into a control group and a experimental group, with 48 patients in each group. There was no statistically significant difference in general information between the two groups of patients (P>0. 05). All patients are required to remain quiet, rest in bed, and receive nutritional support, oxygen inhalation, and treatment to maintain respiratory patency. In addition, the patients in the control group were treated by Levofloxacin for intravenous infusion; the amount is 0. 4 g per time and twice a day. The patients in the experimental group were treated by the combination of Levofloxacin and Cefixime. They are treated by both Levofloxacin for intravenous infusion (0. 4 g per time and twice a day) and Cefixime for oral administration (200mg per time and twice a day). The treatment duration period was two weeks. After two weeks, Zhang Rongrong compared the changes in inflammatory factor levels between two groups of patients before and after 2 weeks of treatment. As shown in Table 2, the total effective rate of the control group was 79. 17% and the total effective rate of the experimental group was 93. 75%. And as shown in Table 3, the adverse effects rate of the control group was 35. 41%, and the adverse effects rate of the experimental group was 10. 42%. All in all, the treatment conducted in the experimental group (The combination therapy with Levofloxacin and Cefixime) is more effective than the treatment conducted in the control group (The therapy with only Levofloxacin). And even the adverse effects rate of the combination therapy with Levofloxacin and Cefixime is lower than the adverse effects rate of the mono therapy with Levofloxacin. The difference is statistically significant (P<0. 05). Therefore, the combination therapy with Levofloxacin and Cefixime is both effective and safe [38].

Table 2. The Comparison of Effective Rate[cases(%)]. [39].

Group	Cases	Significant Effect	Normal Effect	No Effect	Total Effective Rate(%)
Control Group	48	29(60. 41)	9(18. 75)	10(20. 83)	38(79. 17)
Experimental Group	48	38(79. 17)	7(14. 58)	3(6. 25)	45(93. 75)
PS:Compare between the groups, x^2=5. 537, P<0. 05					

Table 3. The Comparison of Adverse Effects Rate [Cases(%)]. [40].

Group	Cases	Hepatic Insufficiency	Leukopenia	Gastrointestinal Reaction	Rash	Total Occurrence
Control Group	48	5(10. 41)	5(10. 41)	4(8. 33)	3(6. 25)	17(35. 41)
Experimental Group	48	1(2. 08)	2(4. 16)	2(4. 16)	0	5(10. 42)
PS:Compare between the groups, x^2=5. 437, P<0. 05						

Zhu Yahong and Tang Xiaoying did a research in Shanghai Pudong Hospital. 106 elderly pneumonia patients who visited the Respiratory Department of Pudong Hospital in Shanghai from February 2014 to February 2015 were randomly divided into an experimental group and a control

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group, with 53 patients in each group. There was no statistically significant difference in general information such as gender, age, pathological classification, course of disease, and severity between the two groups of patients, indicating comparability. All patients should rest in bed, remain quiet, maintain respiratory patency, inhale oxygen, correct water electrolyte acid-base balance, and receive targeted treatment such as nutritional support. In addition, the patients in the control group were given intravenous infusion of Levofloxacin hydrochloride injection, and the amount was 0. 4g per time, twice a day. The patients in the experimental group were treated by the combination of Levofloxacin and Cefixime. They are treated by both Levofloxacin for intravenous infusion (0. 4 g per time and twice a day) and Cefixime for oral administration (200mg per time and twice a day). The treatment duration period was 14 days. After the treatment, Zhu Yahong and Tang Xiaoying collected the data and did the research. According to Table 4, the total effective rate of the control group (only Levofloxacin treatment) is 84. 91%; the total effective rate of the experimental group (The combination therapy with Levofloxacin and Cefixime) is 96. 23%. The difference is statistically significant (P<0. 05). After treatment, the disappearance time of clinical signs such as fever, cough, and lung rales in the experimental group was significantly shorter than that in the control group, and the difference was statistically significant (P<0. 05). There was no significant difference in the rate of adverse effects between the experimental group (7. 55%) and the control group (9. 43%). The adverse effects which had taken place during the research were mild and insignificant. No other adverse effects were observed during the treatment of both groups. All in all, according to the data and comparison, the combination therapy with Levofloxacin and Cefixime is more effective than the mono therapy with Levofloxacin. And there was no significant difference in the rate of adverse effects rate between the two groups. This research also proves that the combination therapy with Levofloxacin and Cefixime is both effective and safe [41].

Table 4. Comparison on clinical efficacies between two groups[41].

Group	n/cases	Cure/cases	Significant effect/cases	Normal effect/cases	No effect/cases	Total Effective Rate%
Control Group	53	20	14	11	8	84. 91
Experimental Group	53	30	16	5	2	96. 23
PS:Compare between the two groups, P<0. 05						

5. Conclusion

All in all, according to the research reviewed before, the combination therapy with Levofloxacin and Cefixime is a very effective approach to deal with pneumonia. Although Levofloxacin is already a very good antibiotic to kill Streptococcus pneumoniae and deal with pneumonia [41]. , the combination therapy is more effective than the mono therapy with Levofloxacin. And its safety and mildness are also pretty good [42]. [43]. Moreover, according to the survey I did in Qilu Hospital of Shandong University, these two antibiotics' prices are both relatively low. It is also an economic-friendly therapy. It's worth popularizing this combination therapy clinically, helping an increasing number of people to get better. I believe this combination therapy will be used clinically more frequently in the future.

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Computational analysis of GCaMP fluorescence data in neuronal activity

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Abstract. The background and motivation of our research is to explore how to use computational methods to analyze GCaMP fluorescence data. GCaMP is a genetically encoded calcium indicator that can be used to observe the activity of thousands of neurons simultaneously using modern fluorescence imaging techniques. Our research includes the application of principles such as basic signal processing, statistical inference, hypothesis testing, and graph theory to help understand raw GCaMP fluorescence data recorded from awake, behavioral mice. The data set includes GCaMP fluorescence trace and correlation coefficient matrix among neurons. Our methods include high-pass filtering, Gaussian mixture model fitting and online active set method peak inference (oasis). By analyzing the correlation between neurons, we can understand the connection and centrality between neurons.

Keyword: Oasis Computational Analysis, GCaMP Fluorescence, Neuronal Activity, Data Deconvolution, Neural Networks

1. Introduction

Calcium imaging is a technique used to observe neuronal activity. It involves labeling calcium ions in neurons using calcium indicators and detecting changes in the fluorescent signal emitted by the indicators.

During neuronal activity, the change of calcium ion concentration is closely related to the electrical activity of neurons. Through calcium indicators, these changes in calcium ion concentration can be translated into changes in the fluorescence signal. Fluorescence microscopes (maikeruoscope) and corresponding imaging systems are used to observe and record these fluorescence signals.

The advantage of calcium imaging is the ability to observe large numbers of neurons simultaneously and to provide spatial and temporal resolution of neuronal activity. This makes calcium imaging a common tool in neuroscience research to study the function of neural circuits, the interactions between neurons, and the activity of the nervous system during different behavioral and cognitive processes.[1]

Calcium imaging analysis is a very complete technology, it includes many steps, such as how to inject calcium indicators, how to extract signals, and data analysis, etc. We only studied a part of it

We have developed a strong calculation method that can be used to analyze GCaMP fluorescent data. GCaMP is a causal coding calcium indicator, the most widely used is to observe the activity of thousands of neural groups at the same time using modern fluor imaging techniques.

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We use some background knowledge of basic information processing, statistical inference, hypothesis testing and basic principles of graph theory. We should use these principles to help interpret the primary GCaMP fluorescence data recorded from the waking rats.

Our initial data were two GCaMP6(special edition of GCaMP) fluorescent data sets containing temporal sequence traces of hundreds of rats primary visual cortex (V1) elements. These elements are located in a three-dimensional volume of about 800 μm x 800 μm x 100 μm. The axial depth is limited by various physical limits. This volume contains a large part of two-thirds of the layers, and many of the higher layers of the large brain are born in the skin.

The GCaMP fluorescent record sample is made as the phase pair modification of the fluorescent phase to the basic fluorescent water level for each celestial element n and each time point m

$$\frac{\Delta F}{F_0}[m] = \frac{F[m] - F_0}{F_0},$$

Where Fo is the estimated baseline fluorescence value.

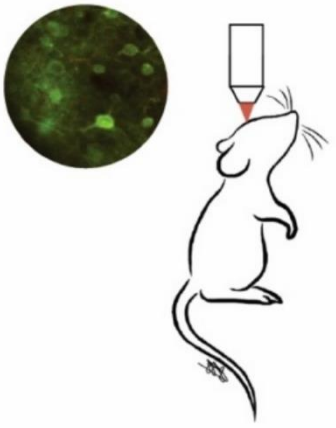

Figure 1. The original data is derived from experimental mice

2. Related work

Calcium imaging, especially using genetically encoded calcium indicators like GCaMP, has become a key method for observing neural activity [2]. Over the years, various methods have been developed and optimized to analyze GCaMP fluorescence data.[3]

Tian et al made significant advancements in improving the GCaMP indicator for observing neural activity in worms, fruit flies, and mice. [4] Their research emphasized the importance of optimizing the sensitivity of GCaMP indicators, highlighting the evolving nature of these tools and their potential to capture neural dynamics in different organisms.

Chen et al highlighted the challenges of threshold fluorescence modes. They laid down fundamental concepts in the field of using GCaMP for neuronal activity imaging. [5] This method, although powerful, often faces challenges distinguishing high-frequency changes from actual neural activity, emphasizing the importance of robust analytical techniques.

In the exploration of optimizing calcium imaging, Dana et al delved deeply into high-performance calcium sensors for imaging neural activity.[6] Their work centered on creating more sensitive and faster variants of GCaMP, elucidating the need for a balance between sensitivity and noise.

There are many ways to analyze calcium imaging data. One of the most notable methods is a toolkit called “EZcalicu”[7]. This toolkit is convenient as it produces results directly. However, EZcalicu has its limitations. Specifically, it only supports the analysis of .tiff files. Data formats like .AVI or .mat are incompatible. Given the frequent challenges of converting complex data formats to either .TIFF or .AVI,

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this posed a significant limitation. Recognizing this challenge, we embarked on the task of developing our own set of codes specifically tailored for similar data analysis, using . MAT data as an example.

Moreover, the work of Eftychios Pnevmatikakis on the MATLAB toolbox, despite being commendable, also has its limitations. While it can efficiently analyze calcium imaging data, it often blurs high-frequency changes, potentially losing key information.

Our approach is largely influenced by the aforementioned research, and we have integrated various elements from them. However, our goal is to address some of these limitations, especially when it comes to preserving high-frequency data without compromises. We adopted a combination of high-pass filtering, Gaussian mixture modeling, and connectivity centrality, ensuring a comprehensive analysis of GCaMP fluorescence data.

3. Research methods

Our workflow is shown in the figure 2

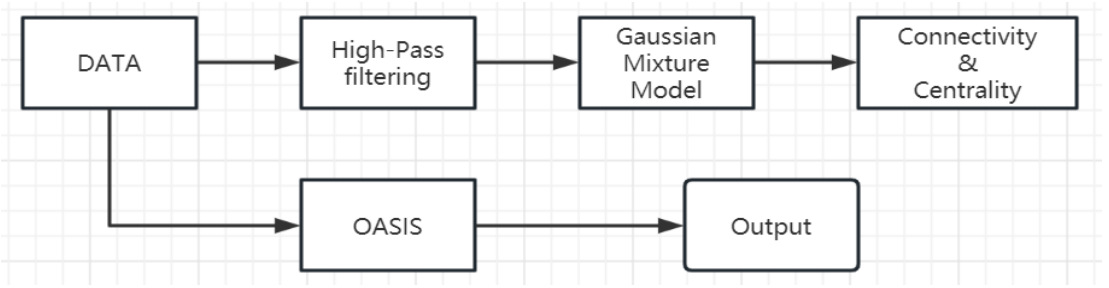

Figure 2. Flow chat

3.1. Filtration

Low frequency base line drift is present in each time sequence track due to motion, image noise, and/or optical bleaching. We are interested in the relative high frequency variation of GCaMP. High frequency variation is the result of calcium inflow and outflow, and is related to the direct discharge of neurons.

We must design a suitable high-pass filter and the filter should be used in tandem data to eliminate the above low frequency drift in each record of the Scriptures. We must ensure that, when designing the filter, only the unwanted low frequency drift induced by the above mentioned error is eliminated and the calcium inflow and outflow fluorescence changes are retained.

First we draw the mean Fourier amplitude spectrum and determine an appropriate cut-off frequency for the logical high pass filter that applies to all GCaMP time sequence traces.[8]

In the frequency domain, the ideal high-pass filter is defined, for some cutoff frequency ωc, as follow

$$H_{\text{ideal}}(e^{j\omega}) = \begin{cases} 0, & \text{if } \omega <= \omega_c, \\ 1, & \text{otherwise.} \end{cases}$$

Note that $\omega_c < \pi$ rad/sec is the normalized angular frequency in this definition.

After analysis and calculation, we determined that ωc = 0.198

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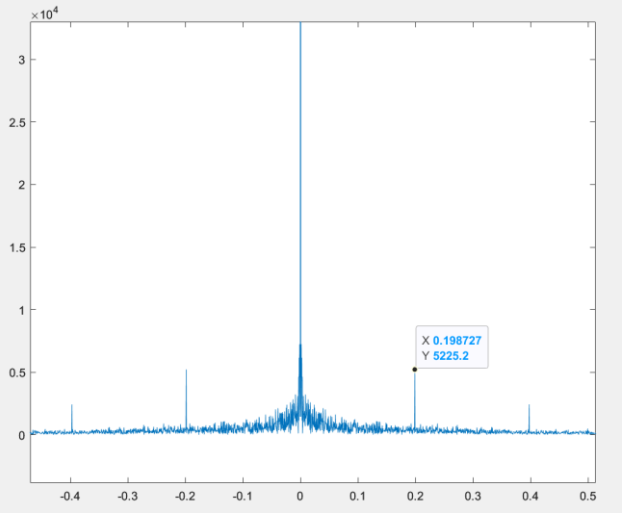

Figure 3. A histogram showcasing the distribution of fluorescence intensities in the collected data

Then we get the raw data and the filter coefficient. Then we can filter the raw data in the time domain resorts to convolving the data with the filter coefficients in the time domain. The result we get after the convolution should the filtered data.

We use Matlab to achieve, the code is as follows

```
%%
% define gate signal function
syms n;
fc = 0.198;
fc_2 = 0.187;
u(n) = piecewise(n < -fc, 1, -fc <= n <= fc, 0, n > fc, 1);
u_2(n) = piecewise(n < -fc_2, 1, -fc_2 <= n <= fc_2, 0, n > fc_2, 1);

% discretization
N = 6.9755 ;
N_2 = 9.344;

start_value = -N;
end_value = N;
step = 0.001;
n_values = start_value:step:end_value;

start_value = -N_2;
end_value = N_2;
step = 0.001;
n_values_2 = start_value:step:end_value;

u_discrete = double(subs(u, n, n_values));
u_discrete_2 = double(subs(u_2, n, n_values_2));
%%
```

```
%%
% Inverse discrete time Fourier transform
u_ifft = ifft(ifftshift(u_discrete));
u_ifft_2 = ifft(ifftshift(u_discrete_2));

x = y0;  % input signal
x_2 = y2;
qwe_0 = sum(data)/233;
qwe_2 = sum(data_2)/453;

y_0 = cconv(qwe_0,u_ifft,length(qwe_0));

p = 0:0.1:(length(y_0)-1)*0.1;
z = abs(y_0);
y_2 = cconv(qwe_2,u_ifft_2,length(qwe_2));
p_2 = 0:0.1:(length(y_2)-1)*0.1;
z_2 = abs(y_2);
%%
```

$$H_{\text{ideal}}(e^{j\omega}) = \begin{cases} 0, & \text{if } \omega <= \omega_c, \\ 1, & \text{otherwise.} \end{cases} \qquad h[\ell] = \frac{1}{2\pi}\int_{-\pi}^{\pi} H_{\text{ideal}}(e^{j\omega})e^{j\omega\ell}\,d\omega$$

Figure 4. Defining and Processing Discrete-Time Fourier Transform with Ideal Gate Signal

And next we did Filtering by convolution:

we using convolution. Filtering data in the time domain resorts to convolving the data with the filter coefficients in the time domain. We design this code

y_0 = cconv(qwe_0,u_ifft, length (qwe_0))

(What this expression means is to convolve qwe_0 and u_ifft cyclically and store the result in y_0. length(qwe_0) is used to specify the length of the convolution, which is the same length as qwe_0.) Our results are shown in the figure 2 and 3 below

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```
%%
%数据1的输出
N = length(data);
fs = FR;
frequencies = (-fs/2:fs/N:fs/2-fs/N);
figure;
subplot(4,1,1);
plot(frequencies, abs(fftshift(y0)));
xlabel('Frequency (Hz)');
ylabel('Amplitude');
title('data1-FFT Spectrum');
%数据2的输出
N_2 = length(data_2);
fs_2 = FR_2;
frequencies_2 = (-fs_2/2:fs_2/N_2:fs_2/2-fs_2/N_2);
subplot(4,1,2);
plot(frequencies_2, fftshift(y2));
xlabel('Frequency (Hz)');
ylabel('Amplitude');
title('data2-FFT Spectrum');
subplot(4,1,3);
plot(frequencies,abs(fftshift(fft(y_0))));
xlabel('time');
ylabel('amplitude');
title('datacase1');
subplot(4,1,4);
plot(frequencies_2,abs(fftshift(fft(y_2))));
xlabel('time');
ylabel('amplitude');
title('datacase2');
```

Figure 5. Code Spectral Analysis of Frequency and Time Domain Using Fast Fourier Transform

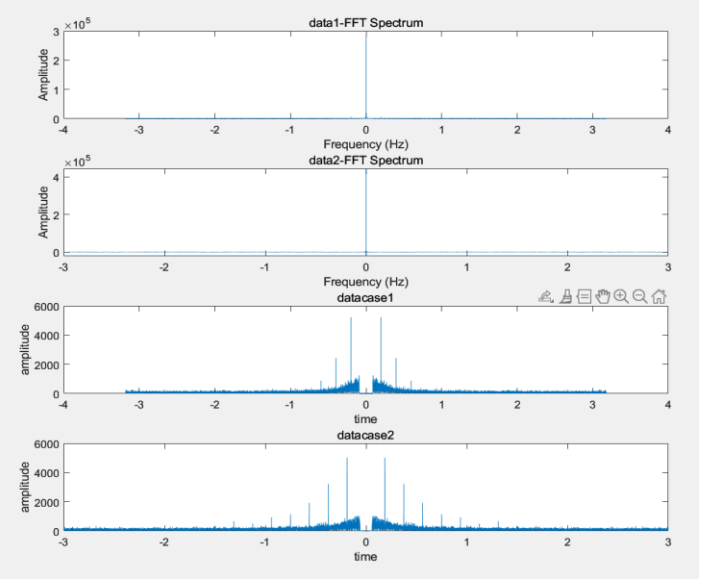

Figure 6. Result 1 Visualization of the Gaussian mixture model's ability to differentiate neuronal states.

3.2. About the Gaussian mixture model

The function of Gaussian mixture model fitting is to model and analyze the activity data of neurons. In our project, Gaussian mixture model is used to fit neuronal data after high-pass filtering. The specific steps are as follows: First, the neuron data after high-pass filtering is taken as a sample, assuming that the sample of each neuron is generated by a Gaussian mixture model with two mixed components. One of the mixed components represents the baseline state and the other represents the excited state.Then, using built-in functions or packages in Python or MATLAB, a Gaussian mixture model is fitted to a sample of each neuron. The goal of fitting is to find the best model parameters so that the model can best describe the sample data. By fitting the Gaussian mixture model, the probability density function of each neuron can be obtained, so that the activity pattern and interaction of neurons can be better understood. These probability density functions can be used for further analysis, such as calculating correlations between neurons, exploring connectivity and centrality between neurons, etc. Therefore, Gaussian

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mixture model fitting plays an important role in studying neuronal activity and the function and structure of neural networks.

But because of time and schedule, the work is still being done.

3.3. OASIS

OASIS (Open Source Imaging Spike Sorting) is a MATLAB toolkit for offline analysis and peak detection of neural activity. [9] It provides a set of functions and tools for processing and analyzing calcium imaging data.

The main features of OASIS-MATLAB include: Data preprocessing, Peak detection, Data analysis, Utility functions.

So, how does the OASIS filter the signal?

Initialization: Firstly, the signal needs to be initialized.

Attenuation Rate Estimation: Next, OASIS distinguishes the desired signal component from the noise component by estimating the attenuation rates of the signal. [10]

Filtering Iterations: In each iteration, OASIS filters the signal based on the estimated attenuation rates.

Convergence Check: After each iteration, OASIS examines the difference between the filtered result and the previous iteration.

Then we using “deconvolveCa” to perform deconvolution analysis on the original calcium imaging data. (code as follow)

```
%%
[c, s, options] = deconvolveCa(qwe_0, 'foopsi', 'ar1', 'smin', -3, 'optimize_pars', true, 'optimize_b', true);
[c_2, s_2, options_2] = deconvolveCa(qwe_2, 'foopsi', 'ar1', 'smin', -3, 'optimize_pars', true, 'optimize_b', true);
%data1
Y = qwe_0;
trueC = c;
trueSpikes = s;
T = length(qwe_0);
firerate = sum(trueSpikes>0)/T*FR;
%data2
Y_2 = qwe_2;
trueC_2 = c_2;
trueSpikes_2 = s_2;
T_2 = length(qwe_2);
firerate_2 = sum(trueSpikes_2>0)/T_2*FR;
%%
```

Figure 7. Code representation of the estimated neural activity and estimated calcium indicator concentration using the deconvolution algorithm

“c”- the estimated neural activity trace

“s” - the estimated calcium indicator concentration signal

“options” - a structure that contains parameter options for configuring the deconvolution algorithm.

This is a cyclic convolution calculation in signal processing that we designed. The deconvolveCa function is used in the code for signal deconvolution operations.

First, the code deconvolveCa deconvolved the signal qwe_0 by calling the deconvolveca function, and assigned the returned results to the variables c, s, and options, respectively. Similarly, the code performs a convolution operation on the signal qwe_2 and assigns the result to the variables c_2, s_2, and options_2.

Then, we define some variables and calculations. Y and Y_2 represent signals qwe_0 and qwe_2, respectively. truec and trueC_2 represent the result c and c_2 after deconvolution, respectively. truespikes and truespikes_2 represent the result s and s_2 after deconvolution, respectively. T and T_2 represent the length of the signal, and firerate_2 represent the frequency of the pulse in the result after deconvolution, respectively.

Next we will plot the result using Matlab. Here is an explanation of our code: First, the code initializes the graph by setting the paper size of the graph to ‘[15, 2.5]’ and calling the ‘init_fig’ function.

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Next, use the 'hold on' command to keep what is already on the graph and allow multiple graphs to be drawn on the same graph. Then, the color variable 'col' is defined, which contains the RGB values for the three colors. Next, plot the fluorescence tracing curve using the 'plot' function. '(1:T)/FR' represents the time point on the X-axis, and 'Y(1,:)' represents the corresponding fluorescence value. The 'o' parameter is used to specify the color, and the 'uint8(col(2))' is used here for the second color. Then, plot the calcium ion trace curve using the 'plot' function. '(1:T)/FR' represents the time point on the X-axis, and 'trueC(:,1)' represents the corresponding calcium ion value. The 'color' parameter is used to specify the color, here 'k' is used for black. Next, use the 'find' function to find the point in time when the pulse occurred, and use the 'plot' function to plot the location where the pulse occurred. '[1,1]*tsp(m)/FR' represents the point in time at which the pulse occurs, and '[0, 1]' represents the starting and ending positions on the Y-axis. The 'color' argument is used to specify the color, and uint8(col(3)) is used here to represent the third color. Then, use the 'axis tight' command to fit the axes to the data range. Next, use 'xlabel' and 'ylabel' to set the labels for the x and y axes, respectively. Then, a legend is drawn using the 'Legend' function, where 'y', 'c', and 's' represent fluorescence tracking, calcium ion tracking, and pulse generation, respectively. Finally, use the 'set' function to set the properties of the current graph object, where 'fontweight' is set to 'bold'. (figure 5)

```
% plot results
figure('papersize', [15, 2.5]);
init_fig;
hold on;
col = {[0, 114, 176];
    [0, 158, 115];
    [213, 94, 0]};

% fluorescence trace
plot((1:T)/FR, Y(1,:)/3, 'o', 'color',  uint8(col{2}));

% calcium trace
plot((1:T)/FR, trueC(:,1)/3, 'color', 'k'); % uint8(col{1}));
% spike train
tsp = find(trueSpikes(:, 1));
for m=1:length(tsp)
    plot([1,1]*tsp(m)/FR, [0, 1], 'color', uint8(col{3}));
end
axis tight;
xlabel('Time');
ylabel('Fluorescence');
legend('y', 'c', 's');
set(gco, 'fontweigth', 'bold');
```

Figure 8. Plotting Fluorescence Trace, Calcium Signal Trace, and Spike Event Time Series

Need attention that Fire rate refers to the frequency at which a neuron generates action potentials, also known as spikes, within a certain period of time.

0.4300 is fire rate of datecase1 and 0.7401 is fire rate of datecase2

Our result is shown in Figure 6

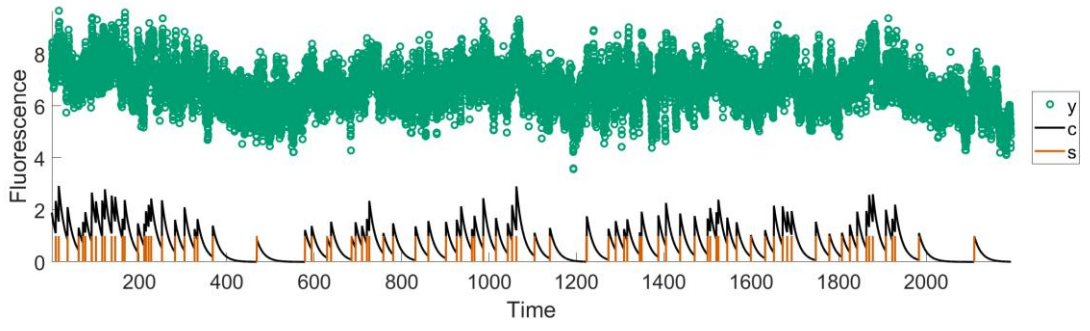

Figure 9. Comprehensive graph showcasing fluorescence trace, calcium trace, and spike train.

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"Y"- flourecence trace
"c" - calcium trace
"s"- spike train

Next we define a few variables and create two empty 2D arrays using the zeros function. We store the randomData value of each data point into a 2D array for subsequent analysis and processing (possible uses include statistical analysis, graph drawing, model training, etc.)

3.4. Connectivity & Centrality

The work of this step is to calculate the correlation coefficient matrix R from the standardized data for each dataset. Now that we have processed and standardized the GCaMP fluorescence time series data for each neuron imaging in each dataset, we can begin to examine the paired interactions between all neurons in the population. The correlation coefficient describes how similar the activity of one neuron is to that of another. Thus, correlation coefficients provide a very simple and easy way to glimpse the connectivity of neural populations. The number of neurons in data 1 and data 2 is 233 and 453, respectively

So we need to get a coefficient matrix 233 by 233 and a matrix 453 by 45

Here are our results (figure 10)

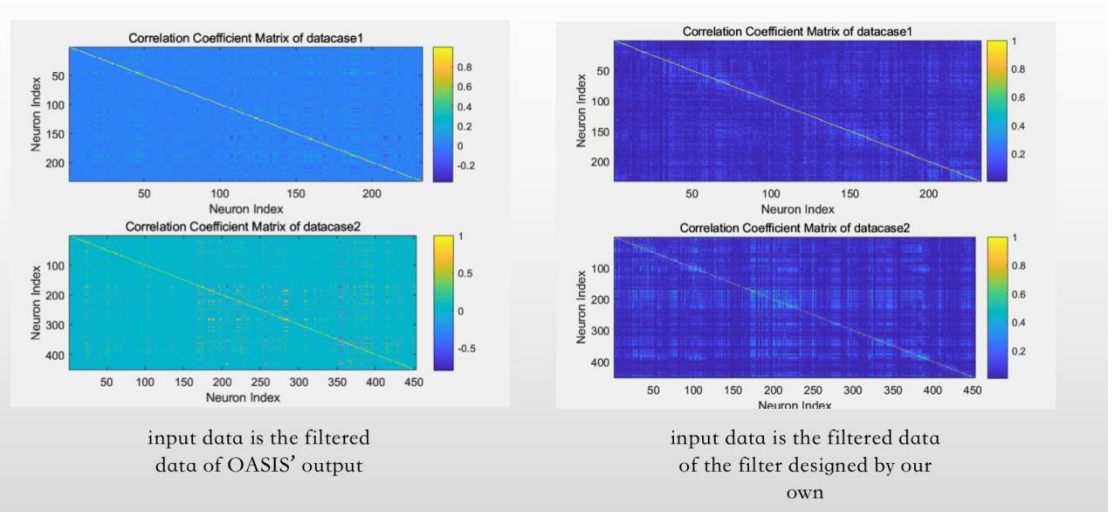

Figure 10. Correlation matrices of the analyzed neuronal populations, indicating the connectivity between different neurons.

4. Conclusion and prospect

Our aim was to explore how GCaMP fluorescence data can be analyzed using computational methods to better understand the patterns and interactions of neuronal activity. By calculating the correlation coefficient matrix between neurons, we can understand the interactions and connections between neurons, and further reveal the structure and function of neural networks. At the same time, the use of high-pass filters can help us eliminate noise and drift, extract the high-frequency changes associated with neuronal activity, and thus more accurately study the behavior of neurons. We use Matlab as our primary tool, along with the application of principles such as basic signal processing, statistical inference, hypothesis testing, and graph theory, to help understand raw GCaMP fluorescence data recorded from awake, behavioral mice. The results have important implications for understanding the patterns and interactions of neuronal activity.

What else can we expand?

Gaussian Mixture Model:

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When we need to analyze a set of data, the Gaussian mixture model (GMM) can help us better understand the distribution of the data. In this case, we can better distinguish between the resting state and the activated state.

Design a better filter:[11]

Since our filter can only filter the low frequencies signals, so the effect of filtering is not very good. We can design a better filter which can also filter the overfrequences.

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Internet-based treatment for Autistic Spectrum Disorder: An overview

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Abstract. Internet-based therapies have emerged as effective alternatives to traditional face-to-face therapies, demonstrating efficacy in treating mental health problems and providing accessible care, especially in this post-pandemic world. Moreover, the unique characteristics of the Internet offer promising opportunities to cater to the unique requirements of patients diagnosed with Autism Spectrum Disorder (ASD) , emphasizing profound impact that can be achieved through internet-based therapy in revolutionizing existing therapeutic paradigms. However, investigation of online therapy for adults and older individuals with ASD is currently limited. This systematic review provides a summary of the status of existing research investigating the application of Internet-based therapies on individuals with ASD, including Virtual Reality-based interventions, Artificial Intelligence-based interventions and other online therapies. The primary objectives are to assess the efficacy of Internet-based therapies as treatments for individuals with ASD. Additionally, potential differences between Internet-delivered therapies and traditional face-to-face therapies are also being explored. By providing a comprehensive analysis of both Internet-based and traditional therapy modalities, this review aims to contribute to our understanding and delivery of more effective therapeutic options available for individuals with ASD. Future directions on better therapeutic choices are discussed, as well as the need for addressing the existing research gap towards elder population.

Keywords: Autism spectrum disorder, internet treatment, VR, AI.

1. Introduction

Internet-based therapy, also referred to as online therapy or teletherapy, has gained substantial attention in recent years, owing to its convenience, accessibility, and potential benefits. After its inception during the early 1990s, this mode of treatments has leveraged the power of technology to remotely deliver therapeutic interventions and support through digital platforms thus bringing many advantages. Especially during the COVID-19 pandemic outbreak where access to offline treatments was substantially curtailed due to restrictions and safety concerns limiting access to offline treatments, the

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pivotal role of internet-based interventions was underscored [1]. Providing care via telepsychotherapy during the pandemic not only reduces travel time and lower health risk but can also be more private, reducing stigma concerns [2].

In contrast to other neurodevelopmental disorders, children with Autism Spectrum Disorder possess a specific pattern of impairments in communication, such as fewer conventional gestures, more echolalia and stereotypical phrases, limited functional use of language, and a reduced inclination to start or engage in verbal interaction [3]. Although the speech of autistic children is often noted to be grammatically correct, it deviates from typical functional language use. This means that while their grammar may be accurate, their speech lacks the expected social and pragmatic functions that are typically expected in communication. Consequently, individuals with ASD may struggle to effectively use language for social purposes, such as initiating conversations, maintaining back-and-forth exchanges, or expressing their needs and desires in a socially appropriate manner. Furthermore, individuals with ASD commonly exhibit intense and focused interests that differ from those without the disorder. These interests often manifest as highly specialized areas of knowledge or expertise, and individuals with ASD may demonstrate exceptional proficiency in these specific domains related to their unique passions.

Computational devices hold immense potential for patients with ASD, offering a stable, rule-based environment in which the speed of tasks can be customized to aligh with individual preferences, thus uniquely appeals to and benefits ASD patients [4]. The internet creates avenues for individuals with ASD to connect and interact with like-minded individuals who share their interests, thus helping them further explore their unique passions [5]. This specific advantage promises novel avenues to cater to the unique requirements of patients with ASD, illustrating the potential of internet-based therapy to revolutionize therapeutic paradigms.

In the subsequent sections of this review, we delve into the specific considerations surrounding internet-based therapy, including VR-based training and AI-supported interventions, for ASD individuals, exploring its feasibility, potential benefits, and the challenges it may surmount. By critically examining existing literature and empirical evidence, we seek to illuminate and bring attention to the transformative potential of this approach in redefining how we conceptualize and deliver therapeutic treatments for individuals with ASD.

2. VR

Virtual reality (VR) stands as an innovative computer-programmed technology that skillfully replicates real-world environments through the integration of vivid images, captivating soundscapes, and tactile feedback. This simulation encompasses a wide range of applications, including interactive video gaming, immersive virtual environments, and multisensory experiences, allowing them to truly immerse themselves in unparalleled digital landscapes [6].

VR has been demonstrated to be an efficient learning method for ASD child, providing an immersive and customizable environment that supports their educational needs [7]. It possesses advantageous features such as controllable input stimuli, modification for generalization, and individualized treatment, creating a primarily visual/auditory world which appears highly compatible with the preferences of effective learning methods for young patients with ASD, given that explaining abstract concepts to individuals with autism has shown effectiveness through the utilization of visual and auditory stimuli, which cater to their unique learning needs [8]. In a study that examines the effects of virtual travel training on children with ASD, a significant statistical increase in their understanding of the bus-riding process was observed. Additionally, the study reported a significant level of achievement in implementing strategies or actions in the game, reaching 93.8% [9]. This result indicates that VR-based serious games can be utilized efficiently to enhance independence of patients with ASD in outdoor activities.

VR has also shown promising results in enhancing performance skills of adult patients in areas such as driving skills, job interviewing and various other activities of daily living (ADLs). Ross et al have conducted studies assessing the use of specific VR-related treatment for driving [10]. The results have shown a notable increase in favorable attitudes and a decrease in unfavorable attitudes, in comparison

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to ASD parent drivers undergoing routine driver training. Burke et al and Smith et al have measured post-VR treatment job interview skills in response to the use of VR training session [11,12]. The findings indicated that adult participants with autism demonstrated notable improvements in various areas, including identifying personal strengths, promoting oneself, advocating for oneself, addressing situational questions, and effectively responding to behavioral and social inquiries. Smith et al have also shown that patients in the VR training group demonstrated greater improvement in their interview performances compared to those who didn't receive VR training [12]. Participants that received VR training also described finding the VR training system easy to manipulate and relatively pleasant, which in turn made them feel more comfortable towards future interviews.

In conclusion, Virtual reality (VR) has become a potent resource for autism intervention, offering unique advantages specifically to ASD patients. By providing immersive, customizable, and multisensory settings, VR caters to the unique learning preferences of individuals with ASD. The effectiveness of virtual reality (VR) interventions has been demonstrated in diverse areas, including driving skills, job interviewing, and social communication, indicating the potential of VR to enhance independence and functional abilities in individuals with ASD. The promising outcomes of VR interventions pave the way for further exploration and utilization of this innovative technology in supporting individuals with ASD, ultimately enhancing their quality of life.

3. AI

Recent advancements in utilizing artificial intelligence (AI) for the early detection and treatment of ASD have yielded promising results. These methodologies involve leveraging algorithms to identify distinctive ASD characteristics and streamline the assessment process by eliminating redundant items. For instance, Liu et al employed facial scanning patterns to differentiate between ASD and non-ASD individuals, achieving an impressive accuracy rate of 88.51% [13]. Notably, the ASD group displayed a preference for left-eye fixation, while the non-ASD group focused more on the right eye. Similarly, Crippa et al highlighted the potential of goal-oriented movement differences as a robust identifier of ASD [14]. In more recent studies, such as the work by Rubio-Martín et al integrated AI model with natural language processing (NLP) techniques have been employed to detect individuals with ASD with an accuracy rate of 84% [15]. This AI-driven approach not only facilitates early ASD detection but also plays a crucial role in enhancing various aspects of ASD individuals' lives.

Christina Whalen et al applied a varity of structured teaching apporaches to alliviate ASD child unappropriate bahviours, and enhance social skills [16]. Specifically, cognitive, social, receptive language and life skills along with non-verbal communication skills, as well in other aged ASD populations [17]. In addition, computer programm been proved useful in teaching child with ASD or Asperger syndrom to recognise and predict others emotins [18], however, the program only category emotions into happy, sad, angry or afraid. this design cannot reflex real world situations, as there are many more complex emotions, e.g., embarrassed, excited or guilty. Furthermore, few studies investigated use computer programm to impove emotion prediction for adults with ASD.

An innovative approach involved utilizing robots for ASD interventions. Farhan et al employed an autonomous humanoid robot named NAO to foster neurological and physical growth in ASD individuals [19]. The study involved multiple sessions, including verbal and non-verbal language, physical activities, and appreciation exercises. The results indicated NAO's effectiveness in improving communication skills among ASD patients. Also, a haptic robot been tested able to improve motor skills in ASD patients, in tasks like writing speed and glyph formation, albeit with limited effectiveness for children under the age of 9 [20]. Recent studies have delved into specific motor skills improvements for ASD individuals. Moorthy et al designed a robot to teach left-right shoe recognition and velcro band closure to ASD children, resulting in improved fine motor skills [21]. So et al compared robot-assisted interventions with human interventions, demonstrating the robot's effectiveness in enhancing motor skills and gestural production, with additional benefits like increased eye contact [22]. Furthermore, AI-supported interventions extend to teachers' roles. Escobedo et al demonstrated how well-designed "smart objects" can aid teachers in object discrimination training for ASD students, reducing their workload and

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enhancing cognitive efficacy [23]. These studies collectively underscore the immense potential of AI-driven interventions in the ASD domain. From early detection to fostering cognitive, social, and motor skills, AI continues to revolutionize ASD treatment methodologies, benefiting individuals, teachers, and families alike. However, those studies focused exclusively on children with ASD, and it remains uncertain whether robots could yield similar improvements in motor skills when applied to older individuals within the ASD population.

4. Other internet-based treatment

The following part summarized three internet-based treatments for ASD: Computer-Based Intervention, App-Based Treatment, and Caregiver Programs.

Computer-Based Intervention (CBI), a method commonly used in special education, serves as a supportive technique for teachers working with ASD children [24] . Notably, Khowaja and Salim demonstrated that CBI improved reading comprehension skills and overall learning in child with ASD [25]. Moreover, CBI has been linked to enhanced attention [26] , literacy skills [27], and learning rates [28] compared to non-CBI conditions [29].

In contrast, app-based treatments have shown mixed results. Whitehouse et al revealed that while these treatments were not superior to standard interventions, they did demonstrate benefits in specific developmental skills like visual reception and fine motor skills [30]. However, the attention span of young children to these apps is a concern which may lower the effectiveness of app- based treatment, with research indicating limited usage (2 minutes per day on average during the second 3 month). Novack et al extended these findings to older children, employing motivational strategies and a comprehensive app, “Camp Discovery,” which aimed at teaching receptive language skills for ASD child, including a variety lesson in a gameplay format, e.g., objects, emotions, sound discrimination, sentences etc [31]. Courses are designed for varying levels of complexity to address matching, sequencing, and/or receiving language skills The results showed the app facilitated gains in receptive language skills over a 4-week period. In addition, participants maintained the skills gained after a one-month delay without additional access to the app [31]. Despite its success, the study’s limited sample size and applicability to various ASD severities and impairments remain notable limitations.

App-based therapies offer cost-effective options and a platform for home-based caregiver programs [30]. This aligns with the last category, caregiver programs, which encompass parent-mediated interventions (PMIs) and parent-child interaction therapy (PCIT). These programs emphasize the interactions between parents and children, supported by therapists or applications, to foster effective parenting practices for ASD. Telehealth-based PMIs have demonstrated the potential to enhance parents’ information, satisfaction, and commitment [32]. Remarkably, PCIT resulted in reduced parenting stress, negative practices, and externalizing behavior problems [33]. The consistent involvement of parents in their child’s life leads to improvements in social skills, communication, general intelligence, work skill, play skill, vocabulary, motor skill and daily living skill, and overall development [32]. In conclusion, these internet-based interventions offer varied approaches to support ASD children and their families, showcasing potential benefits and highlighting the importance of personalized, well-rounded strategies.

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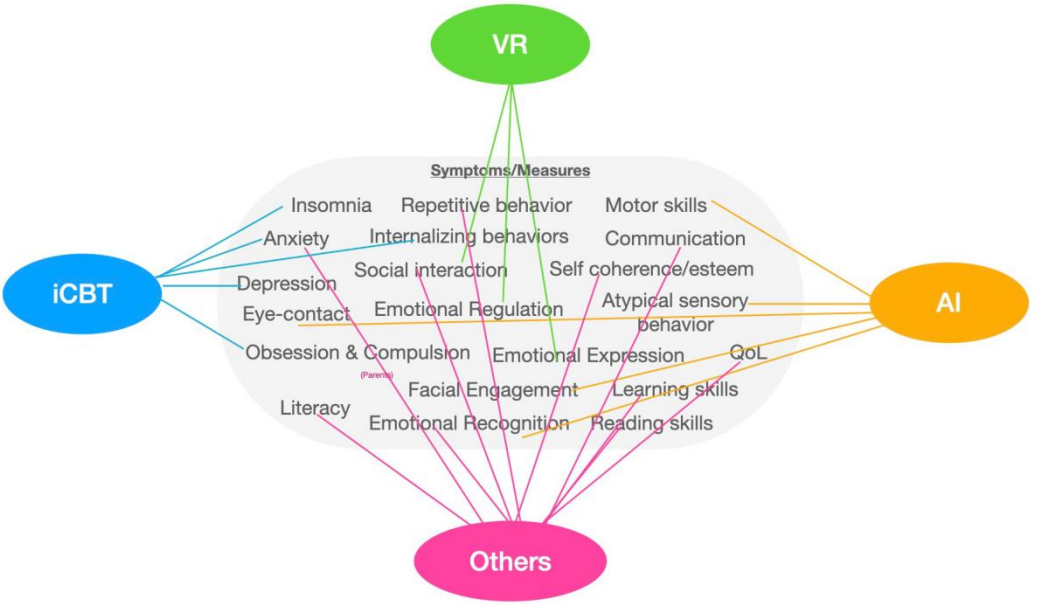

Figure 1. The internet-based treatment for ASD.

Note: There are 4 types of Internet-based treatments (ICBT, VR, AI, and others), each of which targets different ASD symptoms.

5. Discussion

This article presents an in-depth exploration of internet-based treatments as shown in Figure 1 (VR, AI and others) designed for Autism Spectrum Disorder (ASD), delving into their distinctive attributes and limitations. Drawing from a wealth of prior research, internet-based interventions emerge as particularly efficacious in the ASD domain.

Their efficacy extends to early detection and intervention, with a pronounced emphasis on enriching social competencies [19], satisfy educational needs [7](Strickland, 1997), bolstering cognitive acumen [32], refining reading comprehension proficiencies [25], honing communication skills [32], enhancing motor abilities [20,21], and augmenting emotion recognition aptitudes [16,18]. Additionally, noteworthy advancements manifest in the alleviation of parental stress and an enhanced overall quality of life through online therapeutic modalities [33]. The allure of these internet-based therapeutic pathways resides in their undeniable convenience and flexibility. The prospect of receiving therapeutic support within one's domestic haven or a familiar setting resonates significantly, particularly for ASD patients who often grapple with novel environments or transitions. The malleable scheduling options inherent to online therapy cater to demanding routines, offering solace to those beset by time constraints. This characteristic holds even more import for those ensnared by geographical barriers or other encumbrances impeding access to conventional, in-person therapy.

The internet-based treatments discussed are considered as a part of the complementary non-pharmacological interventions, in most cases, supplementing pharmacological interventions. One class of drugs of psychostimulants like methylphenidate are effective in improving comorbid symptoms but cannot intervene in core ASD symptoms including irritability, social withdrawal, and repetitive behaviors. Another common class, atypical antipsychotic drugs like risperidone and Aripiprazole, performs effects on easing ASD's symptoms of irritability and agitation in adults and as well as children [34]. In comparison to traditional treatments, internet-delivered therapy overcomes common treatment barriers like the lack of therapist resources, socioeconomic conditions, and geographical distance, enabling treatment in the period of the pandemic [35].

However, there are few limitations for online treatment. First of all, a discernible gap emerges in the exploration of internet-based therapy for adults and older ASD population. The existing studies have

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primarily revolved around game-based structures and simplistic skill acquisition approaches, potentially neglecting the diverse and nuanced needs of this specific demographic. Use computer programs, vr scenes, robots or apps to treat different ASD symptoms in the form of animations or cartoons, but this approach and treatment symptoms cannot be generalized to adults or older people with ASD. For adult patients, they need to learn more sophisticated knowledge to help them live and work better, such as understanding how emotions work, body movements and interacting with colleagues. Instead of just learning to recognize four emotions [18] , learn to tie your shoelaces [21]or improve your concentration [26]. Consequently, as the field of therapeutic interventions evolves, it becomes evident that there is a pressing need for a shift in focus toward developing internet therapy solutions catering specifically to the older ASD population.

Apart from the evidences listed above, there is still limited empirical research and evidence regarding the effectiveness of online threapy in treating ASD. While some studies have shown positive outcomes, the field is still relatively new, and there is a lack of long-term data on their effectiveness and any potential side effects or unintended consequences. In addition, it will be expensive to design a new programm that fit well with a specific needs ASD patients, making VR and AI less accessible to individuals and families with limited financial resources. Furthermore, it has been argued that online threapy lack of human interaction. Some argue that excessive use of AR technology in therapy may reduce the opportunities for individuals with ASD to interact with real people, potentially hindering social and communication skill development or it is a challenge the skill learned in the setted virtual enviroment would able to genralise to real-world situation. For those threapy focus on care-giver programmes, caregiver involvement in ASD treatment can be demanding and time-consuming. It often requires parents and caregivers to participate in numerous therapy sessions, practice techniques at home, and consistently provide support, which can be physically and emotionally exhausting, and might not statified most ASD families. Finally, internet-based threatment for ASD involoved in collecting sensitive or private datas, which may raise concerns about privacy and data security. Protecting this data and ensuring its confidentiality are important considerations. In addition, as the therapeutic landscape continues to evolve, practitioners must be mindful of the complex consequences of these emerging technologies. While Internet-based therapies offer a wealth of benefits, a sensible balance that balances their advantages with potential drawbacks, such as avoiding excessive use of web-based therapies that reduce opportunities for real-world communication, should guide their seamless integration into the ASD treatment framework.

In conclusion, internet-based treatment for Autism Spectrum Disorder (ASD) has demonstrated significant promise and effectiveness in providing support and intervention for individuals with ASD. This approach offers a convenient and accessible means of reaching out to a wide range of patients, delivering specialized therapies, and enhancing the overall quality of life for those on the spectrum. However, it is important to acknowledge that there are certain limitations associated with internet-based treatment for ASD that must be carefully considered. Internet-based treatment can be a valuable tool in the overall management of ASD, serving as a complementary therapy alongside traditional interventions such as drug therapy or in-person behavioral therapies. This combined approach can provide a more holistic and tailored approach to address the diverse needs of individuals with ASD. In summary, while internet-based treatment for ASD offers numerous benefits, it is essential for patients, caregivers, and healthcare providers to collaborate in making informed decisions about the most appropriate treatment strategy. By doing so, we can harness the advantages of internet-based treatment while ensuring that individuals with ASD receive the comprehensive and individualized care they require.

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