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MAHSA International Medical Sciences and Technology Conference (Mi-MED) 2024

MAHSA University, Malaysia 26 - 27th July 2024

The international conference held by the Faculty of Medicine on July 26-27, 2024, brought together over 400 participants to discuss the latest advancements in medical research and Emerging Trends and New Insights. The event featured 6 keynote speeches, 5 presentation sessions, and two pre-conference workshops, resulting in a robust exchange of ideas and collaboration opportunities.

The Faculty of Medicine organised its annual international conference with the theme 'Health and Disease: Emerging Trends and New Insights'. The event aimed to bridge the gap between research and clinical practice by providing a platform for interdisciplinary dialogue.

Sessions and Speakers:

The conference kicked off with an inspiring keynote by Professor Dato' Dr Hj Abdul Razak Muttalif on 'Health and Disease: Emerging trends and New insights'. This was followed by sessions on various topics, including 'Innovations towards the development of cold chain-free live oral cholera vaccine', 'Paediatric Cancer: Current Perspective, Emerging Trends and New Insights', 'Multi-Omics dissection of Enteroviruses pathogenesis for host-based antiviral therapeutics', 'Nanomaterials for Biomedical Applications' and 'Dissolvable Microneedles for Transdermal Drug Delivery: Current Perspectives and Future Challenges' by the notable speakers included Prof Dr. Manickam Ravichandran, Prof. Dr. Wan Ariffin Abdullah, Assoc. Prof. Dr. Vinod Balasubramaniam, Assoc. Prof. Ir. Ts. Dr. Muhammad Mahyiddin Ramli and Prof. Dr. Azrul Azlan Hamzah.

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The Role of The EphrinB2-EphB4 Bidirectional Signalling on Bone Remodeling: A Review

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Abstract: Postmenopausal osteoporosis is a prevalent disease that can lead to serious outcomes such as fractures. Oestrogen deficiency during menopause and postmenopause increases bone turnover, with elevated bone resorption and formation. However, resorption surpasses formation, resulting in bone loss. Identifying intervention targets in bone physiology to restore bone remodeling balance and normal bone mass is the initial aim in developing new effective therapies. Currently, EphrinB2/EphB4, one of the membrane coupling factors between osteoclasts (OCs) and osteoblasts (OBs), is a prominent topic in bone disease research. However, the regulatory mechanisms of EphrinB2-EphB4 bidirectional signalling on OC and OB and its effects remain incompletely understood. This review seeks to clarify the physiological roles and mechanisms of known EphrinB2-EphB4 bidirectional signalling in bone remodeling, providing insights for future studies on modulating this signalling pathway.

Keywords: EphrinB2, EphB4, Bone remodeling, Osteoclast, Osteoblast.

1. Introduction

Osteoporosis is a metabolic bone disease characterized by reduced bone mass, microstructural deterioration of bone tissue, decreased bone strength, and increased bone fragility, making patients susceptible to fractures (NIH Consensus Development Panel On Osteoporosis Prevention, 2001). Osteoporosis is categorized into primary and secondary types based on the causative factors. Primary osteoporosis is further divided into postmenopausal osteoporosis, which is associated with postmenopausal oestrogen deficiency, and senile osteoporosis, which is linked to aging (Raisz, 2005). Secondary osteoporosis results from various other diseases or as an adverse effect of the treatment of certain diseases (Feng & McDonald, 2011). Maintaining bone health crucially depends on preserving bone remodeling homeostasis, which is the dynamic balance between the bone resorption by osteoclasts and the bone formation by osteoblasts. When factors shift the balance towards increased bone resorption by osteoclasts over bone formation by osteoblasts, continuous bone loss occurs, eventually causing osteoporosis.

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During human development, bones accumulate mass as bone formation exceeds bone resorption. In adulthood, bone remodeling is in dynamic equilibrium, with bone resorption equal to bone formation. In the aging stage, bone mass is lost as bone resorption surpasses bone formation. When bone mass loss reaches a certain level, it can result in osteoporosis. Maintaining bone remodeling homeostasis in the aging stage can reduce the incidence of osteoporosis. Within the bone-remodeling compartment, bone remodeling is carried out by the cells of the basic multicellular units, passing through five steps: activation, bone resorption, reversal, bone formation, and termination (Kenkre & Bassett, 2018). These steps involve the migration and maturation of osteoclast precursors to the damaged bone surface. Mature osteoclasts then perform bone resorption. This is followed by the suppression of osteoclast bone resorption function. Next, osteoblast precursors are recruited and mature at the resorbed bone surface. They produce new bone matrix and mineralize it. Finally, the process concludes (Kenkre & Bassett, 2018). Therefore, the coupling of osteoclast resorption of old bone with osteoblast formation of an equivalent new bone is crucial for precisely maintaining bone remodeling homeostasis. The factors involved in the coupling between osteoclasts and osteoblasts fall into two main categories. First, soluble coupling factors include transforming growth factor-beta (TGF-β) (Tang et al., 2009) and Insulin Growth Factor 1 (IGF1) (Durdan et al., 2022; Xian et al., 2012) from bone matrix, as well as leukemia inhibitory factor (LIF) (Weivoda et al., 2020) and cardiotrophin-1 (CT-1) (Walker et al., 2008) from OCs. Second, membrane coupling factors include EphrinB2-EphB4 (Zhao et al., 2006), semaphorin4D-PlexinB1 (Negishi-Koga et al., 2011; Shindo et al., 2022), and RANKL-RANK signalling (Durdan et al., 2022). For the treatment of osteoporosis, a monoclonal antibody Denosumab (DMAb) targeting the membrane coupling factor RANKL-RANK

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signalling has been developed, which neutralizes RANKL, inhibits osteoclast differentiation and resorptive activity (Khosla & Hofbauer, 2017), and induces multinucleated osteoclast apoptosis to reduce osteoclast numbers (McDonald et al., 2021). However, this drug, besides inhibiting osteoclasts, does not significantly enhance bone formation. Given that most current osteoporosis treatments primarily target anti-resorptive effects, the development of drugs that both counteract bone resorption and stimulate bone formation to restore normal bone mass is increasingly important. Therefore, the EphrinB2-EphB4 bidirectional signalling that exists between osteoclasts and osteoblasts and among osteoblasts has become a significant topic in the research of bone diseases.

Inhibiting excessive bone resorption by osteoclasts and promoting bone formation by osteoblasts is essential for restoring the balance of bone remodeling. This balance is critical for preventing and treating osteoporosis. EphrinB2-EphB4 bidirectional signalling exists between osteoclasts and osteoblasts, as well as among osteoblasts themselves. This makes it a promising therapeutic target that can both inhibit bone resorption and promote bone formation. Understanding how EphrinB2-EphB4 bidirectional signalling affects the generation and function of osteoblasts and osteoclasts is important. It will support conducting animal experiments targeting this signalling pathway. The aim is to explore drugs that can effectively restore the balance of bone remodeling. Successful animal studies will lay the basis for future clinical trials of such drugs.

2. EphB4 and EphrinB2

EphB4 is a member of the Eph receptor family, which belongs to the largest subgroup within the tyrosine kinase receptor family. The Eph receptor family can be divided into two subclasses: EphA and EphB. EphA receptors bind to EphrinA ligands anchored by glycosylphosphatidylinositol (GPI), whereas EphB receptors bind to EphrinB ligands containing transmembrane and cytoplasmic domains (Matsuo, 2010). In humans, there are 14 Eph receptors (EphA1-A8, EphA10, EphB1-B4, and EphB6) and 8 Ephrin ligands (EphrinA1-A5 and EphrinB1-B3) (Kania & Klein, 2016; Liang et al., 2019; Nguyen et al., 2016), with receptor-ligand mixed binding within subclasses (Lindsey et al., 2018). Eph receptors structurally consist of nine domains, including an extracellular N-terminal ligand-binding domain (LBD), a region rich in cysteine residues composed of sushi-like and EGF-like motifs, two fibronectin (FN) domains, a transmembrane domain (TM), a juxtamembrane domain (JM), a tyrosine kinase domain (TK), a sterile alpha motif (SAM) domain, and a C-terminal PDZ domain. Ephrin-As contain only a receptor-binding domain (RBD) and a GPI anchor. In contrast, Ephrin-Bs are integral membrane proteins composed of an extracellular RBD, a transmembrane domain, and an intracellular PDZ domain (Nguyen et al., 2016) (Figure 1). Ephrins and Ephs are membrane-bound proteins. They generate bidirectional signalling upon binding. This affects both the cells expressing the receptor and those expressing the ligand. The signalling through Ephs is termed forward signalling, whereas signalling through Ephrins is termed reverse signalling (Pasquale, 2005). Eph receptors and their Ephrin ligands play essential roles in various cells, regulating cell migration, repulsion, and adhesion in processes such as neuron, vascular, and intestinal development (Arvanitis & Davy, 2008; Pasquale, 2008). They are also closely associated with bone development (Davy et al., 2006) and bone homeostasis (Zhao et al., 2006). EphrinB2 is the preferred ligand for EphB4, showing weak binding to EphrinB1 or EphrinB3, with an affinity for EphrinB2 100 to 1000 times stronger than that for EphrinB1 (Flanagan & Vanderhaeghen, 1998). In bone tissue, osteoclasts express EphrinB2, which is encoded by Efnb2, one of the target genes of NFATc1, and its expression depends on the RANKL-induced c-Fos/NFATc1 transcriptional cascade (Ge et al., 2020; Zhao et al., 2006). Osteoblasts co-express the EphB4

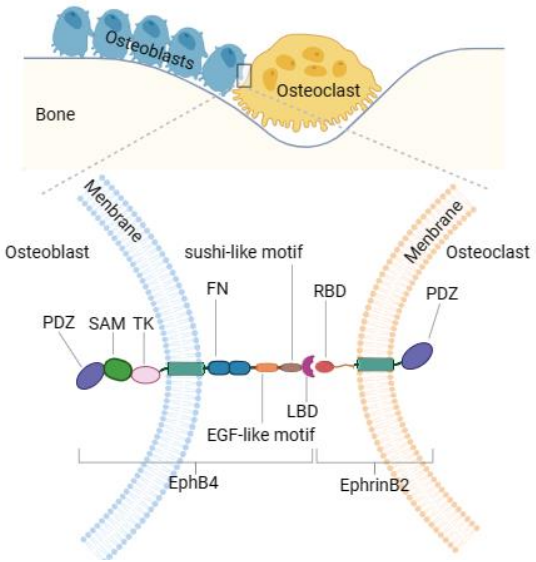

Figure 1. The basic components of EphrinB2 and EphB4

FN: fibronectin domain, LBD: ligand-binding domain, TK: tyrosine kinase domain, SAM: sterile alpha motif domain, RBD: receptor-binding domain.

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receptor and EphrinB2 ligand (Zhao et al., 2006). The reverse EphrinB2-EphB4 signalling between osteoclasts and osteoblasts inhibits osteoclast differentiation, while forward signalling promotes osteoblast differentiation (Zhao et al., 2006).

3. Roles of EphrinB2-EphB4 bidirectional signaling in osteoblast lineage cells

Since the osteoblast lineage expresses EphB4 and EphrinB2, and the osteoclast lineage expresses EphB4, understanding of EphB4-EphrinB2 interaction has evolved over the past 15 years from heterotypic interactions between osteoblast lineage cells and osteoclast lineage cells to homotypic interactions within the osteoblast lineage (Arthur & Gronthos, 2021). However, Blank M et al. argue that the primary role of EphrinB2 in bone is in the osteoblast lineage (Blank & Sims, 2019).

The significance of EphrinB2-EphB4 signalling in the late-stage differentiation and survival of osteoblasts has been demonstrated in several experiments. Results from gain-of-function experiments indicate that overexpression of EphrinB2 in osteoblasts promotes their differentiation, evidenced by increased expression of osteoblast differentiation markers, such as alkaline phosphatase, Col1a1, and osteocalcin (Zhao et al., 2006). Additionally, the expression of distal-less homeobox 5 (Dlx5), osterix (Osx), and runt-related transcription factor 2 (Runx2), which are key transcription factors for osteoblast differentiation, is significantly upregulated (Zhao et al., 2006). Mechanistically, EphrinB2 stimulation may promote osteoblast formation by enhancing ERK1/2 phosphorylation and reducing RhoA activity, but this stimulatory effect is believed to be independent of EphrinB2 cytoplasmic domain (Zhao et al., 2006). Loss-of-function experiments by Tonna S et al. demonstrated that specific deletion of EphrinB2 mediated by Osx-Cre leads to impaired late-stage osteoblast differentiation, accompanied by increased osteoblast and osteocyte apoptosis (Tonna et al., 2014). The compromised osteoblast differentiation and delayed initiation of bone mineralization result in reduced bone strength in adult bones, leading to osteomalacia (high osteoid content) (Tonna et al., 2014). While osteocytes differentiate from osteoblasts embedded in osteoid, they still retain expression of EphrinB2 and EphB4 (Allan et al., 2008; Arthur et al., 2011; Takyar et al., 2013). EphrinB2-specific knockout osteocytes exhibit increased autophagic activity, resulting in enhanced secondary mineralization processes and increased bone fragility as stimulation with EphrinB2-Fc inhibited the increase in autophagosomes in EphrinB2-deficient osteocytes via the RhoA-ROCK signalling pathway (Vrahnas et al., 2019). Treatment of cultured osteoblasts with soluble EphB4 (sEphB4), a specific inhibitor blocking EphrinB2-EphB4 bidirectional signalling, inhibits EphB4 and EphrinB2 phosphorylation. It also decreases the mRNA levels of late-stage osteoblast/osteocyte differentiation markers, such as osteocalcin and sclerostin. Moreover, in vivo administration of sEphB4 increases osteoblast formation and the mRNA levels of early osteoblast markers, such as Runx2 and alkaline phosphatase. This significantly increases the number of osteoblasts. However, there is no significant change in bone formation rate or late-stage markers of osteoblast/osteocyte differentiation. This indicates that EphrinB2/EphB4 signalling within the osteoblast lineage is critical for the late-stage differentiation of osteoblasts (Takyar et al., 2013). These research findings suggest that EphrinB2-EphB4 signalling plays an essential role in the normal differentiation and functional regulation of the osteoblast lineage, but whether these effects are achieved through forward or reverse signalling remains unclear.

Following EphB4-Fc treatment in vivo, immunofluorescence analysis revealed an increase in OPG protein expression and a decrease in RANKL protein expression in osteoblasts, indicating that the reverse signalling of EphrinB2-EphB4 elevated the OPG/RANKL ratio. Examination of the forward signalling of EphrinB2-EphB4 showed that EphrinB2-Fc significantly increased the OPG/RANKL ratio both in vivo and in vitro; however, in vitro stimulation with EphrinB2-Fc resulted in a significant decrease in RANKL mRNA expression in osteoblasts, while the change in OPG mRNA expression was not significant (Ge et al., 2020). These findings suggest a clear distinction in the effects of the forward and reverse signalling of EphrinB2-EphB4 in osteoblasts (Figure 2).

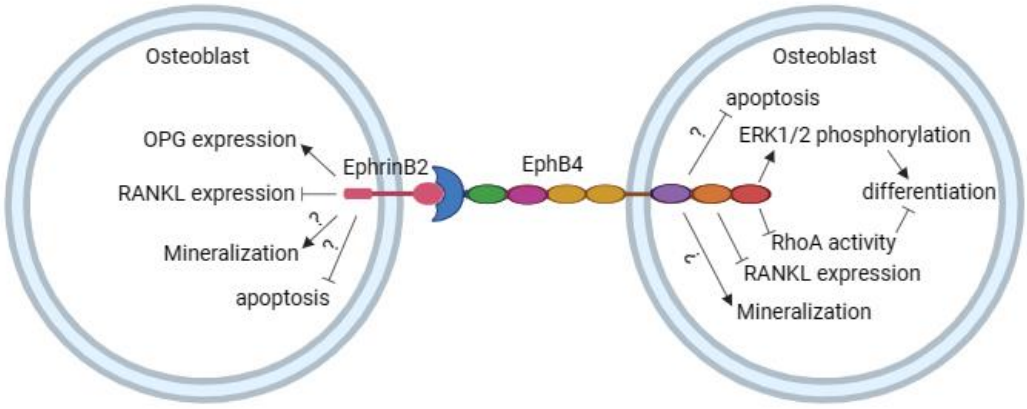

Figure 2. EphrinB2-EphB4 bidirectional signalling in osteoblast lineage cells

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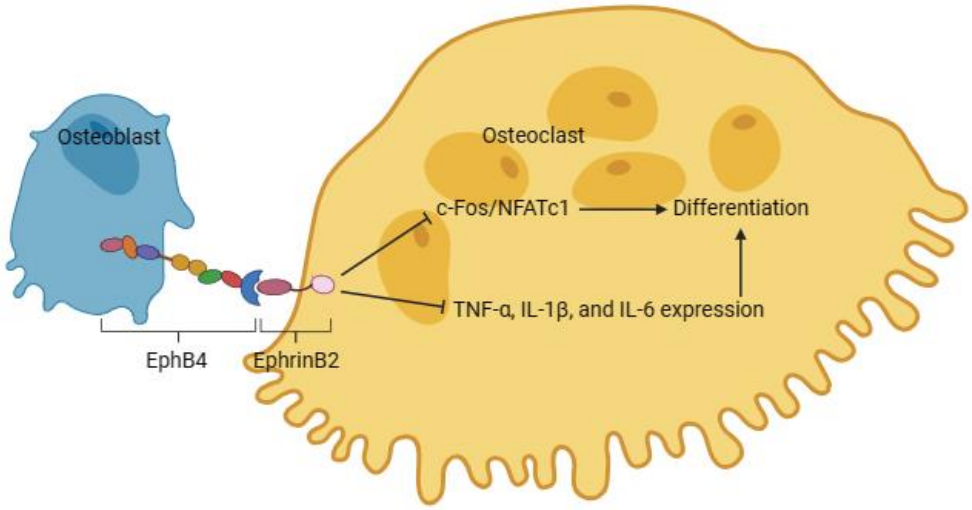

Figure 3. EphrinB2-EphB4 reverse signalling in osteoclasts.

Differentiating the effects of the forward and reverse signalling of EphrinB2-EphB4 in osteoblast differentiation, function, and survival holds potential significance for the treatment of bone diseases.

4. The roles of reverse signaling of EphrinB2-EphB4 in osteoclasts

Osteoclasts have been shown to express the ligand EphrinB2 for the receptor EphB4. Research by Zhao et al. and Baek et al. indicates that the reverse signalling of EphrinB2 entering osteoclast precursors inhibits osteoclast differentiation and maturation by negative feedback suppression of c-Fos and NFATc1 transcription in osteoclasts (Figure 3) (Baek et al., 2018; Zhao et al., 2006). The inhibitory signalling in osteoclasts is mediated by the C-terminal DYKV motif of EphrinB2 intracellular domain binding with PDZ domain proteins (Zhao et al., 2006). The detailed mechanism of EphrinB2-EphB4 reverse signalling in osteoclasts is poorly understood. Experiments using Icariin intervention in a glucocorticoid-induced osteoporotic mouse model have shown that Icariin can counteract osteoporosis via EphB4-EphrinB2 signalling, while also increasing the expression of Grb4 adaptor protein (Huang et al., 2020). This suggests that elevated Grb4 binding with the EphrinB2 intracellular domain amplifies the inhibitory effect of EphrinB2 reverse signalling on osteoclasts. Moreover, it has been found that monomeric EphrinB2 cannot initiate signal transduction (Kertesz et al., 2006). A study revealed that in cells expressing EphrinB2, kindlin2 can interact with the highly conserved NIYY motif in the cytoplasmic tail of EphrinB2, promoting EphrinB2 clustering to regulate EphB4 activation across cells. This indicates that kindlin2 facilitates bidirectional EphB4/EphrinB2 signalling (Li et al., 2022). These research findings suggest that developing a drug that can enhance EphrinB2 clustering may be used to treat osteoporosis by strengthening the EphrinB2-EphB4 bidirectional signalling.

In an in vivo experiment demonstrating the use of EphB4 inhibitors to intervene in orthodontic tooth movement in rats, an increase in tooth movement, a significant increase in bone trabecular separation, a decrease in bone trabecular number, an increase in osteoclast number and activity, and inhibition of osteoblast differentiation and function were observed (Jiang et al., 2023). The researchers believe that by inhibiting EphB4, the reverse signalling of EphrinB2-EphB4 is blocked, leading to a weakening of the inhibition of c-Fos-NFATc1 transcription and osteoclast differentiation, thereby promoting bone resorption on the tension side (Jiang et al., 2023). In addition to in vivo, enhancing osteoclastogenesis by using sEphB4 monomer to block EphrinB2-EphB4 interaction was also reproduced in vitro, but osteoblast presence was necessary. These facts, combined with the result that sEphB4 treatment enhances RANKL expression in osteoblasts, suggest that EphrinB2-EphB4 signalling within the osteoblast lineage limits RANKL production to some extent, thereby suppressing osteoclastogenesis (Takyar et al., 2013). In vitro, treatment with EphB4-Fc inhibited the release of pro-inflammatory factors TNF-α, IL-1β, and IL-6 mediated by titanium particles in BMMs, confirming the inhibition of osteoclast differentiation and bone resorption function through the EphrinB2 signalling pathway by EphB4-Fc(Ge et al., 2020). This suggests that the reverse signalling of EphrinB2-EphB4 can induce a decrease in the expression of factors like TNF-α and indirectly inhibit osteoclast formation (Figure 3).

However, a conclusion drawn by Tonna S and colleagues is that EphrinB2 signalling induced by EphB4 in the osteoclast lineage does not suppress osteoclastogenesis. This conclusion is based on their finding that knocking down EphB4 in Kusa 4b10 cells (pre-osteoblasts) did not change their support for osteoclast formation, and that the number of osteoclasts generated from bone marrow mesenchymal stem cells (BMMs) under conditions with or without EphrinB2 knockout was comparable, and even stimulation with clustered EphB4-Fc did not inhibit osteoclastogenesis (Tonna et al., 2014). The explanation for this finding may relate to the fact that RANKL is a major stimulatory factor necessary for osteoclast differentiation and formation. This is because knocking down EphB4 in Kusa 4b10 osteoblasts may reduce the EphrinB2-EphB4 bidirectional signalling between

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osteoblasts. It caused increased RANKL expression and decreased OPG expression in osteoblasts. Conversely, inhibiting osteoclast differentiation and formation may not be the primary function of EphrinB2-EphB4 reverse signalling between osteoclasts and osteoblasts. The infrequent direct contact between mature osteoclasts and osteoblasts suggests that the interaction of EphrinB2-EphB4 between OC and OB is unlikely to be crucial for normal bone mass, and the role of EphrinB2 expression and signalling in osteoclasts remains unresolved (Vrahnas & Sims, 2015). Considering that during the reversal stage of bone remodeling, it is still unclear how the bone resorption activity of mature osteoclasts terminates, cells leave the resorbing bone surface, or cell apoptosis is initiated. We speculate that the interaction of EphB4 and EphrinB2 in contact between osteoclasts and osteoblasts may relate to this: at the start of the reversal phase, osteoblast precursors migrate to the resorption site of osteoclasts, direct contact between the two leads to the binding of EphrinB2 and EphB4, promoting osteoblast precursor differentiation into mature cells through forward signalling, and inducing cessation of bone resorption activity in mature osteoclasts and initiating the apoptosis program of osteoclasts, preparing for the migration of osteoblasts into the resorption pit.

Furthermore, contrary to previous reports, one study found weak expression of EphB4 in cultured BMMs (Baek et al., 2018). Therefore, further experimental validation is needed on this issue.

5. Prospects of drug development research on EphrinB2-EphB4 signalling

EphrinB2 and EphB4 are expressed in various organs and tissues, including tumor tissues, and their interaction leads to complex biological effects, such as affecting the proliferation, migration, and invasion capacity of tumor cells (Hadjimichael et al., 2022). Many researchers have attempted to study EphrinB2-EphB4 bidirectional signalling as a potential target for intervening in several diseases, such as cardiovascular diseases, neurological disorders, cancer, and bone diseases.

Pharmacological inhibition of EphrinB2/EphB4 interaction impairs osteoblast differentiation both in vitro and in vivo (Allan et al., 2008; Takyar et al., 2013), and intermittent administration of parathyroid hormone (PTH) increases bone mass by upregulating EphrinB2 (Vrahnas & Sims, 2015). This implies that increasing the expression of EphrinB2/EphB4 in osteogenic lineage cells and enhancing EphrinB2-EphB4 bidirectional signalling among bone tissue cells might be a potential research direction for preventing bone loss in osteoporosis and restoring normal bone mass in the future.

Regarding the regulation of EphrinB2/EphB4 expression, Wang R et al. found that silencing Jumonji domain-containing 3 (Jmjd3, a histone demethylase) in osteoblasts inhibits H3K27me3 demethylation in the EphB4 promoter region, reducing EphB4 expression. This also leads to upregulation of RANKL in osteoblasts, with soluble RANKL levels increasing, but RANKL elevation can be suppressed by EphB4 overexpression (Wang et al., 2023). This indicates that Jmjd3 participates in regulating bone resorption and formation by increasing EphB4 expression and inhibiting RANKL levels. Prior research shows that Erythropoietin (EPO) can increase EphB4 expression in ST2 cells (osteoblast precursors) and EphrinB2 expression in RAW264.7 cells (osteoclast precursors) (Li et al., 2015). Additionally, a novel vitamin D analog, Eldecalcitol (ED-71), can prevent bone loss and reduce the number of osteoclasts in Glucocorticoid-Induced Osteoporosis rats and OVX rats in vivo (Rong et al., 2022; Zhang et al., 2022). Moreover, it can also promote new bone formation and osteoblast activity (Rong et al., 2022). The inhibitory effect of ED-71 on osteoclasts was mediated by increasing EphrinB2 expression in osteoclasts, as this effect could be reversed by knocking down EphrinB2 (Zhang et al., 2022). The study also found that ED-71 enhances EphB4 expression in osteoblasts both in vitro and in vivo (Zhang et al., 2022).

In terms of enhancing EphrinB2-EphB4 signalling, Baek JM et al. discovered that Cldn11, the key component of tight junctions (TJs), had the potential to treat bone diseases (Baek et al., 2018). Cldn11 exerted a negative effect on osteoclast differentiation and function by targeting the reverse signalling of EphrinB2 in OCs. It had a positive regulatory effect on osteoblast differentiation by targeting the forward signalling of EphB4 in OBs. Consistent with in vitro effects, subcutaneous injection of recombinant Cldn11 protein in mouse models demonstrated anti-resorptive effects and increased osteogenic activity (Baek et al., 2018). Combined with the research results of Li W et al. showing that kindlin2 promoted EphrinB2 clustering (Li et al., 2022), it provides a clue that factors which promote EphrinB2/EphB4 clustering on the membranes of osteoblasts and osteoclasts or enhance the phosphorylation of the intracellular segment of EphrinB2/EphB4 are also worth investigating.

6. Conclusion

Currently, most clinical drugs for the treatment of primary osteoporosis primarily focus on inhibiting bone resorption. It is difficult to achieve the outcome of reversing bone loss and restoring normal bone mass simultaneously. The development of targeted therapeutic drugs that consider both anti-resorptive and pro-formative effects represents the trend. Further research into the detailed regulatory mechanism of EphrinB2-EphB4 bidirectional signalling in bone resorption and bone formation will help identify key nodes in the signalling process that can significantly enhance osteoblast production, survival, and bone formation. This will pave the way for future interventions in animal models targeting these points, to observe and evaluate their regulatory effects, and establish the foundation for subsequent clinical trials.

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Policy Elements Influencing the Sustainability of the Biosimilar Market in Malaysia: A Review

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Abstract:
Sustaining the market for biosimilars is key to securing the long-term benefits from biosimilar medicines. Policy can influence the achievement of those elements that contribute to the sustainability of biosimilars. Despite being the first country to implement regulatory guidelines for the approval and marketing of biosimilars, the availability and adoption of biosimilars in Malaysia remain suboptimal. This study aims to create the roadmap of policy elements that influence the sustainability of the biosimilar market in Malaysia. The study searched and reviewed articles in PubMed, Google Scholar, National Library of Medicine from 1/1/2003 to 14/04/2024. A total of 3469 results were found, with PubMed contributing 39 records (n=39), Google Scholar contributing 3074 records (n=3074), and the National Library of Medicine contributing 356 records (n=356). The biosimilars policy framework and sustainability measures were adopted from IQVIA Country Scorecards for Biosimilar Sustainability. After eliminating duplicates and applying selection criteria, 20 articles were chosen for review. The selection was justified based on relevance to the five policy domains: regulatory environment and clinical guidelines, awareness and education, incentives, pricing rules and dynamics, and purchasing mechanisms. The 20 selected papers were categorized into five main domains: regulatory environment and clinical guidelines, awareness and education, incentives, pricing rules and dynamics, and purchasing mechanisms. The analysis revealed positive developments in regulatory compliance and clinical guidelines, pricing regulations, and purchasing mechanisms. However, challenges were identified in the areas of awareness and education, pricing rules and dynamics, and incentives. Awareness and education challenges were primarily attributed to limited pharmacist training and physician skepticism regarding biosimilars. Additionally, the absence of strong financial incentives and limited patient education hindered adoption. The "single-winner tendering system" constrained market diversity, restricting competitive pricing. The literature review emphasizes the need for setting up specific prescription targets, promoting competition, and offering incentives to biosimilar manufacturers to increase market appeal. Furthermore, it is critical to enforce strict quality standards for the incorporation of tenders and align the registration requirements with global standards. Education for pharmacists and continuous training for healthcare practitioners are also essential. In Malaysia, these steps are crucial in ensuring the affordability and accessibility of biosimilars. Improving these policy components could help Malaysia create a competitive biosimilar market, increase access to affordable treatments, and eventually raise the standard of healthcare service.

Keywords: biosimilars market Malaysia, sustainability, biosimilars policy, biosimilar regulatory guidelines, literature review.

1. Introduction

Biological therapies, often known as biologics, contain therapeutic proteins and monoclonal antibodies. These are complex and big molecules that are commonly generated in manipulated organisms, such as modified bacteria. Biological therapies are a significant breakthrough in treating chronic, debilitating, as well as fatal diseases such as cancer, neurological disorders, and autoimmune diseases (Baumgart et al., 2019; Chopra & Lopes, 2017). However, they are often associated with high costs and limited patient access (Chopra & Lopes, 2017; Nahleh et al., 2022). For example, while biologics make up only 2% of all prescriptions in the US, they contribute to $120 billion, or 37%, of the entire expenditure on drugs. Additionally, since 2014, they have accounted for 93% of the overall increase in total expenditures (Lexchin, 2020; Makurvet, 2021). Similar observations have been reported in other countries (Simoens & Vulto, 2021). Biologicals made up 35% of Colombia's pharmaceutical business in 2015 (Makurvet, 2021). In Malaysia, like in other nations, the expense of innovative biologics imposes financial restrictions on the healthcare system, leading to limited patient access to these medicines (Khean, 2014; Su-Lyn, 2020). Malaysia faces the multifaceted challenge of improving public standards along with escalating health expenditures, a scenario similar to that of numerous middle- and upper-income countries (Kananatu, 2002). This forces the government to look for viable options for sustainable financing moving forward. In 2010, the estimated per capita health expenditure in PPP international US dollars reached $641, surpassing the median expenditure of $589 for upper-middle-income countries in that year (Khan et al., 2016; WHO, 2012; Yun & Yusoff, 2015). Malaysia does not allocate substantial resources to health expenditures. In 2010, the estimated total health expenditure constituted 4.4% of GDP,

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situating it within the mid-range of high and middle-income nations in Asia, albeit beneath the international average of 6.1% GDP for upper middle-income countries (WHO, 2012; Zamzairee & Muhamad Hanafiah Juni, 2019). The annual growth rate in Total Health Expenditure (THE) has exhibited considerable variability since the year 2000, ranging from 6.4% to 20.8% (WHO, 2012). In 2004, Malaysia achieved a ranking of 59th in the UN Human Development Index, whereas its neighboring countries, Thailand and Indonesia, were positioned at 76th and 111th, respectively (EIU, 2005). According to the most recent data from the Malaysia National Health Accounts (MNHA) Health Expenditure Report, Malaysia spent RM78.2 billion, or 5.1% of GDP, on healthcare in 2021. Total Health Expenditure (THE) for Malaysia from 2011 to 2021 has steadily increased. The proportion of GDP attributed to THE varied between 3.9% and 5.1% across the same time period. The substantial growth in the value of THE in 2021 caused the proportion of THE to GDP to rise (MOH Malaysia, 2021).

In many health systems worldwide, biosimilars are considered a crucial tool for containing growing pharmaceutical spending. The entry of biosimilars into the market has been demonstrated not only to affect pharmaceutical costs but also to improve patient access to biological therapies (Advancing Biosimilar Sustainability in Europe - IQVIA, 2018). The primary goal of biopharmaceutical policy should be to establish a market that is both competitive and sustainable, consisting of off-patent reference biologics, biosimilars, and next-generation biologics. This market should ensure that biologic therapy is accessible to patients at the most affordable price possible (Simoens & Vulto, 2021). The initial regulatory framework for the commercialization of biosimilars was implemented in Europe in 2005, leading to the approval of the first European biosimilar in 2006. As of June 2023, the European Medicines Agency had granted approval to a total of 94 biosimilars (EMEA, 2023). Malaysia became one of the first countries worldwide in 2008 to adopt regulatory guidelines for the approval and marketing of biosimilar medicines. The guiding document and guidelines for the registration of biosimilars in Malaysia were completed in August 2008. The guidance document incorporates the entire set of scientific biosimilar guidelines from the European Medicines Agency (EMA), including both the product-specific guidelines and other relevant guidelines for biosimilars. Some modifications have been made to tailor the guidelines for Malaysian applications (EMEA, 2014; Kang et al., 2021). Policies impacting biosimilars have been investigated in many literature (Arianna Bertolani & Claudio Jommi, 2020; Moorkens et al., 2017; Rémuzat et al., 2017; Simoens et al., 2024; Vogler et al., 2021). It has been observed that the majority of Malaysian hospitals do not have all the biosimilars that have been licensed by the National Pharmaceutical Regulatory Agency (NPRA). This indicates the presence of possible constraints on sustainability within current policy frameworks.

There are several explanations for the necessity of comprehending how policy can enhance the sustainability of biosimilar markets. First and foremost, the problem of insufficient market trust continues to exist. Misunderstandings regarding the safety of biosimilars, which are not identical molecular entities, might lead to distrust among physicians and patients, potentially impacting their utilization (Barbier et al., 2021). Furthermore, policymakers continue to implement laws intended for common small molecules to biosimilars. It is criticized that these rules are not customised to the unique features and market dynamics of biosimilars, which leads to an unsustainable environment (Alnaqbi et al., 2023).

This study aims to establish a literature review of policy elements that influence the sustainability of biosimilars in Malaysia.

2. Methods

2.1 Conceptual Framework

This research adopted the biosimilar policy framework of the country scorecards for biosimilar sustainability (IQVIA, 2020). The IQVIA Institute for Human Data Science and Medicines for Europe has created country scorecards to evaluate the policy aspects necessary for a successful biosimilar market. The scorecards evaluate the positive influence of biosimilar medicines on the long-term viability of healthcare systems and assess the effectiveness of national policy. The sustainability scorecards include five primary policy areas. These include regulatory environment and clinical guidelines, awareness and education, incentives, pricing rules and dynamics, and purchasing mechanisms. By mapping these components for a given country and evaluating the entire impact of biosimilars on the healthcare system, the scorecards may help in evaluating the existing performance and identifying areas for enhancement.

2.2 Paper identification

A comprehensive search strategy was implemented to identify related articles in Malaysia from 1/1/2003 to 14/4/2024. Keywords are used, including “Malaysia biosimilars policy”, “Malaysia biosimilars regulatory environment and clinical guidelines”, “Malaysia biosimilars awareness and education”, “Malaysia biosimilars incentives”, Malaysia biosimilars pricing rules and dynamics”, and “Malaysia biosimilars purchasing mechanisms”. Search engines were PubMed, Google Scholar, and the National Library of Medicine. The reference lists of specific papers and popular web search engines were manually searched to find more relevant research.

2.3 Paper selection and extraction

After identifying the publications, the duplicates were removed. Subsequently, the titles and abstracts were assessed for relevance, using the inclusion criteria. Peer-reviewed papers and publications written in English that describe five essential policy frameworks were selected. Other types were excluded. For a literature review research work, full-text articles were targeted.

2.4 Analysis

The final selected papers were categorized manually based on the content of the research.

3. Results

A total of 3469 records were overall retrieved from PubMed (n=39), Google Scholar (n=3074), National Library of Medicine (n=356), and Additional Searching (n=3) (Figure 1). Ultimately, 20

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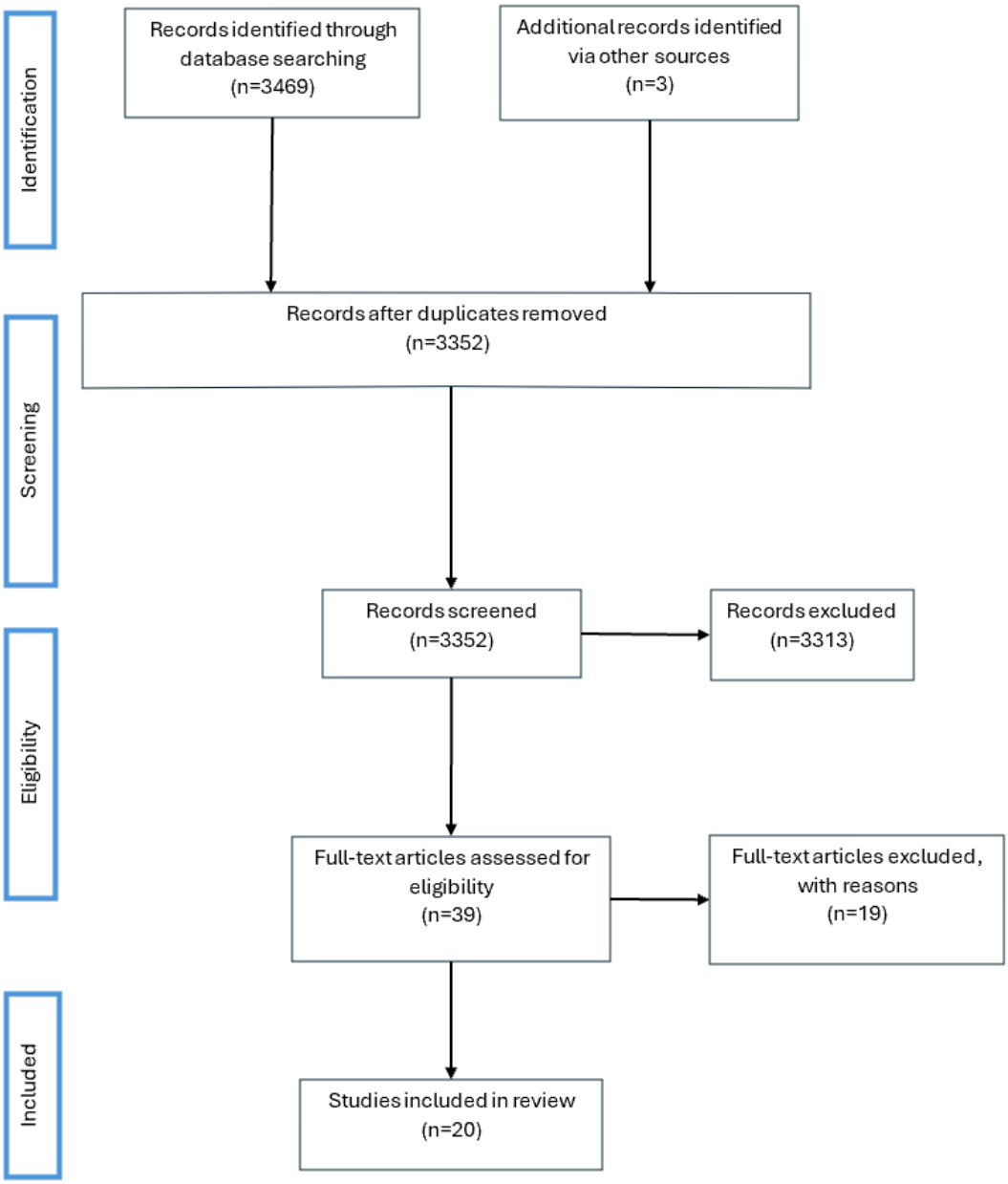

Figure 1. Review process flowchart

articles were chosen, passing all screening procedures, and thematically analysed. The emerged policies and practices were classified under five key policy-related domains (Table 1).

The review process began with an extensive search across multiple databases, identifying 3,469 records through database searches and 3 additional records from other sources. After removing duplicates, 3,352 unique records remained for screening. The initial screening process excluded 3,313 records based on relevance and predefined eligibility criteria. Subsequently, 39 full-text articles were assessed for eligibility. Of these, 19 were excluded due to various reasons, such as lack of direct relevance to biosimilar policies or insufficient data on sustainability factors. Ultimately, 20 studies were selected for the final review. This approach ensured that only the most relevant and high-quality sources were included in the review.

3.1 Category 1: Regulatory environment & clinical guidelines

Researchers identified the regulatory environment and clinical guidelines practices of physicians: time from EMA approval to first biosimilars sales, treatment guidelines for biosimilar use.

3.2 Category 2: Awareness and education

Regarding awareness and education, comprehensive training /education for pharmacists and physicians is noted.

3.3 Category 3: Incentives

This category consists of incentives to promote biosimilar use, and prescription quotas or financial incentives for providers that do not restrict physician choice.

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Table 1. Policy domain and findings

Policy domains	Findings
Regulatory environment & clinical guidelines	Malaysia's biosimilar guidelines were established in 2008, following the principles of the European Medicines Agency. The registration requirements for biotherapeutics (BTPs) and biosimilar products align with international regulatory practices, but there is some unique labeling, package insert requirements, and registration conditions specific to the Malaysian regulatory context. The World Health Organization (WHO) guidelines have played a significant role in establishing regulatory frameworks for biosimilars globally. Biosimilars marketing approval is a complicated process. (Abas, 2011; Bas & Oliu Castillo, 2016; Kang et al., 2020, 2021; Khoo et al., 2017a; Mohd Sani et al., 2020; Wadhwa et al., 2022)
Awareness and education	Pharmacists' knowledge about biosimilars varies by study. Many pharmacists lack of biosimilars training. Physicians' awareness of prescribing biosimilars is noted. (Chong et al., 2022; Mohd Sani et al., 2022a, 2023a, 2024a; Shakeel et al., 2020b)
Incentives	Encouraging biosimilars prescribing, strict quality standards, and procurement pooling for larger volumes are suggested. Local producer incentives and price competition help biosimilars enter earlier. (Godman et al., 2021, 2021; Godman et al., 2021a; Hamzah et al., 2020; Maniadakis et al., 2018a)
Pricing rules and dynamic	Competition between biosimilars and the originators was extreme, and low competition hampered price reduction. The overall procurement price trend was stable until 2014. (Godman et al., 2021a; Hamzah et al., 2020; Kang et al., 2021; Maniadakis et al., 2018a) Cost and strong pharmacovigilance influence biosimilar prescribing. Due to affordability and local production, Malaysia uses biosimilars extensively. (Chong et al., 2022; Godman et al., 2021; Godman et al., 2021a; Hamzah et al., 2020)
Purchasing mechanism	Tendering can reduce costs if supported by strong legal and organizational frameworks and stakeholder management. Government, regional procurement agencies, and international organization's competitive bidding or tendering can affect buyer prices. (Hamzah et al., 2020; Khoo et al., 2017a; Maniadakis et al., 2018a)

3.4 Category 4: Pricing rules and dynamic

Two primary themes were identified regarding pricing rules and dynamics: dynamic price competition between biosimilars and the originators, and factors influencing cost-effectiveness.

3.5 Category 5: Purchasing mechanism

A primary theme that was identified in the purchasing mechanism is the tender/bidding rules.

4. Discussion

This study aimed to present a comprehensive analysis of the policy areas of biosimilars in Malaysia. Thus, the sustainability of biosimilars is estimated. Several interventions were implemented and embraced to promote the utilization of biosimilars, which were deliberated within five overarching domains.

4.1 Regulatory environment & clinical guidelines

In 2008, Malaysia adopted the guidelines of the European Medicines Agency for biosimilar regulations and issued its own guideline (Bas & Castillo, 2016; Sani et al., 2020). While there is a consensus on the fundamental principles regarding the importance of quality assessment in evaluating biosimilars, there is a lack of published discussions providing specific details on this matter (Abas, 2011; Sani et al., 2020). The National Pharmaceutical Regulatory Agency (NPRA) has approved a total of 24 biosimilar products and has documented 499 adverse event (AE) reports. Additionally, NPRA has approved ten Phase III clinical trials in Malaysia, of which four trials are currently in progress (Sani et al., 2020). The registration requirements for novel BTPs and biosimilar products adhere to international regulatory standards. Specific labeling and package insert requirements, as well as registration conditions, are distinct from the regulatory framework in Malaysia (Khoo et al., 2017b). The guidelines established by the World Health Organization (WHO) have played a significant role in establishing the regulatory framework for biosimilars in various countries and promoting regulatory alignment on a global scale (Kang et al., 2020; Wadhwa et al., 2022). Biosimilars pose greater complexities compared to traditional generics, and obtaining marketing approval for them is significantly more intricate (Bas & Castillo, 2016).

4.2 Awareness and education

The variation in awareness among healthcare professionals (ranging from 47% to 86%) suggests that differences in professional training, institutional exposure, and availability of biosimilar-related workshops contribute to the discrepancy.

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Table 2. Positive policy elements, Policy challenges, and Potential policy solutions

Positive Policy Elements
1. High alignment with international best practices, including regulatory frameworks adapted from the European Medicines Agency (EMA) and the World Health Organization 2. Training programs for physicians and pharmacists enhance awareness and professional 3. Reference pricing mechanisms promote competitive pricing and affordability of biosimilars. 4. Government and private hospital procurement contracts ensure market stability and accessibility.
Policy Challenges
1. Lack of localized treatment guidelines and delays in biosimilar market entry due to complex approval processes. 2. Limited patient education initiatives and insufficient outreach efforts. 3. Absence of strong financial incentives for hospitals, prescribers, and patients to encourage biosimilar adoption. 4. Lack of mandatory price reductions for originator biologics, limiting market competitiveness. 5. Single winner tendering systems reduce market diversity and hinder price competition.
Potential Policy Solutions
1. Develop localized biosimilar treatment guidelines to streamline market entry and complement international policies. 2. Launch targeted educational campaigns for both healthcare professionals and patients, using digital platforms and outreach programs. 3. Introduce financial incentives, including reimbursement schemes and prescription targets, to encourage biosimilar uptake. 4. Implement mandatory price reduction policies for originator biologics to promote affordability and competition. 5. Revise tendering systems to support multi-winner models, ensuring fair competition and sustainable procurement practices.

Between 22% and 74% of pharmacists have received training on biosimilars and have obtained information about biosimilars from scientific publications, pharmaceutical companies, and continuing education (Mohd Sani et al., 2022b). Over 80% (n=305) of the participants exhibit a high level of understanding regarding the definition, features, effectiveness and safety, cost considerations, compatibility, and application of BSMs. The majority of papers expressed opposition to the automatic substitution by pharmacists due to the limited evidence supporting this practice (Shakeel et al., 2020a; Mohd Sani et al., 2024b). In other studies, about pharmacists' educational activities, it can be seen that a significant majority (62.8%, n=573) of respondents did not receive adequate training in biosimilars. However, a large proportion (80.6%, n=736) recognized the crucial role that pharmacists play in promoting the prescription of biosimilars (Mohd Sani et al., 2023b).

In other studies, the majority of oncologists (72%, n=26) expressed agreement or strong agreement that switching will not have a substantial impact on the effectiveness of the treatment. However, a smaller percentage agreed or strongly agreed that it will not result in the development of additional negative effects (56%) or harmful immune reactions (64%). The average knowledge score in biosimilar among respondents was 3.81 (± 0.86) out of a maximum possible score of six (Chong et al., 2022).

4.3 Incentives

It is crucial to improve the appeal of the biosimilar insulin market in order to incentivize other biosimilar manufacturers to join the market, especially as the patents for several long-acting insulin analogues expire. This will benefit all important groups involved (Godman et al., 2021). Implementing reimbursement for biosimilar, setting specific prescribing targets, and promoting competition among manufacturers, including incentivizing local production (Godman et al., 2021). Promoting the increased use of biosimilar drugs instead of original and patented medications within a specific category to save resources without

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compromising the quality of care (Godman et al., 2021b). The recommendations for countries with expanding healthcare coverage (CEHCs) suggest implementing stringent quality standards for tender inclusion, consolidating procurement processes to handle larger volumes, and adopting a cautious approach to tendering in order to effectively achieve their objectives in developing their healthcare systems (Maniadakis et al., 2018b). Studies have shown that in Japan, hospitals and clinics meeting biosimilar prescription targets received additional financial support, leading to a 10% increase in biosimilar prescriptions and a 12% reduction in overall healthcare expenditures (Itoshima et al., 2024). A similar model in Malaysia, where financial rewards or subsidies are provided for hospitals exceeding a set biosimilar adoption threshold, could encourage greater usage. To promote the early introduction of generic/biosimilars drugs, enhancing the capacity of local production and offering incentives to local producers to enter the export market is necessary. This can be achieved through agreements to purchase a specified quantity of drugs and other incentives aimed at fostering price competition during the procurement process. A key issue is the pricing disparity between biosimilars and their biologics. While biosimilars are generally priced 20-30% lower than originators, the cost savings have not been substantial enough to incentivize widespread prescribing (Hamzah et al., 2020).

4.4 Pricing rules and dynamic

The overall procurement prices remained relatively stable over the years, except for 2014 when the quantity-weighted average price ratio (QWAPR) and expenditure-weighted average price ratio (EWAPR) decreased by 14% and 9% respectively, compared to 2010. A comparison with International Reference Prices (IRP) revealed that Malaysian government procurement prices were often higher than global benchmarks. For instance, certain medicines had procurement prices exceeding the IRP by up to 21 times, indicating inefficiencies in cost management (Hamzah et al., 2020)**.** The prescribing of biosimilars is primarily influenced by two key factors: cost differences and the implementation of comprehensive pharmacovigilance activities. Cost differences and pharmacovigilance activities primarily influence the prescribing of biosimilars in Malaysia. A survey among Malaysian oncologists found that 40% cited patient preferences and 34% pointed to the non-availability of biosimilars in hospitals as major barriers to their adoption (Chong et al., 2022). Cost and value concerns restricted their usage in Malaysia (Godman et al., 2021). The widespread adoption of biosimilars in Malaysia can be attributed to their affordability and local manufacturing capabilities (Godman et al., 2021). Buyer prices, unlike supplier prices, can be tailored to the government, regional procurement agency, or international organization that conducted the competitive bidding or tender process (Hamzah et al., 2020).

4.5 Purchasing mechanism

There was evidence suggesting that pooling or joint tenders were more effective in attracting bidders and reducing prices. There is a severe lack of research on the effects of procurement through tendering in countries with expanding healthcare coverage (CEHCs), with only six publications found on this topic. Multiple authors concur those efficient tendering leads to a decrease in prices and aids in containing costs in the short term. In Malaysia, tenders are requested for products that surpass a budget of US$250 million per hospital. According to a recent report on tendering, it was found that tendering can help control costs if it is implemented within a strong legal and organizational structure, and if appropriate stakeholder management is in place to protect users from potential risks (Maniadakis et al., 2018b). The majority of the approved product purchase list (APPL) items consisted of local products, accounting for 70% of the total. In contrast, half of the national tenders were for originator products. The Ministry of Health (MOH) facilities, including hospitals, specialized medical institutions, health clinics, and community clinics, are directly provided by the appointed supplier. These facilities are procured through both national tenders and direct purchases made by individual facilities. In 2016, the pharmaceutical spending of MOH was divided as follows: 37% for the approved product purchase list (APPL) (presumably a specific organization), 43% for national tender, and 20% for direct purchases. Although nearly 70% of national tenders consist of imported products, there was no notable disparity in prices between APPL and national tenders when compared to International Reference Prices (IRPs). This could be attributed to the advantages of economies of scale and confidential price discounts associated with national tenders, which allow for prices that are comparable to those of APPL products (Hamzah et al., 2020). In general, the registration requirements for BTPs and biosimilar products align with international regulatory practices (Khoo et al., 2017a).

4.6 Policy Elements Impacting Biosimilar Market Sustainability

The findings, summarized in Table 2, highlight the strengths and challenges of Malaysia's biosimilar policies.

4.7 Positive Policy Elements

Malaysia has made significant progress in biosimilar regulation, aligning its guidelines with international best practices, particularly the European Medicines Agency (EMA) and World Health Organization (WHO) standards (Sani et al., 2020). This regulatory foundation ensures biosimilar safety and efficacy while fostering market confidence (Abas, 2011; Sani et al., 2020). Additionally, structured training programs have been introduced to educate healthcare professionals, improving their understanding of biosimilar prescribing and administration (Chong et al., 2022; Mohd Sani et al., 2023a). The implementation of biosimilars promotes competitive pricing, making biosimilars more affordable (Hamzah et al., 2020). Furthermore, government procurement contracts provide market stability and ensure widespread access to biosimilar treatments in both public and private healthcare settings (Maniadakis et al., 2018a).

The adoption of biosimilars in Malaysia also benefits from the growing global experience in biosimilar use. The increasing number of successful biosimilar cases worldwide, particularly in Europe and the United States, offers valuable lessons that Malaysia can incorporate into its own policies (Paul et al., 2024). By leveraging global best practices, Malaysia can further refine its

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regulatory frameworks to enhance biosimilar acceptance among healthcare providers and patients.

4.8 Policy Challenges

Despite these positive developments, several barriers continue to restrict the full potential of biosimilars in Malaysia. The absence of localized treatment guidelines results in uncertainty among prescribers, which slows adoption rates (Fischer et al., 2016). Delays in market entry due to lengthy and complex approval processes further hinder access to cost-effective biosimilars. (Hoang et al., 2024). Additionally, awareness and education gaps persist; while healthcare professionals receive some training, pharmacists and physicians often lack in-depth biosimilar knowledge, and patient education remains insufficient (Chong et al., 2022; Mohd Sani et al., 2022a, 2023a). Without proactive educational initiatives, skepticism regarding biosimilar safety and effectiveness may continue. The comparison with other Asian countries highlights the need for stronger policy interventions. For instance, in South Korea and Thailand, structured incentive programs and educational initiatives have accelerated biosimilar adoption at a greater pace (Kang et al., 2021).

One of the key concerns among prescribers is interchangeability (Barbier & Vulto, 2021). While biosimilars are rigorously evaluated for safety and efficacy, many physicians remain hesitant to switch patients from originator biologics to biosimilars due to a lack of conclusive real-world data (Chong et al., 2022; Sarnola et al., 2020; Thongpooswan et al., 2024). More extensive post-marketing surveillance and real-world studies could address this uncertainty by providing additional evidence on biosimilar safety and efficacy.

Financial disincentives further complicate adoption. Unlike countries with high biosimilar uptake, Malaysia lacks strong financial motivations for hospitals, prescribers, and patients to choose biosimilars over originator biologics (Mohd Sani et al., 2023a). Without incentives such as rebates, reimbursement schemes, or financial support for local biosimilar manufacturers, biosimilars struggle to gain market traction (Maniadakis et al., 2018a; Mohd Sani et al., 2023a). Additionally, biosimilar pricing policies in Malaysia do not sufficiently encourage competition. The lack of mandatory price reductions for originator biologics upon biosimilar entry reduces the cost-saving potential of biosimilars and limits patient access to these more affordable alternatives.

Pricing and procurement policies also create challenges. While reference pricing helps control costs, Malaysia has not implemented mandatory price reductions for originator biologics upon biosimilar entry. (Chen et al., 2024). This weakens the price competition needed to increase biosimilar adoption. Furthermore, the reliance on single-winner tendering systems limits market diversity, creating monopolistic environments where price reductions are minimal and procurement flexibility is constrained (Mohd Kasim et al., 2024). Internationally, multi-winner tenders have been shown to improve price competitiveness and ensure long-term sustainability (Németh et al., 2023), suggesting that Malaysia could benefit from revising its procurement processes.

4.9 Potential Policy Solutions

To address these issues, several strategic interventions could be considered. First, the development of localized biosimilar treatment guidelines would provide clear, evidence-based directives for prescribers, ensuring consistency in clinical decision-making. By incorporating region-specific treatment data, these guidelines could improve physician confidence in biosimilars and accelerate their adoption (Shubow et al., 2022). Additionally, real-world studies should be prioritized to address concerns about interchangeability and long-term efficacy (Leonard et al., 2019).

Second, targeted educational campaigns should be launched for both healthcare professionals and patients. By leveraging digital platforms, workshops, and professional training programs, knowledge gaps can be reduced, fostering greater confidence in biosimilar usage (Laurisz et al., 2023). Public education campaigns should also emphasize the safety, efficacy, and cost-effectiveness of biosimilars to encourage broader acceptance (Cazap et al., 2018).

Financial incentives should also be introduced to encourage market growth. These could include reimbursement programs for hospitals and prescribers who prioritize biosimilar prescribing, tax incentives for manufacturers to encourage local production, and direct financial support for patients to offset treatment costs (Bond et al., 2023; Itoshima et al., 2024; Lobo & Río-Álvarez, 2021). Implementing mandatory price reduction policies for originator biologics upon biosimilar entry would enhance cost competitiveness, further driving biosimilar adoption.(Chen et al., 2024). Additionally, tiered pricing structures, where biosimilars receive greater price advantages over originators, could further incentivize their use. Addressing biases in the literature review process, the study acknowledges potential selection biases in retrieved articles and calls for real-world studies to complement existing policy assessments. The review process highlights the necessity of transparent evaluation criteria in biosimilar procurement to avoid potential biases in market accessibility.

Finally, procurement reforms should be prioritized. Transitioning from a single-winner to a multi-winner tendering system would increase market diversity, ensure fair competition, and enhance cost-efficiency (Mohd Kasim et al., 2024; Németh et al., 2023). By encouraging multiple suppliers to participate in government tenders, Malaysia can create a more dynamic biosimilar market that benefits both the healthcare system and patients. Encouraging regional procurement collaborations could also help Malaysia negotiate better prices and secure a stable supply of biosimilars.(Hamzah et al., 2020).

5. Conclusion

The sustainability of the biosimilar market in Malaysia is shaped by a complex array of policy factors that govern market entry, adoption rates, and long-term viability. Despite being one of the early adopters of regulatory frameworks for biosimilars, Malaysia's current market continues to encounter significant challenges that impede its comprehensive advancement. To enhance sustainability, policymakers should implement localized treatment guidelines, expand educational initiatives for

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healthcare providers and patients, introduce structured financial incentives, and refine procurement frameworks to encourage market competition. Specifically, integrating biosimilar incentives into Malaysia's National Medicines Policy and adopting multi-supplier tendering models could help optimize market conditions. The review suggests that, even with a strong regulatory framework in place that meets international standards, there are ongoing challenges. These include insufficient awareness among healthcare providers and patients, a lack of adequate financial incentives, and restrictive pricing dynamics, all of which hinder the wider acceptance of biosimilars.

The regulatory advancements in Malaysia have profoundly impacted the biosimilar landscape. However, their effectiveness relies on supplementary approaches, including clearer treatment protocols, continuous monitoring of market trends, and refined approval processes to reduce delays in market access. The improvement of the regulatory framework by implementing uniform biosimilar substitution policies, strengthening pharmacovigilance, and ensuring effective post-market surveillance is crucial for increasing market confidence among healthcare providers and patients. Furthermore, improving alignment with international standards, particularly in terms of pricing and procurement strategies, could play a significant role in cultivating a more competitive and transparent market environment.

An essential element affecting sustainability is the degree of awareness and education present among stakeholders, such as physicians, pharmacists, and patients. Recent investigations indicate varying levels of understanding among healthcare professionals, underscoring that a significant proportion of pharmacists and prescribers lack sufficient training concerning biosimilars. Addressing this issue requires the implementation of targeted educational initiatives designed to enhance confidence in the prescribing and substitution of biosimilars. Furthermore, educational programs focused on patients can address misunderstandings regarding the safety and effectiveness of biosimilars, resulting in enhanced acceptance and use.

Economic incentives significantly influence the sustainability of biosimilars. The lack of robust financial incentives for prescribers and healthcare institutions has led to a more gradual pace of adoption. Policymakers must investigate the potential of competitive price policies, prescription quotas, and reimbursement schemes in order to encourage the larger use of biosimilars. Moreover, encouraging local manufacturing by means of incentives to businesses would greatly increase market competitiveness, hence reducing prices and improving access.

Pricing regulations and procurement mechanisms serve as critical elements that shape market dynamics. Despite Malaysia's adoption of reference pricing strategies for cost management, the prevalence of single-winner tendering systems has limited price competition and diminished market diversity. The shift towards multi-winner tenders and the establishment of long-term procurement contracts could promote a pricing environment that is fair and sustainable. Moreover, regulations requiring price reductions for original biologics might make reasonably priced alternatives more enticing, hence boosting the market for biosimilars in the public and private sectors of medicine.

Overall, the Malaysian biosimilar industry has significant potential; yet, targeted legislative adjustments will help to increase its long-term viability. Enhancing regulatory clarity, broadening educational initiatives, establishing strong financial incentives, and refining pricing and procurement approaches will be crucial for cultivating a competitive and sustainable market. By addressing these gaps, Malaysia has the potential to enhance access to cost-effective biosimilar treatments, lower healthcare expenses, and improve patient outcomes, thereby securing lasting advantages for the healthcare system.

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Assessing Diabetes Screening Outcomes in Klang Valley, Malaysia: A Cross-Sectional Study

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Abstract: Diabetes mellitus, once primarily affecting the elderly, is now increasingly diagnosed in younger individuals due to poor lifestyle habits. Early detection through health screening is essential for effective management. A government agency that has conducted diabetes screening programs since 2013 launched its third initiative, aiming for an 80% detection rate among screened participants using HbA1c (cutoff ≥ 6.3%), rather than reflecting national diabetes prevalence. This cross-sectional study evaluated the program's effectiveness in detecting diabetes among Malaysian adults aged 40 to 59 in Klang Valley. A total of 188 participants attended free screenings at registered clinics between September and December 2023. The cohort included 47.35% men and 52.65% women, with 41.5% Malays, 40.4% Chinese, 14.4% Indians, and 3.7% from other ethnic groups. HbA1c levels ≥ 6.3% were found in 27.12% of participants, with the highest prevalence among Indians (33.3%), followed by Malays (29%), Chinese (23.68%), and others (14.28%). Statistical analysis (ANOVA) showed no significant association between HbA1c levels and age, gender, or ethnicity (p = 0.188). The program did not meet its 80% detection target, suggesting that alternative screening approaches, including revised diagnostic criteria or additional risk assessments, may be needed to improve diabetes detection and intervention efforts.

Keywords: health screening, diabetes, prevalence.

1. Introduction

1.1 Global and Regional Prevalence of Diabetes Mellitus

Diabetes mellitus (DM) is an increasing global public health challenge, affecting approximately 537 million people in 2021, with projections indicating a rise to 783 million by 2045 (Saeedi et al., 2019; International Diabetes Federation, 2021). In 2022, more than 800 million adults were living with diabetes, a fourfold increase since 1990 (World Health Organization, 2024). The global prevalence of diabetes among adults has risen from 7% in 1990 to 14% in 2022, primarily due to obesity, sedentary lifestyles, and unhealthy diets (Magliano et al., 2024).

Countries like China, India, and Pakistan have among the highest numbers of adults aged 20 to 79 years living with diabetes (International Diabetes Federation, 2021).

In the Southeast Asia region, approximately 90 million adults aged 20 to 79 had diabetes in 2021, representing 9.58% of the population. This number is projected to increase to 113 million by 2030 and 151 million by 2045 (International Diabetes Federation, 2021).

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Among the Southeast Asian countries, Malaysia had the highest prevalence at 19% in 2021, significantly higher than the global average (14%) and the regional average (9.58%; The Global Economy, 2023). The diabetes prevalence in Malaysia emphasizes the urgent need for improved prevention, screening, and management strategies to control the rising burden of the disease. Given the rising prevalence trend, focused screening programs and early interventions are crucial to alleviate the long-term effects of diabetes in the nation (Institute for Public Health, 2020).

1.2 Diabetes Contributes to Financial Burden

The financial burden associated with diabetes is substantial, both in terms of direct and indirect healthcare costs, such as productivity loss (Bommer et al., 2017). In Malaysia, diabetes-related healthcare expenses accounted for a significant portion of healthcare spending, with USD3.6 billion spent in 2018 (Ministry of Health Malaysia, 2022). These costs primarily go toward managing complications arising from poorly controlled diabetes (Seuring et al., 2015). Preventive measures and early intervention programs are essential to alleviate this financial strain, as emphasized by the Malaysian guidelines on the management of type 2 DM (T2DM; Ministry of Health Malaysia, 2020). Due to the increasing costs associated with managing DM and its complications, countries worldwide have implemented measures to reduce healthcare expenses.

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1.3 A Comparison of Healthcare Interventions Pertaining to Diabetes

Countries worldwide have implemented various strategies to combat the diabetes epidemic. In the United Kingdom (UK), the National Health Service (NHS) launched the National Diabetes Prevention Program, which emphasizes lifestyle interventions, such as diet and physical activity (NHS England, 2019). In the United States (US), the Exercise is Medicine program integrates physical activity into the healthcare system (American College of Sports Medicine, n.d.). China has implemented the National Standardized Metabolic Disease Management Centre (MMC) to improve access to diabetes care (Li et al., 2020). In Singapore, the War on Diabetes campaign focuses on public awareness and early screening (Ministry of Health Singapore, 2021). In Malaysia, the Ministry of Health has introduced initiatives for glycemic control and medical nutrition therapy to reduce complications, as outlined in the *Clinical Practice Guidelines of Management of Type 2 Diabetes Mellitus* (6th ed.; Ministry of Health Malaysia, 2020).

1.4 The Role of Health Screening in Diabetes Detection

Screening for diabetes is critical in identifying undiagnosed cases and reducing long-term complications. The National Health and Morbidity Survey (NHMS) 2019 reported an 18.3% prevalence of diabetes among Malaysian adults, with 48.6% of these cases being undiagnosed (Institute for Public Health, 2020). The Malaysian guidelines on the management of type 2 DM emphasize routine screening for high-risk populations, particularly those aged 40 and above (Ministry of Health Malaysia, 2020).

1.5 The Health Screening Program (HSP)

The Malaysian Health Screening Program (HSP), introduced in 2013 (Social Security Organization, 2023), is an ongoing measure aimed at enhancing early detection of non-communicable diseases, including diabetes (Social Security Organization, 2023). The program is designed to identify individuals at high risk and encourage early detection of non-communicable diseases such as diabetes (Ministry of Health Malaysia, 2013). Targeting individuals aged 40 to 59, the HSP offers several screening services, including blood tests, urine analysis, and clinical evaluations (Mohamud et al., 2011). The program is designed to identify individuals at high risk and encourage early intervention, contributing to improved long-term health outcomes (Ministry of Health Malaysia, 2013). The success of the HSP is measured by its ability to identify cases of diabetes, with a particular focus on achieving an 80% prevalence of diagnosed cases using HbA1c screening criteria.

HbA1c is a widely accepted biomarker for diabetes screening, providing a three-month average of blood glucose levels (International Diabetes Federation, 2021). The Malaysian CPG recommends an HbA1c threshold of ≥ 6.3%, which is lower than the international standard of 6.5%, to improve early detection among Malaysians (Ministry of Health Malaysia, 2020).

The 80% detection target set by the HSP serves as an internal benchmark for program success. Since the screening program targets a high-risk working population, the expected detection rate should be higher than the national average (18.3%). A similar high detection rate has been observed in previous health screening initiatives (Social Security Organization, 2023). If the program does not meet this target, it may suggest gaps in outreach, participant selection, or screening methods.

1.7 Study Objective

This study evaluates the effectiveness of the HSP in detecting diabetes among Malaysian adults aged 40 to 59, using HbA1c (cutoff ≥ 6.3%). The study also investigates whether age, gender, and ethnicity influence diabetes detection rates.

2. Materials and methods

2.1 Study Design

This is a cross-sectional study that analyzed the HbA1c results of the health screening program using descriptive and inferential statistics to measure the prevalence of DM among the participants in Malaysia.

2.2 Sample and Setting

The sample size was not calculated as the researchers adopted the total population sampling method, a type of purposive sampling wherein the total population with specific characteristics was included. In this study, the specific characteristics were the inclusion criteria. The blood samples, which were processed by the MAHSA laboratory, were those of Malaysians aged 40 to 59 years who attended the screening conducted by a registered panel clinic in Klang Valley. In order to be eligible for this free HSP, the individuals had to fulfil certain criteria. The inclusion criteria were individuals who were Malaysians, aged between 40 and 59 years old, who were active employment insurance contributors with at least one (1) month of contribution in the year 2023, and at least a total of 12 months of total contributions. Exclusion criteria included individuals who did not fulfil the above criteria. The study procured ethical approval for secondary data procurement from the Research Management Centre of MAHSA University (RMC/OCTOBER/2024/EC07). Individual consent was not sought as the participant's information was not exposed at any point in the research. HbA1c data were generated by the laboratory from 1 September2023 to 31 December 2023.

2.3 Data Analysis

The data was analyzed using SPSS version 27, while numerical data was entered into Excel version 2502 build 16. For all tests, a significance level of $p < 0.05$ was set.

3. Results

Of a total population of 199 participants derived from the raw data, 188 were aged between 40 and 59 years. The predominant participants were Malay males aged 40 to 50 years, whereas Chinese ladies constituted the majority within the same age range.

Of the 188 participants between the ages of 40 and 59, 47.35% (n = 89) were men and 52.65% (n = 99) were women. The

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Table 1. Demographic data (gender, age and ethnicity) of the 188 participants.

GENDER	ETHNICITY				
	MALAY	CHINESE	INDIAN	OTHERS	TOTAL
MALE	**N=47**	**N=26**	**N=13**	**N=3**	**N=89**
40 – 50 YEARS	n=37	n=21	n=8	n=3	69
51 – 59 YEARS	10	5	5	0	20
FEMALE	**31**	**50**	**14**	**4**	**99**
40 – 50 YEARS	18	40	12	3	73
51 – 59 YEARS	13	10	2	1	26

ethnicity of the participants was as follows: 41.5% Malays (n=78), 40.4% Chinese (n=76), 14.4% Indians (n=27), and 3.7% others. Of the 188 individuals, 51 (27.12%) had a HbA1c level of ≥ 6.3%.

When disaggregated by ethnicity, 29% of Malays (23 of 78), 23.68% of Chinese (18 out of 76), 33.33% of Indians (9 of 27), and 14.28% of other ethnic groups (1 of 7) exhibited HbA1c levels exceeding 6.3. Table 1 provides the distribution of the 188 participants aged 40 to 59 years, categorized by ethnicity and further divided into two age groups: 40 to 50 years and 51 to 59 years.

The descriptive analysis revealed that only 27.12% of the participants had HbA1c levels within the diabetic range (≥ 6.3%), as defined by the *Malaysian Clinical Practice Guidelines on the Management of Type 2 Diabetes Mellitus* (6th ed.).

Table 2 presents the gender distribution of participants undergoing HbA1c testing, detailing the number and percentage of male and female participants included in the study. The overall mean HbA1c level among participants was 6.56% (N = 188). The findings indicate that while fewer than 80% of individuals had an HbA1c level of ≥ 6.3%, the prevalence was highest among Indians, at 33.3%. The mean HbA1c level among male participants was 6.5%, encompassing nearly all individuals classified within the diabetes group. In contrast, the mean HbA1c level for female participants was 6.6%, only 0.1% higher than that of males.

As the data followed a normal distribution, the mean HbA1c levels between male and female participants were compared using an independent t-test. Levene's test for equality of variances yielded an F-statistic of 0.155 with a non-significant p-

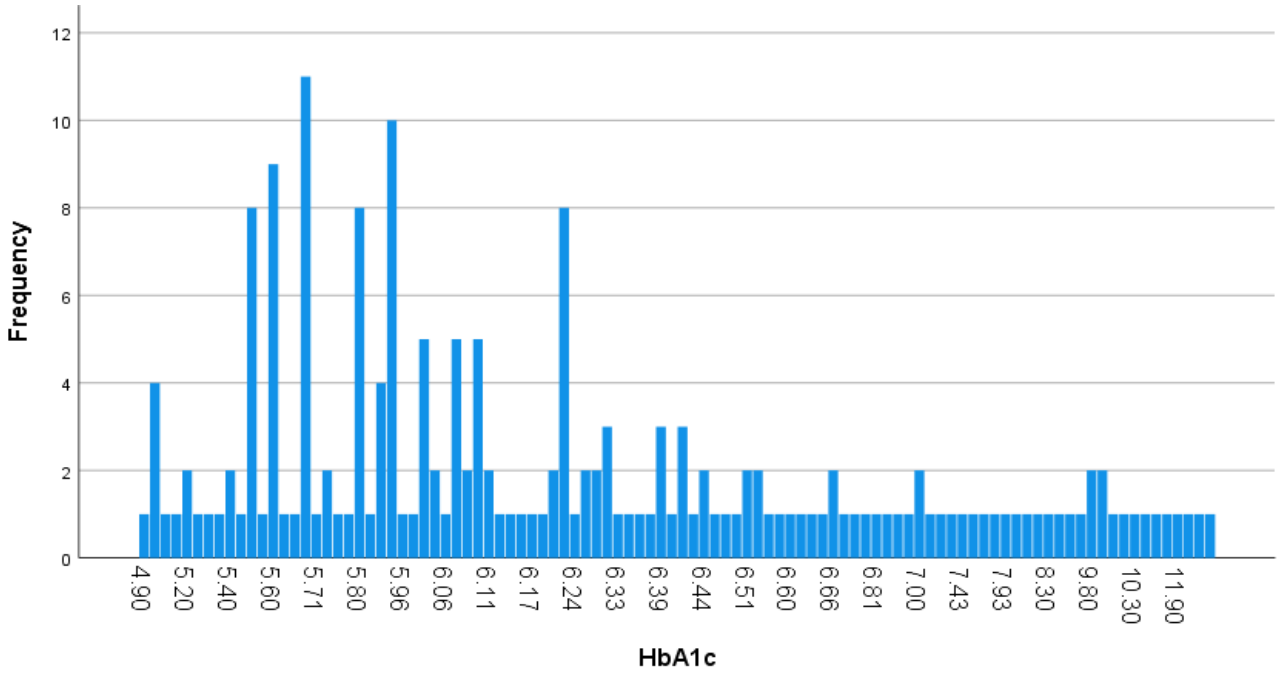

Figure 1. Distribution of HbA1c values among the participants.

Table 2. Mean HbA1c according to gender

	Gender	N (%)	Mean	Std. Deviation	Std. Error Mean
HbA1c	**Female**	99 (52.66)	6.5877	1.71964	0.17283
	Male	89 (47.34)	6.5222	1.50832	0.15988

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value of 0.694, indicating homogeneity of variances. The t-test for equality of means produced a t-statistic of 0.276, with 186 degrees of freedom and a p-value of 0.783, suggesting no statistically significant difference in HbA1c levels between genders. The mean difference was 0.06543, with a 95% confidence interval ranging from -0.40230 to 0.53316, further confirming the absence of a significant gender-based variation in HbA1c levels.

The ANOVA analysis of the model, with HbA1c as the dependent variable, and age, gender, and race as independent variables, indicates that the regression model accounts for some variation in HbA1c levels. Nevertheless, the F-statistic of 1.613 is associated with a p-value of 0.188, which is not statistically significant ($p > 0.05$). This suggests that the model does not effectively explain the variation in HbA1c levels based on the selected predictors.

4. Discussion

This study aims to evaluate the effectiveness of Malaysia's health screening program in identifying the prevalence of type II diabetes within the target population. Although the program aimed to detect diabetes in 80% of participants as an internal benchmark for success, findings from this study indicate that it fell significantly short of this target, with only 27.12% of participants having HbA1c levels ≥ 6.3%. This limitation may be attributed to the study's relatively small sample size, which was confined to a single geographic area, the Klang Valley. Nonetheless, implementing a nationwide screening program is highly relevant given Malaysia's alarming diabetes rates. The country has one of the highest diabetes prevalence rates in the Western Pacific region and ranks among the highest globally. Between 2011 and 2019, diabetes prevalence in Malaysia surged by 68.3%. In 2019, an estimated 3.6 million Malaysians aged 18 and above had been diagnosed with diabetes, while an additional 3.7 million remained undiagnosed. By 2025, the number of Malaysians with diabetes is projected to reach 7 million, translating to a prevalence rate of 31.3%. Reported prevalence rates in Malaysia vary significantly, ranging from 7.3% to 23.8%, depending on the study population and methodology (Ganasegeran et al., 2021).

According to the Demographic Data for Malaysia's fourth quarter of 2022, published by the Department of Statistics Malaysia (DOSM), the country's total population was 33 million. The ethnic composition comprised 17.6 million Malays (57.8%), 6.9 million Chinese (22.7%), 2.0 million Indians (6.6%), 3.7 million Other Bumiputera (12.2%), and 0.2 million individuals classified under other ethnicities (0.7%; Wan Nazaimoon et al., 2013). In this study, the majority of participants were of Malay ethnicity, accounting for 40.4% (n = 76). However, among the Indian participants, 33% (n = 9 of 27) had HbA1c levels exceeding the cutoff point. A 2013 study conducted in Malaysia among 4,341 subjects reported a diabetes prevalence of 22.9%. Consistent with our findings, the study showed that diabetes was most prevalent among Indians (37.9%), followed by Malays (23.8%), and was the lowest among the Chinese (Wan Nazaimoon et al., 2013).

Studies have examined the associations between ethnicity, age, gender, and HbA1c levels among non-diabetic adults across various populations. For example, a community-based cross-sectional study conducted in Northern and Eastern Sudan found significantly higher HbA1c levels in Eastern Sudan compared to Northern Sudan. The study identified ethnicity and body mass index (BMI) as significant factors influencing HbA1c levels, whereas age and gender did not show statistically significant correlations in these regions. These findings highlight the influence of ethnic and regional variations on HbA1c levels, underscoring the importance of considering these factors in diabetes management and prevention strategies in Sudan (Ahmed et al., 2023).

A cross-sectional study in Shenzhen, China, analyzed 18,265 adults without a prior diabetes diagnosis to examine the association between HbA1c levels, age, and gender. The study found that HbA1c levels increase with age and are significantly higher in males compared to females. These findings suggest that both age and gender should be considered when using HbA1c as a diagnostic criterion for diabetes in Chinese populations (Ma et al., 2016).

The finding that Malaysian females and males had similar mean HbA1c levels suggests that gender may not be a significant determinant of blood glucose control in this population. This could be due to similar lifestyle habits, dietary patterns, and levels of physical activity among both genders. Additionally, equal access to healthcare, diabetes awareness, and workplace health programs may contribute to comparable glycemic control. Since the study focused on employed individuals aged 40–59, the participants likely shared similar socioeconomic backgrounds and occupational health support, which may have minimized gender-based differences. While hormonal differences can influence diabetes risk, they may not have had a substantial impact on HbA1c levels in this sample. Therefore, other factors such as age, ethnicity, and lifestyle choices may play a more significant role in influencing blood glucose levels.

Another study involving 8,665 participants from two cohorts (SHIP-0 and SHIP-Trend) aimed to prevent diabetes misdiagnosis in the elderly by establishing age-dependent HbA1c reference intervals. The study found that HbA1c levels increase with age, with the upper reference limit (URL) rising from 42.1 mmol/mol (6.0%) in individuals aged 20 to 39 to 47.5 mmol/mol (6.5%) in those aged 60 and above. These age-dependent reference values for HbA1c, derived from healthy populations, are crucial for improving diabetes diagnosis and care in elderly patients, helping to avoid misdiagnosis and overtreatment (Masuch et al., 2019).

A Malaysian study by Ismail et al. (2000) investigated the factors influencing glycemic control in young diabetic patients across Peninsular Malaysia. The study analyzed various sociodemographic variables, such as age, gender, ethnicity, educational background, and socioeconomic status, to determine their impact on patients' ability to manage blood sugar levels effectively. The findings suggest that socioeconomic and educational factors play a significant role in determining glycemic control among these patients, highlighting the need for targeted interventions to improve diabetes management based on these determinants (Ismail et al., 2000).

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However, in this study, the ANOVA analysis of the model, which examines HbA1c levels based on age, gender, and race, is not statistically significant. The p-value of 0.188 (greater than 0.05) suggests that this explanation is likely due to chance. Therefore, the model may not effectively capture how age, gender, and race impact HbA1c levels, indicating that other factors may be more relevant in explaining the variation.

Another issue that may arise is whether HbA1c is the correct test to do instead of fasting blood glucose (FBG) or random blood glucose (RBG). HbA1c is usually preferred for prevalence studies because it indicates long-term glucose control and is straightforward to use. However, FBG or RBG may be chosen instead in certain scenarios. FBG is often used for immediate diabetes or prediabetes diagnosis, especially in clinical settings requiring precise measurements, in resource-limited environments, or when conditions like anemia affect HbA1c accuracy. RBG is beneficial for rapid confirmation of high glucose levels, especially in emergencies or community screenings where fasting is not possible. Thus, while HbA1c is favored for long-term monitoring, FBG and RBG are selected for immediate, specific, or practical reasons (NCD Risk Factor Collaboration [NCD-RisC], 2023; Ghazanfari et al., 2010).

As an overall prevalence, this study highlights the increase in the prevalence of diabetes in Malaysia, regardless of the diagnostic criteria used (Wan Nazaimoon et al., 2013). It has also elucidated that the HbA1c threshold of ≥ 6.3% as a diagnostic criterion may underestimate the burden of this disease, and the HbA1c with a cutoff point of ≥ 6.3% together with FBG or RBG, is found to give maximal sensitivity (Wan Nazaimoon et al., 2013).

5. Conclusion

The health screening program aimed to assess diabetes mellitus incidence and/or prevalence among the participants. Despite being accessible and free, the program achieved only a 27% diabetes detection rate, falling short of expectations. Contributing factors included the program's short duration, which did not account for aging and increasing life expectancy, self-selection bias, leading to underrepresentation of higher-risk individuals, and its focus on health-conscious individuals, potentially excluding a more diverse population. The social media campaign probably failed to reach the elderly effectively, and the lack of non-fasting tests limited opportunistic screening.

Limitations of this study:

Though the study employed the total population sampling method, the sample was not sufficient to achieve the intended target. It is suggested to conduct this study at various locations with a larger sample size in the future. Alternatively, a study focusing on high-risk individuals would also yield a higher prevalence rate.

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Expression Profile of PmiR-31, Novel npcRNA Of *Proteus Mirabilis* Under Different Growth Phases and Stress Conditions

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Abstract: *Proteus mirabilis* (*P. mirabilis*), known for its swarming motility, is a facultatively anaerobic, rod-shaped, Gram-negative bacterium. It forms elongated swarm cells and moves in a distinctive bull's-eye pattern. Proteus mirabilis significantly causes catheter-associated urinary tract infections. Its virulence factors include flagella, fimbriae, hemolysin, urease, and proteases. Flagella-related motility allows P. mirabilis to colonize the urinary system. The flagellum, composed of about 20 proteins, has a basal body that penetrates the cell wall, a curved hook, and a filament extending several micrometers. Previously, we knocked out npcRNA PmiR-137, expected to regulate flhDC, a master transcriptional activator for flagella synthesis, and performed a differential gene expression analysis. We discovered npcRNA PmiR-31, predicted to influence fliZ production via the Target RNA web tool. We analyzed PmiR-31 expression under various stress and growth phases using northern blot. The PmiR-31 was highly expressed in stress conditions but absent in the mutant strain. Since FliZ regulates flagella assembly, npcRNA PmiR-31 may repress fliZ translation, inhibiting flagella synthesis and promoting biofilm formation to evade host immune responses.

Keywords: Proteus mirabilis, non-protein coding RNA, PmiR-31, flagella biosynthesis

1. Introduction

Proteus mirabilis is a Gram-negative bacterium that accounts for more than 90% of Proteus infections. One of the distinguishing traits of Proteus mirabilis is its ability to differentiate from short vegetative swimmer cells to an elongated, highly flagellated swarmer form. It possesses swarming growth and adhesion factors, making it very adhesive and motile. Proteus mirabilis has the ability to resist capture by evading the host's immune system (Chakkour et al., 2024; Scavone et al., 2023). Proteus mirabilis is widely distributed throughout the environment, particularly in water, soil, and the gastrointestinal tracts of humans and animals. It is an opportunistic pathogen that accounts for less than 0.005% of healthy human gut flora (Jamil et al., 2025). Proteus mirabilis is a common cause of complicated urinary tract infection (UTI) in individuals with anatomical or functional urinary tract abnormalities, particularly in those who have long-term indwelling catheters and may develop catheter-associated UTI (CAUTI). It is responsible for around 3% of hospital infections and 44% of CAUTI in the United States.

Proteus mirabilis has a broad combination of virulence factors to achieve effective motility in the face of a stream of urine, nutrient intake, and host defense system protection. The primary virulence factors of *Proteus mirabilis* involve toxins (HpmAB), iron and zinc absorption systems, proteases, fimbriae, flagella, and urease, which hydrolyzes urea into ammonia and carbon dioxide (Filipiak et al., 2020). The bacteria uses flagella for motility, which enable both swimming and swarming (Yang et al., 2024).

Like many bacteria, it uses flagella to move across liquids and towards chemical gradients (Jamil et al., 2025). In a liquid culture, Proteus mirabilis exhibits a small form and a limited number of peritrichous flagella. Proteus mirabilis, however, divides into extraordinarily long (typically 20 to 80 µm, although cells longer than 100 µm have been reported), non-septate polyploid cells with hundreds to thousands of flagella on a nutrient-rich solid medium (Gmiter & Kaca, 2022). The flhDC genes are known to be the primary transcriptional regulators of bacterial flagella synthesis. Similarly, this master regulator is controlled both positively and negatively in Escherichia coli (E. coli) by directly binding to npcRNAs, indicating that npcRNAs have an important role in bacterial virulence (Takada et al., 2023).

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Messenger RNAs (mRNAs) and non-protein coding RNAs (npcRNAs) are two types of RNA molecules (Wang & Farhana, 2025). Protein translation is carried out by messenger RNAs. N contrast, non- protein coding RNA transcripts are RNA that do not encode proteins yet and are generated by a large number of genome sequences. Despite 90% of bacterial genomes being transcribed, mRNAs account for just a small fraction of RNAs (1-2%), while npcRNAs account for a larger proportion of total RNAs (Kishanraj et al., 2021a). Non-protein coding RNAs are categorized as either regulatory or housekeeping RNAs depending on their functions. Bacterial npcRNAs are a type of small regulatory RNA (sRNA) with less than 500 bp in length that performs important physiological functions in various biological processes by binding to the mRNA or proteins (Ratti et al., 2020). The regulatory actions of npcRNAs affect both bacterial pathogenesis and therapeutic treatment (Kishanraj et al., 2021b).

We previously identified and evaluated Hfq-bound npcRNAs in P. mirabilis, reported 182 npcRNAs, 13 of which bind to the Hfq protein, and validated their expression using Northern blot analysis (unpublished data). According to the target mRNA prediction tool, these 13 npcRNAs regulate the mRNAs associated with P. mirabilis virulence factors, such as flagella protein, which is essential for the bacteria's motility. The goal of this study is to delete the npcRNA PmiR-137 gene from the P. mirabilis genome since it has been shown to play an important role in P. mirabilis virulence. The experiment was then extended to investigate the expression profile of selected novel npcRNA PmiR-31 under various stress conditions, including oxidative stress, heat shock, acidic stress, osmolarity stress, stationary phase, and in wild-type and mutant strains of P. mirabilis. This npcRNA is believed to regulate the mRNA of flagella biosynthesis protein FliZ (fliZ), a protein required for the formation of flagella in P. mirabilis. While several studies have examined the regulatory mechanisms of flagella biosynthesis, the role of npcRNAs in this process remains underexplored. This study investigates the expression of PmiR-31 under different growth and stress conditions to clarify its regulatory function. The findings offer insight into the role of npcRNAs in gene regulation of P. mirabilis.

2. Method

2.1 Bacterial Gene Knock-Out in Single-Step

The npcRNA PmiR-137 was identified in P. mirabilis and selected for the gene knockout experiments because it is more likely to affect and modify the production of bacterial virulence factors. The npcRNA PmiR-137 was knocked out from the P. mirabilis genome using a one-step gene knockout method as described by prior work (Sanniraj et al., 2022).

2.2 Total RNA Extraction under Various Stress Conditions and Growth Phases

The total RNA was extracted from cell pellets of the wild-type strain at the exponential and stationary phases. Several stress conditions were applied to the wild-type during the exponential stage, including oxidative stress, osmotic stress, acidic stress, and heat shock stress. Meanwhile, the total RNA was extracted from the mutant strain during the exponential phase of bacterial growth without exposing it to any stress conditions.

In 10 ml of Luria Bertani (LB) broth, an overnight inoculum of P. mirabilis wild-type and mutant strains was introduced and incubated at 37°C with agitation at 180 rpm. The following day, 250 µl of the overnight culture was added to 250 ml of LB broth under various growth and stress conditions. The cultures were incubated at 37°C at 180 rpm. Total RNA was obtained from both wild-type and mutant cells at the mid-exponential phase (OD600 0.5–0.6).

Oxidative stress was induced in the wild-type cells by adding 5.6 µl of hydrogen peroxide (H_2O_2) during mid-exponential phase and incubating the culture for 30 minutes at 37°C and 180 rpm. To induce heat shock stress, the cell culture at the exponential phase was incubated at 42°C and 180 rpm for 30 minutes. For the osmolarity and acidic stress tests, cultures at OD_{600} 0.5–0.6 were treated with 3 g of sodium chloride and a few drops of hydrochloric acid, respectively, then incubated at 37°C at 180 rpm for 30 minutes. Bacterial cells were harvested at the stationary phase when OD600 reached 0.9–1.0. Total RNA was isolated from bacterial cells under various growth and stress conditions using the Trizol method as directed by the manufacturer. The extracted total RNA's quality and quantity were assessed using the NanoDrop Spectrophotometer and the Qubit 4 Fluorometer, respectively.

2.3 Northern Blot Analysis

The total RNA was isolated from various growth and stress conditions and separated on 8% polyacrylamide denaturing gels containing 7 M urea, then transferred to positively charged nylon membranes (Ambion Ltd, Cambridgeshire, UK) using a semi-dry blotting transfer method (Bio-Rad, USA). Northern blot hybridization was performed using radioactively labeled oligonucleotide probes (Table 1) that were complementary to the npcRNA PmiR-31 as described by Chinni et al. 2010 (Chinni et al., 2010; Kishanraj et al., 2021b).

Table 1. List of DNA oligonucleotide probes designed for Northern blot hybridization

npcRNA	Oligonucleotide sequences (5' to 3')
PmiR-31	GTCGATTGCAAGGTTCTCTT
5S rRNA	TGGCAGTTCCCTACTCTCACAT

3. Results and discussion

3.1 Differential expression of the npcRNA PmiR-31 in wild-type and mutant strains of P. mirabilis under varied environmental conditions.

PmiR-31, a novel npcRNA found in P. mirabilis, was selected to study the expression profile of this npcRNA under different environmental conditions along with the wild-type and mutant strain. In the P. mirabilis genome, the npcRNA PmiR-31 is located between the succinate dehydrogenase iron-sulfur protein (sdhB) and the 2-oxoglutarate dehydrogenase E1 component (sucA) with

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5' and 3' coordinates of 613505-613788 (Figure 1). In our previous study, the npcRNA showed low expression in the log phase but was highly expressed during the stationary phase. However, the expression in the exponential phase was low. Based on Figure 2, the npcRNA PmiR-31 was shown to be expressed during stress conditions.

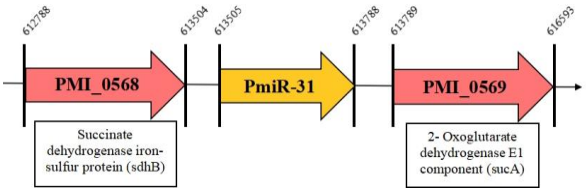

Figure 1. The genomic location of npcRNA PmiR-31 in *P. mirabilis*. Boxes indicate the gene's transcriptional orientation. The orange arrows represent gene locus markers.

According to the published genome of P. mirabilis H14320, the numbers indicate the genomic position of the npcRNA and its flanking genes. The npcRNA gene is represented by the yellow box.

The TargetRNA2 webtool predicts that the PmiR-31 target mRNA is the flagella biosynthesis protein (fliZ) (Table 2). The interaction between npcRNA PmiR-31 and fliZ mRNA occurs with a minimum free energy of -14.43 kcal/mol and a p-value of 0.001. The npcRNA interacts with fliZ from -7 to 9 at the 5'UTR region.

Flagella are complex surface structures that serve as the principal mode of motility for many bacterial species and allow numerous bacterial pathogens to attach, infiltrate, and produce virulence factors (Akahoshi & Bevins, 2022). More than 50 genes are involved in the biosynthesis and function of an E. coli or Salmonella enterica serovar Typhimurium flagellum. FlhDC is a class 1 operon whose products, FlhD4C2 heterohexamers, are essential for the expression of all other flagellar genes. The E. coli FlhD4C2 complex activates class 2 operons, including structural genes for flagella hook-basal-body components (type III secretion system) and the alternative sigma factor fliA (Avelino-Flores et al., 2022; Sassi et al., 2020). In E. coli and Salmonella enterica, the product of the fliA gene, σ28, regulates the transcription of class 3 genes producing filament proteins, hook-associated proteins, motor proteins, and various chemotaxis proteins (Kurniyati et al., 2023). FliZ, encoded in the fliAZY operon, is a FlhD4C2-dependent activator of class 2 gene expression. It promotes the expression of class 2 flagellar genes in Salmonella enterica (Das et al., 2018). Additionally, E. coli FliZ can block RpoS post-translationally, which prevents the development of curli fimbria. The FliZ protein contains a region similar to the core DNA binding domain of phage integrases while exhibiting evident posttranslational effects in Salmonella and E. coli (Ponath et al., 2022; Ravishankar et al., 2024). In Xenorhabdus nematophila, FliZ binds directly to the flhDC promoter to initiate transcription of class 2 flagellar genes (Bientz et al., 2024; Trouillon et al., 2023).

The binding of npcRNA PmiR-31 with the fliZ mRNA suggests that the expression of PmiR-31 suppresses the translation of fliZ mRNA as it might block the ribosome binding site of the mRNA.

The expression of PmiR-31 is high under all various stress conditions as well as in different growth phases of P. mirabilis compared to the mutant strain. The knockout of npcRNA PmiR-137 from the P. mirabilis genome could play a role in the lower expression of npcRNA PmiR-31. Since the absence of PmiR-137 may influence flagella synthesis, the bacteria could experience pressure for survival; thus, it may adhere to the host epithelial cell and start producing biofilm to survive and protect themselves from the host immune system (Sanniraj et al., 2022). This could explain the lower expression of npcRNA PmiR-31 in the mutant strain of P. mirabilis.

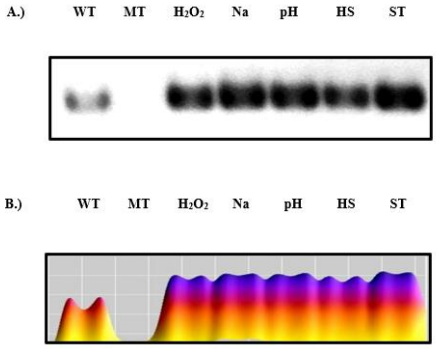

Figure 2. *Proteus mirabilis* npcRNA PmiR-31 expression profile. A.) Northern blot analysis of PmiR-31 expression in *P. mirabilis* under various environmental conditions (WT: Wild-type, MT: Mutant type, H_2O_2: Oxidative stress, Na: Osmolarity stress, pH: acidic stress, HS: heat shock and ST: Stationary phase). B.) ImageJ software was used to construct an interactive 3D surface graphic of the band intensities of the npcRNA northern blot.

Under stress conditions, the expression of PmiR-31 is higher compared to the wild-type strain, which could downregulate the expression of fliZ mRNA. The fliZ showed poor hybridization on the chip and no discernible change in transcription in the presence or absence of chlorine or H2O2 in E. coli (Roth et al., 2022). Our results predict that when the cells are exposed to oxidative stress under severe circumstances, the bacteria protect the cells through reduced permeability and biofilm formation (Shahryari et al., 2021). The expression of PmiR-31 is high during oxidative stress, osmotic stress, acidic stress, and heat shock stress, suggesting a decrease in FliZ protein production. Thus, the cells may proceed to form biofilm for their adaptation and survival.

These results show that npcRNA PmiR-31 expression is slightly higher during the stationary phase compared to the wild-type strain of P. mirabilis. According to Zhuang et al. (2019), the percentage of flagellated bacteria (PFB) further decreased when the culture approached the stationary phase, and the cells actively lost their flagella, as evidenced by the drastic reduction in the number of flagellated cells (Zhuang et al., 2019). The other stages of the E. coli development cycle and the accompanying regulatory circuits are closely linked with the stationary-phase response. At least five RNAP sigma subunits (σ70, σFliA, σS, σE, σ54), as well as the flagellar master regulator FlhDC and the

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second messengers cAMP, (p)ppGpp, and c-di-GMP, are key players in these circuits. For metabolic adaptation to available resources, and regulation of motility, cell shape, stress resistance, and biofilm functions during the exponential, post-exponential, and stationary phases of the growth cycle, the interaction of all these components is essential (Hengge, 2020; Hengge et al., 2023; Kędzierska-Mieszkowska, 2023). Interestingly, Salmonella's fliZ gene may also be directly related to biofilm development (Eran et al., 2020). As mentioned earlier, high expression of PmiR-31 could reduce the translation of the FliZ protein, which is directly associated with FlhDC in flagella formation. As a result, the bacteria may protect themselves to adapt against unfavorable conditions. Although TargetRNA predicts PmiR-31 interaction with fliZ mRNA, further validation using electrophoretic mobility shift assays (EMSA) or ribonucleoprotein immunoprecipitation (RIP) is required. Given that non-coding RNAs in Gram-negative bacteria often regulate multiple virulence pathways, future studies should investigate whether PmiR-31 affects urease expression, fimbriae production, or biofilm formation.

Table 2. Predicted target mRNA for PmiR-31 using TargetRNA2 tool

Target mRNA	Energy (kcal/mol)	P- Value
Flagella biosynthesis protein (fliZ)	-14.43	0.001

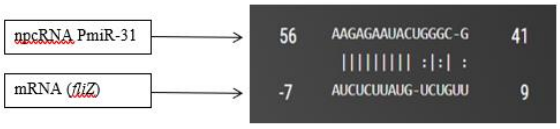

Figure 3. Sequence alignment information of npcRNA PmiR-31 with predicted target mRNA.

4. Conclusion

We have successfully obtained a P. mirabilis strain lacking npcRNA PmiR-137. Northern blot analysis was conducted to examine the differential expression of npcRNA PmiR-31 in both wild-type and mutant strains as well as under various stress conditions. Using the TargetRNA web tool, the potential role of this npcRNA was predicted to regulate the mRNA of the FliZ flagellar biosynthesis protein. Compared to the wild-type of P. mirabilis and under other stress conditions, our results showed that the expression of npcRNA PmiR-31 is significantly increased in stress conditions, as well as during the stationary phase. However, npcRNA PmiR-31 is absent in the mutant strain of P. mirabilis. Instead of prioritizing flagella synthesis in a stressful environment, the bacteria may adopt biofilm formation as an adaptive strategy.

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Unveiling The Roles of npcRNA Modulating the Molecular Mechanism of Pathogenic Bacteria Causing Urinary Tract Infections (UTIs)

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Abstract: Urinary tract infections (UTIs) are among the most prevalent and chronic health problems worldwide, impacting millions of individuals annually and posing a substantial financial burden on healthcare systems. UTIs typically initiate when pathogenic bacteria, originating from the gastrointestinal tract, colonize the urethra or periurethral region. Women are more susceptible to UTIs compared to men because of the differences in female lower urinary tract anatomy and its proximity to reproductive organs. The pathogenic bacteria, which are on the World Health Organization (WHO) priority list and cause both uncomplicated and complicated UTIs, are Escherichia coli (UPEC), Klebsiella pneumoniae, Proteus mirabilis, and Staphylococcus aureus. Different therapeutic approaches are necessary to combat UTIs, as seen by the rise in antibiotic resistance in bacteria. Non-protein coding RNAs (npcRNAs) have emerged as promising candidates for regulating genes involved in bacterial pathogenesis and infection, playing a role in modulating gene expression and host-pathogen interactions. By targeting bacterial virulence factors, biofilm formation, and antibiotic resistance mechanisms, npcRNAs provide a novel approach to disrupting infection pathways while minimizing the risk of resistance development.

Keywords: Pathogenic Bacteria, Multidrug resistance, non-protein coding RNAs, Urinary tract Infections (UTIs)

1. Introduction

UTIs represent significant and persistent health problems worldwide, impacting millions of individuals annually and incurring substantial healthcare costs. UTIs are a common pivotal health issue for all genders (Sgarabotto et al., 2025). Unfortunately, because of the lower urinary system and its proximity to reproductive organs, women are more prone to UTIs than males (Finlayson et al., 2025). In simpler terms, the urethra, a tube that leads from the bladder to the point where urine is excreted from the body, is shorter in length in women than in men, which makes it easier for the pathogen that causes UTIs to enter, disseminate, and ascend to internal organs (Czajkowski K et al., 2021). Approximately 50%-65% of women experience UTIs in their lifetime (Deltourbe L et al., 2022). UTIs also occur more frequently in menopausal women due to their oestrogen level depletion, which harms their urogenital lining. Additionally, the urinary system, such as the kidneys (pyelonephritis), bladder (cystitis), and urethra (urethritis), may easily develop UTIs (Lila et al., 2023).

UTI symptoms can be divided into two categories, which are uncomplicated and complicated infections (Bono et al., 2023). The uncomplicated UTIs usually involve the lower urinary tract system, presenting as cystitis (bladder inflammation). A strong host immune response and the emergence of significant inflammation have been shown to worsen mucosal damage during UTIs (Calin et al., 2024). An acute UTI may become chronically inflamed due to this overreaction of the immune system (Calin et al., 2024). According to Zhou Y et al. (2023), acute UTIs are usually caused by bacteria such as E. coli, and the duration of therapy for these infections is often brief, ranging from three to seven days, depending on the antibiotic provided and the severity of the illness. Meanwhile, the treatment of chronic urinary tract infections might be more complicated and require prolonged antibiotic treatments, sometimes lasting seven to fourteen days or more (Zhou Y et al., 2023; Mak et al., 2024).

UTIs can be caused by numerous microorganisms, including bacteria, viruses, fungi, and molds. However, the vast majority of UTIs are caused by bacteria. Uropathogenic Escherichia coli (UPEC), Klebsiella pneumoniae, Proteus mirabilis, and Staphylococcus aureus are frequently found bacterial species

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involved in UTIs (Zhou Y et al., 2023). Among these bacterial pathogens, Escherichia coli is the most common, responsible for nearly 70-80% of uncomplicated UTIs (Al Lawati et al., 2024). These pathogens are substantial contributors to the occurrence of UTIs worldwide and are prioritized by the WHO (WHO, 2023). While viral and fungal UTIs do occur, they are relatively rare and are typically associated with specific clinical settings, such as immunocompromised states or prolonged use of medical devices like catheters (Lila et al., 2023).

Because of their changing multidrug resistance patterns, these microorganisms present an alarming issue that makes treatment more challenging and increases the threat to global health (Klein RD et al., 2020). The ability of the bacteria to produce and sustain infections in the urinary tract of the host cells is supported by several virulence factors, which are adhesins, toxins, and mechanisms that evade host immune defenses (Klein RD et al., 2020). The bacterial adhesion to the host urothelial cells lining usually starts the infection process. This is followed by the production of biofilms, which enhances bacterial survival and antibiotic resistance. Pyelonephritis, a more complex and potentially fatal form of UTI, can result from an infection that has progressed to the kidneys in more severe forms (Belyayeva et al., 2024).

According to WHO (2023), many UTI-causing bacteria have become resistant to antibiotics. This is because the community overuses antibiotics, which has led to genetic changes in pathogens, allowing them to survive exposure to drugs that were once highly effective against multiple infections. Thus, this will potentially lead to millions of deaths annually by 2050 and underscores its criticality as a leading public health concern if there is no proper solution for these infections (Naghavi et al., 2024). Significantly, the "golden era" of antibiotics is approaching its end, which means that developing alternative therapeutic interventions is needed to prevent these infections (Naghavi et al., 2024).

To address the escalating challenge of antibiotic resistance and the limitations of conventional treatment methods, researchers are increasingly investigating molecular therapeutics as a promising and innovative alternative. Among these, RNA-based therapies have emerged as particularly novel and focused approaches (Al-Fadhli et al., 2024). Moreover, non-protein coding RNAs (npcRNAs), once an unexplored area in genomics, are now recognized for their potential to regulate bacterial virulence and counteract antibiotic resistance (Subhadra et al., 2024). By controlling gene expression, npcRNAs offer new opportunities for enhancing treatment strategies, particularly for difficult infections such as UTIs. As research advances, npcRNAs hold promise for developing new methods to combat bacterial pathogenesis and improve UTI management (Subhadra et al., 2024). In this review, we aim to discuss the regulatory roles of npcRNAs in modulating the molecular mechanisms of pathogenic bacteria that cause UTIs.

2. Factors affecting the development and spread of urinary tract infections

UTIs typically initiate when pathogenic bacteria, originating from the gastrointestinal tract, colonize the urethra or periurethral region (Mancuso et al., 2023). Then, they spread into the bladder and ascend to the organs. This process occurs through specific cell adhesion mechanisms (Mancuso et al., 2023). If the inflammatory response of the host is unstable or unable to eradicate the bacteria, they begin to multiply and secrete toxins as well as enzymes that enhance their survival and persistence within the urinary tract of the host cell. Further issues may arise from this, as the bacteria may spread to the kidneys, where their colonization may result in serious infection (Mancuso et al., 2023).

One significant factor is biofilm formation, where bacteria produce a protective layer that shields them from the immune system and antibiotics of the host, making infections more difficult to treat (Smith et al., 2023). Other contributing factors include urinary stasis, where urine flow is blocked, and the use of catheters, which also contribute to the development of UTIs. On the other hand, intrinsic factors include urinary retention, where the bladder does not fully empty, and vesicoureteral reflux, which allows urine to flow backward from the bladder to the kidneys (Smith et al., 2023). Acquired risk factors include frequent sexual activity, prostatic hyperplasia (enlarged prostate) in men, and vulvovaginal atrophy in women (which involves thinning and drying of vaginal walls), which also contribute to UTI risk. Additionally, having a family history of UTIs can increase susceptibility [Smith et al., 2023; Baimakhanova et al., 2025].

To address these challenges, non-protein coding RNAs (npcRNAs) have emerged as key regulators in the pathogenesis of UTIs. These npcRNAs can influence bacterial gene expression, including genes responsible for adhesion, biofilm formation, and urease activity. These factors are essential for bacterial survival and the development of kidney stones. Additionally, npcRNAs may modulate the host's inflammatory and immune responses, potentially contributing to tissue damage and stone formation (Wang et al., 2021). Figure 2.0 shows the anatomy of the human kidneys, highlighting specific areas affected during UTIs that can lead to the formation of kidney stones.

3. Regulatory roles of non-protein coding RNAs in gene expression

NpcRNAs are a diverse class of RNA molecules that do not translate into proteins but perform significant roles in regulating cellular processes (Nemeth et al., 2024). These RNA species vary in size from 50 to 450 nucleotides and are commonly transcribed from non-coding regions of the genome (Kishanraj et al., 2021). NpcRNAs were once considered to be non-functional by-products of transcription and were informally labeled as "junk RNA." This term originated from the early assumption that most of the genome's non-coding regions, which do not encode proteins, were biologically irrelevant. This view largely stemmed from the prevailing central dogma of molecular biology, which concentrated primarily on the translation of genetic information into functional proteins (Haseltine et al., 2024).

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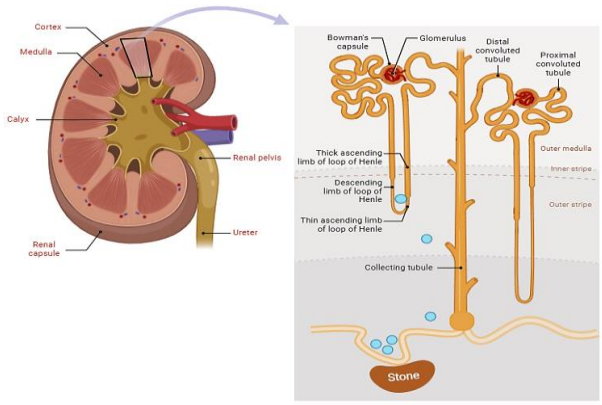

Figure 1. Anatomy of human kidneys, and the specific areas infected and leading to formation of kidney stones (https://www.biorender.com/).

However, this perspective has shifted, and with the emergence of advanced genomic techniques, it has been revealed that npcRNAs play essential roles in cellular regulation, and their functions extend far beyond simple transcriptional noise (Haseltine et al., 2024). NpcRNAs are now acknowledged as key modulators of gene expression and participate in transcriptional and post-transcriptional regulation, as well as in maintaining cellular homeostasis. These molecules typically arise from intergenic regions, which are non-protein coding sequences located between protein-coding genes. The discovery that these intergenic regions serve as a rich source of functional regulatory molecules (Sharma et al., 2024).

Non-coding RNAs generally exert their regulatory effects through sequence-specific interactions with complementary RNA targets, resulting in mRNA degradation or translational repression. The functions of npcRNAs are extensive and multifaceted. They regulate gene expression at various stages, from initiating transcription to modulating mRNA stability and translation. A primary function of npcRNAs is their involvement in post-transcriptional gene regulation. They attach to complementary sequences in the 3' untranslated regions (UTRs) of target mRNAs, causing mRNA degradation or translation inhibition. Besides their role in gene regulation, npcRNAs are crucial for maintaining genome stability (Sharma et al., 2024).

The mechanisms of npcRNAs differ depending on their specific roles. At the post-transcriptional level, cis-encoded npcRNAs can trigger mRNA degradation, inhibit translation, cleave target mRNA (5' UTR overlapping), or induce transcription termination (Chinni et al., 2010). These cis-encoded npcRNAs are transcribed from the same genomic locus as their target mRNA but in antisense orientation, allowing for full complementarity during binding. Conversely, when trans-encoded npcRNA interacts with target mRNA, it can negatively interact with the 5'UTR to prevent the ribosome binding site or promote mRNA degradation with RNase involvement. Both actions suppress translation of the target mRNA. Translation inhibition occurs when a trans-encoded npcRNA binds its target mRNA, forming a structure that blocks the ribosome binding site. Unlike cis-encoded npcRNAs, trans-encoded npcRNAs share only partial complementarity with their target mRNAs. These npcRNAs are encoded at different genomic loci than their targets, enabling them to regulate multiple mRNAs across the genome (Chinni et al., 2010).

Trans-encoded npcRNAs typically require chaperone proteins such as ProQ and Hfq to stabilize their binding to target mRNAs because imperfect base pairing renders the complex susceptible to RNase degradation. ProQ is an RNA chaperone protein primarily present in gram-negative bacteria. It plays an important role in regulating gene expression by binding to RNA molecules, including mRNA and small RNAs (sRNAs). Binding these RNAs stabilizes and protects them from degradation [Mihaita et al., 2025; Singh et al., 2025]. The most studied chaperone is the Hfq protein, which interacts with approximately 40% of npcRNAs in E. coli. According to Schroeder et al. (2016), nearly 50% of bacterial species have trans-encoded npcRNAs that depend on Hfq for stability, with L. monocytogenes being a notable exception, where most trans-encoded npcRNAs function independently of Hfq (Majumder et al., 2022). Unlike trans-encoded npcRNAs, cis-encoded npcRNAs form short, perfectly complementary base pairs with their targets, whereas trans-encoded npcRNAs produce longer imperfect pairings (Chinni et al., 2010).

Similarly, riboswitches are structured elements of npcRNAs classified as cis-encoded RNA elements. They are located in the 5' untranslated region (UTR) of target mRNAs, though they can also occur in the 3' UTR, but less frequently (Olenginski et al., 2024). Riboswitches regulate gene expression by sensing specific metabolite concentrations, making them attractive targets for antibiotic development. Unlike many other regulatory RNAs, riboswitches control transcription and translation without requiring proteins, instead adopting different conformations in response to environmental signals such as temperature changes or the binding of small molecules like ligands or metal ions. Structurally, riboswitches have two main regions: the aptamer region, which binds the ligand, and the expression region, which adopts specific conformations to influence gene expression (Olenginski et al., 2024).

The binding of a ligand to a riboswitch induces structural changes that regulate transcription and translation of the target mRNA. Riboswitches function as molecular "switches," turning gene expression on or off. During transcription, if a ligand binds the aptamer region of the riboswitch, it induces a conformational change that forms a transcription terminator, blocking further transcription and inhibiting gene expression. Conversely, binding a different molecule, known as a linker, can disrupt this terminator, causing anti-termination and allowing transcription to continue. Similarly, in translation, ligand binding can conceal the ribosome binding site (RBS), preventing translation. However, if a linker molecule binds, it can expose the RBS, enabling ribosome attachment and promoting translation (Olenginski et al., 2024).

RNA thermometers/thermosensors (RNATs) also represent a common regulatory mechanism responding to temperature in bacterial pathogens. RNATs are elements typically located in the 5’ untranslated region (UTR) of mRNAs. They function by modifying their secondary structures in response to temperature changes. Alterations in RNA secondary structure significantly

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affect the translation efficiency of the downstream gene due to its proximity to the protein-coding region. RNATs often form stable structures at low temperatures (<30 °C) that block translation of the downstream gene and obscure the ribosome binding site (RBS). Higher temperatures, such as the host body temperature of 37 °C, weaken the RNAT structure due to increased thermodynamic energy. This releases the previously inaccessible RBS and facilitates translation initiation (Loh et al., 2018).

In the urgent effort to combat multidrug-resistant bacteria causing UTIs, innovative therapeutic strategies are critically needed, and molecular biology plays a vital role (Muteeb et al., 2023). Traditionally, molecular biology has focused on elucidating how RNA directs protein production for regulatory functions. However, recent advances in molecular biology, particularly regarding npcRNAs, are transforming this field. NpcRNAs regulate transcription, translation, mRNA stability, and protein interactions. This includes modulating bacterial virulence factors and antibiotic resistance pathways, presenting promising opportunities for developing targeted therapies. Understanding the complex mechanisms of npcRNAs could enable novel treatments that reduce the threat of multidrug-resistant bacteria in UTIs, representing a significant advance in infectious disease management.

4. Bacterial non-protein coding RNA in UTI pathogenesis

4.1 Uropathogen Escherichia coli

Uropathogen Escherichia coli (UPEC), the most common bacterium causing UTIs, attaches to bladder epithelial cells by using type I fimbriae (which promote bacterial adhesion to host tissues) and specifically binds to mannose receptors (Ala-Jaakkola et al., 2022). However, when highly virulent bacteria are present, they can disrupt this balance and weaken the host's defense mechanisms. This disruption can result in urinary tract inflammation, leading to conditions like urethritis, cystitis, and pyelonephritis. Several virulence factors of uropathogenic E. coli (UPEC) contribute to UTIs, including lipopolysaccharides (LPSs), polysaccharide capsules, flagella, outer-membrane vesicles, fimbriae, curli fibers, non-fimbrial adhesins, outer-membrane proteins (OMPs), and iron-acquisition receptors (Zagaglia C et al., 2022).

Based on Zhou (2023), it is highlighted that UPEC involves six phases that lead to UTIs. The process of UPEC infection begins with the bacteria invading and colonizing the periurethral and vaginal areas, using fimbriae and adhesins to attach to host cell surfaces. Additionally, UPEC can ascend into the bladder, where they grow as free-floating cells in the urine. They attach to the bladder lining and interact with host cells. After accumulating, UPEC forms biofilms, which help the bacteria colonize the urinary tract and evade the immune system of the host cell. Biofilms also make the bacteria resistant to certain drugs, leading to chronic and recurring UTIs. When UPEC reaches the kidneys, it releases toxins that kill host tissues, resulting in severe upper UTIs as well as potentially fatal illnesses such as bacteremia, septicaemia, urosepsis, and even death (Zhou et al., 2023).

Based on a clinical report, an elderly 73-year-old woman with type 2 diabetes who had fever, nausea, vomiting, burning, and painful, frequent urination, had evidence of E. coli in UTIs (Ahsan et al., 2024). Her recurring UTI was found to be exacerbated by two strains of E. coli: one that was resistant to several medications and presented treatment difficulties, and another that was responsive to several antibiotics but caused an uncommon infection. Her condition did not improve even after receiving antibiotic treatment with levofloxacin and azithromycin (Ahsan et al., 2024).

From the aforementioned, E. coli stands out as a predominant causative agent of UTIs, being responsible for a significant majority of cases. Its ability to colonize and infect the urinary tract relies on various virulence factors and adaptive mechanisms that enable it to adhere to and invade uroepithelial cells. The genes flhD and flhC in E. coli encode the master regulator of flagellar synthesis (Sun et al., 2022). These genes are co-transcribed by a promoter under the control of multiple transcription factors, each responding to various environmental cues. Sun et al. (2022) highlighted a significant finding involving the npcRNA McaS, which enhances motility and increases the expression of the flhDC operon by binding to the 5' untranslated region (UTR) of its mRNA target. This upregulation results in higher levels of flhDC mRNA and protein production, which are crucial for flagellar synthesis and motility, key factors in the pathogenicity of E. coli in UTIs (Sun et al., 2022).

In contrast, another npcRNA, MicA, also promotes motility but does so independently of flhDC regulation, indicating a different mechanism of motility promotion. This suggests that npcRNAs like McaS and MicA contribute to E. coli's ability to navigate and persist in the urinary tract, potentially influencing the success of infection. The upregulation of motility and flagellar synthesis via these npcRNAs is linked to enhanced virulence, enabling E. coli to better adhere to and invade uroepithelial cells, key steps in UTI development. Through this approach, it is possible to identify the gene and its specific binding regions of the virulence-associated mRNA. Furthermore, gene knockdown studies allow examination of a gene's function by lowering its expression to certain levels and then analyzing the subsequent phenotypic changes. This methodology is an essential tool for validating potential therapeutic targets (Sun et al., 2022).

4.2 Klebsiella pneumoniae

According to Karampatakis (2023), K. pneumoniae is a prevalent pathogen in intensive care unit infections and is the second most common cause of UTIs from both community and hospital sources. Once these bacteria enter the urinary tract, they can adhere to the host epithelial cells lining the urethra and bladder. These bacteria use structures called fimbriae to attach firmly to these cells. Immunocompromised patients can develop serious bloodstream infections from catheter-associated urinary tract infections (CAUTIs), which have high rates of morbidity and mortality (Karampatakis et al., 2023).

Additionally, UTIs are becoming more frequent among residents of long-term care facilities (Karampatakis et al., 2023). The spread of CAUTIs and UTIs caused by K. pneumoniae suggests bacterial factors impair the host immune system. K. pneumoniae

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effectively causes infections by employing numerous virulence factors. Such factors include lipopolysaccharide (LPS), which protects against serum components and aids in survival; capsule prevents host immune cell attacks and modulates immune response; siderophores scavenge iron essential for bacterial growth; urease contributes to catheter encrustation; Type 1 fimbriae form bacterial communities within cells; Type 3 fimbriae assist biofilm development on surfaces; biofilms resist immune responses and antibiotics; and carbapenem resistance restricts treatment options for these infections (Li et al., 2023). According to WHO (2023), carbapenemase-producing K. pneumoniae strains are already present worldwide, with some regions experiencing prevalence rates above 50% (WHO, 2023).

According to another clinical report, it describes a fatal case of septic shock in a 44-year-old patient with compromised immunity, stemming from a UTI caused by multidrug-resistant Klebsiella pneumoniae, specifically extended-spectrum β-lactamase (ESBL)-producing strains. Despite initial negative blood cultures in the ICU, urine tests detected K. pneumoniae. The pathogen was resistant to antibiotics such as amoxicillin and clavulanic acid, which the patient had used prior to hospitalization (Braczkourska et al., 2020).

Based on Kwok (2024), the study explores the posttranscriptional regulation of virulence in hypervirulent Klebsiella pneumoniae (hvKp), a pathogen capable of infecting healthy individuals and posing a significant threat due to its potential to acquire carbapenem resistance, resulting in difficult-to-treat infections. While npcRNA is known to regulate bacterial virulence, its role in K. pneumoniae remains underexplored. Using RIL-seq, the authors investigated the RNA-RNA interaction (RRI) network of hvKp, revealing a prominent role for npcRNAs, including several novel species that were experimentally validated. Among the findings, a stringent subnetwork of RRIs involving virulence-associated genes highlighted the capsule gene loci as a central regulatory hub. The capsule is the primary virulence factor in K. pneumoniae. In UTIs, the capsule is critical for resisting phagocytosis and innate immune responses. One particularly notable npcRNA, OmrB, suppresses capsule production and hypermucoviscosity traits linked to virulence by base-pairing with the kvrA gene (Kwok et al., 2024).

Furthermore, OmrB base pairs within the kvrA coding region, partially suppressing the translation of the capsule regulator KvrA. OmrB, a small RNA, binds to the kvrA gene within its coding sequence (CDS) at positions +81 to +88, disrupting the sequence where the 30S ribosomal subunit binds typically. While it was once believed that npcRNA binding in the CDS region was unlikely due to the ribosome's helicase activity, recent studies suggest such interactions are more common than previously thought. This regulation may depend on features like weak translation initiation signals, such as a non-optimal Shine-Dalgarno sequence or start codon. The kvrA gene in K. pneumoniae shows similar characteristics, with translation initiation possibly occurring at a suboptimal start codon. Although the precise mechanism by which OmrB represses kvrA translation is not fully understood, the findings emphasize growing evidence that npcRNAs can regulate gene expression by binding to CDS regions, affecting virulence-associated phenotypes. Modulation of kvrA expression by OmrB may influence key bacterial factors, such as capsule production and hypermucoviscosity, which are critical for the pathogenicity of K. pneumoniae in the urinary tract [Kwok et al., 2024; Kot et al., 2023].

4.3 Proteus mirabilis

P. mirabilis is a prevalent Gram-negative bacterium that is well-known for causing challenging UTIs, particularly in people who have urinary catheters or urinary tract anomalies. The primary virulence factors linked to various stages of infection, such as flagella, pili or adhesins, urease, hemolysins, and others, are mediated by a catheter into the urethra, bladder, and kidney, resulting in UTIs (Norsworthy et al., 2017). One of the key factors contributing to its prevalence in these infections is its production of diverse types of fimbriae, such as mannose-resistant Proteus fimbriae (MRP). These fimbriae allow the bacteria to attach and colonize in the bladder and kidneys, enhancing their ability to infect uroepithelial cells. P. mirabilis also utilizes two autotransporters, TaaP and AipA, which aid in binding to specific proteins like collagen and laminin found in the urinary tract of host cells. This binding increases the capacity of P. mirabilis to adhere to and invade tissues, leading to the severity of UTIs. Furthermore, P. mirabilis poses a significant challenge in catheter-associated urinary tract infections (CAUTIs) due to its ability to produce urease, which is an enzyme that breaks down urea into ammonia and carbon dioxide (CO_2). According to Wasfi et al. (2020), this process raises the pH of urine, which causes calcium and magnesium phosphate crystals to develop and form a protective biofilm layer on catheters. External antibiotics and the host immune system are unable to penetrate the biofilm, which makes the therapy more difficult to cure (Tian L, et al., 2024).

P. mirabilis urease participates in the formation of urinary stones, which obstruct proper urine flow and can lead to reflux, worsening the infection and potentially causing pyelonephritis (kidney infection) and septicemia (bloodstream infection). This bacterium rapidly attaches to and colonizes the surface of newly inserted urinary catheters, using surface structures like fimbriae and other adhesins (Armbruster et al., 2018). Additionally, P. mirabilis produces toxins such as hemolysin (HpmA) and Proteus toxic agglutinin (Pta), which damage host tissues and promote the spread of the bacteria to the kidneys, leading to acute pyelonephritis. These toxins also facilitate nutrient release from host cells, supporting bacterial growth and survival. P. mirabilis possesses mechanisms to neutralize antibodies, which limit their ability to effectively combat the bacteria (Armbruster et al., 2018).

A clinical report revealed that an 82-year-old woman from Caracas with chronic health issues, including hypertension, diabetes, and recurrent UTIs, was hospitalized after presenting with fever and behavioral changes. She was diagnosed with a UTI caused by P. mirabilis, treated initially with ceftriaxone, but later developed septic shock following femur fracture surgery. The bacteria in her urine were susceptible to several antibiotics, and her blood infection showed specific antibiotic resistance patterns. Further research is needed to discover and explore more about the antibacterial immune responses against P. mirabilis, which

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could potentially lead to new strategies for managing and treating UTIs caused by this bacterium (Maldonado et al., 2022).

Based on Abirami (2022), the author identified and characterized a group of npcRNAs in P. mirabilis that bind to the Hfq protein, a key player in RNA regulation. Thirteen npcRNAs were specifically bound to Hfq using Northern blot analysis, indicating their active expression. These npcRNAs were predicted to target mRNA molecules associated with virulence factors such as fimbriae and flagella proteins. This is necessary for P. mirabilis bacteria to attach to the urothelial cells and initiate infections. This gene has been shown to significantly impact the virulence of P. mirabilis, influencing its ability to cause UTI infections. The npcRNA discussed in these studies is PmiR-137. The removal of the PmiR-137 npcRNA resulted in notable physiological changes in P. mirabilis. These changes included alterations in mobility, biofilm formation, and the ability to respond to environmental stress conditions. This study highlights the regulatory role of npcRNAs in bacterial virulence and provides insight into potential targets for therapeutic interventions against P. mirabilis infections (Abirami et al., 2022).

4.4 Staphylococcus aureus

Staphylococcus aureus is a versatile pathogen causing various infections, from minor infections to severe conditions (Eman et al., 2023). Hospitals frequently have both Staphylococcal infections and UTIs, but S. aureus-induced UTIs are rare, making up only 0.021% to 1.53% of cases. The prevalence of methicillin-resistant S. aureus (MRSA) has increased in hospitals and communities as a result of the increased usage of antibiotics. Therefore, MRSA-induced UTIs that are resistant to common antibiotic treatments have become more frequent, particularly in immunocompromised patients or those with urinary catheters [Yamamoto H, et al., 2022; Gopinath et al., 2022]. S. aureus produces a variety of toxins, such as hemolysins, which can damage host cells and contribute to tissue injury and inflammation. The biofilm matrix formed by S. aureus strains significantly enhances their ability to adhere to host cells and horizontally transfer genetic material through conjugation or transformation (Alshomrani et al., 2023). A resistome and virulome can develop as a result of this process, which promotes the transfer of genes associated with virulence factors and antibiotic resistance. This bacterium is known to build a biofilm and break down urea to produce ammonia, which increases its capacity to form biofilms and adhere to the epithelium, thus promoting its growth and persistence (Xu K, et al., 2023).

According to Delgado (2024), S. aureus can still infect the urinary tract and adapt to the low-iron environment of host tissues. Like other pathogens, S. aureus relies on various mechanisms to acquire iron, an essential nutrient for growth and virulence. The study demonstrates S. aureus uses the ferric uptake regulator (Fur) and npcRNA IsrR to control iron acquisition and metabolism. Specifically, the findings suggest that IsrR plays a significant role in increasing iron uptake, promoting the growth of S. aureus under iron-restricted conditions. This iron acquisition process is important during urinary tract infections, where the host tightly regulates iron levels to limit pathogen growth. During a UTI, the host uses various mechanisms to sequester iron from pathogens in a process known as 'nutritional immunity.' For example, proteins like lactoferrin and transferrin bind iron and prevent bacteria from accessing it. Additionally, the acidic environment and reduced availability of free iron in the urinary tract further limit bacterial proliferation. IsrR's ability to regulate the expression of genes involved in the tricarboxylic acid (TCA) cycle and iron metabolism may play a significant role in the survival and virulence of S. aureus in the urinary tract. In this environment, iron is often limited, making it more difficult for the bacteria to obtain the necessary nutrients for growth. Thus, by controlling genes related to energy production and iron acquisition, npcRNA IsrR helps S. aureus adapt to these nutrient-scarce conditions, improving its ability to persist and cause infection (Delgado et al., 2024).

5. Potential RNA-based targets for treating these urinary tract infections (UTIs)

In contemporary medicine, RNA-based targets have become an innovative approach that is ushering in a new era of personalised and targeted treatment for a wide range of human diseases (Zhu Y, et al., 2022). RNA-based targets have established themselves as an essential tool through groundbreaking discoveries and persistent innovation, providing hope where conventional methods have failed. By specifically targeting disease-related mRNA molecules, RNA-based targets effectively prevent the production of virulence proteins, marking a revolutionary advancement in medicine. To act at the molecular level, this novel therapeutic approach employs a wide variety of RNA molecules, such as oligonucleotides (ASOs), siRNAs, aptamers, mRNAs, ribozymes, and CRISPR/Cas9 (Kim et al., 2022).

Antisense oligonucleotides (ASOs) are short synthetic RNA or DNA molecules. They are designed to bind to particular mRNA sequences and alter their gene expression. The FDA approved the first ASO, Fomivirsen, in 1998 to treat cytomegalovirus retinitis in patients with impaired immune systems (Lauffer et al., 2024). Moreover, Mipomersen is another well-known ASO, authorised in 2013 to treat familial hypercholesterolaemia by inhibiting ApoB-100 mRNA translation (Lauffer et al., 2024). Additionally, FDA-approved siRNA treatments are currently available for acute hepatic porphyria and hereditary transthyretin-mediated amyloidosis. Small interfering RNAs (siRNAs) act by targeting mRNA to reduce protein production (Motamedi et al., 2024).

The application of nucleic acid-based technology has shown promise in combating bacterial infections. Small nucleic acid molecules called aptamers specifically bind to target molecules and show promise in targeting and detecting bacteria such as S. aureus and E. coli (Chen et al., 2022).

When combined with CRISPR technology, aptamers have enhanced bacterial detection techniques, allowing for faster and more accurate diagnosis (Bingshuo Yan et al., 2024). Additionally, messenger RNA (mRNA) therapies offer a promising approach to preventing infectious diseases, such as the COVID-19 vaccines, which use mRNA to instruct cells to produce a particular viral protein that stimulates an immune response [Qin et al., 2022; Deal et al., 2021].

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The potential of ribozymes, RNA molecules that act as enzymes, to cleave mRNA sequences has also been explored. This method targets and silences virulence genes associated with infections (Chen et al., 2024). In conclusion, the capacity of CRISPR/Cas9 gene-editing technology to precisely modify DNA has attracted significant attention due to its promise for genetic repair. Novel strategies to directly target and alter bacterial genomes are currently being evaluated for bacterial infections using CRISPR-Cas9-based methods (Aljabali et al., 2024). This could enable accurate disruption of bacterial pathogens, including antibiotic-resistant strains, providing a promising alternative to conventional antibiotic treatments.

6. Conclusion

UTIs remain a significant public health concern. These bacteria possess complex mechanisms that enable them to colonize and persist within the urinary tract, often leading to recurrent infections and increasing multidrug resistance. As research continues to unravel the potential encoded within npcRNA, the future holds promising advancements that aim to redefine the landscape of healthcare, bringing us closer to effective treatments and cures for previously untreatable conditions. The identification of novel npcRNAs represents a substantial advancement in our understanding of bacterial pathogenesis. These npcRNAs have the potential to fundamentally transform diagnostic and therapeutic strategies for infectious diseases. Integrating npcRNA research into diagnostic methodologies could substantially improve the precision and speed of identifying species-specific pathogens. The development of innovative biomarkers and biosensor technologies based on npcRNAs holds potential for achieving earlier and more reliable infection detection. This approach has the potential to enhance therapeutic interventions and stimulate the emergence of a new market for npcRNA-based drug development. Such progress could yield significant economic benefits, generating revenue and contributing to national economic growth through advanced healthcare solutions and improved public health outcomes.

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A Patient with Platelet Transfusion Refractoriness

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Abstract: Platelet transfusion refractoriness is a less-than-expected increase in platelet count following platelet transfusions. We report a case of platelet transfusion refractoriness with identification of anti-HLA class I alloantibody. In patients with thrombocytopenia, even when multiple established aetiologies are present, it is essential to recognise platelet transfusion refractoriness and to perform further laboratory investigations, including platelet immunology test. This approach enables identification of additional contributing factors to thrombocytopenia and clarification of the underlying causes of platelet refractoriness, thereby guiding appropriate therapeutic strategies.

Keywords: Alloantibody, platelet immunology test, platelet transfusion refractoriness, thrombocytopenia.

1. Introduction

Platelet transfusion refractoriness is characterized by a less-than-expected increase in platelet count after platelet transfusions (Cohn, 2020). We report a case of platelet transfusion refractoriness with identification of anti-HLA class I alloantibody in a patient with relapsed B-cell acute lymphoblastic leukaemia (B-ALL) post-transplant.

2. Case Report

In May 2017, a 39-year-old woman presented with anaemia, leucocytosis, and thrombocytopenia (platelets 29 x 10^9/L) and was diagnosed with B-ALL. She was married with two children. After receiving chemotherapy, she achieved complete remission.

In April 2018, she underwent allogeneic haematopoietic cell transplantation (allo-HCT) (human leucocyte antigen (HLA)-matched brother donor; both blood group B+). Secondary graft failure occurred on day +36 post-transplant. She received a second stem cell infusion in May 2018 and achieved complete remission. However, since allo-HCT, she had persistent thrombocytopenia and mild leucopenia.

In July 2021 (3 years+ post-allo-HCT), her disease relapsed. In September 2021, she was admitted for autologous chimeric antigen receptor T cell (CAR-T) therapy. She experienced febrile neutropenia and grade 1 cytokine release syndrome. Her platelet count prior to CAR-T therapy (day -6) was 53 x 10^9/L. During her hospital stay for CAR-T therapy, platelet counts displayed a decreasing trend (Figure 1).

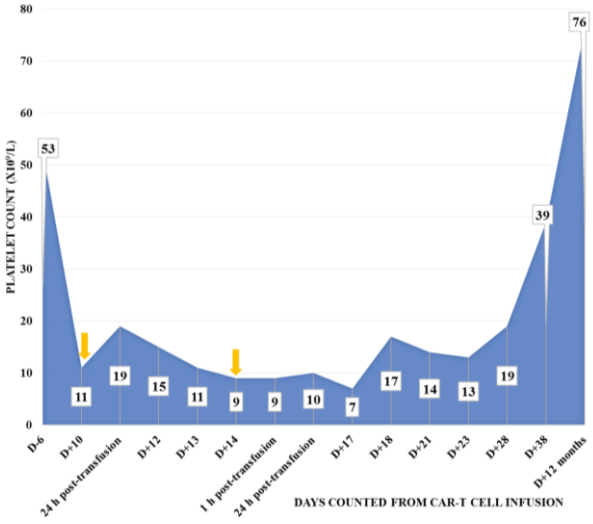

Figure 1. Trend of platelet counts in our patient. (Yellow arrow indicates platelet transfusion)

At day +10 post-CAR-T therapy, her platelet count dropped to 11 x 10^9/L, but she had no bleeding manifestations. Since she was a hospitalized patient with febrile neutropenia, she received platelet transfusion. The platelet count at 24 hours post-transfusion was 19 x 10^9/L, which did not meet the expected increment (Table 1; Figure 2).

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Table 1. Assessment of platelet increment after platelet transfusions in our patient

Parameter therapy	Value at Day+10 post-CAR-T cell
Body weight	58 kg
Height	157 cm
Body surface area (Du Bois Method)	1.58 m^2
Platelet count before transfusion	11 $x10^9$/L
Platelet count at one hour post-transfusion	-
Platelet increment at one hour post-transfusion	-
Corrected count increment at one hour post-transfusion	-
Platelet count at 24 hours post-transfusion	19 $x10^9$/L
Platelet increment at 24 hours post-transfusion	8 $x10^9$/L
Corrected count increment at 24 hours post-transfusion	4,213

CCI-1h: corrected count increment within 10 minutes to one hour after transfusion. CCI-24h: corrected count increment at 24 hours after transfusion; PC: platelet count; PI: platelet count increment.

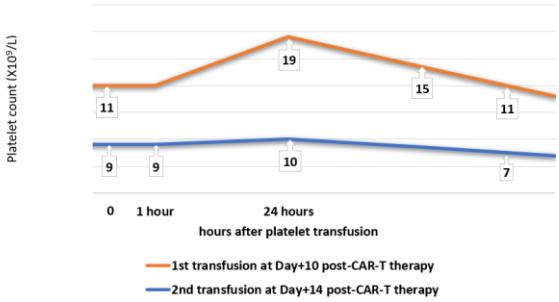

Figure 2. Response pattern to platelet transfusions in our patient.

The lack of the expected platelet count increase following transfusion led to suspicion of refractoriness to platelet transfusion.

Platelet count at day +14 post-CAR-T therapy was 9 x 10^9/L, but she had no bleeding manifestations. Apheresis platelets (one unit) were transfused, but a suboptimal response to platelet transfusion was observed (platelet counts were 9 x 10^9/L at one hour and 10 x 10^9/L at 24 hours after transfusion) (Table 1, Figure 2).

With the recognition of platelet refractoriness, her blood sample was sent to the laboratory to identify the alloimmune cause of platelet refractoriness. Platelet immunology test reported a positive result, with detection of platelet alloantibody with HLA class I specificity when testing the patient's serum against an allogeneic platelet antigen panel. During her hospital stay, she had no bleeding manifestations, and her platelet levels remained stable; hence, watchful observation was adopted with no prophylactic platelet transfusion. Platelet counts gradually increased over time (Figure 1).

Platelet count at day +28 post-CAR-T therapy was 19 x 10^9/L. Disease assessment at day +28 post-CAR-T therapy showed complete remission with incomplete haematologic recovery (CRi) and measurable residual disease (MRD) negativity.

Platelet count at 12 months post-CAR-T therapy was 76 x 10^9/L. Her disease status was complete remission with MRD negativity, and she was well.

3. Discussion

Our patient had pre-existing thrombocytopenia after allo-HCT, despite achieving complete remission. During her hospital stay for CAR-T cell infusion, the possible causes of thrombocytopenia were multifactorial: disease-related (relapsed B-ALL), therapy-related (chemotherapy-induced myelodysplasia, recent leucodepleting chemotherapy, and CAR-T therapy), and platelet refractoriness (anti-HLA class I alloantibody). Anti-HLA class I antibody confirmed an alloimmune cause, although non-immune factors such as fever may also have been present.

3.1 Platelet Transfusion

Prophylactic platelet transfusions are administered to reduce the risk of bleeding in patients with thrombocytopenia, undergoing chemotherapy or haematopoietic cell transplantation for haematologic malignancy, when the platelet count declines to 10 × 10^9/L (McCullough, 2010).

The standard dose of platelets is one unit of apheresis platelets or four to six units of pooled (random) platelets (McCullough, 2010). An apheresis unit contains ~3 x 10^{11} platelets, while a pooled unit contains ~0.55 x 10^{11} platelets (Davis et al., 1999). Transfusion of 1 × 10^{11} platelets in a 70-kg adult increases counts by ~10 × 10^9/L within one hour, and 3–4 × 10^{11} platelets raise counts by ~30–40 × 10^9/L (McCullough, 2010).

3.2 Response Patterns to Platelet Transfusion

For a normal response, platelet count increases after transfusion and gradually declines by approximately three days.

In non-immune refractoriness, an initial rise in platelet count is observed, but transfused platelets are rapidly removed from circulation, causing the count to return to baseline within 24 hours. This indicates normal platelet recovery with reduced survival. A pattern where the initial count rises but then decreases to baseline within 24 hours suggests a non-immune cause.

In alloimmune refractoriness, the platelet count shows little or no increase post-transfusion, indicating alloimmune destruction of transfused platelets. Little or no increase in platelet count after transfusion suggests an alloimmune cause. Our patient showed minimal increment post-transfusion, consistent with alloimmune refractoriness (Figure 2).

3.3 Platelet Transfusion Refractoriness

Platelet transfusion refractoriness is the failure to achieve the expected increase in platelet count after platelet transfusions on at least two consecutive occasions (Cohn, 2020). In our patient, platelet count increments were lower than expected after transfusions on two sequential occasions, and platelet refractoriness was identified.

Causes of platelet refractoriness are classified into alloimmune causes, such as anti-HLA antibodies or anti-HPA (human platelet antigen) antibodies, and non-immune causes, including fever, infection, bleeding, medications (e.g., Amphotericin B), graft-versus-host disease, or splenic sequestration (Cohn, 2020).

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3.4 Evaluation of Platelet Transfusion Refractoriness

Platelet increment (PI) represents a rise in platelet count after transfusion.

PI = post-transfusion platelet count − pre-transfusion platelet count (1)

A PI of < 10×10^9/L on two consecutive transfusions suggests the presence of platelet refractoriness**.**

Corrected count increment (CCI) adjusts for patient body size and the number of transfused platelets (Davis et al., 1999; Cohn, 2020).

If the exact number of transfused platelets is unknown, 3×10^{11} platelets can be used.

$$CCI = PI \times \text{body surface area } (m^2) / \text{Platelets transfused } (\times 10^{11}) \quad (2)$$

In the TRAP study (Trial to Reduce Alloimmunization to Platelets Study Group, 1997), platelet transfusion refractoriness was defined as a ≤4-hour CCI of less than 5,000 following two consecutive transfusions of ABO-compatible platelets, at least one of which had been stored for no longer than 48 hours. Platelet transfusion refractoriness is considered present if two sequential CCIs are both below 5,000. Although most studies adopt a CCI threshold of 5,000, some authors accept a cut-off of less than 7,500 to define refractoriness (Cohn, 2020).

Analysing platelet count within 10 minutes to one hour after transfusion and platelet count at 24 hours after transfusion helps distinguish between immune and non-immune causes of platelet refractoriness. Typically, non-immune causes suggest a normal increase in platelet count immediately after transfusion (CCI-1h above 7,500), but a significant reduction in platelet count by 24 hours (CCI-24h below 5,000). Immune causes often show a lower immediate increase in platelet count (CCI-1h below 7,500) and a continued decrease at 24 hours. Our patient's CCI-1h and CCI-24h after two sequential platelet transfusions were less than 5,000, indicating alloimmune platelet transfusion refractoriness (Table 1).

3.5 Alloimmune Platelet Refractoriness

Alloimmunization involves the development of antibodies against antigens on transfused blood cells. This process can be triggered by prior exposure through pregnancy, blood transfusion, or haematopoietic cell transplantation. The antigens most frequently involved are those of the HLA system, expressed on platelets and leucocytes.

Platelet refractoriness is identified when a patient exhibits a suboptimal response to platelet transfusions on at least two occasions. When this inadequate response is caused by alloantibodies, it is classified as alloimmune platelet refractoriness.

Not all HLA alloimmunization causes refractoriness to platelet transfusion. Data from the TRAP study (n=530) showed that alloimmunization (HLA class I) occurred in 17% to 45% of patients depending on the platelets transfused. Platelet refractoriness was observed in 7% to 16%, and the subset with alloimmune refractoriness ranged from 3% to 13% (Trial to Reduce Alloimmunization to Platelets Study Group, 1997).

Alloimmunization accounts for a minority of cases of refractoriness to platelet transfusion. In the platelet dose (PLADO) trial, refractoriness to platelet transfusion developed in 14% of patients who received at least two platelet transfusions (102 of 734 patients) (Hess et al., 2016). Alloimmunization was present in only 8% (8 of 102) of documented cases of platelet refractoriness, suggesting that non-immune causes of platelet refractoriness were frequent. In contrast, alloimmunization contributed to platelet refractoriness less commonly (Hess et al., 2016).

Here, we report the occurrence of alloimmune platelet refractoriness with identification of anti-HLA class I alloantibody in a patient with relapsed B-ALL post-transplant.

3.6 Strategies to Prevent Platelet Refractoriness

Interventions to prevent platelet refractoriness include: (1) avoiding unnecessary blood transfusion, (2) transfusing ABO-identical or ABO-compatible platelets, (3) pre-storage leukoreduction of blood components, and (4) treating underlying conditions that contribute to platelet consumption and decreased platelet survival.

4. Conclusion

Even when multiple established causes are identified in patients with thrombocytopenia, it is essential to recognise platelet transfusion refractoriness and conduct further laboratory investigations, including platelet immunology test. This approach supports the identification of additional contributory factors to thrombocytopenia and clarifies the underlying causes of platelet refractoriness.

5. Acknowledgement

We acknowledge the CAR-T UKM research group, led by Prof. S. Fadilah Abdul Wahid, the Director of Plutonet Sdn. Bhd. (the principal sponsor of the CAR-T clinical trials) and his team, as well as the staff of Pusat Terapi Sel, MAKNA, and all haematologists involved, for their excellent teamwork in managing the patient. We thank the patient for providing informed written consent for the use of clinical data.

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A Patient's Journey with Immunoglobulin Light Chain (Al) Amyloidosis

Wint Wint Thu Nyunt[1a*] and S Fadilah S Abdul Wahid[2b]

Abstract: A 64-year-old man presented with progressively worsening difficulty in breathing and was subsequently diagnosed with immunoglobulin light chain (AL) amyloidosis. However, he was keen to seek a second opinion, causing a delay in initiating definitive treatment. His clinical manifestations included heart failure, chronic diarrhoea, symmetric lower extremity peripheral neuropathy, and autonomic neuropathy (postural hypotension). As he was non-transplant eligible and had financial constraints, he was initially treated with conventional chemotherapy [two cycles of cyclophosphamide + thalidomide + dexamethasone, followed by seven cycles of melphalan + prednisolone (MP)]. Throughout his disease course, he experienced chronic diarrhoea and profound oedema of lower limbs. He had to relocate to his son's residence in Kuala Lumpur to enable frequent and regular hospital visits. He had impaired health-related quality of life (HRQoL); however, he had excellent family support. After nine cycles of conventional chemotherapy, the optimal response was not achieved. Bortezomib was added to MP therapy [bortezomib + melphalan + prednisolone (VMP) regimen]. Following three cycles of VMP, he achieved complete haematologic response (CR), resulting in symptomatic improvement and his eventual return to his hometown. He continued the same treatment regimen to control the disease. His hospital admissions decreased, and his HRQoL improved, although no organ response was noted. Three years later, he developed decompensated cardiac failure and passed away. His overall survival was five years and two months. This case report highlights that achieving CR leads to prolonged overall survival and improved long-term clinical outcomes, including HRQoL.

Keywords: AL amyloidosis, clinical outcomes, complete haematologic response, survival outcome.

1. Introduction

Immunoglobulin light chain amyloidosis (also known as amyloid light chain amyloidosis, AL amyloidosis, or systemic light chain amyloidosis) is a monoclonal plasma cell proliferative disorder that is rare and associated with a poor prognosis, particularly when cardiac involvement is present (Palladini & Merlini, 2016).

In AL amyloidosis, there is uncontrolled proliferation of monoclonal plasma cells that secrete excessive amounts of abnormal immunoglobulin light chains. These misfolded light chains aggregate to form insoluble amyloid fibrils that subsequently deposit extracellularly within various tissues, ultimately impairing organ function, and in advanced cases, resulting in organ failure. Common sites of involvement include the heart (~75%), kidneys (~65%), liver (~15%), soft tissues (~15%), peripheral nerves/ autonomic nervous system (~10%), and gastrointestinal tract (~5%) (Palladini & Merlini, 2016). The clinical presentation and disease severity are heterogeneous and largely depend on the organs involved and the extent of amyloid deposition.

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We report a case of AL amyloidosis to emphasize that (1) the holistic management approach is essential for optimising clinical outcomes and improving health-related quality of life (HRQoL) in this debilitating and incurable disease, and (2) the depth of haematgic response significantly affects clinical outcomes and overall survival.

2. Case Report

In September 2017, a 64-year-old man, with hypertension and dyslipidaemia, presented with progressively worsening difficulty in breathing. He subsequently underwent percutaneous coronary intervention (PCI) to left anterior descending artery. During evaluation at that time, he was diagnosed with AL amyloidosis. However, the patient was keen to seek a second opinion, causing a delay in initiating definitive treatment. He lived in another district and traveled to Kuala Lumpur for a second opinion.

In December 2017, the patient presented with a five-month history of bilateral leg swelling and worsening shortness of breath. His clinical manifestations included New York Heart Association (NYHA) class III heart failure, chronic diarrhoea, symmetric lower extremity peripheral neuropathy, and autonomic neuropathy (postural hypotension).

Echocardiography revealed global biventricular hypertrophy, interatrial septal thickening, and ejection fraction of 50%.

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Cardiac magnetic resonance imaging (MRI) findings were consistent with infiltrative cardiomyopathy, strongly suggestive of cardiac amyloidosis (diffuse enhancement of right ventricle, left ventricle and interatrial septum post-gadolinium, thickened interatrial septum, pericardial effusion, and right pleural effusion).

Chest X-ray (CXR) revealed right-sided pleural effusion (Figure 1). Computed tomography scan (CT) of thorax demonstrated pericardial effusion and pleural effusion (more on the right) (Figure 1).

Additional investigations were performed, and the results are shown in Table 1.

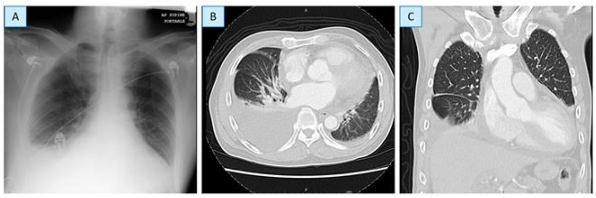

Figure 1. (A) Chest X-ray showing right-sided pleural effusion. (B&C) Computed tomography scan of thorax demonstrating pericardial effusion and pleural effusion.

Investigations excluded hypercalcemia, renal function impairment, lytic bone lesions, and extramedullary involvement.

In December 2017, the diagnosis of systemic AL amyloidosis was established based on the following:

Table 1. Results of investigations performed in our patient

Investigations	Results
Rectal biopsy	Chronic proctitis with amyloidosis Microscopic examination showed amorphous eosinophilic acellular material. Positive Congo red stain
Bone marrow aspirate and trephine biopsy examination	Clonal plasma cells (14%)
Serum protein electrophoresis, Serum immunofixation electrophoresis, Urine protein electrophoresis, Urine immunofixation electrophoresis	Paraproteinaemia (8.9 g/L, IgG lambda) No detectable paraprotein in the urine
Serum free light chain (FLC) assay	kappa (κ) FLC — normal (12.7 mg/L) lambda (λ) FLC — elevated (227 mg/L) kappa : lambda ratio — abnormal (0.06)
Full blood count	Normochromic normocytic anaemia (Hb 11.2 g/dL) White cell count — normal Platelet count — normal
Serum troponin I level	Elevated (65.9 pg/mL)

(1) amyloid-related systemic syndrome, including involvement of the heart, peripheral nerves, autonomic nervous system, and gastrointestinal tract;

(2) positive Congo red stain in rectal biopsy indicating the presence of amyloid;

(3) monoclonal plasma cell proliferative disorder, evidenced by clonal plasma cells in bone marrow, paraproteinaemia, and abnormal serum free light chain (FLC) ratio.

He was a small business owner but could not work due to illness. His son works in a restaurant. He had financial constraints, resulting in limited access to certain medications (bortezomib).

Since he was non-transplant eligible, he received conventional chemotherapy [two cycles of cyclophosphamide + thalidomide + dexamethasone, followed by seven cycles of melphalan + prednisolone (MP)].

Throughout his journey with immunoglobulin light chain (AL) amyloidosis, he experienced chronic diarrhoea and profound oedema of the legs. He needed to relocate to his son's house in Kuala Lumpur to enable frequent and regular hospital visits. His health-related quality of life (HRQoL) was impaired; however, he had excellent family social support.

After nine cycles of conventional chemotherapy, an optimal response was not achieved.

With support from the patient assistance programme, bortezomib was added to MP therapy [bortezomib + melphalan + prednisolone (VMP) regimen].

In March 2019, after three cycles of VMP, he achieved complete haematologic response (CR) (negative serum and urine immunofixation; normal FLC ratio), resulting in symptomatic improvement that allowed him to return to his hometown.

He continued the same treatment regimen to control the disease. His hospital admissions decreased, although no organ response was detected. His HRQoL improved.

Three years later, in January 2023, he succumbed to decompensated cardiac failure.

3. Discussion

Our patient achieved CR but no organ response, with an overall survival of five years and two months. Adding bortezomib to conventional chemotherapy enabled rapid and deep complete haematologic response, resulting in prolonged overall survival and improvement in HRQoL.

3.1 Haematologic Response and Survival Outcome

Response to therapy in AL amyloidosis can be assessed by evaluating haematologic response and organ responses.

Haematologic response is assessed by measuring levels of monoclonal proteins, FLCs, FLC ratio, and the difference between involved and uninvolved FLCs (dFLC). Criteria for complete haematologic response (CR) include normalisation of FLC levels and ratio (when FLC ratio is not within the reference range, the uninvolved FLC concentration must be greater than the involved FLC concentration), and negative serum and urine immunofixation (for monoclonal protein) (Palladini et al., 2020).

In AL amyloidosis, the CR rate was lower with conventional chemotherapy (melphalan plus dexamethasone) compared to bortezomib-based therapy (bortezomib, melphalan and dexamethasone) (Wechalekar et al., 2023).

In AL amyloidosis, bortezomib-based therapy has demonstrated superior CR rate compared with conventional chemotherapy: 8% (4/53) in the bortezomib, melphalan and dexamethasone (BMDex) group versus 4% (2/56) in the melphalan and dexamethasone (MDex) group ($p = 0.012$) (Kastritis et al., 2020).

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Clinical trials have demonstrated that failure to achieve CR correlates with poorer survival outcomes in AL amyloidosis (Kastritis et al., 2020; Wechalekar et al., 2023). This case report supports existing evidence indicating that achieving CR is associated with improved overall survival in AL amyloidosis.

3.2 Organ Response

Organ-specific responses in AL amyloidosis are evaluated by measuring specific organ biomarkers for organ involvement.

Organ response (heart) is assessed by measuring N-terminal pro-B-type natriuretic peptide (NT-proBNP). Criteria for organ response (heart) include NT-proBNP response (30% and >300 ng/L decrease over the starting value in patients with baseline NT-proBNP ≥650 ng/L) or NYHA class response (≥2 class decrease in subjects with baseline NYHA class III or IV) (Palladini et al., 2020; Comenzo et al., 2012).

Organ response (kidney) is assessed through quantification of proteinuria and estimation of glomerular filtration rate (eGFR). Criteria for organ response (kidney) include 30% decrease in proteinuria or drop of proteinuria below 0.5 g per 24 hours in the absence of renal progression (defined as ≥25% decrease in eGFR) (Palladini et al., 2020; Comenzo et al., 2012).

Criteria for organ response (liver) include 50% decrease in abnormal alkaline phosphatase value, decrease in liver size radiographically by at least 2 cm (Comenzo et al., 2012).

Criteria for organ response (peripheral nervous system) include improvement in electromyogram nerve conduction velocity (Comenzo et al., 2012).

Unlike haematologic responses, cardiac responses are less frequent and often delayed. Our patient's lack of cardiac improvement despite CR is consistent with the literature, where cardiac amyloidosis remains a major determinant of mortality (Palladini & Merlini, 2016).

3.3 Impact of Delay in Initiation of Treatment and Financial Constraints

The delay in diagnosis and initiation of definitive treatment is not uncommon in AL amyloidosis, thereby worsening prognosis. Early intervention remains critical to prevent irreversible organ damage.

Financial barriers restricting access to novel therapies (e.g., bortezomib, daratumumab) constitute a recurring issue in real-world settings.

3.4 Recent Advances and Future Directions

The addition of bortezomib to conventional chemotherapy resulted in CR, leading to favourable long-term clinical outcomes with prolonged overall survival (Kastritis et al., 2020).

In this era, a paradigm shift with emerging treatment, the addition of daratumumab to bortezomib-based therapy (daratumumab, bortezomib, cyclophosphamide and dexamethasone), resulted in deeper responses [53.3% (104/195) in the daratumumab group versus 18.1% (35/193) in the control group (bortezomib, cyclophosphamide and dexamethasone); $p < 0.001$] and delayed major organ deterioration in patients with newly diagnosed AL amyloidosis (ANDROMEDA trial) (Kastritis et al., 2021).

However, clinical trials with novel agents are urgently required to improve rapid, deep, and durable haematologic response rates and organ response rates, with the ultimate goal of achieving a cure in AL amyloidosis.

3.5 Implications for Clinical Practice

A comprehensive and timely diagnostic work-up, including tissue biopsy, bone marrow biopsy examination, serum and urine protein electrophoresis with immunofixation, and serum FLC assay, is essential for establishing early diagnosis of this rare and often underdiagnosed disorder. Early identification and prompt initiation of definitive treatment are crucial for improving clinical outcomes.

For patients with newly diagnosed AL amyloidosis, first-line therapies such as bortezomib-based or daratumumab-based regimens result in deeper responses and higher rates of CR.

Addressing socioeconomic barriers (e.g., through patient assistance programmes) may enhance access to novel therapies.

Multidisciplinary care involving cardiology, nephrology, neurology, and supportive care is essential for the holistic management of this systemic disorder.

Clinical trials evaluating anti-fibril therapies, such as monoclonal antibodies targeting amyloid deposits, may further improve haematologic responses, organ responses, and optimise clinical outcomes.

4. Conclusion

This case report highlights that achieving CR leads to prolonged overall survival and improved long-term clinical outcomes, including HRQoL. Achieving a rapid and durable complete haematologic response is essential for optimising outcomes and improving survival in AL amyloidosis. An integrated and holistic management strategy incorporating novel therapies is crucial to improve patients' HRQoL in addition to achieving rapid, deep, and durable haematologic response and organ responses, with the ultimate goal of achieving a cure in immunoglobulin light chain (AL) amyloidosis.

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We acknowledge the staff of Pusat Terapi Sel and the Haematology Unit, Hospital Canselor Tuanku Muhriz UKM, as well as all haematologists involved, for their outstanding teamwork in managing the patient and for their invaluable support.

We also extend our sincere gratitude to the patient and his family for providing written informed consent for the use of the clinical data.

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Bioinformatics Analysis of Potential Biomarkers for Lupus Nephritis

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Abstract: Lupus Nephritis (LN) is a complication of Systemic Lupus Erythematosus affecting the kidney. The purpose of this study was to identify signalling pathways and hub genes involved in the pathogenesis of LN. Methods: The mRNA expression profiles of LN were obtained from the Gene Expression Omnibus database, and differentially expressed genes (DEGs) were identified using the online tool GEO2R. Enrichment analysis was conducted in DAVID. The Protein-Protein Interaction network of DEGs was constructed in STRING, and hub genes were identified with Cytoscape. The hub genes were validated using differentially expressed proteins (DEPs) from proteomics data to identify potential biomarkers for LN. Results: A total of 138 DEGs were identified, primarily associated with immune response, neutrophil chemotaxis, and antimicrobial humoral immunity. In KEGG analysis, the NOD-like receptor signalling pathway and the Cytokine-cytokine receptor interaction pathway were mainly involved. Nine hub genes of LN, including *Ifit1, Ifit3, Ifih1, Ifi44, Irf7, Irf9, Oasl1, Stat1*, and *Usp18* were identified. Conclusion: *Ifi44* and *Stat1* were expressed in both DEGs and DEPs. *Ifi44* and *Stat1* may be potential biomarkers and therapeutic targets for LN.

Keywords: Lupus Nephritis, bioinformatics, proteomics, biomarker.

1. Introduction

Lupus Nephritis (LN) is a complication of Systemic Lupus Erythematosus (SLE) associated with kidney damage. As the most common and serious complication of SLE, the pathological mechanism of kidney damage in LN remains complex (Lech & Anders, 2013; Nicolaou et al., 2020). Among patients with SLE, kidney disease-related mortality is significantly higher in patients with LN than in patients without nephritis (Parikh et al., 2020). Renal biopsy is the gold standard for diagnosing LN. The renal biopsy results can help assess renal pathology, disease activity, and prognosis (Giannico & Fogo, 2013; Saxena et al., 2011), but it is an invasive procedure that has the potential to cause harm (Mou et al., 2024). Therefore, exploring new biomarkers in the blood of LN patients is particularly important for diagnosing and treating LN.

In the past decade, multiple omics studies, such as genomics, transcriptomics, proteomics, and metabolomics, have examined the diagnosis, treatment, and prognostic analysis of kidney diseases (Zhou et al., 2023). Currently, integrating bioinformatics with renal and urinary proteomics studies has been developed as a method for diagnosing renal diseases (Paul et al., 2020). In a study of cross-species transcriptional networks of LN-prone mice and human LN, analysing this bidirectional information provides a new approach for diagnosing and treating LN (Berthier et al., 2012). Additionally, in an association study using single-cell RNA sequencing and proteomics, COL6A3 was identified as a biomarker and therapeutic target for diagnosing LN (Mou et al., 2024). Through multi-omics studies, the pathogenesis and progression of LN can be revealed from a novel perspective, aiding further exploration of new diagnostic and treatment strategies for LN.

In this study, differentially expressed genes (DEGs) from transcriptomics in mice with LN were validated using differentially expressed proteins (DEPs) from proteomics data to identify potential biomarkers for LN.

2. Materials and methods

2.1 Overview of Datasets Collection

The keywords Lupus Nephritis were searched in the Gene Expression Omnibus (GEO) database (https://www.ncbi.nlm.nih.gov/geo/); the datasets must have both LN and control groups. We obtained 4 LN gene expression profile datasets from the GEO database. GSE27045 was submitted by Bethunaickan et al. (2011), and GSE86423, GSE86424, and GSE86425 were submitted by Gardet et al. (2016). The gene expression profiles of renal macrophages in 6 pre-nephrotic control mice and seven nephritis NZB/W mice were obtained in GSE27045. GSE86423 included gene expression profiles of 3 control mice and 30 nephritis NZB X NZW F1 mice. GSE86424 included 40 control mice, 40 Pristane-induced nephritis SNF1 (SWR X NZB) mice, and 8 SNF1 (SWR X NZB F1) mice with spontaneous nephritis. GSE86425 included 51 control mice and 44 Pristane-induced nephritis SNF1 (SWR X NZB F1) mice.

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The proteomic data were uploaded by Wen et al. (2024) on https://pubs.acs.org/doi/10.1021/acs.jproteome.3c00558. The raw data were stored in Proteomexchange (https://www.proteomexchange.org). The search number was PXD046815. This study included 40 female mice that were divided into four groups (n = 10): normal C57BL/6 control group; untreated MRL/lpr lupus; prednisone positive control MRL/lpr lupus and artesunate-treated MRL/lpr lupus groups.

2.2 Identification of common differentially expressed genes (CDEGs)

GEO2R is an online analysis program based on the R language in NCBI. We divided the samples into a control group and an LN group, and selected DEGs in each chip according to the default scheme of the platform. P value < 0.05 and |logFC|≥1 was set as the threshold for screening DEGs. The DEGs of 4 datasets were intersected by a Venn diagram to screen the CDEGs in the four datasets.

2.3 Enrichment analysis of CDEGs

Enrichment Analysis of CDEGs using the online tool DAVID (Huang et al., 2009) for Gene Ontology (The Gene Ontology Consortium et al., 2021) and the Kyoto Encyclopedia of Genes and Genomes pathway (Kanehisa et al., 2021), and the online tool bioinformatics (https://www.bioinformatics.com.cn) (Tang et al., 2023) was used for visualization of the results.

2.4 Construction of Protein-Protein Interaction (PPI) network and identification of hub genes

The CDEGs were imported into the online database String (https://string-db.org/) (Szklarczyk et al., 2021) to construct the PPI network and perform cluster analysis, and the visualization of the PPI network was created in Cytoscape (Version 3.10.2). The MCODE plugin of Cytoscape was used to identify the significant gene modules in CDEGs, and the cytoHubba plugin was used to identify the TOP10 genes (Betweenness) in CDEGs. The intersection of these two methods was considered as hub genes in LN.

2.5 Identification of differentially expressed proteins (DEPs)

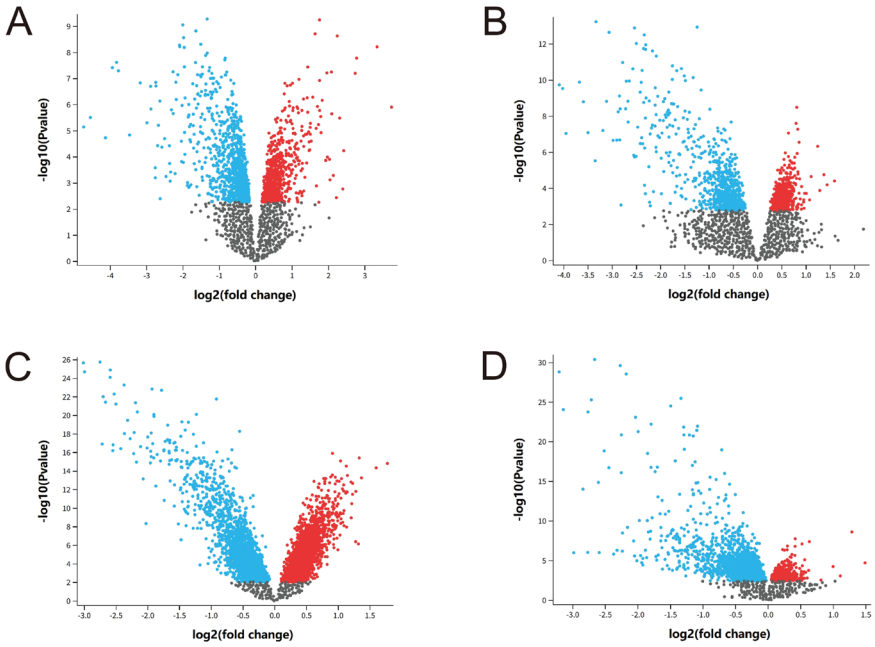

Figure 1. The volcano map of DEGs between LN and normal control of 4 datasets. (A) Volcano maps of GSE27045. (B) Volcano maps of GSE86423. (C) Volcano maps of GSE86424. (D) Volcano maps of GSE86425. Red dots represent up-regulated gene expression; blue dots represent down-regulated gene expression; black dots represent that these genes have no significant difference in LN. The horizontal axis indicates the fold change of gene expression in LN compared to normal control, and the vertical axis indicates the significance of the P. Value.

P <0.05 and | log2FoldChange |> 1 were set as thresholds for screening proteomic data results. DEPs were screened between the LN and the normal control.

2.6 Screening for potential biomarkers

The intersection of hub genes and DEPs was used to screen the potential biomarkers for LN.

2.7 Construction of mRNA-miRNA interaction

The hub genes were imported into the NetworkAnalyst (Zhou et al., 2019) database to predict the mRNA-miRNA interactions. The miRNAs interacting with the hub genes were identified.

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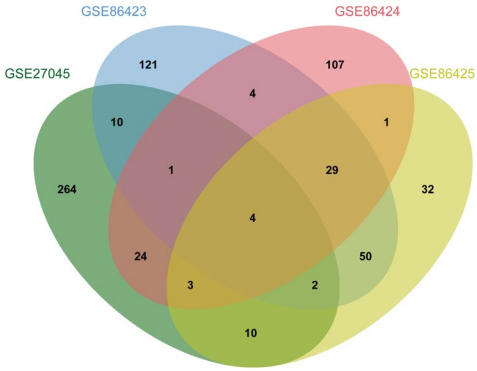

Figure 2. The Venn diagram of the CDEGs.

results showed that these CDEGs were primarily associated with immune response, neutrophil chemotaxis, and antimicrobial humoral immunity (Figure 3).

3.4 KEGG pathway enrichment analysis

The enrichment analysis of the KEGG pathway for CDEGs was performed, and 40 signaling pathways were obtained. These signal pathways were analyzed by KEGG secondary classification (Kanehisa et al., 2023). Five types of signaling pathways were obtained, including genetic information processing, environmental information processing, cellular processes, organic systems, and human diseases (Figure 4).

3.5 PPI network

The PPI network of the CDEGs was obtained by String, and the PPI network was used with Cytoscape plug-ins for visualization (Figure 5).

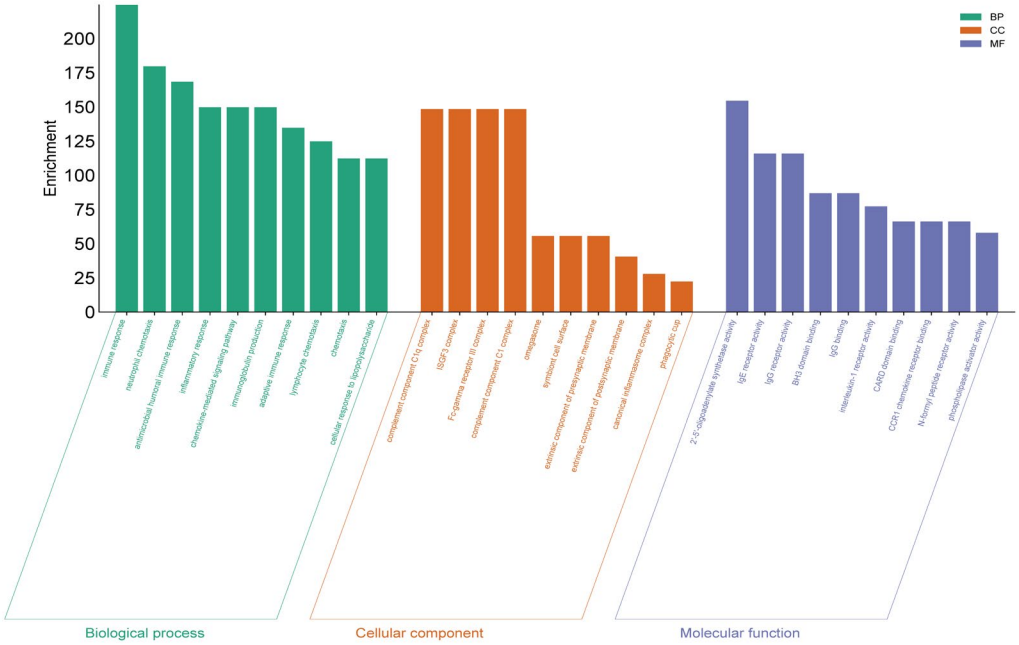

Figure 3. The GO enrichment analysis of CDEGs: The horizontal axis indicates the name of Biological Process, Cell Component and Molecular Function, and the vertical axis indicates the number of Biological Process, Cell Component and Molecular Function.

3. Results

3.1 The volcano map of DEGs

There are 318 differentially expressed genes (DEGs) in GSE27045, 221 DEGs in GSE86423, 173 DEGs in GSE86424, and 131 DEGs in GSE86425 (Figure 1).

3.2 Common differentially expressed genes (CDEGs)

The screening thresholds were set as $P < 0.05$ and $|\log FC| \geq 1$. The DEGs of the four datasets were intersected by a Venn diagram, and a total of 138 CDEGs were identified (Figure 2).

3.3 GO enrichment analysis

GO enrichment analysis includes biological processes, cell components, and molecular functions. The enrichment analysis

3.6 Hub gene of LN

The Cytoscape's MCODE plugin found three significant modules with 65 genes. Moreover, the cytoHubba plugin identified the TOP 10 genes. A Venn diagram was used to analyze the overlapping genes. Nine hub genes were identified, including *Ifit1, Ifit3, Ifih1, Ifi44, Irf7, Irf9, Oasl1, Stat1* and *Usp18* (Figure 6).

3.7 Potential biomarkers of LN

Through the analysis and selection by the setting thresholds, 211 DEPs were identified from the proteomic data. The nine hub genes and 211 DEPs were used in a Venn diagram to analyze the overlapping genes, and *Ifi44* and *Stat1* overlapped in both CDEGs and DEPs. *Ifi44* and *Stat1* were considered as the potential biomarkers of LN (Figure 7).

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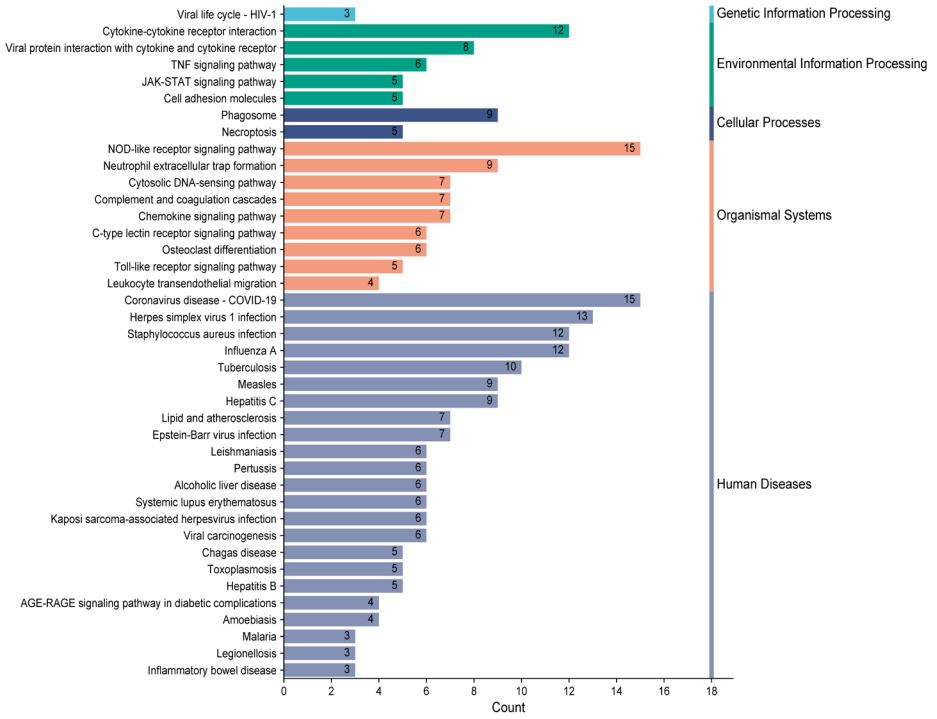

Figure 4. The secondary classification KEGG enrichment analysis of CDEGs. The horizontal axis indicates the count of signalling pathway, the left vertical axis indicates the name of signalling pathway, and the right vertical axis indicates the name of secondary classification.

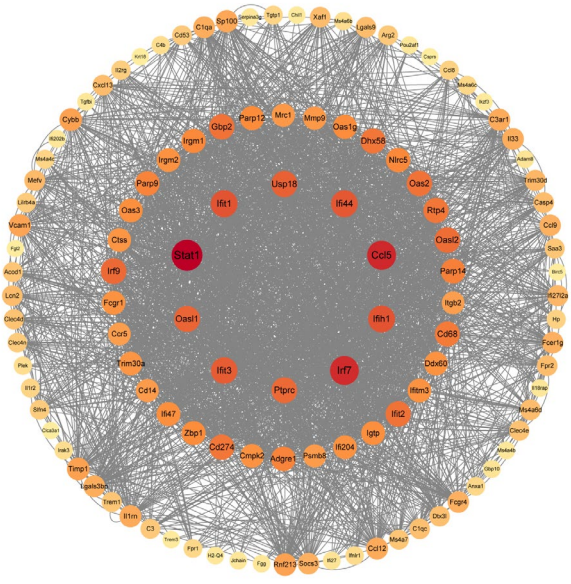

Figure 5. The PPI network of the CDEGs.

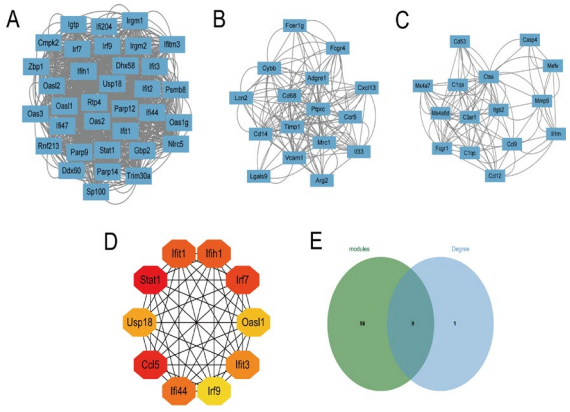

Figure 6. The CDEGs PPI network analysis. (A) Top 1 module identified by MCODE. (B) Top 2 module identified by MCODE. (C) Top 3 module identified by MCODE. (D) Top 10 genes identified by cytoHubba. (E) Hub genes identified by Venn diagram, the green circle represents the genes of MCODE three modules and the blue circle represents the cytoHubba Degree TOP10 genes.

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3.8 Identification of mRNA-miRNA interaction

The mRNA-miRNA interactions were constructed by importing *Ifi44* and *Stat1* into the NetworkAnalyst database. The mRNA-miRNA network was developed, and *Ifi44* interacted with nine miRNAs, including mmu-miR-574-5p and mmu-miR-599, while *Stat1* interacted with 22 miRNAs, such as mmu-miR-3082-3p and mmu-miR-351-5p (Figure 8).

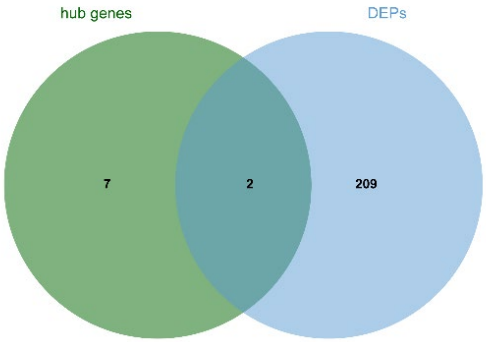

Figure 7. Venn analysis of hub genes and DEPs. The green circle represents the hub genes and the blue circle represents DEPs. The intersection of these two circles represents the potential biomarkers.

4. Discussion

LN is the most common and serious complication of SLE, and the incidence of LN is closely associated with increased mortality in SLE patients (Davidson et al., 2015; Lin et al., 2023). At present, single-omics studies have been widely employed in the study of LN, but they remain insufficient to reveal the complex mechanisms of LN fully. Therefore, integrating multi-omics techniques to investigate the pathophysiological mechanism of LN will contribute to a more comprehensive and deeper understanding of LN.

In this study, we identified 138 CDEGs associated with LN. These CDEGs mainly involve biological processes such as immune response, neutrophil chemotaxis, and antimicrobial humoral immune response. LN is characterized by autoimmune response and inflammation of the kidney, and its onset is closely related to the abnormal activation of the immune system (Tang et al., 2023; Arazi et al., 2019). Neutrophil dysregulation is involved in the immune disturbance and vascular injury in SLE; the immune complex can induce neutrophil activation, which leads to the aggravation of inflammation and renal tissue injury (Mistry et al., 2019; Yu et al., 2022). Humoral immunity mediates the initiation and expansion of inflammatory response through autoantibodies, and the activated immune response and inflammation play an important role in the development of LN (Arora et al., 2017; Conti et al., 2011).

In the secondary classification results of the KEGG pathway, we found two key signalling pathways after excluding human diseases: the NOD-like receptor signalling pathway and Cytokine-cytokine receptor interaction. Studies have shown that the NOD-like receptor signalling pathway is related to the production of inflammatory factors; the inhibition of the NLRP3 signalling pathway can reduce the expression of inflammatory factors, thereby alleviating kidney injury (Zhang et al., 2018; Zhao et al., 2013). Cytokine-cytokine receptor interaction regulates the immune response, inflammation, and intercellular communication through the binding of cytokines and their receptors. Interleukin (IL) and tumour necrosis factor (TNF) are common cytokines; previous reports have shown that IL-35 affects the JAK-STAT signalling pathway, involved in the regulatory process of LN development (Cai et al., 2021). The activation of the TNF signalling pathway triggers the production of inflammatory cytokines, increasing kidney injury in LN (Qing et al., 2018).

Through the PPI network of CDEGs, we identified nine hub genes associated with LN, including *Ifit1, Ifit3, Ifih1, Ifi44, Irf7, Irf9, Oasl1, Stat1*, and *Usp18*. Studies have shown that the genes *Ifit1*, *Ifit3, Ifih1, Irf7,* and *Irf9* in lupus mice can induce the production and response of interferon, participating in the development of LN (Funabiki et al., 2014; Hu et al., 2016; Ikeda et al., 2017; Miyagawa et al., 2016; Thibault et al., 2008). The imbalance of macrophages is involved in the pathogenesis of SLE; researchers found that the expression of USP18 in monocytes of SLE patients was higher than that of normal controls. Further research found that *Usp18* can mediate cell polarization through M1 signalling (Lai et al., 2024). Another study showed that in the peripheral blood mononuclear cells of LN, the expression of USP18 is associated with disease activity (Shen et al., 2022). *Oasl1* is a member of the OAS gene family, participating in the immune response through interferon (Elkhateeb et al., 2016). In a chronic viral infection mouse model, the IFN-I negative regulator Oasl1 can inhibit T cell function and plays an important role in virus clearance (Lee et al., 2013). However, there are no reports of Oasl1 in LN.

In addition, *Ifi44* and *Stat1* were differentially expressed in DEPs and DEGs. IFI44 is a type I interferon (IFN)-inducible gene implicated in the pathogenesis of autoimmune diseases. In a TLR3-deficient mouse model infected with Friend retrovirus, the expression of the IFN-stimulating gene *Ifi44* was significantly decreased, suggesting that *Ifi44* is involved in the immune response to retrovirus infection by mediating the IFN pathway (Gibbert et al., 2014). The expression of IFI44 shows significant specificity in LN patients; studies have shown that IFI44 can be used as a candidate biomarker for the diagnosis of LN (Shen et al., 2021). Additionally, it has been reported that in naive CD4+T LN patients, they have higher expression of IFI44 due to DNA demethylation (Coit et al., 2015), and Mok et al. (2016) reported similar results, showing that methylation of IFI44 in CD4+T cells is significantly associated with LN development.

Stat1 is an important transcription factor involved in the interferon signalling pathway. In LN mice, knocking down Stat1 can inhibit the NLRP3 inflammasome and reduce inflammatory markers such as IL-1β and IL-18, thereby alleviating kidney injury

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in LN mice (Zheng et al., 2024). Additionally, it has been reported that Stat1 is highly expressed in the kidneys of LN mice (Deng et al., 2021), while inhibiting Stat1 expression can alleviate glomerular proteinuria (Yiu et al., 2016). Studies have confirmed that STAT1 is significantly correlated with LN; the overexpression of miR-145 can inhibit STAT1 expression and participate in interferon-mediated signalling pathways (Lu et al., 2012). In another study, lncRNA RP11-2B6.2 participates in the pathogenesis of LN by mediating the IFN-I signalling pathway through the SOCS1 gene. Knocking down lncRNA RP11-2B6.2 promotes SOCS1 expression and inhibits STAT1 phosphorylation in the IFN-I pathway (Liao et al., 2019).

We constructed mRNA-miRNA networks to further explore the diagnostic value of Ifi44 and Stat1. MiRNAs mainly regulate gene expression at the post-transcriptional level. Previous studies have demonstrated that the LncRNA XIST/miR-381-3p/STAT1 axis may serve as a potential biomarker for LN (Chen et al., 2024). In another study, miRNA-155 promotes podocyte apoptosis by activating the JAK1-STAT1 signalling pathway (Pang et al., 2025). To our knowledge, there have been no reports on Ifi44 and miRNA in LN. Therefore, the mRNA-miRNA network constructed in this study enhances the understanding of Ifi44 and Stat1 in LN. On one hand, the expression of Ifi44, Stat1, and miRNAs such as mmu-miR-574-5p, mmu-miR-599, mmu-miR-3082-3p, and mmu-miR-351-5p can be used as biomarkers for diagnosing LN. On the other hand, by regulating the activity of miRNAs, new therapeutic methods can be developed, such as modulating Ifi44 and Stat1 expression by interfering with miRNA expression.

5. Conclusion

This study used bioinformatics combined with proteomics to identify key signalling pathways and hub genes involved in LN. The results show that Ifi44 and Stat1 may be potential biomarkers and therapeutic targets for LN. However, the findings of this study require confirmation through experimental verification.

5.1 Data availability statement

The Lupus Nephritis datasets GSE27045, GSE86423, GSE86424 and GSE86425 were downloaded from the Gene Expression Omnibus (GEO) database. Proteomics data is available on the website https://pubs.acs.org/doi/10.1021/acs.jproteome.3c00558. The raw data was stored in proteomexchange (https://www.proteomexchange.org), serial number is PXD046815.

6. Author contributions

This work was conceived by Zhao Liang and Zhiming Tang. Zhao Liang performed the experiments with the help from Suresh V Chinni and conducted the data analysis. Zhao Liang wrote the manuscript and Suresh V Chinni edited and revised the manuscript. All authors contributed to the article and approved the submitted version.

7. Declaration of competing interest

The authors have no conflicts of interest to disclose.

8. Acknowledgements

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Safety Assessment of One Day Treatment of Liquorice Extract in Female Sprague Dawley Rats

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Abstract: This study aimed to provide preliminary data on the safe use of liquorice extracts for herbal product consumers. Female Sprague Dawley rats received a single-day treatment with liquorice extract (50–2000 mg/kg) or distilled water (control). Liver and kidney functions were evaluated through blood biochemical analysis, gross, and histological evaluation. This animal study adhered to the OECD Test Guideline 423. Fifteen female SD rats, aged 16 weeks, were randomly assigned to five groups (n = 3). The control group received distilled water, while treatment groups T1, T2, T3, and T4 were administered liquorice extract at doses of 2000, 200, 100, and 50 mg/kg via oral gavage. Body and organ weights (liver, kidney, heart) were measured. Blood samples were collected to assess serum biochemical markers, including alanine aminotransferase (ALT), gamma-glutamyl transferase (GGT), creatinine, and urea levels. Data were analysed using Dunnett's tests, with $p < 0.05$ denoting statistical significance. No significant changes in body or organ weights occurred across groups. Liquorice extract had no effect on ALT or GGT. Serum creatinine decreased ($p < 0.05$) at 200 mg/kg, and serum urea decreased at 50 mg/kg compared with controls. A single-day liquorice extract treatment (50–2000 mg/kg) was safe and caused no hepatic or renal toxicity in female SD rats.

Keywords: Liquorice extract, hepatic function, renal function, serum biochemical marker

1. Introduction

Herbal treatments are increasingly recognized as potential alternatives for treating various diseases worldwide. One such herb is liquorice, scientifically known as Glycyrrhiza glabra (Sharifi-Rad et al., 2021). It was the most prescribed herb in Ancient Egyptian, Roman, Greek, East China, and the West from the Former Han era. Various species of liquorice are cultivated in Europe, the USA, southwestern Asia, and Central Africa, the Middle East, Afghanistan, and the northern part of India. In addition, England, Spain, Iraq, Turkey, China, and Sicily commercially cultivate liquorice. Other countries that also produce liquorice include Pakistan, Azerbaijan, Turkmenistan, and Uzbekistan (Wahab et al., 2021).

Liquorice extract has received significant attention in research due to its potential benefits, including anti-inflammatory, immunostimulatory, and antimicrobial properties and hepatoprotective effects. Adverse effects have mainly been associated with high doses, primarily due to glycyrrhizin, which leads to cortisol accumulation and can cause pseudohyperaldosteronism, resulting in health issues such as elevated sodium levels, decreased potassium levels, and water retention (Murray, 2020).

Although the literature on the gross and histological effects of liquorice extract on vital organs in rats remains limited, existing studies suggest promising biological activity, particularly in promoting wound repair. For instance, Assar et al. (2021) reported that administration of liquorice alcoholic extract significantly increased total and differential leukocyte counts, enhanced neutrophil phagocytic function, and upregulated antioxidant biomarkers such as superoxide dismutase (SOD), glutathione peroxidase (GPx), and reduced glutathione (GSH), while decreasing malondialdehyde (MDA), a marker of oxidative stress. Histopathological examination revealed complete re-epithelialization and increased collagen formation. Immunohistochemical analysis showed elevated expression of α-smooth muscle actin (α-SMA), platelet-derived growth factor receptor alpha (PDGFR-α), fibroblast growth factor receptor-1 (FGFR1), and cytokeratin 14 in treated groups—findings that

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support the extract's role in enhancing angiogenesis and tissue regeneration (Assar et al., 2021). In contrast to previous studies that focused primarily on wound healing and inflammatory responses (Assar et al., 2021), our investigation provides additional insight into the systemic safety profile of liquorice extract, specifically its effects on liver and kidney function. The absence of significant changes in ALT, GGT, and organ weights suggests short-term tolerance. Furthermore, observed reductions in serum creatinine and urea levels may indicate a potential renoprotective effect. However, variations in experimental design, such as dosage, exposure duration, and animal species, highlight the need for further research to establish comprehensive safety and dosing protocols.

Liquorice extract, including herbal teas, capsules, tablets, and liquid extracts, is commonly consumed orally. It is known for its potential soothing and sweet flavour and is used for digestive health and respiratory relief. Topically, it is applied in creams, ointments, and gels for skin conditions such as eczema and psoriasis. For respiratory issues, it is available as lozenges, syrups, or inhalation products (Wang et al., 2022).

The phytochemical analysis of liquorice extract from Glycyrrhiza species reveals a rich mixture of bioactive compounds, particularly in the roots. It contains sugars, starch, bitters, resins, essential oils, tannins, and nitrogenous compounds. The key constituents of Glycyrrhiza glabra are triterpenoid saponins, flavonoids, coumarins, alkaloids, amino acids, volatile components, and polysaccharides, with over 60 compounds identified (Dang et al., 2024). More than 300 flavonoids, such as liquiritin and isoliquiritin, give liquorice its yellow colour, while bioactive flavonoids like licochalcone C and glycosides enhance its therapeutic potential. Triterpenoid saponins like glycyrrhizin are notable for their role in healing gastric ulcers, while coumarins such as liqcoumarin exhibit antiviral properties. Liquorice's essential oils also contain α-pinene and β-caryophyllene (Husain et al., 2021). In summary, liquorice extract is a complex mixture of bioactive compounds with diverse pharmacological activities, supporting its use in various medicinal applications when properly dosed.

The COVID-19 pandemic has sparked considerable interest in liquorice, particularly its active component, glycyrrhizic acid, which has shown potential in combating COVID-19 due to its antiviral and anti-inflammatory properties. Research is ongoing to assess its health benefits. The pressing need for effective and well-tolerated treatments has led to growing interest in traditional herbal remedies as potential adjuncts for viral diseases, including COVID-19 (Gomaa & Abdel-Wadood, 2021). Due to these pre-existing and recent developments and the need for updated, significant evidence, further research is necessary to determine its safety and effectiveness in humans.

Since most recent studies focus primarily on the pharmacological characteristics and chemical composition of liquorice extract, the current research gap in the literature becomes evident. As a result, there is a scarcity of research examining its impact on the biochemistry and histology of rats. These findings emphasise the importance of preclinical animal studies to understand the underlying mechanisms, even though human case reports have highlighted instances of adverse effects, such as hypertension, hypokalaemia, and metabolic disturbances, as mentioned in the cited study (Sharifi-Rad et al., 2021). Exploring the histological outcomes in different organs is vital for gaining insights about the safety and efficacy of liquorice as a therapeutic agent (Sharifi-Rad et al., 2021). This study aims to address this gap by investigating the less-explored adverse aspects of liquorice extract, providing a more comprehensive understanding of its impact on human health and assisting in assessing the potential risks and benefits of its consumption.

The main aim of this study is to investigate and determine the safe administration of liquorice extract treatments. To achieve this aim, we have set objectives to guide us towards the aim. Our primary objective is to observe and compare the biochemical and histological changes of vital organs in female Sprague Dawley (SD) rats given liquorice extract treatments after 1 day, as compared with the distilled control group treated with water. In this way, we can determine if there are significant differences between the groups, which allows us to conclude. Our secondary objective is to study the effects of various doses of liquorice extract treatment (2000 mg/kg, 200 mg/kg, 100 mg/kg, and 50 mg/kg concentrations) on the vital organs of rats after 1 day. The results obtained from this study will be significant to the community regarding the use of liquorice extract treatments on individuals.

In conclusion, although prior research has yielded numerous benefits and some drawbacks, further investigation is necessary to provide a comprehensive understanding of the possible pathological impacts on vital organs at the histological and biochemical levels. With liquorice's rich history and diverse health benefits, this study seeks to contribute to the growing interest in traditional herbal remedies and their role in promoting overall well-being and addressing pressing health challenges.

2. Materials and methods

2.1 Raw Materials and Extractions

Liquorice roots were purchased from a local traditional Chinese medicine supplier in Klang Valley. The dried and pulverized liquorice roots were weighed and macerated using distilled water from an ultrapure system in a conical flask with percolation in boiling water for 30 minutes in a hot water bath maintained at 100°C. The mixture was allowed to cool to room temperature and filtered after 24 hours using sterile filter paper. The extract was freeze-dried to obtain a constant weight of fine powder and stored in an airtight container for further use.

2.2 Selection of Animals

A total of 15 healthy female Sprague Dawley (SD) rats with 150 g ± 20 g body weight were used for this study. All groups of rats were fed a standard rodent pellet diet and maintained in the animal holding room under a 12-hour light/dark cycle at 25 ± 2ºC. The animal work was conducted according to the procedure approved by the Animal Ethics Committee of UTAR (U/SERC/253/2022).

2.3 Study Design

A total of 15 randomly selected female Sprague Dawley rats were divided into five groups, each consisting of three rats (n=3).

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Table 1. Body weights

Body Weight (g)	Control (distilled water) [n=3]	T1 (2000 mg/kg) [n=3]	T2 (200 mg/kg) [n=3]	T3 (100 mg/kg) [n=3]	T4 (50mg/kg) [n=3]
Body weight (g)	220.5 ± 14.7	222.4 ± 15.2 (ns)	221.7 ± 21.6 (ns)	221.0 ± 13.6 (ns)	220.6 ± 7.7 (ns)

Data were expressed as mean ± SD; Analysed with Dunnett's test
ns: non significant difference compared with the control

The groups included one control group, which received distilled water orally, and four experimental groups (T1, T2, T3, and T4). The experimental groups received one day of liquorice extract at doses of 2000 mg/kg, 200 mg/kg, 100 mg/kg, and 50 mg/kg, respectively. Animal care and handling followed OECD 423 guidelines.

2.4 Blood Sampling and Biochemical Analyses

After 24 hours of the last treatment, blood samples were collected via cardiac puncture under anaesthesia using carbon dioxide to evaluate liver and kidney function. Liver function tests measured ALT (U/L) and GGT (U/L), while kidney function tests assessed creatinine (mmol/L) and urea (mmol/L) within a diagnostic laboratory. Relative organ weights were calculated using the formula: average organ weight divided by average body weight.

2.5 Histopathological Study

The vital organs of the rats from both the control and the liquorice treatment groups were removed after the rats were sacrificed. The stomach was opened along its greater curvature, flattened, and pinned onto a corkboard with the mucosal surface facing upwards. Great care was taken to avoid damaging the mucosal surface. Tissue samples from each group were obtained and fixed in 10% formaldehyde for 24 hours. The fixed specimens were dehydrated, cleared, and embedded in paraffin. Each section was 5 microns thick, and sections were obtained using a rotary microtome (brand: RWD Life Science). Using a water bath at 37℃, individual wax sections were flattened onto glass slides and allowed to dry overnight in an incubator. The sections were dewaxed with xylene, rehydrated through descending grades of alcohol, and washed in distilled water. Subsequently, the sections were stained using routine Harris's haematoxylin and eosin technique, mounted with Dibutylphthalate Polystyrene Xylene (DPX), and examined by light microscopy. Histological changes in the organs were compared between the control and treatment groups.

2.6 Statistical Analysis

The results for body weight, relative organ weights, and blood biochemical tests were presented as mean ± standard deviation. Dunnett's test was applied to determine significant differences between experimental and control groups. A p-value of <0.05 was considered statistically significant.

3. Results

Compared with the control group, there were no significant changes in body weight (Table 1) and relative organ weight for liver, kidney, and heart in all liquorice treatment groups (Table 2). Female rats treated with a single dose of liquorice extract did not show significant changes in serum levels of ALT and GGT compared to the control group (Table 3). A significant ($p<0.05$) decrease in serum levels of creatinine and urea was observed in rats treated with 200 mg/kg and 50 mg/kg of liquorice extract, respectively, compared to the control group (Table 3).

There are no observable changes in heart tissue on the histopathological slide. The lack of necrosis, fibrosis, or inflammation indicates that liquorice extract did not cause cardiotoxicity at this dose. Kidneys showed occasional thinning of Bowman's space in a few glomeruli and haemorrhages. The liver showed minimal cytoplasmic vacuolation of hepatocytes surrounding the central vein. Mild lymphocytic infiltration was observed in the portal triad with intact architecture and absence of fibrosis. Hence, these histopathological findings are consistent with the biological outcomes of our investigation.

4. Discussion

Changes in body weight and organ weight are both important indicators of drug toxicity; however, changes in organ weight show higher sensitivity in this regard, especially if alterations in body weight are inevitable (Lazic et al., 2020). Based on the results obtained, single-day usage of liquorice did not significantly affect the body weights or the relative organ weights of the heart, kidneys, and liver. These findings imply a lack of toxicity after one day; however, biochemical and histological assessments of the aforementioned organs were conducted to enhance the sensitivity of this evaluation. The serum samples of the rats were tested for levels of Alanine Transaminase (ALT), Gamma-glutamyl Transferase (GGT), creatinine, and urea. ALT levels in the serum increase when structural damage to the liver occurs, causing this enzyme to leak out of damaged cells into the bloodstream (Thakur et al., 2024; Moriles & Azer, 2020). Serum GGT, alternatively, may be used to indicate bile duct cell necrosis or bile duct hyperplasia (GGT – EClinpath, n.d.; Sun et al., 2021). In our study, rats treated with liquorice extract showed no significant differences in ALT or GGT levels compared to the control group, suggesting no hepatotoxic effects following a single day of treatment. The observed decrease in creatinine and urea levels may indicate enhanced kidney function, possibly due to improved blood flow or changes in the filtration process, although our histological research revealed renal haemorrhage in

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Table 2. Relative organ weights

Organ Index (g/100 g *b.w.*)	Liquorice Extract (mg/kg *b.w.*)				
	Control (distilled water) [n=3]	T1 (2000 mg/kg) [n=3]	T2 (200 mg/kg) [n=3]	T3 (100 mg/kg) [n=3]	T4 (50mg/kg) [n=3]
Liver	2.73 ± 0.03	2.78 ± 0.02 (ns)	2.78 ± 0.02 (ns)	2.74 ± 0.02 (ns)	2.75 ± 0.02 (ns)
Kidney	0.50 ± 0	0.52 ± 0 (ns)	0.56 ± 0 (ns)	0.56 ± 0.01 (ns)	0.53 ± 0.01 (ns)
Heart	0.31 ± 0.02	0.31 ± 0 (ns)	0.31 ± 0 (ns)	0.32 ±0.01 (ns)	0.33 ± 0.01 (ns)

Data were expressed as mean ± SD; Analysed with Dunnett's test
ns: non significant difference compared with the control

Table 3. Effect of 1 day of Oral Administration of Liquorice Extract on Serum Biochemical Parameters

Parameters	Control (distilled water) [n=3]	T1 (2000 mg/kg) [n=3]	T2 (200 mg/kg) [n=3]	T3 (100 mg/kg) [n=3]	T4 (50mg/kg) [n=3]
Liver Function					
ALT (U/L)	115.5 ± 79.90	68 ± 4.24 (ns)	1.39 ± 2.83 (ns)	67.5 ± 3.54 (ns)	41.5 ± 3.54 (ns)
GGT (U/L)	4.5 ± 0	4 ± 0 (ns)	5 ± 0 (ns)	4 ± 0 (ns)	4 ± 0 (ns)
Renal Function					
Creatinine (mmol/L)	23.35 ± 2.3	22.9 ± 0.57 (ns)	13.25 ± 0.07* *(p < 0.05)*	26.05 ± 1.48 (ns)	26.6 ± 1.27 (ns)
Urea (mmol/L)	7.5 ± 0.7	7.1 ± 0.07 (ns)	6.75 ± 0.35 (ns)	7.2 ± 0 (ns)	5.8 ± 0.14* *(p< 0.05)*

Data = mean ± standard deviation; Analysed with Dunnett's test
ns: non significant difference compared with the control; *p<0.05: significant difference compared with the control

response to a 200 mg/kg dosage. This action may be explained by the capacity of glycyrrhizin, a major component in liquorice, to mimic certain hormones and influence renal function. Even though this reduction could appear beneficial, additional research is necessary to exclude any underlying damage or negative effects. Serum creatinine and urea are important indicators for renal function concerning their glomerular filtration and are usually elevated in renal impairment (Brookes & Power, 2022; Gounden & Jialal, 2024). Our study showed a decrease in serum creatinine and urea in specific groups of rats. However, variations in these parameters may also be caused by non-renal factors, such as muscle mass and diet ("Biomarkers in Acute Kidney Injury," 2020); thus, these findings may be unrelated to the rats' treatment with liquorice extract, particularly as reduced serum creatinine and urea were only found in a single group of rats, respectively.

While the biochemical assessment suggests a lack of toxicity, histological examination was performed on the heart, liver, and kidneys of the rats to verify this with greater sensitivity. Upon histological analysis, the kidneys and liver after a single day of usage of liquorice showed minimal alterations. These findings were also seen in the control group, indicating that these histological changes may not be related to liquorice use but might have potentially resulted from handling conditions of the rats or environmental stressors. In another study in 2020 (Kim et al., 2020), the administration of liquorice extract in various dosages and periods produced no biochemical or histological alterations, and organ body weights remained within normal limits, which aligns with the results of our investigation.

This study evaluated the acute oral toxicity of liquorice extract after a single day. This is useful in determining the safe dose level of liquorice extract, its potential to cause acute toxicity, and possible target organ toxicity (Pawar et al., 2023). However, the duration of one day may be insufficient to detect the full extent of any acute and chronic effects of liquorice extract on the organs studied, as adverse effects related to acute toxicity typically manifest within 14 days (Pawar et al., 2023). Therefore, further studies should be conducted to observe the effects of liquorice extract on the organs of rats after longer durations, e.g., 30 days. Additionally, controlled clinical trials may prove valuable to assess the effects of liquorice extract consumption specifically in humans.

Water is a highly suitable solvent for plant extraction due to its non-toxic, safe, and environmentally friendly nature, making it ideal for food, pharmaceuticals, and cosmetics applications without leaving harmful residues (Lajoie et al., 2022). It is widely

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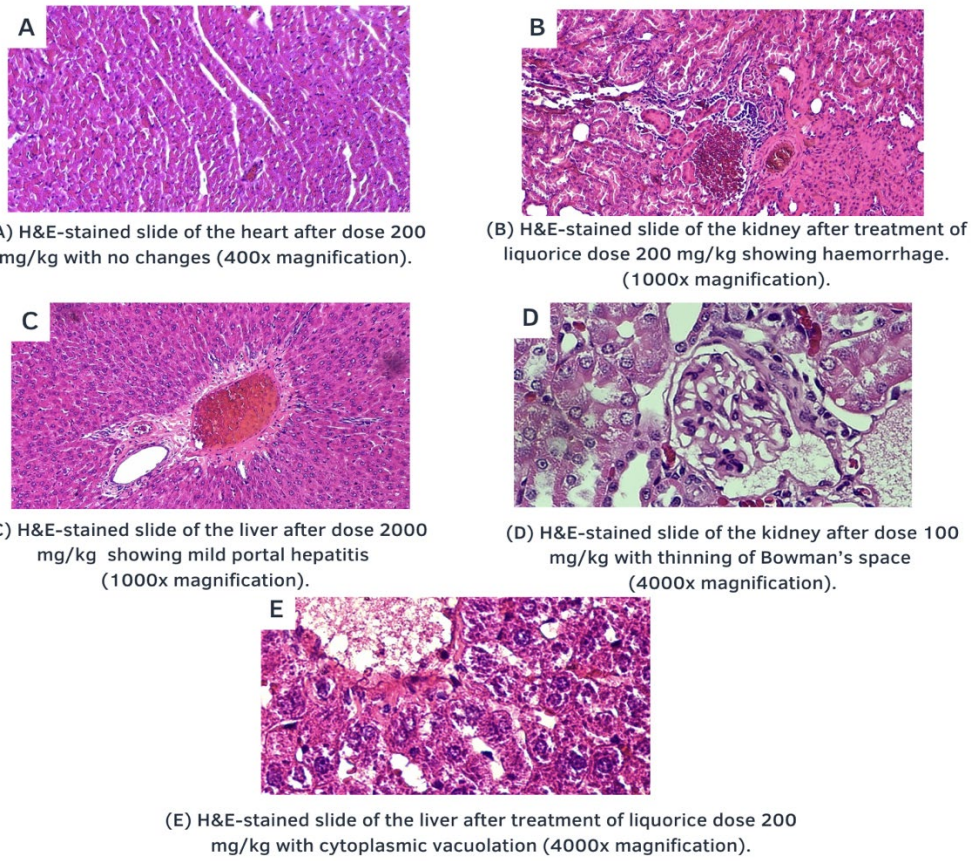

Figure 1. Histological changes in the vital organs of the rats after 1 day treatment of liquorice extract

available and cost-effective, making it practical for large-scale extractions. Water efficiently extracts polar bioactive compounds such as phenolics, flavonoid glycosides, and tannins, which are recognized for their antioxidant and health-promoting properties. The extraction efficiency can be further improved by acidification, such as acetic or hydrochloric acid (Plaskova & Mlcek, 2023). Water can also be combined with organic solvents, such as ethanol, in binary mixtures to expand the range of extracted compounds and increase overall yield. Additionally, water-based extraction is gentler, reducing the risk of thermal degradation or oxidation, making it suitable for heat-sensitive bioactive compounds (Plaskova & Mlcek, 2023). As a result, water provides a flexible, sustainable, and efficient method for plant extraction in various industries, making it an optimal solvent for the extraction of liquorice in this study.

5. Conclusion

One-day treatment with liquorice extract from 50 mg/kg to 2000 mg/kg did not produce any toxic effects on the vital organs of the rat and was safe to administer. Additionally, the unexpected reduction in creatinine and urea levels may indicate improved renal function, although further investigations are required to confirm this finding and exclude any potential adverse effects. However, it is important to acknowledge that the study's short duration and limited sample size present notable limitations. Larger, long-term studies are necessary to establish the comprehensive safety profile of liquorice extract and assess its potential therapeutic or toxic effects.

6. Acknowledgement

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A Rare Case of Fibrosing Obliterative Appendicitis in a Young Patient: Case Report

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Abstract: Fibrosing obliterative appendicitis is an uncommon inflammatory condition of the appendix that can lead to significant diagnostic challenges, particularly in younger patients. In a patient presenting with abdominal pain at the emergency department, appendicitis must be considered and ruled out. However, due to various causes of abdominal pain, the diagnosis of appendicitis may be challenging. Therefore, thorough history taking and a careful clinical examination are essential for identifying appendicitis and ensuring prompt treatment. This study describes a young patient who presented with a complaint of right lower abdominal pain for the past 4 days. A 14-year-old male presented to the emergency department with right lower abdominal pain, without associated symptoms. He had a similar episode three months earlier and was treated conservatively. Ultrasound suggested mesenteric lymphadenitis. Diagnostic laparoscopy was initially performed, but was then converted to open surgery due to an invisible appendix. The procedure revealed an inflamed, slender appendix adhering to the cecum with enlarged mesenteric lymph nodes. Appendectomy and lymph node excision were performed. Histopathology confirmed fibrosing obliterative appendicitis with reactive lymphadenitis. The patient was discharged after two days in stable condition. This case highlights the diagnostic challenge of chronic appendicitis in paediatric patients. Diagnostic laparoscopy proved valuable in identifying fibrosing obliterative appendicitis, allowing for effective surgical intervention.

Keywords: chronic appendicitis, obstructive appendicitis, appendiceal inflammation, mesenteric lymphadenitis

1. Introduction

Fibrosing obliterative appendicitis is an atypical and often challenging form of appendicitis, distinguished by the gradual replacement of normal appendiceal tissue with fibrous tissue, resulting in progressive obstruction of the lumen. Simple forms of acute appendicitis have classical symptoms including fever, nausea, vomiting, and localized abdominal tenderness; conversely, symptoms associated with fibrosing obliterative appendicitis may be less apparent or unusual, leading to an even more complicated diagnosis. This form of appendicitis is particularly rare in children and may be mistaken for gastrointestinal disease. The exact pathogenesis of this lesion is unclear; however, the etiology of the complaint is believed to be secondary to recurrent inflammatory processes, which lead to neuroendocrine cell hyperplasia in the submucosa and lamina propria of the wall of the appendix. The recurrent cycles of subclinical inflammation lead to fibrosis and scarring over time, and as such, chronic inflammation progresses to fibrosis and scarring, resulting in a narrowed or totally obliterated appendiceal lumen (Zarghami et al., 2024). The fibrotic appendix itself can also form adhesions, particularly if the appendix is adherent to other structures in the surrounding area, which can complicate the diagnosis as well as the surgical management based on visualization. In general, fibrosing obliterative appendicitis has a subtle presentation and could be mistaken for mesenteric lymphadenitis or other chronic abdominal pain disorders. Advanced imaging studies, such as ultrasound, may not often show the characteristic presentation of acute appendicitis and may complicate diagnosis. If the diagnosis is unclear and further evaluation is needed, diagnostic laparoscopy is helpful for either diagnosis directly through visualization or therapeutic treatment.

This article presents a rare case of fibrosing obliterative appendicitis seen in a young patient that highlights the difficulty in the diagnosis and management of this clinical problem. This case highlights the importance of including fibrosing obliterative appendicitis in the differential diagnosis of chronic or recurrent abdominal pain when standard imaging studies and clinical evaluations suggest a different diagnosis.

Fibrosing obliterative appendicitis differs from typical appendicitis. Appendicitis is defined as inflammation of the appendix, which is located at the terminal end of the ileum. One of the most frequent abdominal surgical emergencies is appendicitis, which affects 10% of the general population and most frequently affects patients between the ages of 10 and 30. (Lee et al., 2021). This inflammation occurs due to obstruction of the lumen by faecal matter, parasites, and food (Shahba et al.,

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2024). Without prompt treatment, the obstruction can cause perforation of the gastrointestinal tract. The terminal ileum and duodenal C-loop are the most frequent sites of gastrointestinal tract perforation due to accidental ingestion of sharp foreign bodies. One case report has highlighted a rare case of appendicitis that perforated due to ingestion of a fish bone, and the fish bone was found in the appendix (Uchihara et al., 2022). Appendicitis can be divided into two types: acute and chronic. Acute appendicitis is a common surgical emergency with a lifetime risk of approximately 7%, requiring surgical treatment (Ljubas et al., 2024). Conversely, chronic appendicitis (CA) is an uncommon medical disorder that lasts for months or even years and is defined by less intense and intermittent abdominal pain as well as a longer-lasting clinical picture than 1-2 days (Ljubas et al., 2024). Therefore, it is difficult for physicians to diagnose chronic appendicitis because it is not always appropriate to consider it as a first diagnosis (Kothadia et al., 2015). The clinical manifestations of acute and CA in any patient vary from one another. However, one common chief complaint that can be identified is abdominal pain. Abdominal pain is the main complaint in any patient suspected of having appendicitis and one of the most common and challenging symptoms to report, particularly in children. The majority of children who come to the hospital with abdominal pain that lasts longer than three days comprise 5.1% (Kurian et al., 2024). In cases of chronic abdominal pain with no clear cause, diagnostic laparoscopy (DL) may be performed to identify the underlying cause. This minimally invasive procedure often reveals conditions such as adhesions, chronic appendicitis, hernias, or enlarged mesenteric lymph nodes. Current evidence supports DL as a safe and effective approach for uncovering organic aetiologies of persistent abdominal discomfort (Zhao et al., 2020). This method not only assists in diagnosis but also allows for immediate treatment in many cases.

2. Case profile

A 14-year-old male patient, Master Sanjay, came to the emergency department with complaints of right lower abdominal pain. The patient revealed no associated symptoms such as fever, nausea, vomiting, migratory pain, diarrhoea, or urinary issues. Notably, the patient had experienced similar abdominal pain three months ago, which was managed conservatively. An abdominal ultrasound suggested mesenteric lymphadenitis. The three-month delay from the initial presentation to surgery occurred due to several factors. Initially, the patient's symptoms were mild and did not include fever or nausea, which are expected in acute appendicitis. Instead, a conservative approach was applied. An ultrasound indicated possible mesenteric lymphadenitis, a self-limiting process typically improved by conservative treatment, leading us not to pursue surgery at that time. Because the symptoms remained stable, and the patient did not show alarming signs, he was closely monitored, and conservative management continued. Eventually, when the symptoms persisted without improvement, the decision was made to attempt a diagnostic laparoscopy. Mesenteric lymphadenitis was the working diagnosis based on the initial symptoms and imaging studies. The patient was a 14-year-old male, presenting with right lower quadrant abdominal pain, a common complaint seen in numerous gastrointestinal conditions, including appendicitis and mesenteric lymphadenitis. Particularly, the absence of additional classic appendicitis symptoms (fever, nausea, vomiting, migratory pain, etc.) made it necessary to consider alternative causes of abdominal pain. Further questioning of mesenteric lymphadenitis was an abdominal ultrasound that showed enlarged mesenteric lymph nodes, commonly associated with mesenteric lymphadenitis, an inflammatory response typically caused by viral or bacterial infection. Mesenteric lymphadenitis is frequently associated with abdominal pain, especially in the right lower quadrant, and is often misdiagnosed or mistaken for appendicitis due to the clinical overlaps of symptoms. The ultrasound findings of enlarged lymph nodes indicated this diagnosis, resulting in an initial diagnosis of mesenteric lymphadenitis as the cause of her symptoms. However, in light of the ultrasound findings, the patient continued with recurrent pain and no improvement, which warranted further evaluation for the cause of her symptoms. This ultimately resulted in diagnostic laparoscopy being performed, which finally identified fibrosing obliterative appendicitis. The diagnosis can be somewhat elusive by imaging alone, as it is not always definitive of acute appendicitis and can clinically and radiographically mimic other conditions, such as mesenteric lymphadenitis.

Given the persistent nature of the symptoms, a diagnostic laparoscopy under general anaesthesia was planned. During the procedure, the appendix was not immediately visible, requiring conversion to an open surgery. Upon exploration, an inflamed, slender appendix was found adherent to the cecum. The mesoappendix was difficult to separate, and enlarged mesenteric lymph nodes were observed.

During the surgical procedure, the observation of "appendicitis adherent to the mesoappendix" indicates a challenging clinical situation. The inflamed appendix, rather than being loose and movable, is firmly attached to the mesoappendix, the fold of peritoneum supporting the appendix and containing its blood supply. This adhesion often results from chronic inflammation, which can lead to scar tissue formation and

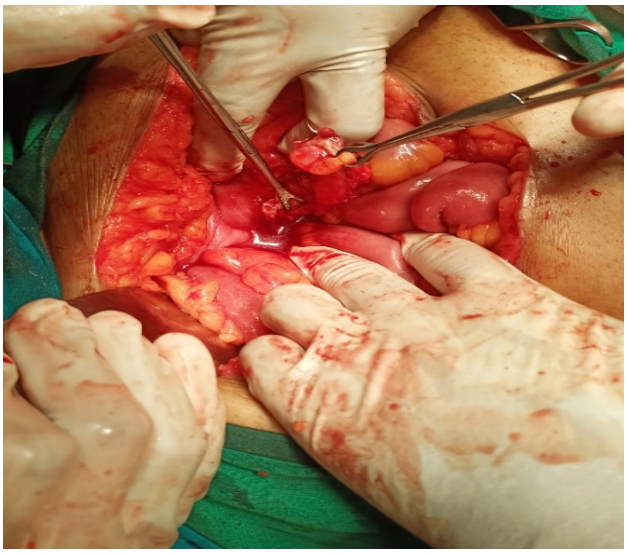

Figure 1. Intraoperatively appendicitis adherent to mesoappendix

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complicate the surgical approach. The presence of such adhesions may suggest a more advanced or complicated appendicitis, necessitating careful dissection to prevent damage to surrounding structures and vessels. This finding highlights the importance of thorough intraoperative assessment and may influence the surgical technique employed to ensure safe and effective removal of the appendix while minimizing complications. With considerable technical difficulty, an appendectomy was performed, along with excision of one lymph node for biopsy (Figure 1).

In Figure 2, both the inflamed appendix and the attached mesoappendix are excised during the appendectomy. This method is commonly used when appendicitis occurs with significant inflammation or adhesion to the mesoappendix, requiring precise resection to achieve complete removal of the infected tissue. Additionally, division of the mesoappendix, which contains the blood vessels and lymphatics supplying the appendix, reduces the risk of residual inflammation or infection, thus preventing complications such as abscess formation or persistent pain after surgery. This comprehensive approach ensures a clearer surgical field and contributes to a more effective resolution of appendicitis, ultimately improving postoperative outcomes for the patient.

Histopathological examination of the specimens disclosed features that are consistent with fibrosing obliterative appendicitis and reactive lymphadenitis. These findings offer insights into the chronic nature of the patient's symptoms and the challenges encountered during surgery. The patient recovered well from the surgery, and he was discharged in stable condition two days after the procedure.

3. Discussion

Fibrosing obliterative appendicitis is a rare form of appendiceal pathology that poses significant diagnostic challenges due to its unusual presentation. In contrast to classical acute appendicitis, which typically presents with well-defined symptoms and imaging findings, fibrosing obliterative appendicitis can manifest insidiously, frequently mimicking other gastrointestinal conditions. The cause of fibrosing obliterative appendicitis remains poorly understood (Agha et al., 2020; Al-Janabi et al., 2022; Greenson, 2019; Molina et al., 2020; Scoazec, 2010), although it may be associated with chronic inflammation, previous infections, or foreign body reactions. The initial ultrasound suggesting mesenteric lymphadenitis highlights the limitations of imaging studies and emphasizes the need for careful clinical correlation (Choi et al., 2014). In this case, ultrasound imaging is an important tool for differentiating between appendicitis and mesenteric lymphadenitis (ML) in children. This approach has proven highly accurate in diagnosing appendicitis, with a 78% positive predictive value. Characteristic ultrasound findings in appendicitis cases include closed-loop patterns and free fluid between intestinal loops, which are observed less frequently in ML.

Enlarged lymph nodes on ultrasound are more commonly present in appendicitis than ML. These distinct imaging features are necessary for rapid differentiation between appendicitis and ML, which is essential as ML typically resolves with conservative treatment, avoiding unnecessary surgical procedures. The surgical challenges encountered, including the necessity to convert from laparoscopy to open surgery and the adherent nature of the appendix, demonstrate the difficulties in managing chronic appendicitis cases. In rare cases, it may result from an autoimmune process that leads to persistent inflammation and fibrosis of the appendiceal wall (Choi et al., 2014). This chronic inflammatory response can form adhesions between the appendix and adjacent structures, such as the mesoappendix, complicating surgical intervention. During surgery, the identification of an appendix adherent to the mesoappendix highlights the need for careful dissection to prevent injury to surrounding tissues. The decision to excise the mesoappendix along with the appendix is critical in ensuring complete removal of all inflamed tissue and minimizing the risk of postoperative complications. By excising both the appendix and the associated mesoappendix, surgeons can reduce the likelihood of complications such as abscess formation or chronic pain.

The recurrent right lower abdominal pain is an example of diagnostic difficulties associated with chronic appendicitis in paediatric patients. [14] The unique presentation, lacking classic symptoms such as fever, nausea, vomiting, and migratory pain,

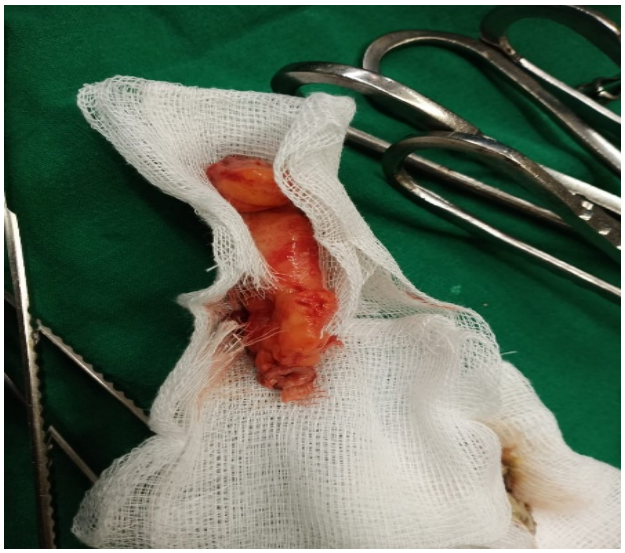

Figure 2. Excised appendicitis along with mesoappendix

underscores the importance of considering chronic appendicitis in cases of recurrent abdominal pain. The histopathological diagnosis of fibrosing obliterative appendicitis with reactive lymphadenitis provides a clear explanation for the patient's chronic symptoms and represents a rare pathology, especially in young patients. This case emphasizes the value of diagnostic laparoscopy in cases of chronic abdominal pain with unclear aetiology, as it allowed for both diagnosis and treatment in a single procedure. This case report highlights that chronic appendicitis poses a significant diagnostic challenge for clinicians. This condition frequently manifests with nonspecific symptoms and may present ambiguous findings on physical examination. In such cases where the clinical picture is unclear, yet symptoms persist, surgical intervention appears as a viable diagnostic and therapeutic option (Amadea & Jurnalis, 2022).

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One study reports a case of a young man with abdominal pain and signs of acute appendicitis, in which an appendix-dependent tumour was discovered during surgery. Histopathological examination showed lympho-plasmocytic infiltrate, storiform fibrosis, and obliterative phlebitis (Cabrales-Escobar et al., 2021). In this case, it was fibrosing obliterative appendicitis, which developed without typical signs of appendicitis and was delayed by 3 months.

In a study conducted by Jenkin et al., a 75-year-old female with a history of chronic, intermittent abdominal pains presented to the general surgery clinic after abnormal thickening of the appendix was discovered on abdominal and pelvic computed tomography imaging. The patient underwent a laparoscopic appendectomy for suspicion of malignancy. The histologic evaluation of the specimen demonstrated a diverticulum at the distal end of the appendix with fibrosing obliteration of the lumen. Fibrosing obliterative appendicitis (FOA) is a rare and unusual form of appendicitis that can occur in children. It is characterized by chronic inflammation, fibrosis, and obliteration of the appendiceal lumen, meaning that the appendix becomes scarred and narrowed, potentially leading to complete obstruction (Jenkins et al., 2022).

In one report, a single Caucasian woman aged 21 presented to the hospital complaining of dull, intermittent abdominal pain localized in the upper portion of her abdomen, where the pain was not referred to any other part of the body. This pain lasted a few days (1-2 days) and was experienced about two times a week. The pain was regular and accompanied by fever, and on two or three occasions was also associated with nausea and vomiting. Ultrasonic imaging disclosed an appendix that was thickened, hypoechoic, hyperemic, and also edematous in the neighbouring fat region. Confirmation was provided by a CT scan performed with clinical suspicion of chronic appendicitis, revealing both segmental and circumferential sanguineous thickening of the appendix. No course of antibiotics was administered. Elective surgery was planned for the patient; however, because the abdominal pains worsened, the patient required emergency surgery one and a half months after the initial hospital visit, approximately three and a half months after symptoms first appeared (Holm et al., 2022). Similarly, in our study, the operation was delayed from the initial visit.

This approach not only allows for direct visualization and assessment of the appendix but also offers the opportunity for definitive treatment if pathology is identified. Further studies are needed to investigate the underlying mechanisms of this condition and to establish standardized treatment protocols that can assist in the timely identification and management of fibrosing obliterative appendicitis.

4. Conclusion

In conclusion, this report is both distinctive and interesting because it describes an uncommon form of appendicitis, fibrosing obliterative appendicitis, in a young patient. This contrasts with the typical acute presentation of appendicitis, which generally presents with acute symptoms such as fever and nausea. Fibrosing obliterative appendicitis has a more gradual onset and often manifests as chronic or recurrent abdominal pain, which made the diagnosis challenging, particularly in a paediatric patient (initially, a diagnosis of mesenteric lymphadenitis was being considered). The patient exhibited persistent symptoms for several months. The initial imaging did not report appendicitis but suggested criteria for mesenteric lymphadenitis. Diagnosis was ultimately confirmed during diagnostic laparoscopy, where an inflamed and fibrotic appendix was found, with firm adhesion to the mesoappendix. The need to convert from laparoscopy to open surgery for adequate visualization of the appendix highlights the difficult nature of this unusual and chronic form of appendicitis. This article emphasizes the importance of including fibrosing obliterative appendicitis in the differential diagnosis of chronic abdominal pain, particularly when conventional diagnoses fail, and underscores the necessity of laparoscopy in unusual paediatric cases for both diagnosis and treatment.

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Integrated Analysis of Cytoskeleton-Associated lncRNAs and Their Regulatory Networks in Mouse Oocyte Maturation

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Abstract: With the increase in maternal age and the impact of environmental stress, the decline in ovarian reserve and oocyte quality has emerged as a primary cause of infertility. Dysfunction of cytoskeletal proteins plays a central role in this process. This study aims to examine the differential expression and regulatory functions of cytoskeleton-associated long non-coding RNAs (lncRNAs) during the development of mouse oocytes at the germinal vesicle (GV) and metaphase II (MII) stages. This study employed bioinformatics analyses and machine learning techniques to analyze publicly accessible data from the Gene Expression Omnibus (GEO) database, which comprised 13 samples of Germinal Vesicle (GV) stage oocytes and 15 samples of Metaphase II (MII) stage oocytes. Differential expression analysis, weighted gene co-expression network analysis (WGCNA), and interaction network construction were performed to screen for lncRNAs closely related to oocyte development. A total of 338 differentially expressed lncRNAs (DE-lncRNAs) with statistical significance were identified, including 136 upregulated and 202 downregulated lncRNAs, indicating their potential roles in the transition from the GV to the MII stage during oocyte development. WGCNA further identified modules strongly correlated with cytoskeletal proteins by integrating these results with the differentially expressed lncRNAs. A total of 47 candidate lncRNAs were shortlisted. Subsequently, LASSO regression and random forest algorithms were applied to identify six key lncRNAs from the candidate set. Combined with miRNA prediction and target gene analysis, a lncRNA-miRNA-mRNA regulatory network was constructed, revealing that these key lncRNAs may indirectly regulate downstream target gene expression through specific miRNAs. Furthermore, Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analyses indicated that these key lncRNAs are primarily involved in cytoskeletal remodeling, cell proliferation, and differentiation, and may play critical roles in follicle structure formation and oocyte development. This study systematically mapped the regulatory network of lncRNAs during oocyte development and elucidated the lncRNA-miRNA-mRNA interactions. The results emphasize the key roles of lncRNAs in cytoskeletal remodeling and oocyte maturation, providing valuable insights for the diagnosis and treatment of ovarian disorders.

Keywords: Cytoskeletal proteins, long non-coding RNAs, mouse oocyte development, ceRNA, reproductive health

1. Introduction

In modern society, the postponement of childbearing age and the increasing impact of environmental stressors have presented women with challenges such as diminished ovarian reserve and reduced oocyte quality, which have become major contributing factors to infertility (Hart, 2016; Barragán et al., 2017). Moreover, the rising prevalence of malignancies in young women has made fertility preservation for cancer patients a pressing concern (Rodriguez-Wallberg et al., 2021). These challenges highlight the need for improved understanding of the molecular mechanisms governing oocyte development, as they are directly related to reproductive health and fertility outcomes. Understanding the molecular regulatory mechanisms of oogenesis holds significant scientific and clinical importance for improving reproductive health and advancing assisted reproductive technologies (ART).

The cytoskeleton is essential to maintaining the structure and function of oocytes throughout maturation. Composed mainly of microtubules, actin filaments, and intermediate filaments, it participates in key biological processes during oocyte maturation, including spindle assembly, chromosome segregation, and cytoplasmic reorganization (Roeles & Tsiavaliaris, 2019). Microtubules are critical for spindle formation and chromosome segregation in oocytes. For example, p21-activated kinase 4 (PAK4) is a serine/threonine kinase vital for regulating microtubule stability (Wu et al., 2022). Inhibition of PAK4 in murine oocytes results in destabilization of microtubules, which subsequently causes defective spindle assembly and erroneous chromosome segregation (He et al., 2019). RAB35 GTPase also regulates microtubule stability in mouse oocytes; its deficiency results in spindle formation defects and asymmetric division failures (Y. Zhang et al., 2019).

Actin filaments are essential for polar body extrusion and spindle migration during oocyte maturation. The interaction

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between actin filaments and microtubules, which involves specific molecular mechanisms such as the coordinated action of motor proteins and the regulation of filament dynamics, ensures correct spindle positioning and successful polar body extrusion. In human oocytes, this interaction is fundamental for spindle assembly and accurate chromosome segregation (Roeles & Tsiavaliaris, 2019). Moreover, the organization of the actin network can influence microtubule behavior, affecting the overall oocyte maturation process (Colin et al., 2018). In summary, the dynamic remodeling and coordination of the cytoskeleton are indispensable for oocyte maturation. Any disruption to these structures may lead to oocyte maturation abnormalities.

Although the importance of the cytoskeleton in oocyte maturation is well-established, the regulatory mechanisms of its dynamic remodeling remain incompletely elucidated. In recent years, the discovery of non-coding RNAs (ncRNAs) has provided new insights. Long non-coding RNAs (lncRNAs) can act as competing endogenous RNAs (ceRNAs) by binding to miRNAs, regulating the expression of protein-coding genes (Salmena et al., 2011; Mattick et al., 2023). Accumulating evidence shows that lncRNAs are involved in various reproductive processes, including primordial germ cell development and migration (Jiao et al., 2018), oocyte maturation, and ovarian cell apoptosis and proliferation (L. Zhang et al., 2023). For example, the lncRNA PWNR2 functions as a ceRNA, inhibiting the interaction between miR-92b-3p and its target mRNA TMEM120B, and thus plays a crucial role in oocyte maturation (Wei et al., 2022). Additionally, studies have revealed significant differences in lncRNA profiles between MII oocytes and granulosa cells (GCs), with higher lncRNA expression in GCs compared to MII oocytes (Ernst et al., 2018).

LncRNAs can also influence gene expression by interacting with chromatin. They can act as scaffolds or guides for chromatin-modifying complexes, targeting specific genomic loci and modulating their epigenetic states (Mangiavacchi et al., 2023). During oogenesis, lncRNAs may regulate the expression of cytoskeleton-related genes through these mechanisms, contributing to cytoskeletal remodeling.

However, the molecular mechanisms by which lncRNAs in cytoskeletal remodeling through the lncRNA-miRNA-mRNA regulatory network remain largely unexplored. This study seeks to elucidate the role of the lncRNA-miRNA-mRNA regulatory axis in the dynamic remodeling of the cytoskeleton during oogenesis. We specifically hypothesize that lncRNAs, through their interactions with miRNAs and mRNAs, are pivotal in regulating cytoskeletal remodeling during oocyte maturation. By identifying novel competing endogenous RNA (ceRNA) regulatory pathways associated with oocyte development, this research aims to establish a theoretical foundation for the development of innovative therapeutic strategies to enhance oocyte quality and fertility outcomes, thereby contributing to the reduction of reproductive disease incidence.

2. Materials and methods

2.1 Data Collection and Preprocessing

All datasets used in this study were publicly available and downloaded from the Gene Expression Omnibus (GEO) database (https://www.ncbi.nlm.nih.gov/geo/). We searched the database for species limited to *Mus musculus*. The GSE137458 dataset was selected due to its provision of lncRNA expression data from both Germinal Vesicle (GV) stage oocytes (prophase I-arrested immature state with intact nuclear envelope) and Metaphase II (MII) stage oocytes (metaphase II-arrested mature state after polar body extrusion), which are directly pertinent to our research focus on the developmental transition from the GV to the MII stage. The GSE141190 dataset was selected because of its extensive data on mouse oocyte development, offering additional valuable information for a comprehensive analysis. From GSE141190, we selected seven raw GV-stage oocyte samples (GSM4196729 to GSM4196735) and eight MII-stage oocyte samples (GSM4196770 to GSM4196777) as control samples to complement the data from GSE137458, increasing the sample size and potentially improving the reliability of our analysis.

The lncRNA expression profiles of mouse oocytes at the germinal vesicle (GV) stage were retrieved and downloaded using the "GEOquery" package in R. The two datasets were merged. To correct for batch effects caused by non-biological technical variations, the ComBat function from the "sva" R package was applied (Leek et al., 2012). ComBat utilizes an empirical Bayes framework to adjust gene expression data by estimating and removing batch-specific effects, ensuring that observed differences in expression reflect biological variation rather than technical inconsistencies. Before merging, we carefully examined the data formats of the two datasets. Both had gene expression values in a tabular format, but the column headers and some metadata labels differed slightly. We standardized the column headers to ensure consistency. Regarding the data, GSE137458 had a relatively smaller number of samples compared to GSE141190. To address this, we did not perform any data subsampling but instead weighted the contribution of each dataset during the batch correction process to avoid overemphasizing the larger dataset. After batch correction, principal component analysis (PCA) was performed to assess its effectiveness. As a result, we obtained a new integrated dataset comprising 13 GV-stage and 15 MII-stage oocyte samples, which was used for all subsequent analyses. This study adhered to the data access policies of each database utilized.

A total of 102 human cytoskeleton-related genes were obtained from the GeneCards database. These genes were mapped to their mouse homologs through homology mapping, resulting in 98 mouse cytoskeleton-related genes, which were subsequently used for downstream analyses.

We also conducted a data quality assessment of the public database data. For the GSE137458 and GSE141190 datasets, the missing value proportion was below 5%, which was acceptable. The sample quality control metrics, such as RNA integrity number (RIN), were available for most samples and indicated that the RNA quality was relatively high, with an average RIN value above 7.0, ensuring the reliability of the gene expression data.

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2.2 Screening and Analysis of Differentially Expressed lncRNAs

To identify lncRNAs most closely associated with cytoskeletal proteins, the GTF annotation file provided by the R package AnnoProbe (version 0.1.6) was used to differentiate between mRNAs and lncRNAs. Expression matrices for mRNAs and lncRNAs were separately extracted for further analyses.

Differential expression analysis of the lncRNA expression matrix was conducted using the limma package (version 3.50.0) (Ritchie et al., 2015). The comparison was made between the MII-stage group (n = 15) and the GV-stage group (n = 13). Differentially expressed lncRNAs (DElncRNAs) were identified based on the following criteria: |log2Fold Change| > 0.25, p-value < 0.05.

The identified DElncRNAs were then used for subsequent analysis. Hierarchical clustering analysis was performed using the pheatmap package in R. Euclidean distance and hierarchical clustering methods were applied to produce heatmaps and visualize expression patterns. In the heatmap, the color scale represents the relative expression levels of lncRNAs. Red indicates high expression, and blue indicates low expression. This color scheme is clearly defined to assist readers in interpreting the expression patterns of different lncRNAs.

2.3 Weighted Gene Co-expression Network Analysis (WGCNA) and Identification of Significant Modules

The WGCNA package (version 1.70-3) in R was employed to construct a weighted gene co-expression network using the WGCNA algorithm (Langfelder & Horvath, 2008). Pearson correlation coefficients were calculated to evaluate the similarity among gene expression profiles. These correlations were then raised to a power function to achieve an approximate scale-free topology in the network.

We used the PickSoftThreshold function to determine the optimal soft-thresholding power (β). After testing various β values, we found that when β = 3, the average connectivity approached zero, and the scale-free topology fit index exceeded 0.85 (Figure 2A). This indicated that the constructed network conformed to the properties of a scale-free network. We chose this parameter because it provided the best balance between network stability and the ability to detect meaningful gene modules. Other values of β led to a network that was too sparse or too dense, making it difficult to interpret the co-expression relationships accurately.

Gene modules are clusters of densely interconnected genes within the co-expression network. WGCNA applies hierarchical clustering to identify gene modules, which are color-coded for visualization. The dynamic tree cut approach was utilized to detect distinct gene modules. During module detection, the adjacency matrix (which measures topological similarity) was converted into a topological overlap matrix (TOM), followed by hierarchical clustering to identify gene modules.

To explore the relationship between gene modules and cytoskeletal proteins, Pearson correlation analysis was performed between the module eigengenes (MEs)—which represent the first principal component of each module, reflecting the overall expression pattern—and the expression of cytoskeletal protein-related genes. Modules that showed significant correlations with cytoskeletal proteins were identified as key modules.

The structure of the co-expression modules was visualized through heatmaps of gene network topological overlap. Furthermore, a hierarchical clustering dendrogram of module eigengenes, along with the corresponding heatmap, was used to summarize the associations among the identified modules.

Finally, cytoskeletal protein-related differentially expressed lncRNAs (cytoskeletal protein-related DElncRNAs) were identified by taking the intersection between DElncRNAs and the lncRNAs present in the cytoskeletal protein-related modules.

It should be noted that the WGCNA algorithm has several limitations. It is sensitive to data noise, and outliers in the gene expression dataset can potentially affect module identification. We pre-processed the data to mitigate this by removing genes with extremely low expression levels. We performed multiple rounds of analysis with different data subsets to ensure the identified modules' consistency.

2.4 Identification of Hub lncRNAs Using LASSO Regression and Random Forest Analysis

To compute and select linear models while retaining valuable variables, the LASSO (Least Absolute Shrinkage and Selection Operator) regression was performed using the glmnet package in R. A binomial distribution was applied for LASSO classification, and the model was established by choosing the lambda value corresponding to the minimum cross-validated error. This approach resulted in a model with optimal performance and included 10-fold cross-validation to ensure reliability.

Subsequently, random forest (RF) analysis was conducted using the RandomForest function in R. Ultimately, the ntree parameter was set to 1000, and the computation of the proximity matrix was activated (proximity = TRUE). According to the importance measures, namely, Mean Decrease Accuracy (MDA) and Mean Decrease Gini (MDG), the top 30 cytoskeleton-related DElncRNAs were identified as key genes selected by the random forest algorithm.

By integrating the results from both the LASSO regression and random forest analysis, the most significant feature lncRNAs were identified and selected as hub lncRNAs in this study.

LASSO regression and random forest analysis may face challenges when dealing with high-dimensional data, such as overfitting. To address this, we performed feature selection prior to applying these algorithms to reduce the dimensionality of the data. Additionally, we used cross-validation techniques to optimize the model parameters and evaluate the performance of the models.

2.5 LncRNA-miRNA-mRNA Regulatory Network Analysis

In this study, six hub lncRNAs were selected for further analysis. To predict their potential interacting miRNAs, four established bioinformatics tools were employed: Miranda, PITA, TargetScan, and RNAhybrid. Each of these tools has its own algorithmic principle. Miranda uses a seed-based pairing algorithm to predict miRNA-target interactions, which is sensitive

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to the sequence complementarity in the miRNA seed region. PITA predicts miRNA-target binding based on the free energy change of the miRNA-mRNA duplex formation, considering the local RNA secondary structure. TargetScan focuses on conserved miRNA-binding sites in the 3'-UTR of mRNAs, and RNAhybrid predicts hybridization between miRNA and mRNA by calculating the minimum free energy of the duplex. Each tool has its own advantages and limitations. Miranda can identify many potential targets but may have a relatively high false-positive rate. PITA's consideration of RNA secondary structure makes its predictions more biologically relevant, but it may miss some targets due to the complexity of structure prediction. TargetScan's reliance on conserved binding sites may lead to omission of non-conserved but functional targets, and RNAhybrid's accuracy may be affected by the precision of the energy calculation model.

We integrated the prediction results from all four algorithms to identify candidate miRNAs. Only the intersecting miRNAs were retained to improve the reliability and specificity of the predictions.

Subsequently, to further investigate the downstream regulatory networks of these candidate miRNAs, five widely recognized algorithms were used to predict their potential target mRNAs. These included TargetScan, Miranda, miRmap, PITA, and PicTar. The intersection of these prediction results was taken to identify a set of high-confidence target genes, ensuring reliability and minimizing false-positive results in the subsequent construction of the lncRNA-miRNA-mRNA regulatory network.

We compared the prediction results of different tools. For example, TargetScan and Miranda often predicted overlapping targets, but there were differences as well. TargetScan was more likely to identify conserved targets, while Miranda could detect some non-conserved ones. The differences were mainly due to their different algorithmic principles and data sources. By taking the intersection of multiple predictions, we aimed to obtain a more reliable set of candidate molecules.

2.6 GO and KEGG Pathway Enrichment Analysis

Gene Ontology (GO) analysis is a commonly used method for large-scale functional enrichment investigations, encompassing three main categories: Biological Process (BP), Molecular Function (MF), and Cellular Component (CC). In this study, the target genes of cytoskeleton-related differentially expressed lncRNAs were subjected to GO and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis using the ClueGO plugin in Cytoscape software (Bindea et al., 2009).

For the GO and KEGG enrichment analysis results, when terms such as "cytoskeleton organization" and "MAPK signaling pathway" were significantly enriched, we further examined their relationships with oocyte development and cytoskeletal remodeling.

2.7 Ethical Statement

This study did not involve human subjects or animal experiments. All experimental data were obtained from publicly available databases (Gene Expression Omnibus, GEO), specifically datasets GSE137458 and GSE141190. The use of these datasets complies with the access policies and terms of use of the GEO database and has been authorized by the relevant institutions. Throughout the research process, strict adherence to data privacy and ethical guidelines was maintained to ensure the lawful use and analysis of the data.

2.8 Statistical Analysis

All statistical analyses in this study were conducted using R software (version 4.1.2). Spearman's correlation test was employed to evaluate the association between two variables. The Wilcoxon rank-sum test was applied to compare differences between two groups, while the Kruskal-Wallis test was used to compare three or more groups. A two-sided p-value of less than 0.05 was considered statistically significant.

3. Results

3.1 Differentially Expressed lncRNAs Related to Oocyte Development

By comparing the lncRNA expression profiles between the GV group and the MII control group, a total of 338 significantly differentially expressed lncRNAs (DElncRNAs) were detected ($p < 0.05$, $|Log2FC| > 0.25$), as shown in Figure 1A. Among these, 136 lncRNAs were upregulated, and 202 lncRNAs were downregulated. The heatmap in Figure 1B further displays the top 10 lncRNAs with the most significant differential expression. In the volcano plot (Figure 1A), the x-axis depicts the log2 fold change in lncRNA expression between the MII-stage and GV-stage groups, while the y-axis shows the -log10 of the p-value. Data points above the horizontal dashed line (representing a p-value threshold of 0.05) and beyond the vertical dashed lines (representing a log2 fold change threshold of ±0.25) are considered differentially expressed lncRNAs. The distribution of these points illustrates the overall pattern of differential expression, with some lncRNAs being notably upregulated and others downregulated. In the heatmap (Figure 1B), each row corresponds to an lncRNA, and each column represents a sample. The color gradient from blue to red indicates a gradual rise in expression levels, allowing for a visual comparison of lncRNA expression patterns across different samples.

3.2 Construction of Weighted Gene Co-expression Network and Identification of Cytoskeleton-Related Modules

In this study, the WGCNA method was applied to analyze lncRNAs related to cytoskeletal proteins. The results of the scale-free topology and mean connectivity analysis showed that when the soft-thresholding power (β) was set to 3, the average connectivity approached zero, and the scale-free topology fit index exceeded 0.85 (Figure 2A), indicating that the constructed network conformed to the properties of a scale-free network. A total of eight co-expression modules were identified using WGCNA. lncRNAs that did not cluster into any of the modules

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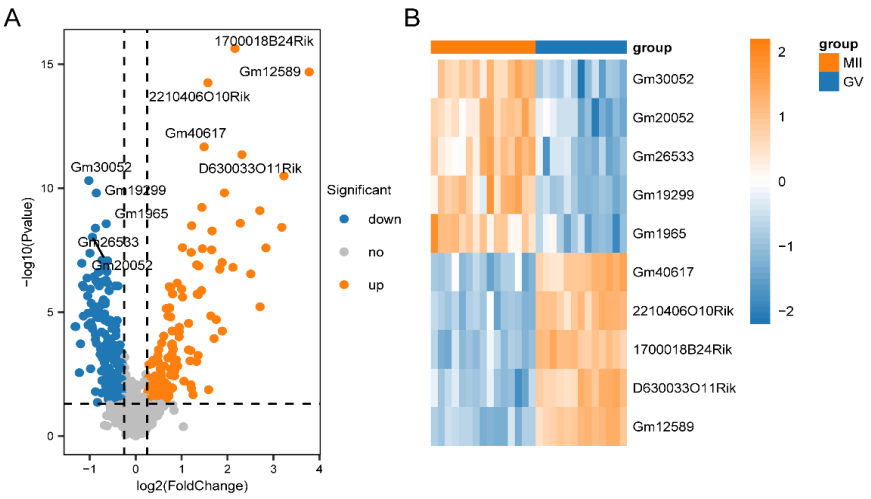

Figure 1. Identification of Differentially Expressed lncRNAs (DElncRNAs) During Oocyte Development. (A) Volcano plot illustrating the distribution of DElncRNAs between the GV group and the MII control group (B) Heatmap showing the top-ranked DElncRNAs with the most significant differential expression

were assigned to the grey module, which was excluded from further analysis (Figure 2B).

To explore the relationships between modules and assess their association with cytoskeletal proteins, correlation analyses were conducted between the module eigengenes (MEs), and the results were visualized as a heatmap to illustrate the network of module relationships (Figure 2C). By correlating the MEs of the eight modules with cytoskeleton-related genes, the blue module, comprising 264 lncRNAs, was found to show the strongest positive correlation with cytoskeletal proteins ($r = 0.8401$, $P < 0.05$), as indicated in the module-trait relationship heatmap (Figure 2D). Given its statistical significance, subsequent analyses primarily focused on the blue module, which most likely represents the regulatory roles associated with cytoskeletal proteins.

Furthermore, an intersection analysis was performed between the differentially expressed lncRNAs (DElncRNAs) and the lncRNAs within the cytoskeleton-related module, resulting in the identification of 47 lncRNAs closely linked with cytoskeletal proteins (Figure 2E). Wilcoxon rank-sum tests further confirmed that these lncRNAs exhibited statistically significant expression differences between the GV and MII groups ($p < 0.05$, Figure 2F). In the module-trait relationship heatmap (Figure 2D), each cell represents the correlation coefficient between a module eigengene and cytoskeleton-related genes, with the p-value shown in parentheses below. The color legend clearly indicates the strength and direction of the correlations, with red representing positive correlations and blue representing negative correlations. This format allows readers to easily identify the modules most strongly associated with cytoskeletal proteins.

3.3 Identification of Key lncRNAs Using Two Machine Learning Algorithms

To further identify key cytoskeleton-related lncRNAs, this study applied two machine learning algorithms: LASSO regression and random forest analysis. Through LASSO regression analysis, six key lncRNAs were ultimately identified (Figure 3A-B). In the random forest analysis, the top 30 cytoskeleton-related lncRNAs with significant feature importance were selected based on two evaluation metrics: Mean Decrease Accuracy (MDA) and Mean Decrease Gini (MDG) (Figure 3C-D). By integrating the results from both methods and taking the intersection, a total of six key cytoskeleton-related lncRNAs were finally determined as hub lncRNAs for subsequent analyses. These six lncRNAs are: BC023719, 1700026F02Rik, 4930567H12Rik, Gm20319, Gm46355, and 6430573P05Rik (Figure 3E). In the LASSO regression coefficient profiles (Figure 3A), the horizontal axis represents the logarithm of the lambda parameter, and the vertical axis shows the coefficients of the independent variables. As the lambda value changes, the coefficients of the lncRNAs are shrunk towards zero. The point where the coefficients stabilize and the cross-validation error is minimized (Figure 3B) indicates the optimal set of lncRNAs selected by the LASSO regression. In the random forest analysis, the error rate plotted against the number of classification trees (Figure 3C) shows how the model's performance improves as more trees are added. The top 30 lncRNAs ranked by MDA and MDG (Figure 3D) represent the most influential features in the model, and the intersection with the LASSO-selected lncRNAs (Figure 3E) yields the final set of hub lncRNAs.

3.4 Validation of Hub lncRNAs

Further validation of the expression patterns of the identified hub lncRNAs was performed using a heatmap. The results show that all hub lncRNAs exhibited low expression levels at the GV stage, which increased progressively during oocyte development (Figure 4).

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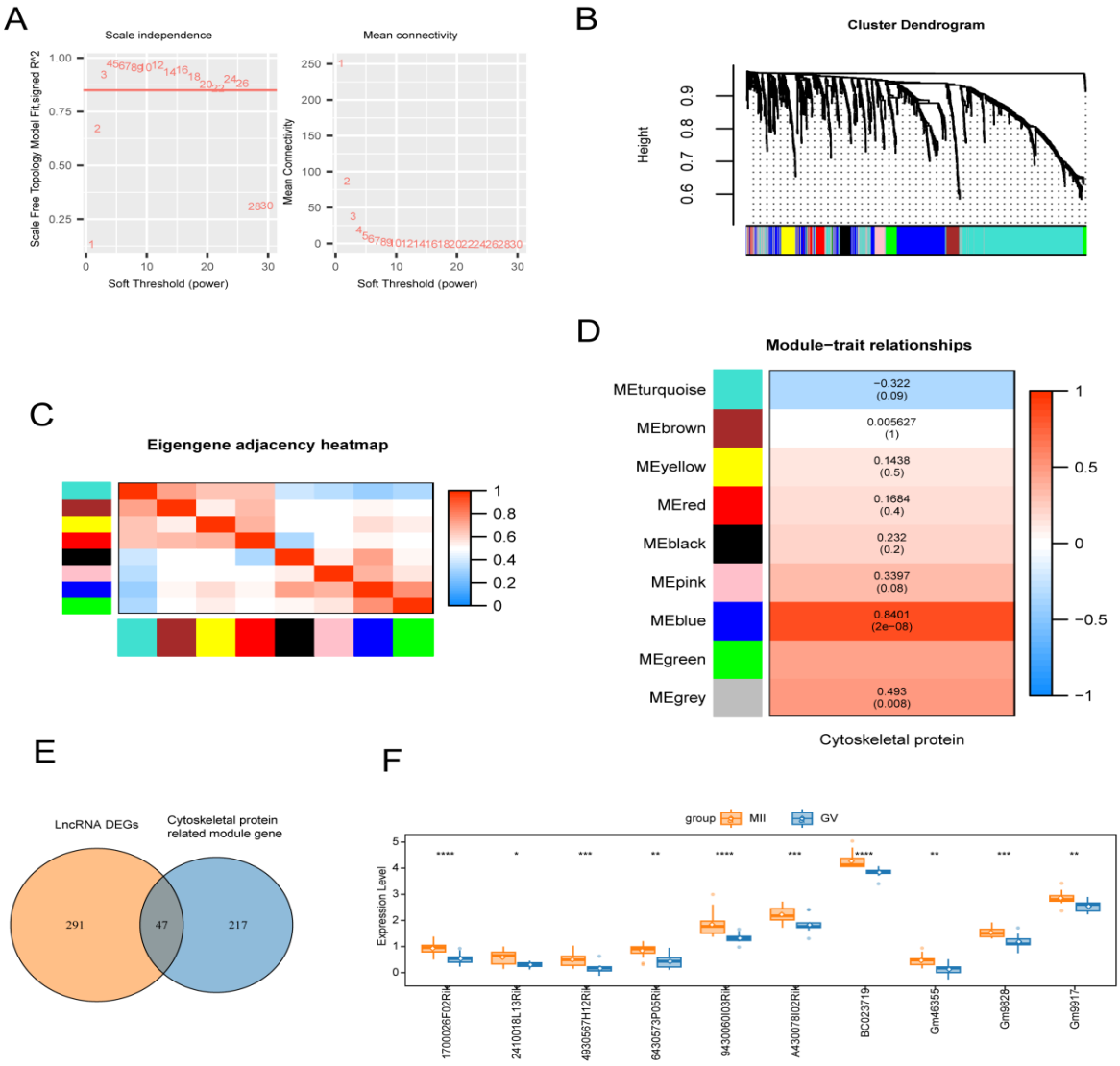

Figure 2. Construction of the WGCNA Co-expression Network

(A) Scale-free topology model fit index (R^2) when the soft-thresholding power β = 3. The high R^2 value above 0.85 indicates that the constructed weighted gene co-expression network adheres to the scale-free property, which is crucial for analyzing gene-gene relationships in a biological context. (B) Identification of distinct co-expression modules from the lncRNA expression network in the GV stage. A different color represents each module. The clustering of lncRNAs into modules based on their co-expression patterns helps in understanding the coordinated functions of these non-coding RNAs during oocyte development. This visual representation provides insights into the similarities and differences in the expression trends of different modules. (C) Relationships among the identified modules. Top: Hierarchical clustering dendrogram of module eigengenes (MEs), summarizing the clustering of modules. Branches of the dendrogram (meta-modules) group positively correlated module eigengenes. Bottom: Heatmap showing the correlations between module eigengenes. Each row and column represent an eigengene of a module (color-coded). In the heatmap, red indicates high adjacency, and blue indicates low adjacency. Red squares along the diagonal correspond to meta-modules. (D) Heatmap showing the correlations between consensus module eigengenes and cytoskeleton-related genes. Each row corresponds to a consensus module, and each column corresponds to a trait (cytoskeletal proteins). The numbers represent correlation coefficients, with p-values shown in parentheses below. The color legend indicates the strength and direction of the correlations. The strong positive correlation of the blue module with cytoskeletal proteins (r = 0.8401, P < 0.05) suggests its significant role in cytoskeletal regulation during oocyte development. (E) Venn diagram illustrating the intersection between differentially expressed lncRNAs (DElncRNAs) and lncRNAs within the blue module. The identification of 47 lncRNAs at this intersection implies that these lncRNAs are likely to be involved in both the differential expression events between GV and MII stages and the regulation of cytoskeletal proteins. (F) Box plots showing the expression differences of the top 10 lncRNAs between GV and MII control groups. Statistical significance was assessed by the Wilcoxon rank-sum test. Asterisks indicate p-values: ****p < 0.0001, ***p < 0.001, **p < 0.01, *p < 0.05. The significant differences in expression levels of these lncRNAs further support their potential importance in oocyte development.

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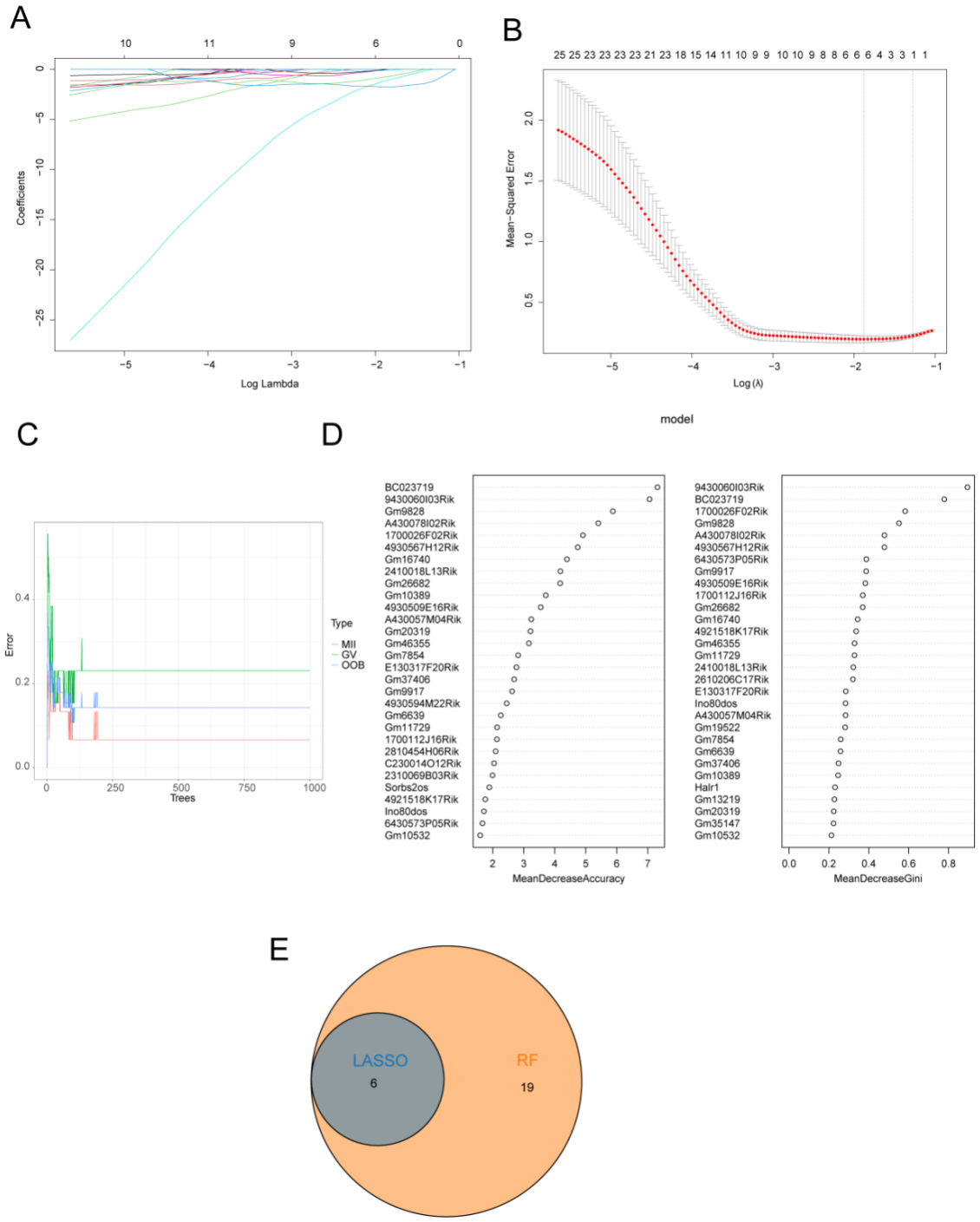

Figure 3. Selection of Candidate Diagnostic Biomarkers for Oocyte Development Progression Using Machine Learning Approaches

Note: (A) LASSO regression coefficient profiles of the candidate variables. The horizontal axis represents the logarithm of the lambda parameter, and the vertical axis represents the coefficients of the independent variables. As the lambda value changes, the coefficients of the candidate lncRNAs are shrunk, and the most relevant lncRNAs are retained in the model. (B) The cross-validation curve for the LASSO regression model shows the confidence intervals for each lambda value. The optimal lambda value is selected based on the minimum cross-validation error, ensuring the model's reliability and generalization ability. (C) Random forest model error rates plotted against the number of classification trees, highlighting the key lncRNAs identified. As the number of trees increases, the error rate stabilizes, and the importance of different lncRNAs can be evaluated based on metrics like MDA and MDG. (D) The top 30 cytoskeleton-related differentially expressed lncRNAs (DElncRNAs) ranked by two importance measures in the random forest analysis: Mean Decrease Accuracy (MDA) and Mean Decrease Gini (MDG). These rankings help identify the most influential lncRNAs in the context of cytoskeletal regulation during oocyte development. (E) Venn diagram illustrating the intersection of lncRNAs identified by machine learning algorithms (LASSO regression and random forest). The six lncRNAs at the intersection are considered the most significant and are selected as hub lncRNAs for further in-depth analysis.

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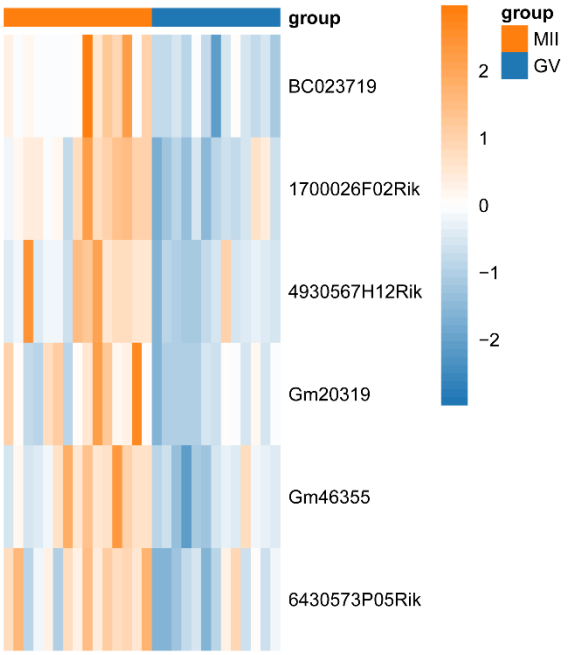

Figure 4. Heatmap Showing the Expression Levels of Hub lncRNAs in GV and MII Stages. The heatmap clearly shows the differential expression of the six hub lncRNAs between the GV and MII stages. The upward trend in expression from GV to MII stages indicates their potential involvement in the regulatory processes associated with oocyte maturation.

We also generated box plots to demonstrate the distribution of hub lncRNA expression levels in various sample groups. The box plots illustrate the median, interquartile range, and any outliers in the data, providing a more comprehensive view of the expression variability.

3.5 Analysis of the LncRNA-miRNA-mRNA Regulatory Axis

To further demonstrate the regulatory mechanisms of the key lncRNAs, this study focused on the six most critical cytoskeleton-related lncRNAs and predicted their potential interacting miRNAs. Using four bioinformatics tools for miRNA prediction, 370 candidate miRNAs potentially interacting with the six lncRNAs were identified by taking the intersection of the results (Figure 5A). To explore the downstream regulatory roles of these miRNAs, five additional prediction tools were applied to identify their target mRNAs. By intersecting the prediction results, 608 potential target genes were obtained (Figure 5B). These findings suggest that the main lncRNAs may form lncRNA-miRNA-mRNA regulatory axes by interacting with specific miRNAs, thereby indirectly regulating gene expression in ovarian development.

We constructed a more detailed lncRNA-miRNA-mRNA regulatory network diagram. In this diagram, each lncRNA is represented as a circular node, miRNAs as triangular nodes, and mRNAs as rectangular nodes. The edges connecting the nodes represent the predicted interactions, with arrows indicating the direction of regulation. The thickness of the edges can be adjusted to represent the confidence level of the prediction, based on the number of algorithms that predicted the interaction. This diagram offers a more intuitive understanding of the complex regulatory relationships.

3.6 Key Regulatory Roles of the LncRNA-miRNA-mRNA Axis in Follicular Development and Its Biological Processes

To further elucidate the regulatory mechanisms of key lncRNAs in follicular development, this study constructed lncRNA-miRNA-mRNA regulatory axes and conducted an in-depth functional analysis of the target genes of the associated miRNAs (Figure 6). GO functional enrichment analysis revealed that the miRNA target genes play essential roles in multiple key biological processes during follicular development, particularly in cell proliferation, differentiation, signal transduction, and cytoskeletal dynamics remodeling. Among the enriched pathways, terms such as "positive regulation of developmental process," "positive regulation of cell differentiation," and "positive regulation of cell population proliferation" emphasized the potential involvement of these miRNA target genes in promoting the rapid proliferation and differentiation of follicular cells.

For example, in the context of cell proliferation, the target genes may regulate the cell cycle by interacting with key cell-cycle-related proteins. In cell differentiation, they may modulate the expression of transcription factors that are crucial for determining the fate of follicular cells. Regarding cytoskeletal dynamics remodeling, the target genes might influence the polymerization and depolymerization of actin filaments and microtubules, which are essential for maintaining the structural stability of follicular cells and facilitating their movement.

In addition, reproductive system-related pathways, such as "reproductive system development" and "reproductive structure development," further suggest that these miRNA target genes may play important roles in ovarian development and functional maturation. Moreover, signaling pathways, including the Wnt signaling pathway and the regulation of the MAPK cascade, revealed a complex interaction between cytoskeletal dynamics and intracellular signal transduction. These pathways are likely to be crucial for coordinating the communication between oocytes and granulosa cells, ensuring synchronized follicular development and intercellular interaction within the follicular microenvironment. Importantly, the organization and dynamic regulation of the cytoskeleton are indispensable across various stages of follicular development. The enrichment of pathways such as "cytoskeleton organization," "actin cytoskeleton organization," and "regulation of cytoskeleton organization" indicates that the miRNA target genes may regulate cytoskeletal remodeling. These processes are vital for maintaining follicular structural stability, establishing oocyte polarity, and ensuring accurate chromosome segregation. Beyond providing mechanical support, the cytoskeleton may also coordinate with signaling networks to influence the proliferation, differentiation, and functional coordination of cells within the developing follicle.

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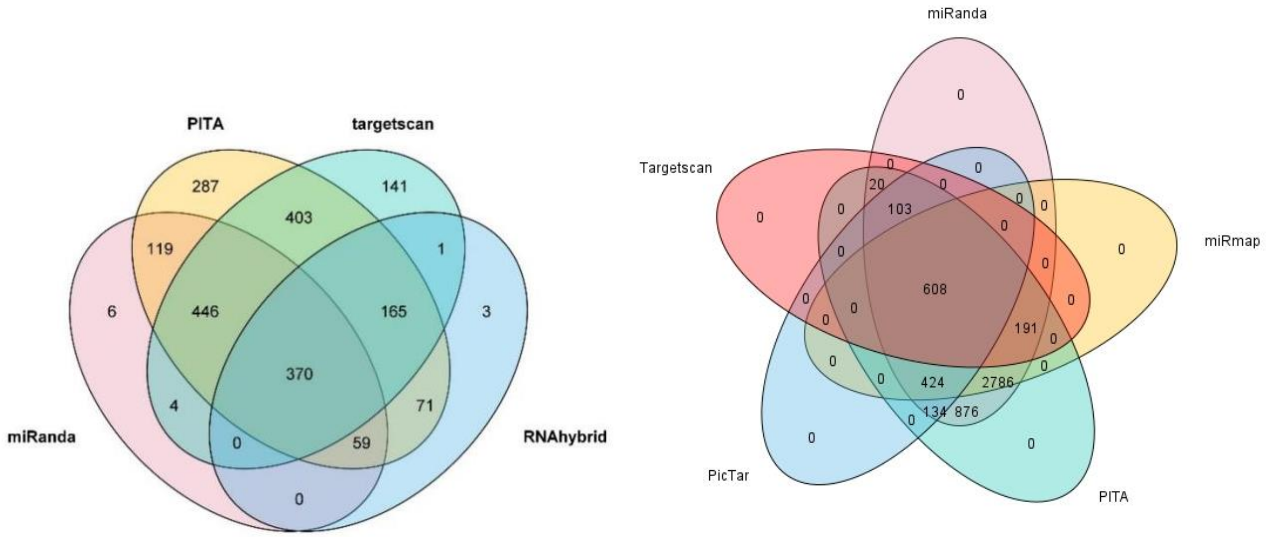

Figure 5. Identification of High-Confidence lncRNA-miRNA Interactions and Identification of miRNA Target Genes

Note: (A) The interaction between lncRNA and miRNA was predicted using four bioinformatics tools (Miranda, PITA, TargetScan, and RNAhybrid). By integrating the predictions from these four tools, 370 candidate miRNAs were identified, which are more likely to interact with six key lncRNAs. (B) The downstream target genes of the 370 candidate miRNAs were predicted using five bioinformatics tools (TargetScan, Miranda, miRmap, PITA, and PicTar). By analyzing the intersection of the results from these tools, 608 potential target genes were identified.

3.7 *Signaling Pathways and Cytoskeletal Dynamics Regulated by the LncRNA-miRNA-mRNA Axis During Follicular Development*

To further elucidate the mechanisms by which the lncRNA-miRNA-mRNA regulatory axis influences follicular development, KEGG pathway enrichment analysis was conducted to investigate its potential biological functions. The results demonstrated that the miRNA target genes were significantly enriched in several key signaling pathways, including the MAPK, PI3K-Akt, Notch, and Hippo signaling pathways. These pathways are likely involved in granulosa cell proliferation and differentiation, oocyte maturation, cell-cell communication, and the regulation of follicular reserve.

For instance, in the MAPK signaling pathway, activation can lead to the phosphorylation of various downstream targets, which in turn regulate processes such as cell proliferation and differentiation in granulosa cells. In the PI3K-Akt signaling pathway, activation promotes cell survival and growth, which is crucial for the development and maintenance of follicles. The Notch signaling pathway plays a role in cell fate determination and cell-cell communication between granulosa cells and oocytes. The Hippo signaling pathway regulates organ size and tissue homeostasis, which may be relevant to follicular growth and maturation.

In addition, the enrichment of Focal adhesion and Tight junction signaling pathways suggests that granulosa cells may preserve follicular structural integrity through interactions with the extracellular matrix (ECM). The enrichment of VEGF and Chemokine signaling pathways indicates potential roles in angiogenesis and local microenvironment regulation, which are essential for follicular growth and immune balance.

Notably, the significant enrichment of the Regulation of Actin Cytoskeleton signaling pathway underscores the critical role of cytoskeletal dynamics during follicular development. By providing essential mechanical support, cytoskeletal remodeling may contribute to sustaining oocyte maturation as well as the structural and functional stability of the follicle.

4. Discussion

In this study, the construction of a lncRNA-miRNA-mRNA regulatory axis, combined with several analytical approaches, systematically clarified the functions of key lncRNAs in mouse follicular development, particularly their roles in cytoskeletal dynamic remodeling and the regulation of signaling pathways. Through an integrated analysis involving differential expression analysis, weighted gene co-expression network analysis (WGCNA), LASSO regression, and random forest algorithms, we identified core lncRNAs associated with cytoskeletal regulation and further examined their potential regulatory mechanisms.

Firstly, we employed WGCNA to investigate the co-expression relationships between differentially expressed lncRNAs and cytoskeleton-related genes. In recent years, WGCNA has been widely used in biological research for mining co-expression patterns from high-dimensional gene expression data to identify key regulatory modules and candidate genes (B. Zhang & Horvath, 2005). In our study, WGCNA identified eight co-expression modules, among which the blue module showed the strongest positive correlation with cytoskeleton-related genes ($R > 0.8$, $P < 0.01$). This method's reliability and biological relevance have been

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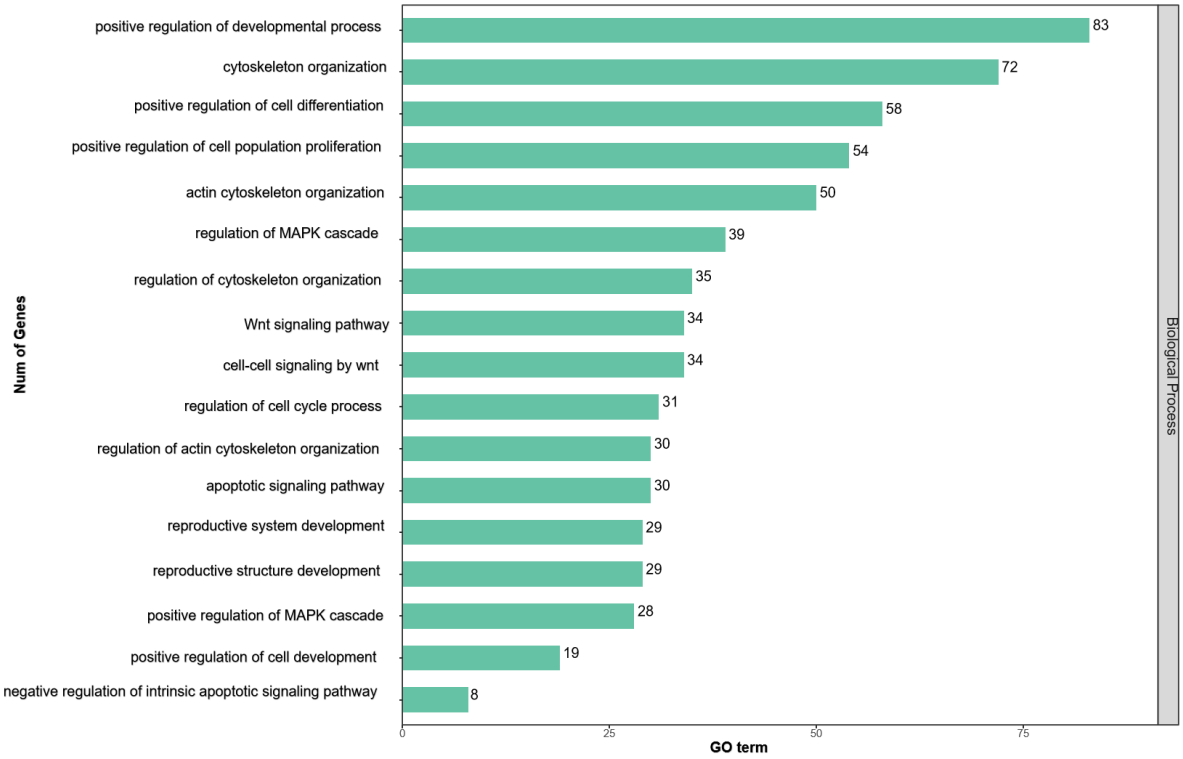

Figure 6. Biological Process Regulation of the LncRNA-miRNA-mRNA Regulatory Axis in Follicular Development.

Note: The figure visually represents the various biological processes in which the lncRNA - miRNA - mRNA regulatory axis is involved during follicular development. The enrichment of these processes in the miRNA target genes indicates the significance of this regulatory axis in orchestrating follicular growth and maturation. The figure depicts the key signaling pathways in which the miRNA target genes are enriched. These pathways are intricately linked to the processes of follicular development, and the lncRNA - miRNA - mRNA regulatory axis appears to play a central role in modulating these pathways.

validated in other models, including identifying key lncRNAs in cancer and cardiovascular diseases (Yin et al., 2020; Song et al., 2023). By intersecting the differentially expressed lncRNAs (DElncRNAs) with those in the blue module, we identified 47 cytoskeleton-related DElncRNAs, which are likely to play essential roles in oocyte polarity establishment, cytoskeletal remodeling, and cytokinesis.

To further pinpoint the most functionally significant lncRNAs, we applied LASSO regression and random forest methods. Through these two machine learning techniques, we ultimately identified six hub lncRNAs, including BC023719, Gm20319, and 1700026F02Rik, which are likely to play dominant roles in regulating follicular development and were subjected to further regulatory network analysis.

Gene Ontology (GO) enrichment analysis revealed that the lncRNA-miRNA-mRNA regulatory axis was significantly enriched in biological processes such as "cytoskeleton organization", "actin cytoskeleton organization", and "regulation of cytoskeleton organization", emphasizing the central role of the cytoskeleton in follicular development. Cytoskeletal dynamic remodeling is necessary for oocyte polarity establishment and meiotic division, particularly in chromosome segregation, maintenance of oocyte morphology, and granulosa cell migration (Mao et al., 2014; Roeles & Tsiavaliaris, 2019). Additionally, GO analysis highlighted the role of the cytoskeleton in cell proliferation and differentiation, as shown by pathways such as "positive regulation of cell differentiation" and "positive regulation of developmental process", indicating that dynamic cytoskeletal changes regulate cell morphology and interactions, enabling the transition of follicles from the primary stage to the dominant follicle stage (Irles et al., 2016; Mogollón García et al., 2024).

KEGG pathway enrichment analysis further revealed several classical signaling pathways involved in cytoskeletal regulation and follicular development. Notably, pathways such as "Regulation of Actin Cytoskeleton", "Focal Adhesion", and "PI3K-Akt Signaling Pathway" demonstrated that cytoskeletal dynamics not only provide mechanical stability for granulosa cells but also coordinate cell migration, polarity, and proliferation through integrated signal transduction, thereby establishing the foundation for follicular development (Gardel et al., 2010; Greig & Bulgakova, 2020). Moreover, the MAPK signaling pathway regulated cell adhesion and intercellular communication between granulosa cells, ensuring the organized and coordinated development of follicular cells (Long et al., 2021; Shen et al., 2023). Of particular note, the enrichment of the VEGF signaling pathway suggests that cytoskeletal regulation may contribute to follicular angiogenesis, ensuring an adequate supply of nutrients and energy to the oocyte (Ortega Serrano et al., 2016; Guzmán et al., 2023). The complex interplay between these signaling

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pathways and the cytoskeleton highlights the dual role of the cytoskeleton as both a structural scaffold and a key center for signal transduction and developmental regulation during

key lncRNAs could potentially serve as novel diagnostic biomarkers. By quantifying the expression levels of these lncRNAs in ovarian tissue or biological fluids such as follicular fluid, it may

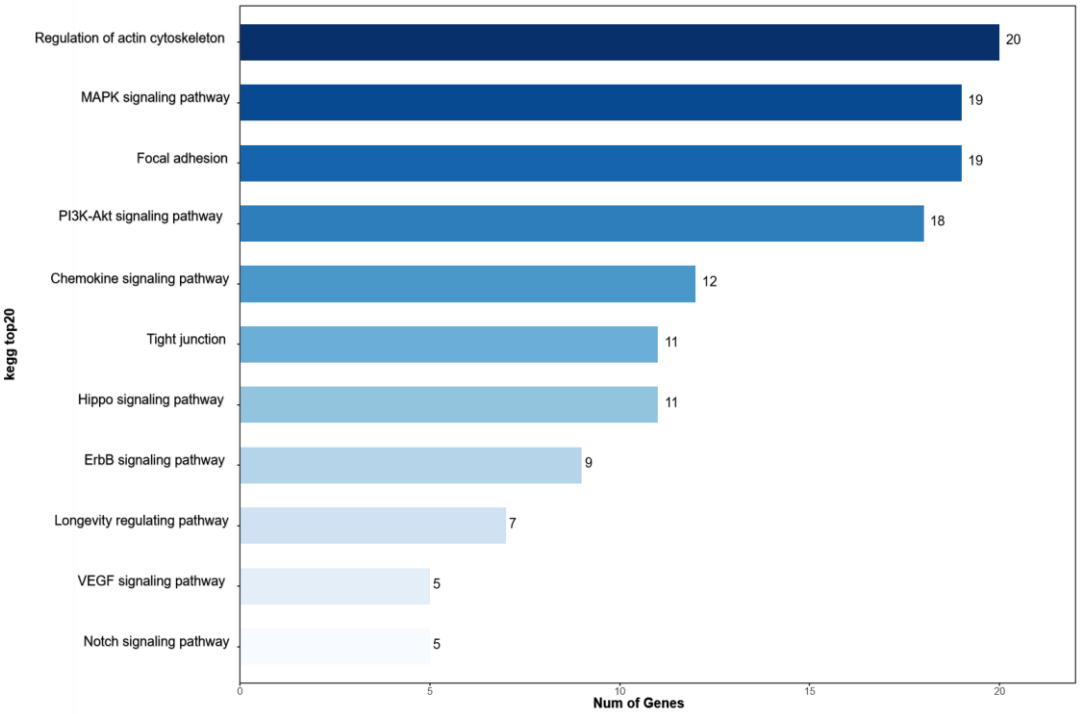

Figure 7. Signaling Pathway Regulation Mediated by the LncRNA-miRNA-mRNA Regulatory Axis During Follicular Development

follicular growth. By constructing the lncRNA-miRNA-mRNA regulatory axis, this study further clarified the mechanisms by which the cytoskeleton is regulated during follicular development. Core lncRNAs may regulate the dynamic remodeling of the cytoskeleton indirectly by modulating downstream miRNAs and mRNAs. For example, the Notch and Hippo signaling pathways play essential roles in the interaction between granulosa cells and oocytes by stabilizing the cytoskeleton and controlling its associated functions (Hu et al., 2019; Hubbard et al., 2019). Similarly, the Focal Adhesion and Tight Junction pathways preserve follicular structural integrity by controlling granulosa cell adhesion and intercellular connections (Yamada et al., 2013; Campbell et al., 2017). These regulatory processes collectively ensure the coordinated organization and functionality of the cytoskeleton during follicular development.

However, it is important to recognize the limitations of this study. The predictions of miRNA-target interactions and the constructed regulatory network are derived from bioinformatics algorithms, which may present certain levels of false-positive and false-negative rates. The data employed in this study were obtained from public databases. Although we performed quality assessments, the sample size remained relatively limited, which may influence the generalizability of the results. Additionally, the biological roles of the identified lncRNAs require further verification through experimental investigations.

In the context of ovarian disease diagnosis and treatment, our findings have relevant implications. For example, the identified

be possible to evaluate the quality of oocytes and the health of the ovarian follicles. This could provide valuable information for early detection of ovarian disorders related to oocyte maturation defects, such as polycystic ovary syndrome (PCOS) and premature ovarian insufficiency (POI) (D. Li et al., 2021; Y. Li & Tan, 2021).

Furthermore, targeting these lncRNAs or components of the lncRNA-miRNA-mRNA regulatory network might represent a promising therapeutic approach. For instance, if a specific lncRNA is demonstrated to play a critical role in promoting abnormal cytoskeletal remodeling in diseased ovaries, developing small molecule inhibitors or RNA-based therapies to modulate its expression could potentially restore normal oocyte development and improve fertility outcomes. However, before translating these findings into clinical practice, further pre-clinical and clinical studies are necessary to validate the efficacy and safety of such interventions.

To progress toward clinical application, future research should emphasize the experimental validation of these findings, particularly through in vivo studies examining the functional roles of the six identified hub lncRNAs. Overexpression and knockdown experiments should be conducted to evaluate how these lncRNAs regulate cytoskeletal dynamics and affect oocyte development. By manipulating the expression of these lncRNAs in mouse models, we can explore their direct effects on spindle formation, chromosome segregation, and overall oocyte maturation. Additionally, assessing how these lncRNAs interact with their target miRNAs and mRNAs in vivo will provide essential insights

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into their mechanistic roles during oogenesis. These experimental validations are crucial for confirming the clinical relevance of these lncRNAs and their potential as therapeutic targets in reproductive medicine.

This study identifies key lncRNAs significantly associated with cytoskeletal regulation during oocyte development, particularly between the GV and MII phases. Through various computational methods, including WGCNA, LASSO regression, and random forest analysis, we identified six hub lncRNAs—BC023719, Gm20319, 1700026F02Rik, 4930567H12Rik, Gm46355, and 6430573P05Rik—that play central roles in regulating cytoskeletal dynamics. Gene ontology and KEGG pathway enrichment analyses further support the involvement of these lncRNAs in important pathways such as "cytoskeleton organization," "MAPK signaling," and "PI3K-Akt signaling." Additionally, the construction of a lncRNA-miRNA-mRNA regulatory network suggests that these lncRNAs may indirectly affect follicular development by modulating downstream miRNA and mRNA targets.

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Rare Case of High Voltage Electric Injury Resulting in Bowel Perforation: A Successful Management with Diversion Colostomy

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Abstract: Electrical injuries are categorized into low-voltage injuries (<1 kilovolt) and high-voltage injuries (>1 kilovolt). Electrical injuries usually present with an entry wound at the site of contact and an exit wound where the current leaves the body. Bowel perforation caused by an electric current is a rare but serious complication. We report a case of a high-voltage electric burn with entry wounds on both hands and an exit wound on the right foot, complicated by descending colon perforation, which presented late with peritonitis. The patient was successfully treated with a diversion colostomy and subsequent takedown. This case highlights the potential for significant internal injuries, such as bowel perforation, following high-voltage electrical injuries and emphasizes the importance of timely surgical intervention for such complications.

Keywords: Electric injury, descending colon perforation, diversion colostomy

1. Introduction

Electrical burns account for 2–5% of all burn admissions and may cause extensive internal damage despite minimal external injury (Williams et al., 2010). Based on voltage, electrical burns are classified as low-voltage (<1,000 volts) and high-voltage (>1,000 volts), with the latter more frequently resulting in deep tissue necrosis and systemic complications. Visceral involvement following electrocution is uncommon, and gastrointestinal perforation is an exceptionally rare but serious consequence. The underlying mechanisms include direct thermal injury, vascular thrombosis, and delayed ischemic necrosis of the bowel wall. Since clinical manifestations may appear several days after the initial injury, diagnosis is often delayed, necessitating a high index of suspicion (Lee, 1997). We present a rare case of descending colon perforation secondary to high-voltage electrical injury, successfully managed with a diversion colostomy, highlighting the importance of early surgical intervention to prevent catastrophic outcomes.

2. Case presentation

A 52-year-old construction worker sustained a high-tension electric current injury involving both hands and his right foot while using a crowbar that accidentally made contact with a high-tension electric wire. Physical examination revealed second-degree burns on the palmar aspects of both hands and third-degree burns affecting the great, second, and third toes of the right foot (Fig.1). There was no limitation in joint movement.

On clinical examination, he appeared dehydrated, with tachycardia (110/min), blood pressure of 100/60 mmHg, and a respiratory rate of 25/min. ECG findings were normal, and urine output was sufficient. He received intensive monitoring and treatment.

On the third day, he complained of left-sided abdominal pain and distension. Physical examination revealed a distended abdomen with tenderness, rebound tenderness, and absent bowel sounds, indicating peritonitis. Abdominal X-ray revealed pneumoperitoneum, and CT scan showed thickening of the descending colon with fat stranding, free fluid in the peritoneal cavity, and basal atelectasis.

An emergency exploratory laparotomy was conducted, revealing a large 2 cm perforation in the lower descending colon with peritonitis. A diversion colostomy with Hartmann's procedure was performed. He was initially cared for in the ICU and transferred to the ward on the fourth day.

Subsequently, his right foot developed gangrenous changes, and the vascular surgeon recommended amputation of the necrotic toes. He recovered well and was discharged on the 12th postoperative day.

Patients with injuries caused by high-voltage electrocution typically have both an entrance and an exit burn wound, each with different complications. The entrance burn wound generally occurs where electrical contact with the body first occurred, usually on the hands, arms, or feet. This wound may extend into deeper tissues, resulting in severe thermal burns, tissue necrosis, and damage to underlying muscles or bones.

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The exit burn wound or wounds are located where the electric current exited the body, typically affecting the feet or hands and often causing more severe, full thickness burns. Exit wounds can lead to extensive burns, deep tissue necrosis, fractures, and muscle injury.

High-voltage electrocution can also cause significant internal complications. Cardiac arrhythmias, neurological injuries, and bowel perforations are serious concerns, with bowel perforations potentially presenting later with peritonitis.

The intraoperative findings indicated perforation of the descending colon [Fig.2 (A)] and a congested intestine [Fig.2(B)], both of which were significant injuries resulting from electrocution. The perforation of the descending colon [Fig.2(A)] is particularly important, as it is a known complication that may arise due to the damaging effects of electric current on the bowel wall. Without prompt surgical intervention, this could lead to secondary complications such as peritonitis or sepsis.

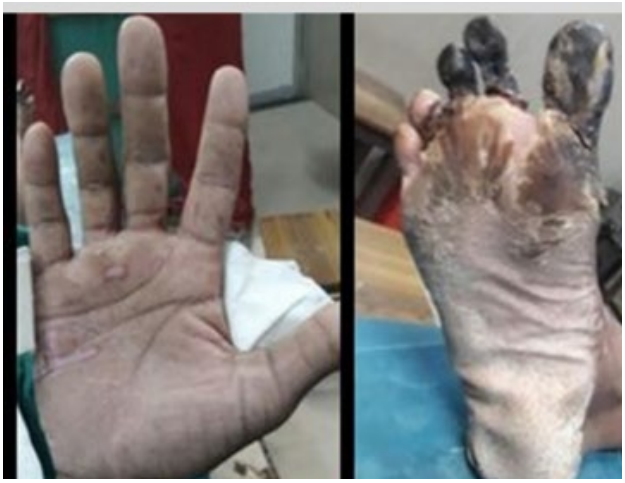

Figure 1. Entry and exit burn wound due to high voltage electrocution

Additionally, the congested intestine [Fig.2(B)] was indicative of ischemia and compromised blood flow, possibly due to the thermal effects of the electric current. These effects can lead to inflammation, vascular compromise, and swelling of the affected bowel segments. Immediate surgical treatment, such as a diversion colostomy, is required to manage the perforation and minimize the risk of further complications, including infection and organ failure.

The surgical procedure of a diversion colostomy [Fig.3] involves redirecting a portion of the colon to an external opening in the abdominal wall, resulting in a stoma (a small external opening) through which stool can pass into a colostomy bag. This procedure is generally performed to divert stool away from a diseased or damaged segment of the colon, allowing the affected area to heal or rest.

In cases of trauma, such as bowel perforation from electrocution, a diversion colostomy is commonly performed to manage contamination, prevent peritonitis, and avoid the passage of stool through the compromised bowel. The diversion colostomy may be temporary if the intent is to reverse the procedure once the injury has healed, or permanent, depending on the extent of bowel injury and overall patient condition (e.g., severe bowel ischemia).

3. Discussion

Electrical burns account for approximately 5% of hospital admissions in major burn centers. Electric current can cause internal injury through both direct and indirect mechanisms, with the severity influenced by several factors, including voltage, current intensity, the pathway of the current, duration of exposure, tissue resistance, contact surface, and the presence of underlying medical comorbidities.

During electrical burns, the electric current travels through underlying tissues, leading to coagulative necrosis and cell membrane rupture. The extent of injury varies based on individual susceptibility, the type of electrical source (AC vs. DC), and the quality of initial medical treatment. While external burns

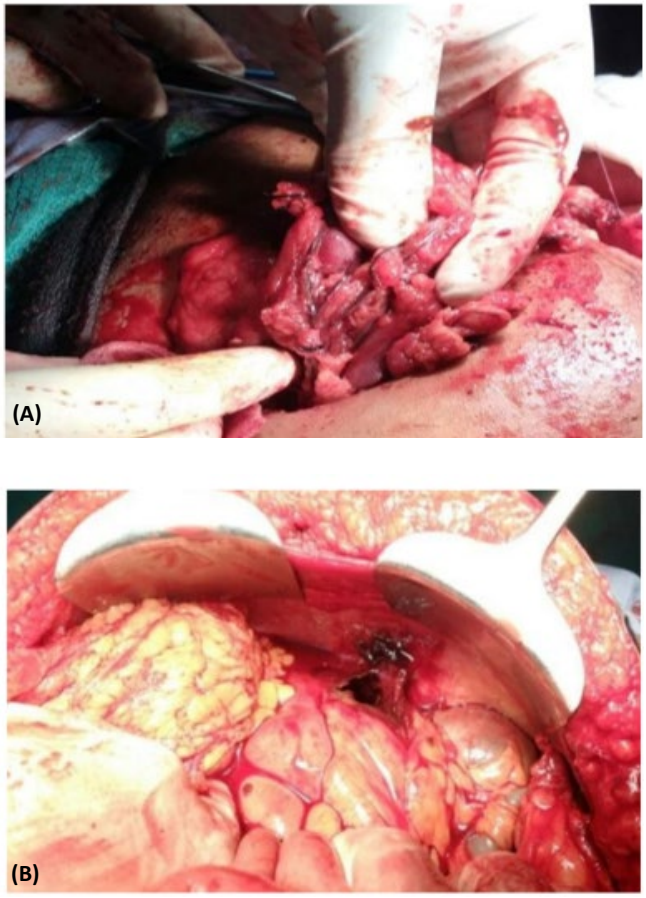

Figure 2. Intra op findings showing (A) perforation of descending colon and (B) the congested intestine

may be visible, internal injuries are often more severe and may progress insidiously, sometimes leading to delayed complications.

Visceral injuries are among the most severe consequences of high-voltage electrical burns and often require surgical intervention (Haberal et al., 1996). The most frequently affected organs are the colon and small intestine, while less commonly involved organs include the heart, esophagus, stomach, pancreas, liver, gallbladder, lungs, and kidneys. According to the literature,

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most bowel perforations caused by electric current involve the colon, particularly the descending or sigmoid colon, although the exact mechanism for this predilection remains poorly understood (Sharma et al., 2015). Some theories suggest that these regions may be more vulnerable due to their vascular supply, direct electrical conduction pathways, or proximity to sites of electrical grounding.

Delayed bowel perforation is a well-documented but common complication of electrical injuries, sometimes occurring days after the initial trauma. In one study, a patient developed bowel perforation despite hospitalization following an upper arm electrical injury (Goyal et al., 2020). Research conducted by Handaya et al. (2024) has emphasized intestinal perforation as a rare but important late complication of electrical injuries, necessitating high clinical suspicion for timely diagnosis and management (Handaya et al., 2024).

In one study, a 42-year-old male with a history of electric shock developed an ulcer in the right iliac fossa, which expanded and began extruding fecal matter. A CT scan revealed a full-thickness abdominal wall defect with prolapsed ileum, indicating evisceration, perforation, and bowel gangrene. An emergency laparotomy was performed, followed by bowel resection and end-to-end anastomosis (Reddy et al., 2023).

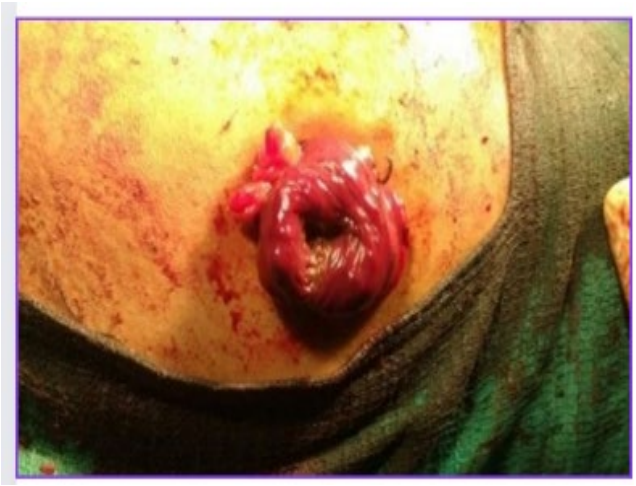

Figure 3. Diversion colostomy

Colonic perforation following burns was most frequently observed in middle-aged male patients, many of whom had a history of mental health conditions. These perforations primarily occurred on the right side of the colon, typically after the second week of hospitalization. Right-sided perforations were linked with a higher mortality rate compared to left-sided ones (Fadel et al., 2021).

Abdominal pain is one of the most significant features of post-burn gastrointestinal complications (Lopez et al., 2018). Many studies indicate that abdominal complications resulting from electrical injuries are uncommon; however, they should be considered if digestive symptoms appear (Buja et al., 2010).

A study conducted in Yemen reported that lightning strike injuries are relatively common natural events. However, cases of lightning-induced perforation of hollow viscera are extremely rare. Further tests confirmed bowel injury, requiring surgical repair of the small bowel perforation and removal of a hematoma from the omentum (Nasr et al., 2025).

A retrospective study in China (2020–2023) documented six male patients with penetrating high-voltage electrical burns to the thoracoabdominal wall. One patient had defects involving the gastric wall and diaphragm, two had gastric wall perforations, and three had small intestinal perforations. Three patients with gastric perforations underwent subtotal gastrectomy and anastomosis, with one additionally requiring diaphragmatic repair. The three patients with small intestinal perforations underwent resection and anastomosis (Ai et al., 2024).

According to the literature, the mortality rate for electrical injuries ranges from 2.7% to 5.3% (Butler & Gant, 1977; Kasana et al., 2016). Causes of death may include the electrical injury itself (such as cardiac arrest), wound-related complications, or systemic complications (Sokhal et al., 2017).

The treatment of intestinal perforation depends on factors such as the extent of injury (localized versus diffuse), anatomical site, and presence of peritonitis. In this case, an exploratory laparotomy revealed a descending colon perforation, which was managed with a diversion colostomy and Hartmann's procedure. Early recognition and prompt surgical intervention are essential to reducing morbidity and improving patient outcomes in such cases.

4. Conclusion

Electric injury can cause intestinal perforation through direct thermal damage and secondary effects on tissue viability. High-voltage electric currents (above 1,000 volts) produce intense heat, leading to immediate tissue coagulation and necrosis. Although the gastrointestinal tract is less frequently affected, it can sustain substantial injury when the current path involves the abdomen.

Electric burns disrupt blood flow, resulting in ischemia and delayed perforation. Symptoms frequently present late and include abdominal pain, fever, and peritonitis. Diagnosis typically involves imaging techniques such as X-rays or CT scans, which may reveal free air suggestive of perforation.

Prompt diagnosis and active treatments are crucial. Electrical burns presenting with colonic perforation within 12 hours can often be effectively managed with primary repair. However, a diversion colostomy followed by a subsequent takedown is the preferred management strategy in delayed presentations.

Multidisciplinary care involving surgeons, intensivists, and burn specialists is essential for improving outcomes in patients with electric injury-induced intestinal perforation.

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Behavioral and Neuronal Alterations Following Oral Naphthalene Exposure in Rats

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Abstract: Naphthalene (NP), a widely used polycyclic aromatic hydrocarbon, is present in various commercial products and environmental pollutants. Despite its established toxicity, its impact on neuronal integrity and behavior remains relatively underexplored. This study investigates oral NP-induced behavioral and neuronal alterations in Sprague Dawley rats. Twenty-five animals were divided into five groups and oral NP was administered at varying doses (200 mg/kg and 400 mg/kg) for 28 days, with post-treatment evaluations up to 42 days. Behavioral assessments using the open field test revealed initial hyperactivity, followed by a progressive decline in locomotion and increased anxiety-related behavior in high-dose groups. Cresyl violet staining of the basolateral amygdala showed significant neurodegeneration, with pyramidal neuronal damage more pronounced in high-dose groups. Statistical analysis was conducted using one-way ANOVA, and post-hoc Duncan's test was applied to confirm a dose-dependent decrease in neuronal viability ($p < 0.05$). Post-treatment observations indicated partial behavioral recovery but no significant reversal of neuronal damage. The findings suggest that oral NP exposure induces anxiety-associated behavioral changes and neurotoxicity in the amygdala, potentially affecting emotional regulation. Further investigation is required to assess the long-term effects of oral NP exposure on brain function.

Keywords: Napthalene; toxicity; neuronal alteration; behavioral alteration; neurotoxicity

1. Introduction

Naphthalene (NP) is a polycyclic aromatic hydrocarbon, characterized as a white solid that readily sublimates into gas. It is primarily obtained from coal tar. NP is also present in various types of smoke, including cigarette smoke, forest fire emissions, and automobile exhaust (Angu Bala Ganesh et al; 2024).

NP is widely used in the manufacture of commercial goods, most notably as the active component in mothballs (naphthalene balls) or crystals that release toxic vapours to repel insects and animals. Naphthalene was first documented as a pesticide in the United States in 1948 (CDC, 2005). It also produces toilet deodorant blocks, pesticides, leather tanning agents, dyes, resins, and PVC polymers (Baker et al., 2011; Preuss et al., 2003).

NP can be introduced and absorbed through four distinct pathways: orally, by ingesting contaminated water with NP; dermally, by contact with fabrics treated with NP; via inhalation, through low concentrations present in indoor and outdoor air, including emissions from factories during the production and handling of commercial goods, as well as smoke from tobacco, wood, and coal; and through ocular exposure, either from NP vapours or by touching the eyes with contaminated hands (Mugweru et al., 2020; Wang et al., 2020). The absorbed NP metabolites are distributed via the bloodstream to many organs, particularly the heart, lungs, liver, kidneys, and spleen, and are ultimately eliminated through urine and faeces (Waidyanatha et al., 2020; Mugweru et al., 2020).

The basolateral nucleus of the amygdala (BLA) engages in two-way communication with brain regions that influence cognition, motivation, and stress responses, including the prefrontal cortex, hippocampus, nucleus accumbens, and hindbrain areas that activate norepinephrine-mediated stress responses. The hyperexcitability of BLA principal neurons is related to behavioral disorders characterized by excessive fear and anxiety (Etkin et al., 2004, Angu Bala Ganesh., 2024).

Many studies have been conducted and shown that NP toxicity produces histological changes in multiple organs of animal models, such as hyperplasia of hepatic bile ducts (Radoslaw Świercz & Maciej Stępnik, 2011), thickening of the inter-alveolar septa and hepatocellular necrosis (Fang Zhang et al., 2016), vacuolisation in Clara cells (Ching-Yu Lin et al; 2015).As a result of naphthalene-induced hemolysis, newborn neonates are at risk for

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permanent neurological impairment known as kernicterus. Convulsions and, in rare cases, death occur when brain cells absorb more bilirubin. Survivors often suffer from motor abnormalities and dementia. It has been reported that kernicterus occurred in eight of twenty-one Greek newborns who had hemolysis due to naphthalene exposure (Valaes et al., 1963).

More recent case reports have reaffirmed this association, particularly in infants with glucose-6-phosphate dehydrogenase (G6PD) deficiency, who are especially vulnerable to oxidative damage from naphthalene exposure through ingestion of naphthalene-containing mothballs (Bölükbaş et al., 2021; Koca, N., & Doğan, M., 2022).

Human case studies have documented neurological symptoms associated with naphthalene ingestion, including disorientation, altered sensorium, listlessness, lethargy, and vertigo. Individuals who ingested naphthalene exhibited severe symptoms such as convulsions, muscle fasciculations, reduced reactivity to painful stimuli, and coma prior to death. During autopsy, the brain exhibited edema, accompanied by histological signs of axonal disconnection and inflammation of the myelin sheaths (Sharma et al., 2024). Cerebral oedema, which was most likely caused by abrupt hemolysis, could be the cause of the neurologic symptoms observed after naphthalene exposure (Shaikh et al., 2023). However, previous research that did not focus on behavioral and neuronal changes due to NP toxicity was inconclusive. Hence, this study was conducted to analyse the effect of naphthalene on behavior and neuronal cell integrity.

2. Materials and methods

Chemicals: Naphthalene balls were obtained from Research Lab Fine Chemical Industries. All other chemicals were obtained from a local supplier of standard quality.

Animals: Male Sprague Dawley rats less than 9 weeks old were obtained from Invivo Biosciences, No. 23, Kodigehalli village, Eshwanthpur hobli, Bangalore North taluk, Bangalore. All animals were kept under 12-hour light and dark conditions. All experimental animals were randomly assigned to polypropylene cages and bedded with autoclaved paddy husk. The cages were covered with stainless-steel grid tops. Cages were changed on alternate days, and cage grilling occurred once a week. The animals were fed commercial rodent pelleted feed obtained from M/s. VRK Nutritional Solutions, Pune, and water ad libitum. The Institutional Animal Ethical Committee (IAEC) granted ethical permission via ethics certificate IAEC/60/SRIHER/674/2019. All experiments complied with IAEC and Sri Ramachandra Institute of Higher Education and Research procedures.

Test item preparation: Naphthalene balls were placed in a mortar, crushed, and ground into fine powder. The required test material was weighed, mixed with a small amount of vehicle, and formed into a fine pasty liquid using a pestle. This pasty liquid was transferred to a graduated measuring cylinder and adjusted to the desired volume by adding vehicle.

Induction of Naphthalene Toxicity: A total of 25 experimental animals were allowed five days of acclimatisation before being randomly assigned to one of five groups. The experimental period lasted 42 days (28 days for the main group and 14 days for post-treatment observation for satellite groups). The test item was administered daily as described below, with the dosage determined by body weight.

Dose Volume = (Body weight of the animal (g) × Dose) / (1000 × Concentration (mg/ml)). Control animals received the vehicle at a 5 ml/kg body weight dose. The test item was delivered orally through gavage.

The LD50 value, which represents the lethal dose that causes death in 50% of a set of test animals, is a crucial factor in toxicology studies. Naphthalene has an oral LD50 of over 2000 mg/kg/bw in rats. In this study, the LD50 value for naphthalene in rats was used to determine safe doses, with selected doses being 1/10 and 2/10 of the LD50 value. Table 1 presents the experimental design in this work.

Table 1. Experimental Design

S.No	Animal Groups	Treatment,	No	Dose and Duration
1	Group 1	(Control- Vehicle)	5	5ml corn oil/kg/for the period of 28 days
2	Group 2	Low Dose Naphthalene	5	200mg/kg/ for the period of 28 days
3	Group 3	High Dose Naphthalene	5	400mg/kg/day for 28 days
4	Group 4 (Satellite- Control Vehicle)	(Satellite Control- Vehicle)	5	5ml corn oil/kg/day for 28 days and 14 days post treatment observation period.
5	Group 5 (Satellite- High Dose)	(Satellite- High Dose Naphthalene)	5	400/mg/kg/ for the period of 28 days and 14 days of post treatment observation period.

2.1 Behavioural test

Open field test

Open field tests were conducted for all animals on days 0, 7, 14, 21, and 28 for the leading group and on days 35 and 42 for the satellite groups.

The open field apparatus consisted of a wooden box with dimensions of 60x60x60 cm and painted black except for the analysis area, which was equally divided into sixteen squares by drawing lines on the floor region, where the central analysis area included four squares. At the time of open field testing, the animals were placed in the right arena corner, and their movement around the analysis field was recorded. The experimental arena was cleaned with 70% alcohol after each group, and the following animal behaviour was analysed using the recorded video.

Locomotion measurement: Number of lines crossed by each animal during the given 5-minute interval.

Central latency: The time taken (in seconds) by an individual rat to enter the central square of the apparatus, defined as head entry and placement of two front paws, was measured in seconds.

After completion of 28 days, the rats in groups 1 to 3 were euthanised by deep anaesthesia with intraperitoneal injection of ketamine (50 mg/kg, b.wt) + xylazine (5 mg/kg, b.wt); death was confirmed via fixative perfusion with 10% neutral buffered

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formalin and the cessation of heartbeat. In groups 4 and 5 (satellite groups), the same procedure was followed for euthanasia after completion of 42 days.

2.2 Sample collection and Preparation

The excised rat brain tissues were immersed in ice-cold saline. The brain tissues intended for histological analysis were thoroughly washed with normal saline and then immersed in 10% formalin solution for one week. The brain samples were subsequently processed to reveal histological changes. Using a rotary microtome, tissue sections of 5 µm thickness were obtained, after which they were stained with Cresyl Violet to observe the neurodegenerative cells in the deep temporal region of the basolateral nucleus of the amygdaloid region.

Cresyl Violet Staining

The portions were deparaffinized and rehydrated in distilled water. The slides were incubated with a 0.1% cresyl violet stain for several minutes and washed with distilled water. The specimen was rapidly dehydrated in absolute alcohol, cleared in xylene, and mounted in synthetic resin. The cresyl violet-stained sections of the basolateral amygdala were examined using a 40x objective lens, and a random selection procedure quantified the neurons through the microscope.

Statistical Analysis

The open-field test results for central latency and locomotion were presented as mean ± SD. ANOVA was conducted for G1, G2, and G3, whereas paired t-tests were applied for the satellite groups (G4 and G5). Cresyl violet results were presented as mean ± SD with one-way ANOVA and post-hoc Duncan's test for group comparison. All analyses were statistically significant at $p < 0.05$.

3. Results

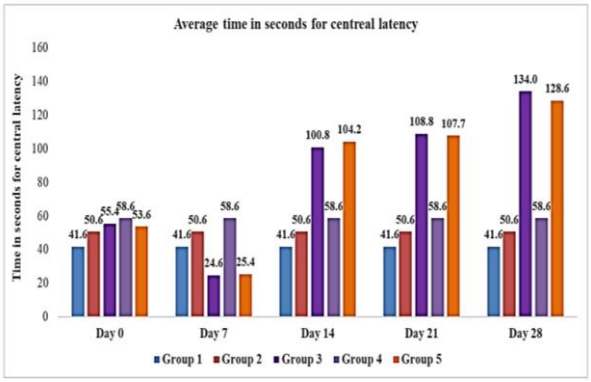

Figure 1. Behavioural changes in time in seconds for central latency in rats induced with naphthalene toxicity. Represented values are expressed as average (Mean) for five rats in each group. p<0.05* denotes significantly different compared to normal control rats.

3.1 Effect of Naphthalene on behaviour and locomotion

On the seventh day, central latency was shorter in G3 and G5 compared to G1, G2, and G4, and on the 14th, 21st, and 28th day, a gradual decrease in the central latency, i.e., delay in time to enter the central square, was noted in G3 and G5 (Figure 1).

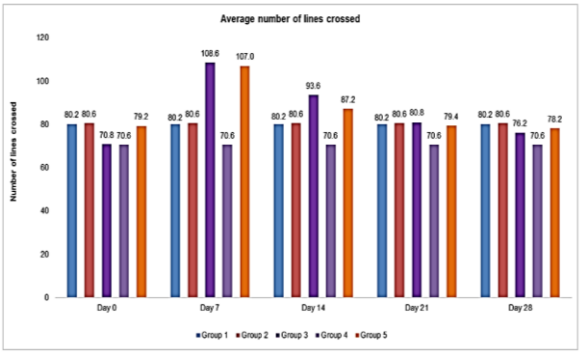

Figure 2. Behavioural changes in the number of lines crossed in rats induced by naphthalene toxicity. Represented values are expressed as average (Mean) for five rats in each group, and p<0.05* denotes significantly different compared to normal control rats.

It was found that, on day 7, there was an increase in the locomotor activity in G3 and G5 compared to G1, G2, and G4, and on the 14th, 21st, and 28th day, the mean number of lines crossed gradually decreased compared to day 7, which was noted in G3 and G5 (Figure 2).

Post-Treatment Observation: Central latency was shorter in both groups on the 42nd day than on the 35th day. G4 and G5 locomotor activity increased on the 42nd day compared to the 35th day. In the post-treatment observation period, no delayed withdrawal effects were noted in all the parameters of the open field test on G5.

3.2 Effect of Naphthalene in the Depth of the Temporal lobe in the basolateral amygdaloid region

The standard control group shows intact neuronal cell bodies with the typical morphological characteristics of pyramidal neuronal cells present in the depth of the temporal lobe in the basolateral amygdaloid region. At the same time, mild toxicity was observed in low-dose toxicity groups with subtle morphological abnormalities on the pyramidal cells. In the case of high-dose naphthalene toxicity, degenerated pyramidal cellular morphology with cell death was observed. The satellite control group was similar to the control group. Necrotic neurons were observed in the delayed toxicity group, similar to the naphthalene toxicity group (Figure 3).

Post-hoc Duncan's test for group comparison (pairwise comparison of groups) shows a mean difference in the total number of surviving neurons across all pairs. However, a statistically significant (p<0.05) mean difference was found between all pairs except for G1 and G4 and G3 and G5 of animals (Figure 4). It indicates that low, high, and satellite-high doses reduced the number of surviving neurons. In the post-treatment

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observation period, minor and delayed withdrawal effects were noted in the satellite-high doses.

4. Discussion

Naphthalene is well known for its cytotoxicity and has even been used as a therapeutic agent for its cytotoxic properties (Anwar et al., 2021). When naphthalene is administered as a drug,

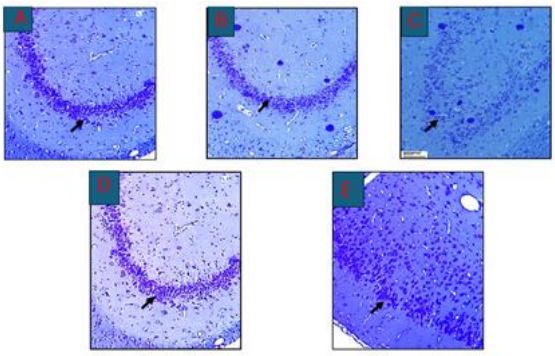

Figure 3. Cresyl violet staining analysis of naphthalene toxicityrats (magnification 40x). G1(A). G2(B), G3(C), G4 (D), G5 (E)

it converts into its reactive metabolites, like NP epoxides and NP-quinones. These active breakdown products are associated with cysteine residues of several intracellular proteins and cause toxicity. Primarily, NP oxides interact with the sulfhydryl group of cysteine and produce naphthoquinones (Jing et al., 2020).

Naphthalene, which is commonly used in households for pest control and deodorizing, has become an important yet problematic substance due to its widespread domestic use and subsequent emergence as a major environmental pollutant. This highlights the necessity for extensive toxicity testing in mammals (Pannu & Singla, 2020). Chlorinated forms of naphthalene enter edible products, especially animal food, which is considered an early exposure source in the general population. In human biological samples such as plasma, serum, milk, and adipose

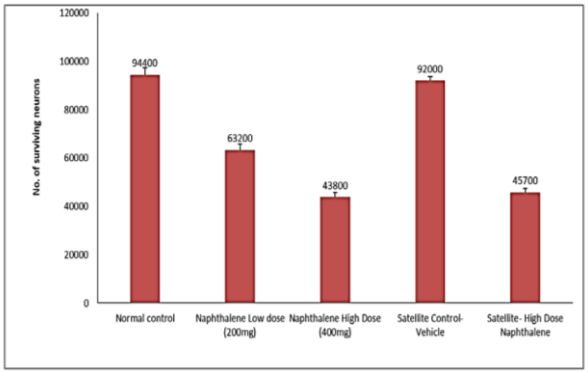

Figure 4. Quantification of surviving neurons across all groups using cresyl violet staining. Data are presented as Mean ± SD (n = 5 rats per group).

tissue, the chlorinated form of naphthalene has been detected (Jin et al., 2019; Li et al., 2021; Waheed et al., 2020).

The open field test is a well-established method for studying anxiety-like behavior and evaluating drug-related toxic effects on animal behavior (Zimcikova et al., 2017). This test can also be used to assess the levels of anxiety effects on movement activity under drug influence. On repeated exposure to the open field test, animals adapt to the open field area, and their movements tend to decrease (Choleris et al., 2001).

The total number of entries to the central square, the time spent in the central square, and the latency to enter the central square are indicators of exploratory behavior and anxiety (Zimcikova et al., 2017). Figure 2 illustrates the change in central latency in rats subjected to NP toxicity. It was found that, on day 7, central latency was shorter in G3 and G5 compared to G1, G2, and G4. Moreover, on days 14, 21, and 28, a gradual reduction in the central latency (in seconds), i.e., delay in time to enter the central square, was observed in the G3 and G5 groups.

On the seventh day of the test, the high-dose and high-dose satellite groups exhibited increased exploratory behavior and a quicker time to enter the central square. On days 14, 21, and 28, exploratory behavior decreased, and the time to enter the central square was prolonged. On days 35 and 42, the satellite high-dose group showed a slight increase in exploratory behavior.

To examine the neurotoxic effects, measures such as the number of line crossings and depressive and anxiety behaviors in animals were assessed using the open field test. Figure 3 shows the change in locomotor activity (average number of lines crossed) in rats induced with naphthalene toxicity. It was found that, on day 7, locomotor activity increased in G3 and G5 compared to G1, G2, and G4, and on days 14, 21, and 28, the mean number of lines crossed gradually decreased compared to day 7 in G3 and G5 groups. Prolonged NP exposure in animals and humans can cause pathological changes in brain function, resulting in maladaptive behavior (Lupien et al., 2018; Mineur et al., 2006).

These effects are demonstrated by studying animals' responses to stress stimuli in novel environments (Ramos & Mormède, 1998). Glutamatergic neurons and inhibitory interneurons are located in the BLA (Krabbe et al., 2018).

BLA encompasses a dynamic interaction between excitatory glutamatergic neurons and inhibitory interneurons, both essential for modulating emotional and behavioral responses. Naphthalene exposure, recognized for inducing oxidative stress and neuroinflammation, may disrupt the excitatory-inhibitory balance within the BLA. This disruption may contribute to the behavioral changes observed in this study, including symptoms of anxiety and cognitive impairments. Injury to glutamatergic neurons or disturbance of interneuronal inhibition may contribute to the abnormal brain signaling linked to naphthalene neurotoxicity (Moriceau et al., 2006). The principal neurons are pyramidal-like projection neurons with small dendrites that use glutamate as an excitatory neurotransmitter. In contrast, nonpyramidal neurons of the basolateral amygdala are spine-sparse interneurons that use the amino acid GABA as an inhibitory neurotransmitter. The majority (~80%) of neuronal cells are principal glutamatergic, and

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the remainder (~20%) are GABAergic inhibitory interneurons (Spampanato et al., 2011). Stress in any form affects pyramidal neurons in multiple ways, mainly altering the morphology of the dendritic tree, dendritic process length, and synapse spine density (Chocyk et al., 2013b; Muhammad et al., 2011; Monroy et al., 2010).

The deep temporal region of the basolateral part of the amygdala in naphthalene-treated animals exhibited intense neurodegeneration. These changes occurred due to naphthalene exposure, scattered with asymmetric neurons stained dark by cresyl violet. The degenerative cells appeared shrunken with distorted morphology and stained darker compared to normal cells.

The neuronal arrangement of the basolateral amygdala region appeared symmetrical in control rats. Normal and healthy cells were identified by spherical morphology with a visible nucleus. The low toxicity group showed pyramidal cells with minor abnormalities in morphology compared to the standard controls. The high naphthalene toxicity group demonstrated that more pyramidal cells of the basolateral amygdala exhibited degenerated morphology, indicating cell death. It was also observed that the pyramidal cells of the basolateral amygdala were replaced by abundant cells of unidentified origin, resembling inflammatory cells based on morphology. The satellite control group resembled the control group. Necrotic neurons were observed in the delayed toxicity group, similar to the naphthalene toxicity group. From the results obtained, extensive neurodegeneration occurred following treatment with naphthalene. These types of changes were studied three decades earlier, revealing that neuronal damage features condensed neurons scattered darkly in all brain regions (Sugimoto et al., 1990). That study also identifies three main characteristics for damaged neurons: uneven cellular outlines; increased chromatin density in both cytoplasm and nucleus; and profoundly and consistently stained nuclei. All these characteristics were observed in the neurons of naphthalene-treated basolateral amygdala region rats. The morphological abnormalities in pyramidal cells after a high dose of naphthalene confirm neurodegeneration.

5. Conclusion

The open field test revealed that the high dose and high dosage satellite groups exhibited increased anxiety-like behaviour on days 14, 21, and 28. In contrast, the high-dose satellite group showed a slight reduction in anxiety-like behaviour during post-treatment observation periods on days 35 and 42. There were no delayed withdrawal effects in any open-field test parameters for the high-dose and high-dosage satellite groups. Cresyl violet staining indicated that apoptosis and the number of surviving neurons decreased in the low, high, and satellite high dosage groups. The satellite high-dose group experienced no significant reversible or delayed withdrawal effects following treatment. This study concluded that naphthalene oral toxicity damages numerous amygdala basolateral nuclei cells, resulting in anxiety or mood changes. More studies are necessary to validate structural abnormalities associated with oral naphthalene intake and neurological disorders in other brain regions.

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Therapeutic Potential of Cannabidiol in Alleviating Cognitive Decline and Hippocampal Damage in a Rat Model of Alzheimer's Disease

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Abstract: Alzheimer's disease (AD) is a common neurodegenerative disorder marked by progressive cognitive decline. Due to its effects on cognitive functioning and hippocampal integrity, the combined treatment of D-galactose (D-gal) and Aluminium chloride (AlCl3) in rats is a widely used model for producing AD-like symptoms. Previous studies demonstrated that Cannabidiol (CBD) exhibits neurotherapeutic effects. This study examines the efficacy of CBD in reducing cognitive deficits and brain ultrastructural damage induced by D-gal and AlCl3. Male Wistar rats were treated with D-gal (60 mg/kg body weight/day) and AlCl3 (200 mg/kg body weight/day) for 10 weeks to induce AD-like symptoms, followed by CBD administration at doses of 20, 40, and 80 mg/kg/day. Donepezil (1 mg/kg body weight/day) served as a positive control. Cognitive performance was evaluated using the modified elevated plus maze and T-maze spontaneous alternation tests. Ultrastructural changes in the hippocampus were examined using transmission electron microscopy. Rats exposed to D-gal and AlCl3 exhibited significant cognitive impairments, including deficits in spatial learning and memory, as well as hippocampal ultrastructural damage. The results indicated that D-gal and AlCl3 exposure produced notable cognitive deficits and structural alterations in the hippocampus. Administration of CBD at all doses significantly enhanced cognitive function and reduced pathological changes, providing protective effects comparable to donepezil. These findings support CBD's potential as a neurotherapeutic compound for mitigating cognitive decline and hippocampal damage associated with AD.

Keywords: Alzheimer's disease, Cannabidiol, Cognitive deficits, Hippocampus, Neurotherapeutic effects.

1. Introduction

Alzheimer's disease (AD) is a neurodegenerative disorder that primarily affects the elderly, characterized by progressive memory impairment (Safiri et al., 2024). As life expectancy increases and populations age, AD has become a major global healthcare challenge (Zhang et al., 2021). AD is marked by neurofibrillary tangles (NFTs) formed from hyperphosphorylated tau protein and senile plaques (SP) composed of beta-amyloid protein (Ju & Tam, 2022). The progression of AD and other neurodegenerative diseases is believed to be strongly influenced by oxidative stress and reactive oxygen species (ROS) (Buccellato et al., 2021). Studies suggest oxidative stress may contribute to the onset and progression of AD. An imbalance in the production and removal of ROS can accelerate the initiation and advancement of AD by causing widespread and persistent damage to the central nervous system (CNS) (Ganguly et al., 2021; Olufunmilayo et al., 2023; Plascencia-Villa & Perry, 2023).

Chronic treatment with D-galactose (D-gal) is widely accepted to accelerate aging processes by causing modest neuronal damage and cognitive deficits, which are typical of the early stages of AD (Flores-Cuadra et al., 2021). As a result, animals treated with D-gal are frequently used as models to study the molecular causes of aging, including memory impairments and neurodegeneration, as well as to test potential anti-AD treatments (Xu et al., 2023). Aluminium (Al) is a toxic metal that is widely dispersed and primarily affects the brain, bones, liver, and spleen. Al accumulation in the brain can cause dementia by increasing malondialdehyde (MDA) levels, acetylcholinesterase (AChE) activity, and producing oxidative stress (Pankaj Bhargava et al., 2021). Consequently, rats exposed to Al over long periods serve as useful models for evaluating anti-AD therapies (Xia et al., 2023). Given that both D-gal and Al are established neurotoxins, researchers have investigated their combined effects (Luo et al.,

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2024). In mice, it was observed that the combination of D-gal and aluminium chloride (AlCl3) causes cognitive impairment, increased amyloidogenic proteins, cholinergic system impairments, and the formation of neurofibrillary tangles (NFTs) and senile plaques (SP) (Mahdi et al., 2021). Thus, treating rats with D-gal and $AlCl_3$ has been shown to be a viable and cost-effective approach for developing AD models to evaluate potential anti-AD therapies (Xu et al., 2023).

Cannabis is a plant recognized for its psychoactive and therapeutic properties, which are primarily due to the presence of a wide variety of chemical components such as cannabinoids, terpenes, and flavonoids (Fordjour et al., 2023). Cannabinoids such as tetrahydrocannabinol (THC) induce the well-known "high" and alter mood and pain perception, while cannabidiol (CBD) provides therapeutic benefits without psychoactivity (Pagano et al., 2022; Prenderville et al., 2015; Valeri & Mazzon, 2021). Research conducted to investigate the effect of CBD on hyperphosphorylation of tau and amyloid pathways in AD models of rodents induced by AlCl3 and injecting beta amyloid demonstrated neuroprotective and anti-inflammatory effects (Bhunia et al., 2022; Tambe et al., 2023). CBD may help reduce some AD symptoms by lowering oxidative stress, inflammation, and neurodegeneration, all of which contribute to the disease's progression (Hickey et al., 2024). CBD has been shown in studies to improve cognitive function and reduce behavioural symptoms in AD models (Trojan et al., 2023). Its capacity to interact with the endocannabinoid system may also support neurotransmitter regulation and brain cell protection (Kamaruzzaman et al., 2023).

Despite the pharmacological benefits attributed to CBD, it is uncertain if CBD can potentially reduce neurotoxicity caused by D-gal and $AlCl_3$, and effectively prevent cognitive decline and neurodegeneration in rats. This study aims to investigate the potential therapeutic effects of CBD against D-gal and $AlCl_3$-induced cognitive impairments and structural brain alterations. The study also compared CBD's effects to those of donepezil, an FDA-approved medication for cognitive deficits associated with AD. However, the limitations of donepezil include only symptomatic relief, no impact on disease progression, and usefulness only in mild cases. Other side effects include gastrointestinal distress, muscle weakness, and bradycardia.

2. Materials and methodology

2.1 Animals

The study included 36 male albino Wistar rats (180-250g, 2-3 months old) purchased from Takrif Bistari Enterprise, Seri Kembangan, Malaysia. The rats were housed in climate-controlled cages with free access to food and water during a 12-hour light/dark cycle. The Institutional Animal Ethics Committee (UPM/IACUC/AUP-R017/2023) approved the study procedure, and it was conducted according to their authorized parameters.

2.2 Chemicals

CBD was obtained from Aktin Chemicals (China), and D-gal, AlCl3, and donepezil from Sigma-Aldrich (USA). All chemicals were of analytical grade. For intraperitoneal (i.p.) administration, D-gal was dissolved in distilled water (Chiroma et al., 2018); CBD was dissolved in Tween 80 (Feng et al., 2021). For oral dosing, AlCl3 and donepezil were dissolved in distilled water (Jagadeesan et al., 2019).

2.3 Design of the experiment

After seven days of acclimatization, the rats were randomly divided into six groups (n=6), as shown in Figure 1.

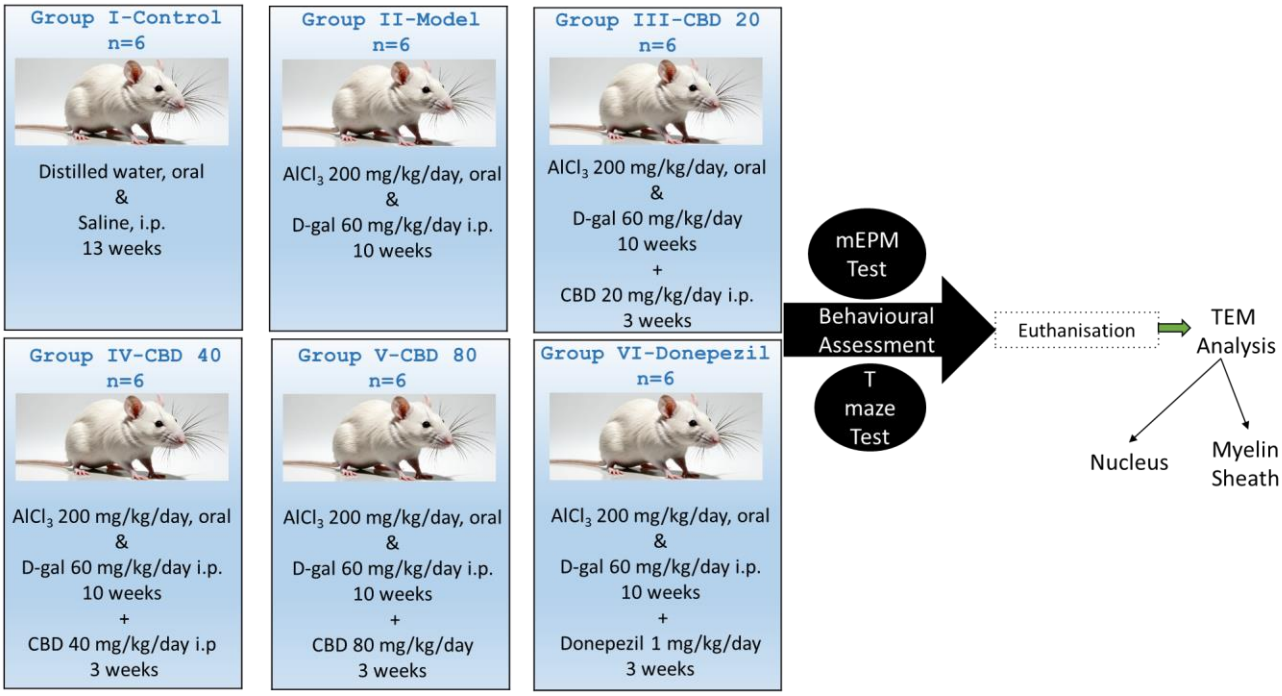

Figure 1. Experimental design; mEPM-Modified elevated plus maze.

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The dosages of D-gal, AlCl3, and CBD used in this study were selected based on previous research and published literature (Chiroma et al., 2019; Khan et al., 2024). After a 91-day treatment period, the rats underwent behavioural testing, which included the modified Elevated Plus Maze Test and the T Maze Spontaneous Alternation. At the end of 13 weeks, the rats were sacrificed, and their hippocampal tissue was collected for TEM analysis to examine the nucleus and myelin sheath.

2.4 Apparatus

The experimental apparatus used in this study, consisting of a modified elevated plus maze and a T-maze, was constructed in the workshop of the Department of Human Anatomy, Faculty of Medicine and Health Sciences (FMHS), Universiti Putra Malaysia (UPM).

2.5 Modified elevated plus maze Test (mEPM):

The apparatus used in the experiment was a plus-shaped piece of dark plexiglas with two open arms (50 cm long, 10 cm wide) and two enclosed arms (50 cm long, 10 cm wide, 40 cm high) positioned opposite each other (Nabeshima & Kameyama, 1990). It was elevated 50 cm off the ground with a central square platform (10 × 10 cm) connecting the arms. The room was dimly illuminated by a 60 lx red halogen bulb. On the first day of the acquisition phase, each rat was placed at the end of an open arm, facing away from the centre, and allowed 90 seconds to explore and enter one of the enclosed arms. The time taken to enter was recorded as the Initial Transfer Latency (ITL). Once inside, rats remained for 20 seconds (Mutlu et al., 2011). If a rat did not enter an enclosed arm within 90 seconds, it was gently guided in. A rat was considered to have entered when all four paws crossed the entrance line. Retention sessions were conducted 24 hours (TL1) and 7 days (TL2) after the initial trial. If the rat did not enter within 90 seconds, TL1 or TL2 was recorded as 90 seconds, marking the test's end. Longer latencies suggested possible memory impairment, while shorter latencies indicated better memory (Raghavendra & Kulkarni, 2001). The apparatus was cleaned with 70% alcohol between tests to eliminate scent cues. Data were recorded using ANY-maze software.

2.6 T maze Test for spontaneous alternation:

The T-maze apparatus was constructed from dark Plexiglas, comprising a start arm (50 cm long, 16 cm wide) and two goal arms (50 cm long, 10 cm wide), extending perpendicularly. Each arm was enclosed by 30 cm high walls, and the maze featured an open top. Three guillotine doors were placed at the junctions of the arms. A central barrier extended 15 cm into the start arm, compelling rats to choose between the left or right goal arm (d'Isa et al., 2021). During testing, rats were placed at the start arm and permitted to choose a goal arm. After 30 seconds, the chosen arm was closed with a guillotine door, the middle barrier removed, and the rat gently returned to the start. The rat then faced away from the arms and was provided another choice. A correct choice occurred if the rat alternated arms, and only choices where all four paws entered the goal arm were recorded. Each rat performed the test 11 times, with 30-second intervals, resulting in ten spontaneous alternations (Deacon & Rawlins, 2006). Data were recorded using ANY-maze software.

2.7 Transmission Electron Microscopy:

Transmission electron microscopy (TEM) was employed to investigate the neuronal ultrastructure following neurodegeneration induced by a combination of D-gal and AlCl3, as well as to assess the neurotherapeutic effects of CBD. Hippocampal tissue was promptly extracted from the brains of decapitated rats using a cold plate (Lam et al., 2021). A 1 × 1 mm segment of the hippocampus was dissected and preserved in a 5% glutaraldehyde solution at 4 °C for 12 hours for conventional electron microscopy (Abdelmeguid et al., 2021). The hippocampal tissues were prepared and examined following the methods described by Ojo and Barsoum (Peng et al., 2013). The tissue samples were analysed using a TEM LEO LIBRA-120. Images of the hippocampus were captured from at least twelve random fields per rat group.

2.8 Statistical analysis:

The data were analysed using one-way ANOVA in GraphPad Prism version 6 software (ISI, USA). Tukey's post hoc test was performed after ANOVA to determine statistical significance among groups. A significance level of $p < 0.05$ was chosen. The results were presented as mean values with their corresponding standard deviation (mean ± SD), representing the central tendency and variability in the data. This comprehensive methodology ensured that the experimental results were rigorously assessed and that statistical interpretations were accurate.

3. Results

3.1 CBD Mitigates Spatial Learning Deficits Induced by D-gal and $AlCl_3$:

The therapeutic effects of CBD on spatial learning and memory impairment induced by D-gal and AlCl3 were first assessed using the mEPM. No significant differences in initial transfer latency (ITL) were observed among the various rat groups [$F = 0.6052$, $p = 0.6964$] (Fig. 2A). However, one-way ANOVA revealed significant differences in first transfer latency (TL1) values [$F (5, 30) = 6.396$, $p = 0.0004$] (Fig. 2B). Tukey's post hoc test showed a significant reduction in TL1 for the control group and CBD-treated groups (CBD 20: $p = 0.0056$; CBD 40: $p = 0.0087$; CBD 80: $p = 0.0013$) and donepezil group ($p = 0.0040$) compared with the model group of rats ($p = 0.0003$). Similarly, significant differences were observed in second transfer latency (TL2) values [$F (5, 30) = 6.018$, $p = 0.0006$] (Fig. 2C). Tukey's post hoc test indicated significant reductions in TL2 for the control, CBD groups (CBD 20: $p = 0.0150$; CBD 40: $p = 0.0064$; CBD 80: $p = 0.0009$) and donepezil group ($p = 0.0055$) compared to the model group ($p = 0.0009$).

3.2 CBD attenuates hippocampal dysfunction induced by D-gal and $AlCl_3$ in the T-Maze spontaneous alternation test:

Rats with hippocampal lesions generally scored less than 60% across several trials. Control groups generally achieved more than 80% accurate alternations in the T-maze when the right strains of rats were used, and the right conditions were met. The control group in this trial obtained 83.33% correct alternations, followed

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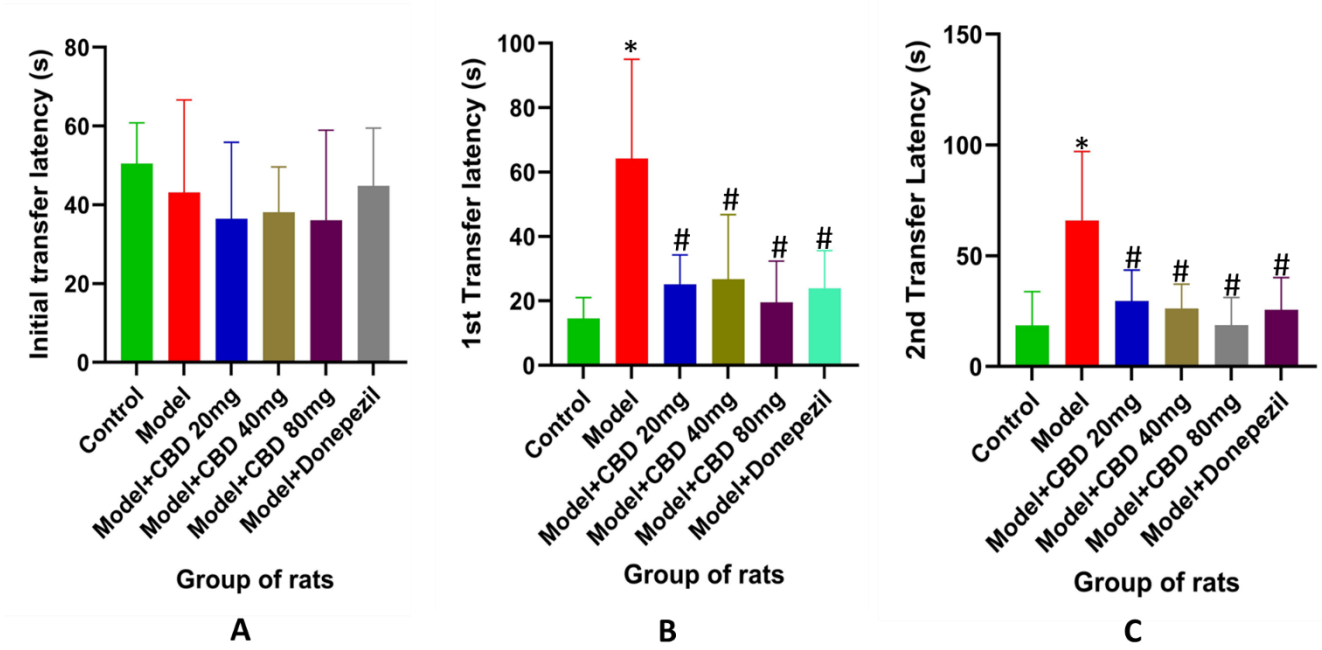

Figure 2: Modified Elevated Plus Maze. Effects of *CBD* on spatial learning and memory in rats induced with D-galactose and aluminium chloride. Panel A shows the initial transfer latency recorded on the first day of testing. Panel B displays the first transfer latency measured 24 hours after the initial trial, while Panel C illustrates the second transfer latency assessed 7 days post-initial trial. Data are presented (n = 6). Significant differences are indicated by *p < 0.05 compared to the Model group.

by the model group at 43.33%, the donepezil group at 70%, the CBD 20 group at 63.33%, the CBD 40 group at 73.33%, and the CBD 80 group at 80%. According to Tukey's post hoc test, significant variations in alternation rates were found using a one-way ANOVA test ($F(5, 26) = 22.76$, $p < 0.0001$) (Fig. 3).

In comparison to the model group (43.33 ± 4.30, $p < 0.0001$), the control group (83.33 ± 3.0), CBD 20 group (63.33 ± 2.65, $p = 0.0057$), CBD 40 group (73.33 ± 4.47, $p = 0.0002$), CBD 80 group (80.0 ± 3.79, $p < 0.0001$), and donepezil group (73.33 ± 3.79, $p < 0.0002$) all showed significantly higher accurate alternations, according to Tukey's post hoc test.

3.3 CBD Alleviates Ultrastructural Morphological Alterations in the Hippocampus Induced by D-gal and $AlCl_3$:

TEM investigations were conducted to confirm the impact of CBD on neuronal ultrastructure following rats' hippocampal D-gal and AlCl3-induced neurodegeneration.

3.4 Nucleus:

Electron photomicrographs of the hippocampus nucleus from each group of rats are presented in Figure 4. The nucleus in the control group (Fig. 4A) showed normal features, including double-layered nuclear membranes, intact nucleoli, and evenly distributed chromatin. Conversely, pyknosis, crescent formation, degraded chromatin, damaged nucleoli, and deformed nuclear membranes were among the morphological changes observed after treatment with D-gal and AlCl3 in the nucleus of pyramidal neurons (Fig. 4B). Some of these nuclear abnormalities were attenuated by treatment with CBD or donepezil (Fig. 4C–F).

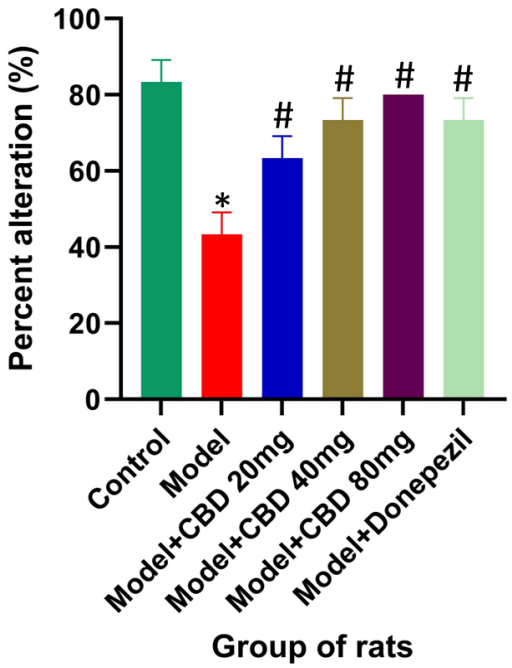

Figure 3: T Maze Spontaneous Alternation. Effects of *CBD* on hippocampal dysfunction caused by D-galactose and aluminium chloride in rats. Data are presented as mean ± SEM (n=6). # indicates *p < 0.05 for comparisons between Control, CBD 20, CBD 40, CBD 80 and Donepezil groups versus Model group.

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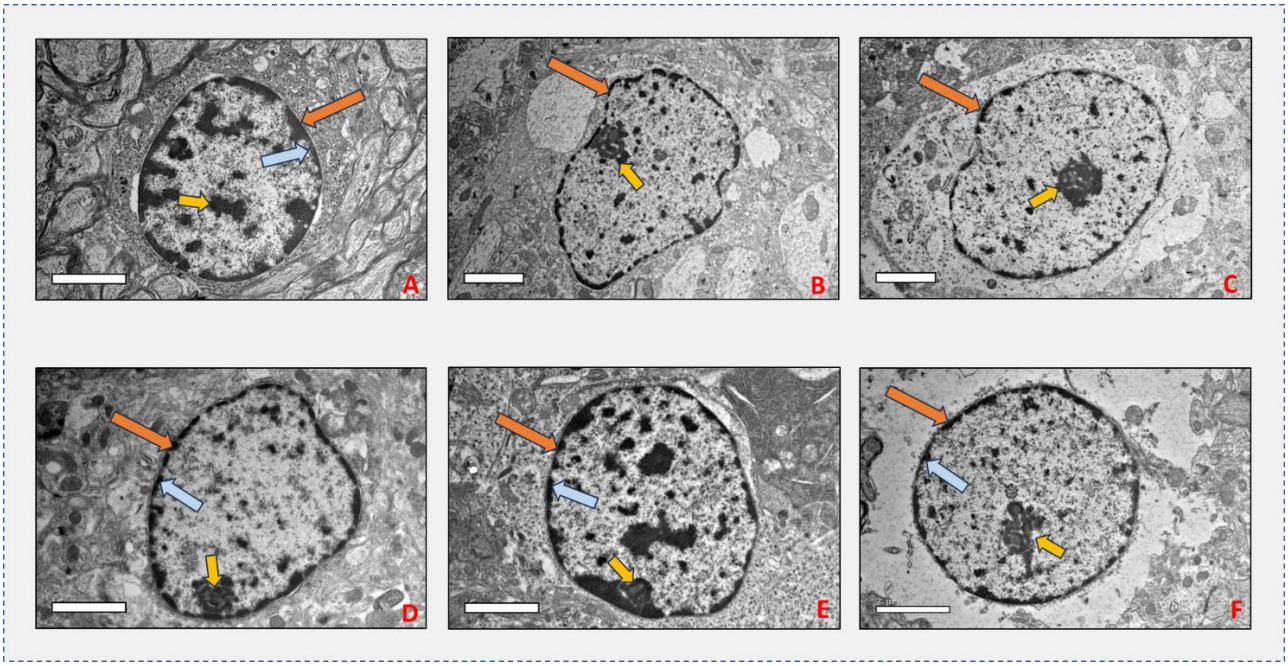

Figure 4: TEM photomicrographs of the rat's hippocampus showing the nucleus and its components. A) Control group of rats showing nucleolus (yellow arrow), evenly distributed chromatin and double nuclear membrane (orange and blue arrows), B) Model group of rats showing pyknotic nucleus and crescent formation and distorted nuclear membrane, C-E) CBD 20, 40 & 80 group of rats showing normal nucleus with double nuclear membrane F) Donepezil group of rats showing nucleus with normal nuclear membrane and nucleolus

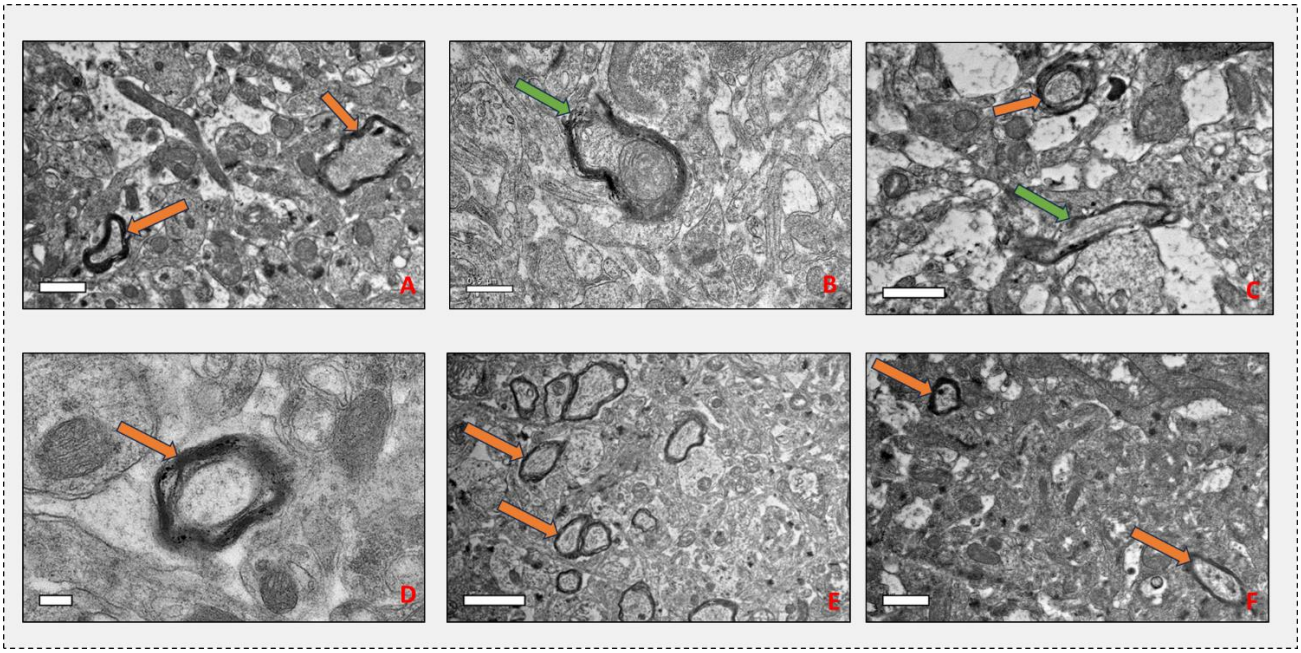

Figure 5: TEM photomicrographs of rat hippocampus showing myelin sheath, A) The control group of rats showed normal myelin sheaths that appeared dense, thick, and tightly wrapped around their axons (orange arrows). B) The model group of rats showing discontinuous myelin (green arrow). C-E) CBD 20 group shows both normal myelin (Orange arrow) and slightly disrupted myelin sheath (green arrow), D-F) CBD 40, 80, and donepezil group of rats showing normal myelin sheath tightly wrapped around an axon.

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The therapeutic effects of CBD against neurotoxicity caused by D-gal and AlCl3 were assessed by analysing the ultrastructure of the myelin sheath. The myelin sheaths in the control rats were continuous, thick, highly electron-dense, and firmly wrapped around the axonal membranes (Fig. 5A). In contrast, animals given D-gal and AlCl3 treatments exhibited various myelin abnormalities. Among the prominent myelin defects observed were myelin sheath disintegration (Fig. 5B); CBD and donepezil administration helped in reducing these defects (Fig. 5C-5F).

4. Discussion

Chronic administration of D-gal or AlCl3 in rats induces aging-like changes, including cholinergic dysfunction, cognitive impairments, oxidative stress, and advanced glycation end product accumulation (Chiroma et al., 2018; Stanciu et al., 2020; Di Benedetto et al., 2022). This combination causes cognitive deficits and hippocampal pyramidal neuron degeneration (Khan et al., 2024). In this study, rats treated with D-gal and AlCl3 exhibited hippocampal ultrastructural alterations and learning and memory impairments, making this rat model valuable for studying Alzheimer's disease (AD)-related conditions. Additionally, the co-administration of CBD significantly reduced cognitive deficits and ameliorated the morphological abnormalities caused by D-gal and AlCl3.

To further assess cognitive function, the mEPM test was employed to evaluate anxiety, spatial learning, and memory in rats (Walf & Frye, 2007). Previous studies have demonstrated that D-gal and AlCl3 impair spatial learning and memory in rats (Chiroma et al., 2018). The mEPM results in this study corroborated these findings, showing that CBD significantly improved spatial memory and learning in rats, comparable to the effects of donepezil. These results indicate CBD's potential in enhancing cognitive performance.

The T-maze test was also utilized to evaluate cognitive function, particularly to identify hippocampal dysfunction in spontaneous and rewarded alternation tasks (Deacon & Rawlins, 2006; d'Isa et al., 2021). Rats treated with D-gal and AlCl3 performed poorly on the T-maze spontaneous alternation task, with less than 60% correct alternations. However, the CBD and donepezil groups achieved scores higher than 60%, with the 80 mg/kg CBD group demonstrating results comparable to donepezil (Tournier et al., 2021). These findings align with previous studies indicating that CBD enhances learning and memory retention (Peres et al., 2016; Singh et al., 2023; Watt, 2020).

Electron microscopy (TEM) analysis provided clear evidence of the neurotoxic effects of D-gal and AlCl3 treatment and the neurotherapeutic potential of CBD. The control group's hippocampal nuclei displayed normal characteristics, including intact nucleoli and evenly distributed chromatin. In contrast, the D-gal and AlCl3-treated group exhibited severe nuclear abnormalities, including pyknosis, crescent formation, and disrupted nuclear membranes, consistent with previous research on the neurotoxic effects of these substances (Chiroma et al., 2019). However, CBD and donepezil administration mitigated these nuclear abnormalities, suggesting their potential therapeutic roles in maintaining cellular health and nuclear integrity.

The therapeutic effects of CBD were further corroborated by the analysis of myelin sheath integrity. Rats treated with D-gal and AlCl3 showed significant myelin abnormalities, including swelling, disintegration, and detachment from axons. In contrast, the control group's myelin remained intact. Treatment with donepezil and CBD decreased these myelin abnormalities, with the 80 mg/kg CBD group showing the most favorable results. This aligns with prior studies indicating that CBD possesses myelin-protective properties, potentially reducing damage caused by neurotoxic substances like D-gal and AlCl3 (Navarrete et al., 2021). These findings emphasize the neurotherapeutic potential of CBD in preventing neurodegeneration and maintaining myelin integrity.

Together, these results suggest that CBD provides significant neuroprotective benefits by improving cognitive function, preserving neuronal integrity, and mitigating the neurotoxic effects of D-gal and AlCl3. The combination of CBD with established treatments such as donepezil may offer a promising strategy to alleviate the effects of Alzheimer's disease and related neurodegenerative disorders.

5. Conclusion

This study shows that continuous administration of D-galactose and aluminium chloride to rats causes severe cognitive deficits as well as histological and ultrastructural abnormalities in the hippocampus. The results indicate that Cannabidiol (CBD) effectively reduces these cognitive deficits, as demonstrated by improved performance in the modified Elevated Plus Maze test and greater alterations in the T-maze spontaneous alternation test. Furthermore, CBD treatment diminished the histopathological hippocampal abnormalities detected and confirmed by transmission electron microscopy. The findings of this study suggest CBD has potential as an alternative treatment for AD. Further research is needed to investigate the other pathways involved in the pathogenesis of AD.

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