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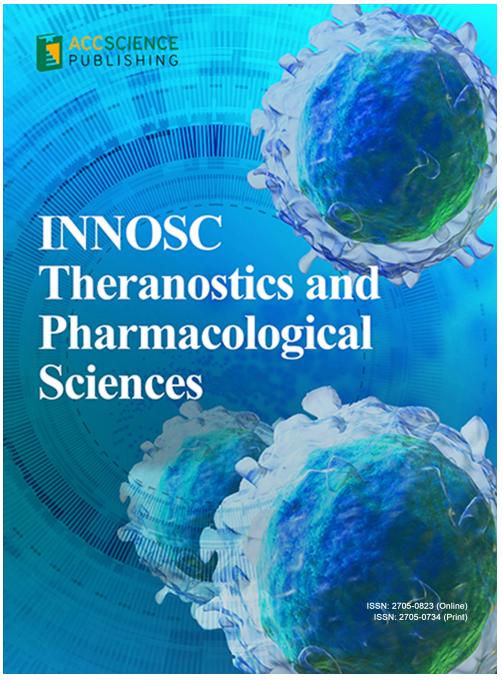

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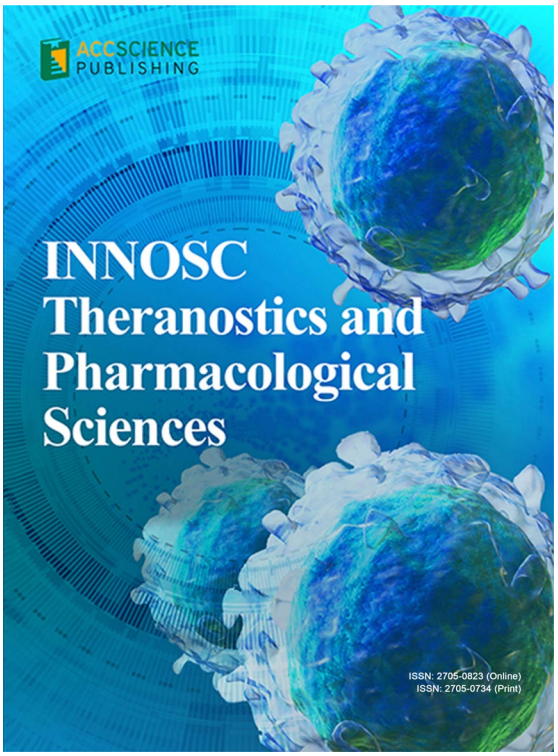

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INNOSC Theranostics and Pharmacological Sciences

REVIEW ARTICLE

The role of AMPK activation in metabolic regulation, energy homeostasis and aging: A comprehensive overview

George Jîtcă*

Department of Pharmacology and Clinical Pharmacy, Faculty of Pharmacy, George Emil Palade University of Medicine, Pharmacy, Science and Technology of Târgu Mureş, 540139 Târgu Mureş, Romania

Abstract

5′ Adenosine monophosphate-activated protein kinase (AMPK) is a metabolic sensor responsible for maintaining homeostasis, regulating metabolic control, monitoring energy status, and balancing energy production with consumption. The most important protein kinases that activate AMPK are liver kinase B1 and calcium-calmodulin-dependent protein kinase. AMPK significantly impacts physical performance by accelerating recovery periods and restoring energy stores through several key mechanisms. It regulates muscle glycogen content, ensuring readily available energy during physical exertion. In addition, a high number of mitochondria enable the utilization of fatty acids as an energy source, thereby improving endurance efforts. Furthermore, rich vascularization in the muscles enhances the delivery of nutrients and oxygen, optimizing performance and speeding up recovery. AMPK is also important for maintaining muscle homeostasis. It decreases insulin resistance, stimulates mitochondrial biogenesis, and exerts an antioxidant effect. As a regulator of energy homeostasis, AMPK plays a key role in linking cellular energy levels with the aging process. It acts as a modulator of cellular senescence and has the potential to extend life expectancy, particularly in the context of caloric restriction. By activating AMPK, caloric restriction (activated with a 30 – 70% reduction in nutrient intake) helps to maintain energy balance in cells, which may slow down aging and support healthier cellular function over time. In conclusion, the involvement of AMPK in metabolic regulation and maintenance makes it an important therapeutic target for the treatment and prevention of several diseases, both age-related and otherwise.

Keywords: AMPK; Oxidative stress; Physical effort; Exercise; Metabolism

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1. A short overview of AMPK

With advances in medicine, life expectancy has significantly increased. These advances are attributed to the emergence of the concept of personalized medicine and the development of new pharmacological agents, all aimed to improve quality of life, prolong life expectancy, and enhance physical condition. The purpose of the present review is to highlight the role of 5' adenosine monophosphate-activated protein kinase (AMPK) in certain processes (carbohydrate and lipid metabolism, autophagy, mitochondrial biogenesis, oxidative stress), and to explore how these mechanisms benefit athletes.

In 1987, researchers David Carling and Grahame Hardie hypothesized the existence of two protein kinases that inhibited enzymes involved in the *de novo* synthesis of fatty acids (acetyl-CoA carboxylase [ACC]) and cholesterol (hydroxymethylglutaryl-CoA reductase [HMG-CoA reductase]). Subsequent studies revealed that both kinases were, in fact, the same protein.[1,2] Studies on experimental animals have presented data confirming that physical exercise decreases the muscle concentration of malonyl-CoA formed during the ACC pathway, which contributes to more efficient utilization of energy substrates.[3] Under normal physiological conditions, malonyl-CoA inhibits carnitine palmitoyltransferase I, blocking the transport of fatty acids into the mitochondria where they would otherwise be oxidized. Consequently, a decrease in malonyl-CoA concentration promotes fatty acid transport into mitochondria, facilitating their use for ATP production.[4]

AMPK is a metabolic sensor responsible for maintaining homeostasis, metabolic control, and energy balance by monitoring energy status and matching energy production to its consumption. Following favorable metabolic outcomes after AMPK activation, it has been identified as an important therapeutic target.[5] Its activation is triggered when low energy levels are detected, indicated by reduced adenosine triphosphate (ATP) levels, which can occur during fasting, hypoxia, or when various toxic substances impact the electron transport chain (ETC) and inhibit oxidative phosphorylation (OXPHOS).[6,7]

AMPK exists as a heterotrimeric complex, consisting of a catalytic α subunit (α_1, α_2) encoded by *PRKAA1*; regulatory β subunits (β_1, β_2) encoded by *PRKAB1* and *PRKAB2*; and γ subunit (γ_1, γ_2, γ_3) encoded by *PRKAG1*, *PRKAG2,* and *PRKAG3*. These subunits are encoded in the genomes of all cells, suggesting that the AMPK heterotrimer arose very early in the evolution of eukaryotic cells.[8,9] One of the most important event in this evolution was the endosymbiotic capture of aerobic bacteria, which gave rise to mitochondria. After this event, the newly formed organism developed new abilities to convert adenosine diphosphate (ADP) into ATP and established signaling pathways to monitor ATP availability. In evolved organisms, AMPK's role in ATP regulation, mitochondrial biogenesis, mitophagy, and fission underscores its significance as a link between mitochondria (aerobic bacteria) and the host cell (anaerobic bacteria).[10-13] While AMPK initially served a primordial role in energy regulation, it has evolved to also regulate the energy balance of the whole organism through hormone-mediated responses in the hypothalamus.[3,14] The adaptability of the eukaryotic cell ensures cellular homeostasis and survival under adverse conditions. In athletes, AMPK activation may confer advantages by promoting mitochondrial gene expression and increasing mitochondria numbers. One of these regulators of multiple mitochondrial genes is peroxisome proliferator-activated receptor-γ coactivator-1α (PGC-1α), which, in muscle, promotes the conversion of type IIb muscle fibers to type I and type IIa fibers, both characterized by increased mitochondrial content.[15,16]

The γ subunit of AMPK contains two Bateman domains, which serve as binding sites for adenosine monophosphate (AMP) and ATP. Therefore, when the cell does not detect an energy deficiency, ATP molecules bind to the appropriate site on the enzyme, maintaining it in an inactive state. Conversely, if the AMP/ATP ratio exceeds unity, AMP molecules bind to the Bateman subunit, triggering the activation of the enzyme through three mechanisms:

- Allosteric activation;
- Stimulation of AMPK activating proteins, which phosphorylate the α catalytic subunit;
- Preventing dephosphorylation as a result of AMP binding to the γ subunit which extends the phosphorylation of the α subunit.

The primary protein kinases that activate AMPK are liver kinase B1 (LKB1) and calcium-calmodulin-dependent protein kinase α and β (CaMKK).[17,18] In response to energy-related stress, such as an energy deficit or depletion, skeletal muscle shows increased LKB1 activity. Conversely, the absence of this protein is associated with reduced physical endurance. CaMKK exists in two isoforms, α and β, which are 70% similar. These isoforms become active in the presence of Ca^{2+} and calmodulin and are capable of phosphorylating AMPK in the brain, endothelium, lymphocytes, and striated muscles. Given that the enzyme has multiple subunits with multiple isoforms, it can be inferred that AMPK also exists in multiple forms, as shown in Table 1. Among them, the $\alpha_2\beta_2\gamma_3$ isoform is activated under conditions of short-term physical exercise, while the $\alpha_2\beta_2\gamma_1$ isoform is present in long-term effort.[2]

2. AMPK activators and physical activity

For most compounds that activate AMPK, the activation occurs by inhibiting the ETC, leading to an increase in ADP and/or AMP levels. Another important aspect to consider is the functional differences between AMPK isoforms.

A study of transgenic mice deficient in AMPKα_2 showed that in the absence of this enzyme, the contractility of the heart is impaired, indicating that the absence of the α_2 isoform is correlated with reduced physical capacity. In contrast, mice injected with 5-Aminoimidazole-4-carboxamide ribonucleoside (AICAR, a known AMPK modulator) showed improved running capacity, enhanced endurance, increased oxygen consumption, and decreased

Table 1. Activation of the AMPK enzyme complex depending on the intensity and duration of physical exercise

Type of exercise	High intensity, short duration	Moderate intensity, medium duration	Low intensity, long duration
AMPK activator	LKB1		CaMKK
AMPK complex α subunit	$\alpha_2\beta\gamma$		$\alpha_1\beta\gamma$
Activated AMPK isoforms	$\alpha_2\beta_2\gamma_3$	$\alpha_2\beta2\gamma_1$	$\alpha_1\beta_2\gamma_1$

Abbreviations: AMPK: 5' Adenosine monophosphate-activated protein kinase; LKB1: Liver kinase B1; CaMKK: Calcium-calmodulin-dependent protein kinase.

body mass. These effects are due to the phosphorylation and activation of target genes under the control of AMPK, such as transcription factors and co-activators of the p53 gene, cyclic-AMP response element-binding protein, mammalian target of rapamycin (mTOR), and PGC-1α pathways. However, there is also evidence showing a protective role for AMPK in cancer cells, which are susceptible to oxidative stress. These events occur because the LKB1-AMPK system helps maintain cellular homeostasis under stress.[7] Preclinical studies suggest that combinations of agents that activate AMPK produce stronger therapeutic effects. Thus, by targeting AMPK, the *de novo* synthesis of fatty acids, a hallmark of several cancers, can be prevented. A study by O'Brien *et al.*,[19] suggests that the combination of metformin and aspirin decreases the survival rate of prostate and lung cancer cells.

In vitro studies on cardiomyocyte cell lines reveal translocation of the fatty acid translocase transporter (FAT/CD36) to the cell membrane with increased fatty acid uptake and oxidation rates. Other studies state that when muscle glycogen stores are low, fatty acid oxidation becomes more intense. Activation of AMPK during physical effort inhibits triglyceride synthesis by suppressing glycerol-3-phosphate acyltransferase activity.[4,20,21] Moreover, AMPK prevents the incorporation of palmitic acid into ceramides, lipids involved in the generation of reactive oxygen species (ROS). AMPK is also involved in glucose uptake through the TBC1D1 protein, facilitating translocation of the glucose transporter GLUT4. It inhibits glycogen synthetase, an enzyme that catalyzes an energy-consuming anabolic process.[22,23] Glycogen is broken down to glucose by glycogen phosphorylase. While it was originally thought that AMPK regulates both glycogen phosphorylase and glycogen synthase, studies show that only glycogen synthesis is affected. Specifically, glycogen synthesis is regulated through phosphorylation of glycogen synthase by the α2 isoform of AMPK. In skeletal muscles, endurance training activates AMPK, which favors glycogen synthesis by increasing glucose uptake and its conversion to glucose-6-phosphate, an allosteric activator of glycogen synthase. This activation can offset the inhibitory effects of AMPK on glycogen synthase. During feeding after a period of fasting, insulin activates AMPK, which in turn influences genes involved in gluconeogenesis, including cyclic AMP-regulated transcriptional co-activator 2 (CRTC2) and class IIA histone deacetylases (HDACs).[24,25] This activation can benefit athletes by enabling more efficient use of energy reserves and improving their replenishment during rest and recovery periods.

Given the role in the regulation of energy consumption and maintenance of carbohydrate and lipid metabolism homeostasis,[26,27] as well as its involvement in mitochondrial biogenesis,[28] AMPK is considered a therapeutic target not only in metabolic diseases (diabetes, obesity) but also in other pathologies. Thus, there are studies in the literature that highlight the role of AMPK in suppressing tumorigenesis.[29,30] In addition, AMPK activation induces G1 cycle arrest, an effect associated with p53 activation, followed by cell cycle inhibition through p21. It is also important to note that AMPK has the ability to protect tumor cells against cytotoxic medication and hypoxic conditions.[5]

Another important role of AMPK is its crucial role in autophagy processes, which rely on the catabolic activity of lysosomes. In this context, AMPK regulates the activity of two proteins involved in the initiation of autophagy, such as Unc-51-like autophagy-activating kinase (ULK1) and the phosphatidylinositol 3-kinase catalytic subunit type 3 (PI3KC3/VPS34).[31,32]

Last but not least, the role of AMPK in redox homeostasis and its involvement in increasing antioxidant activity should be highlighted.[13] Analysis of a study published in 2008 during the Beijing Olympic Games demonstrated that AICAR significantly enhanced sports performance by increasing the physical endurance capacity of mice, even in the absence of prior physical training. Following these findings, the World Anti-Doping Agency (WADA) was notified, leading to the prohibition of AMPK activators, such as AICAR and their inclusion in the list of "hormones and metabolic modulators". This raises the question on whether all AMPK activators should be included in the list of prohibited substances or is the improvement of sports performance is only correlated with certain activators of the enzyme.[2]

From the perspective of physical exercise, AMPK influences performance, accelerates recovery, and restores

energy reserves through several mechanisms. These include the presence of glycogen in muscles, a high number of mitochondria that facilitate the utilization of fatty acids as an energy source, and extensive vascularization to ensure efficient nutrient and oxygen delivery during physical activity.[33] In addition, the use of AMPK modulators decreases insulin resistance in skeletal muscles and allows glucose utilization as an energy substrate by translocating the GLUT4 to the cell membrane. In terms of regenerative and recovery capacity, AMPK also plays a role in responding to muscle inflammatory processes. During the first phase, M1 macrophages secrete pro-inflammatory cytokines that result in ROS, which are necessary for muscle recovery. Subsequently, the recruitment of M2 macrophages is necessary to combat inflammation. Muscle regeneration involves the migration, proliferation, and fusion of myoblasts, as well as their interaction with immune cells, to form myotubules. Combating the inflammatory process is extremely important, as disruptions can impair the regeneration capacity of the skeletal muscles.[34] In degenerative myopathies, an inflammatory response is commonly observed, similar to the condition seen in muscle tissues deficient in AMPK. Both AICAR and metformin have shown anti-inflammatory effects, demonstrating potential therapeutic benefits in these conditions.[35-37]

Among the benefits of physical exercise, sports, and movement are reduced incidence of cardiovascular diseases, cancer, neurodegenerative diseases (Alzheimer's, Parkinson's, Huntington's), and other neurological conditions. Neurogenesis in the hippocampal region and increased neuronal plasticity are associated with improved memory and cognitive abilities. However, a direct link between these benefits and muscle metabolism has not been demonstrated.[38] To mimic the effects of physical activity, compounds have been developed and extracted that activate regulatory pathways and stimulate genes involved in skeletal muscle remodeling.[39-41] However, from an ethical perspective, this raises the question of whether such modulators of physical movement might open the door to controlled forms of sports doping.

AICAR is known to promote angiogenesis and vascularization by inducing vascular endothelial growth factor gene expression, similar to the effects of physical exercise.[42,43] In doses of 500 mg/kg administered to small, young, and adult rodents for 1 and 2 weeks, respectively, AICAR improved spatial memory and coordination. These effects are unlikely to result from direct central effects, as AICAR is poorly permeable to the blood-brain barrier. Notably, treatments longer than 14 days in young mice do not provide any neuronal benefits.[44] However, central effects have been observed in the hippocampal dentate gyrus, where increased levels of brain-derived neurotrophic factor (BDNF) were found following short-term treatment.[45]

A hypothesis suggesting that chronic treatment does not provide benefits supports the idea that prolonged use increases cytokine levels, leading to the onset of an inflammatory process followed by oxidative stress. Based on these observations, it can be stated that AICAR improves physical effort parameters, but only with short-term use. Like AICAR, metformin activates AMPK through an indirect mechanism by blocking complex I of the respiratory chain, which results in ATP depletion and an increase in AMP levels, with the possibility of similar effects.[46]

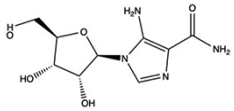
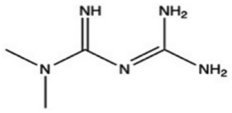
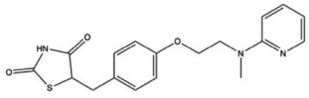
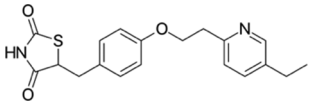
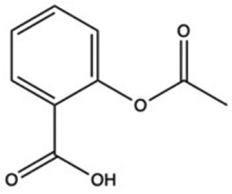
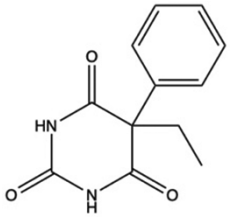
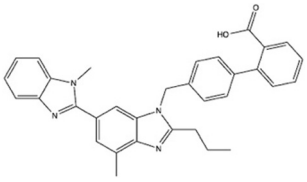
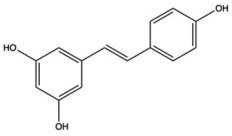
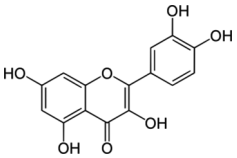
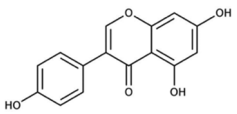
In conclusion, AMPK is extremely important for the regulation of muscle homeostasis with extension to other types of cells and tissues by decreasing insulin resistance, stimulating mitochondrial biogenesis, and exerting an antioxidant effect.[28,33,47,48] Table 2 lists the main molecules known to activate AMPK and their mechanism of activation.

3. Unraveling aging: The interplay between AMPK, caloric restriction and autophagy

One of the most interesting and bold hypotheses, proven by evidence in some studies, is the "free radical theory", which suggests that the uncontrolled generation of free radicals is the main driving force of biological aging. Following ROS accumulation, the senescence process accelerates due to the loss of mitochondrial integrity, resulting from increased membrane permeability.[74-76] In addition, factors such as increased adipose tissue and metabolic imbalances trigger inflammatory processes, leading to mitochondrial lesions, which favors the acceleration of the senescence rate.[77-79]

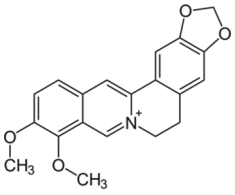
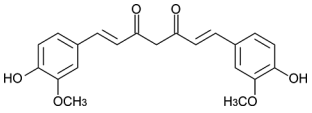
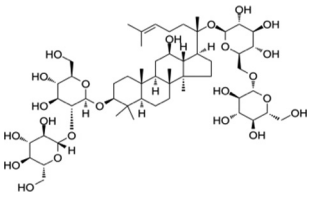
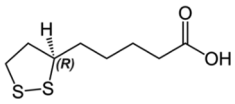
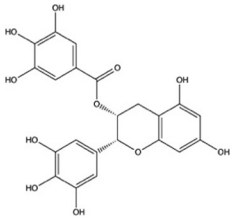
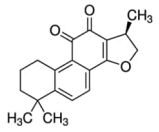
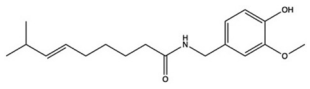
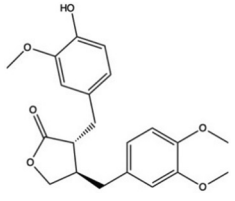
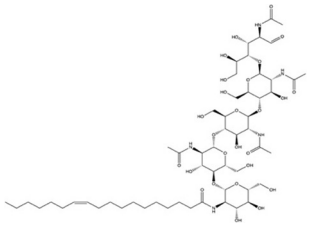
As aging is an inevitable (temporary) process that is characterized by the loss of tissue functionality and an increased risk of age-related diseases, the use of AMPK modulators is an alternative. Studies suggest the activation of AMPK is associated with increased life expectancy by maintaining cellular homeostasis, enhancing stress resistance, and promoting apoptosis and autophagy. Thus, activation of AMPK in *Caenorhabditis elegans* and rodents has been shown to extend lifespan, with numerous studies in *C. elegans* suggesting a possible link between AMPK activation and caloric restriction.[80] Irregularities in mitochondrial dynamics with abnormal mitochondrial morphology are key characteristics of aging and play a significant role in the development of various age-related neurodegenerative diseases, including Alzheimer's disease and Parkinson's disease.[81-83] In an animal model of Parkinson's disease induced in mice, treatment with metformin (500 mg/kg) for 21 days increased the level of BDNF and reduced oxidative stress in the substantia

Table 2. AMPK activators, mechanism of action, and impact on exercise capacity

Compound	Chemical structure	AMPK activation mechanism	References
AICAR		ZMP binding to the γ-subunit of AMPK	5
Metformin		Respiratory chain blockage (complex I) Increase in AMP/ATP ratio	49
Rosiglitazone		Respiratory chain blockage Increase in AMP/ATP ratio	50
Pioglitazone		Respiratory chain blockage Increase in AMP/ATP ratio	51
Salicylates		Allosteric activator (AMPK binding, β1 subunit)	52
Phenobarbital		Respiratory chain blockage Increase in AMP/ATP ratio	53
Telmisartan		PPARγ-dependent mechanism	54
		Increase in AMP/ATP ratio (action on mitochondrial F1F0-ATPase/ATP synthase)	55
Quercetin		Increase in AMP/ATP ratio (action on mitochondrial F1F0-ATPase/ATP synthase)	56,57
Genistein		Increase in AMP/ATP ratio (action on mitochondrial F1F0-ATPase/ATP synthase)	57,58

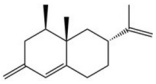
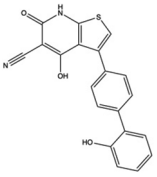
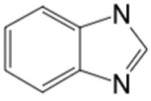
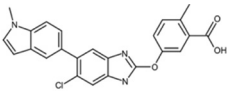
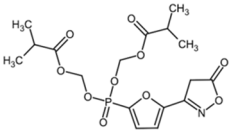
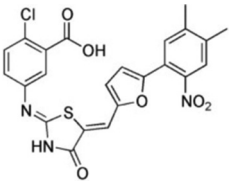
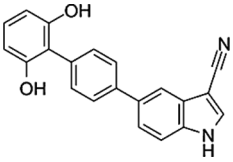
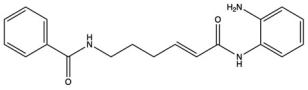
(Cont'd...)

Table 2. ***(Continued)***

Compound	Chemical structure	AMPK activation mechanism	References
Berberine		Respiratory chain blockage (complex I) Increase in AMP/ATP ratio	59
Curcumine		Increase in AMP/ATP ratio (action on mitochondrial F1F0-ATPase/ATP synthase)	60
Ginsenoside Rb1		Increase in AMP/ATP ratio (unknown mechanism)	61
α-lipoic acid		Unknown mechanism	62
EGCG		Respiratory chain blockage. Increase AMP level	63
Cryptotanshinone		Increase ROS (unknown mechanism)	64
Capsaicin		Not specified	65
Arctigenin		LKB1/CaMKK	66
Chitooligosaccharides		Not specified	67

(Cont'd...)

Table 2. ***(Continued)***

Compound	Chemical structure	AMPK activation mechanism	References
Nootkatone		Not specified	68
Thienopyridone (A-769662)		Allosteric activator (AMPK binding, β1 subunit)	69
Benzimidazole (Compound 911)		Allosteric activator (AMPK binding, β1 subunit)	5
Compound 991		Not specified (AMPK binding, β1 subunit)	70
Compound-13		Allosteric activator (AMPK binding, α1 subunit)	71
PT-1		Allosteric activator (AMPK binding, γ1 subunit)	72
MT 63-78		Allosteric activator (AMPK binding, β1 subunit)	73
R118		Respiratory chain blockage	69

Abbreviations: AICAR: 5-Aminoimidazole-4-carboxamide ribonucleoside; ZMP: 5-Aminoimidazole-4-carboxamide-1-β-D-ribofuranosyl-5′-monophosphate; AMPK: 5' adenosine monophosphate-activated protein kinase; EGCG: Epigallocatechin-3-gallate; LKB1: Liver kinase B1; CaMKK: Calcium-calmodulin-dependent protein kinase; ROS: Reactive oxidative species; AMP: Adenosine monophosphate; ATP: Adenosine triphosphate.

nigra pars compacta (SNpc), a region involved in the development of specific symptoms.[84] However, long-term treatment has been shown to negatively influence intellectual capacities, with the exact mechanism remaining unknown, though interference with vitamin B_{12} absorption is a likely factor.[85-87] Mitochondrial dynamics regulate many key cellular functions, including metabolic plasticity, mitochondrial turnover, and organelle communication, but the mechanisms by which these functions are affected by perturbations of mitochondrial dynamics during aging remain unclear. However, the appearance of swollen and fragmented mitochondria has been reported across all species studied, suggesting that mitochondrial fragmentation is a senescence-promoting factor.[88-90]

AMPK, as a regulator of energy homeostasis, links energy levels with the rate of aging and can be considered a modulator of senescence and lifespan extension, particularly through mechanisms such as caloric restriction (activated with a 30 – 70% reduction in the initial amount nutrient

consumed). Another interesting observation is the low level of leptin following metformin treatment, which may explain its anorexic effect. This effect could be due to the sensitization of the hypothalamus to leptin, potentially through an increase in the number of specific receptors in this region. The hypothalamus plays a crucial role in regulating energy balance, making it a key region for this effect.[91] Regarding the anti-inflammatory effect of metformin, various studies show a positive effect, following a reduction in cytokines such as interleukin-6, tumor necrosis factor-α, nuclear factor-kappa B, but this remains a hypothesis at the research stage.[91-93]

A mechanism for eliminating aged cellular organelles or damaged proteins can be achieved through autophagy. This process can be activated through the administration of exogenous agents, such as rapamycin, resveratrol, nicotinamide derivatives (Vitamin B_3), metformin, urolithin A or spermidine, compounds thought to delay aging, and even extend lifespan.[94,95]

Instead, the body has several defense and preservation mechanisms, with autophagy, which represents a process of "purification". This involves recycling outdated or damaged protein structures, including cellular organelles, by forming autophagosomes, which later fuse with lysosomes.[96,97]

Autophagy is the cell's survival mechanism, regulated by various signaling pathways, with the AMPK-mTOR system being one of the most important.

There are three types of autophagy described:
- Macro-autophagy
- Micro-autophagy
- Chaperone protein-mediated autophagy

In macro-autophagy, the autophagosome represents the catalytic unit. It is formed from an insulating membrane in which a small part of the cytoplasm, soluble molecules, and cell organelles are included. The next step involves fusion with lysosomes to form autolysosomal units, where the degradation of internal contents take place.

Micro-autophagy, on the other hand, occurs through the engulfment of small components by the lysosome. Thus, significant amounts of cytosolic material, including protein molecules are incorporated into this complex.

The third type of autophagy, chaperone protein-mediated autophagy, does not involve membrane reorganization. Instead, cytosolic proteins are selectively recognized and translocated to the lysosomal membrane by the chaperone protein Hsc7 together with its co-chaperones. The unfolded proteins are then transported to the lysosome through a multimeric complex.[98]

Experimental models in *C. elegans*, *Drosophila*, and rodents have demonstrated that mTOR growth factor inhibitors or caloric restriction led to similar results, significantly increasing the life expectancy of those organisms.[99,100] In contrast, continuous mTORC1 blockade can lead to the onset of cardiomyopathy and rapid progression to heart failure, hence impairing life expectancy.[101]

Thus, the autophagic process is crucial for promoting health and longevity. Disruptions in this mechanism can contribute to the development of age-related diseases.[102-104]

Substances known to be longevity promoters are divided into three categories according to their mode of action:
- Those that have demonstrated anti-aging effect but lack conclusive evidence for this ability;
- Those suggested to prolong youth by preventing or delaying the onset or progression of age-related diseases, though their influence on the aging process itself has not been proven;
- Those that reverse the aging mechanism, at least under certain conditions, thereby prolonging youth;

4. AMPK at the hypothalamus level

AMPK in the central nervous system is known to control nutrient intake and restore energy balance which is activated under pathophysiological conditions. The neuroprotective effect of metformin is thought to result from increased neuronal sensitivity to insulin, as poor insulin signaling in the brain is associated with an increased risk of neurodegenerative diseases.[105] AMPK integrates peripheral signals, such as metabolites and hormones, through neural networks. Following the integration of signals at the level of the hypothalamus, it causes the sensation of hunger, the production of heat (thermogenesis) and energy by mobilizing brown and white adipose tissue.

Studies have identified specific hypothalamic nuclei where AMPK is expressed, including the arcuate, dorsomedial, paraventricular, ventromedial, and lateral hypothalamic nuclei. Unlike AMPK in the rest of the body, hypothalamic AMPK activation increases food intake and causes weight gain, while its inhibition reduces appetite and weight.[3]

Using genetic models, it was shown that by blocking the dominant negative isoform of hypothalamic AMPKα decreases the messenger RNA (mRNA) expression of orexigenic neuropeptides, such as agouti-related protein (AgRP) and neuropeptide Y (NPY). Conversely, increased expression of the active isoform of AMPKα increases AgRP and NPY synthesis in the arcuate nucleus.[106] New data also indicate that AMPK modulates autophagy, thereby controlling the production of both NPY and proopiomelanocortin (POMC).[107] Both orexigenic and anorexigenic hormones control appetite through their

influence on AMPK activity. Anorexigenic hormones, such as leptin, glucagon-like peptide (GLP-1), estradiol, insulin, and ciliary neurotrophic factor (CNTF), inhibit AMPK activity, while orexigenic hormones, including ghrelin, adiponectin, glucocorticoids, cannabinoids, and AgRP, stimulate it.[108] AMPK is also involved in regulating thermogenesis in brown adipose tissue through its influence on the sympathetic nervous system, which in turn activates to increase heat production, thereby contributing to body temperature regulation and energy expenditure. Estradiol centrally binds to α-type estrogen receptors, selectively decreasing AMPK activity in the ventromedial nucleus, which increases the thermogenic capacity of brown adipose tissue. Variations in estradiol levels during the estrogenic cycle and pregnancy further modulate the AMPK pathway, establishing its important role for in energy balance and thermoregulation.[109]

5. AMPK and the endocannabinoid system

The metabolic effects associated with excess glucocorticoids are correlated with steps controlled by AMPK. As previously mentioned, activation of hypothalamic AMPK increases appetite.[110] In addition, there appears to be a link between AMPK and metabolic effects involving the endocannabinoid system.[111] These effects of the cannabinoid system are mediated by cannabinoid type 1 (CB1) receptors, which are also expressed in the hypothalamus.[112]

Activation of hypothalamic CB1 receptors stimulates appetite.[113] Concurrently, glucocorticoids induce changes at the hypothalamic level that are correlated with the increase in endocannabinoid content, suggesting an interdependence between endocannabinoids, glucocorticoids, AMPK, and appetite regulation. However, the effects of glucocorticoids and cannabinoids on AMPK can vary depending on tissue type. For example, in the hypothalamus (stimulation) and adipose tissue (inhibition), the effects are the same, whereas in the liver and heart, they differ.[114] In the heart, cardiac AMPK activation by cannabinoids appears to have cardioprotective effects, whereas reduced CB1 receptors expression is associated with negative effects.[115] The content of endocannabinoids at the hypothalamic level varies with caloric intake. During fasting, the concentration of endocannabinoids increases, and they decrease during feeding periods, mediating both the anorexigenic effects of leptin and orexigenic effects of ghrelin.[116,117]

Caloric restriction has additional implications, particularly in modulating nociception (perception of pain). Caloric restriction has been shown to modulate pain sensitivity, potentially reducing pain perception and influencing the body's response to injury or inflammation. Thus, reduced caloric intake is correlated with pain relief, as shown in clinical studies on patients with rheumatoid arthritis or fibromyalgia.[118] In addition, this pain relief correlates with increased levels of endocannabinoid mediators. Specifically, caloric restriction considerably increases the levels of anandamide (AEA) and 2-arachydonoyl glycerol (2-AG) in certain areas of the brain.[116] Through the same mechanism, activation of AMPK by caloric restriction increasing endocannabinoid levels is proposed to reduce nociception.[119] However, studies suggest conflicting effects of the endocannabinoid system on AMPK activity. Findings indicate a reduction in AMPK activity following endocannabinoid system activation.[120,121] Furthermore, a gradual and moderate reduction in caloric intake is more effective to activate these mechanisms, because the sudden and drastic caloric restriction can lead to unwanted effects, such as malnutrition. Intermittent fasting has been shown in both human and animal studies to increase levels of endocannabinoid mediators.[122]

In contrast to the previously mentioned approach, prolonged periods of moderate caloric restriction correlate with reduced AEA and 2-AG levels.[123] The observed analgesic effect and increased 2-AG level are correlated with low leptin level and/or decreased leptin sensitivity.[124]

Finally, the antinociceptive response mediated by the endocannabinoid system during caloric restriction is attributed to an increase in the number of CB1 receptors.[125]

6. Conclusion

Due to its central role in metabolic processes, AMPK represents an important therapeutic target for the treatment and prevention of several diseases, including age-related conditions. However, questions remain regarding its mechanism of action, activation pathways, and its diverse roles throughout the body. A multitude of studies consider AMPK as a metabolic "switch" that is inactive under sufficient energy supply and activated when energy sources are limited. AMPK appears to coordinate heart metabolism through protein interactions to generate targeted effects, with the goal of restoring cellular homeostasis. Systemic activation of AMPK, as discussed earlier, can influence the level of the hypothalamus and food consumption. In addition, the existence of multiple AMPK isoforms adds complexity, as the activation of AMPK may benefit one organ while disadvantaging another. For example, a gain-in-function mutation in the γ subunit is correlated with greater glycogen storage in skeletal muscles (γ_3) and in the heart (γ_2). This can lead to glycogen storage cardiomyopathy, cardiac hypertrophy, and arrhythmias, which are disadvantages for non-athletes, especially athletes. It is important to note that the activation

of AMPK by some compounds does not improve sports performance, as the outcomes depend on various factors, such as the type of physical activity (aerobic or anaerobic) and its intensity.

Future studies are essential for evaluating the impacts of AMPK-activating substances on sports performance, with particular attention to their mechanism of action and physiological outcomes. An additional critical consideration is the development of isoform-specific AMPK activators to ensure targeted effects and minimize potential adverse consequences. Until such advancements are achieved, regulatory frameworks, including "The Prohibited List" remain pertinent and subject to ongoing updates.

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REVIEW ARTICLE

Current view on photodynamic therapy in medicine

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Abstract

Light has been used for medical purposes for centuries, but the first steps toward photodynamic therapy (PDT) were taken in the early 20th century. PDT is an innovative therapeutic approach that involves three key components: A photosensitizer (PS), molecular oxygen, and visible light. The destruction of diseased tissues and cells in PDT occurs through the activation of a PS by near-infrared or visible radiation. This activation, in the presence of molecular oxygen, generates singlet oxygen and other reactive oxygen species. PDT has been successfully applied to treat various types of cancer, particularly superficial ones. This review outlines the principles of PDT and discusses its application in cancer treatment, specifically in the context of pancreatic and esophageal cancer. While PDT is effective, it can also have adverse effects on the human body, such as changes to cell and organelle membranes. PDT is a modern, non-invasive treatment modality utilized for both non-malignant conditions and also various types of tumors in diverse locations. Enhancing the efficacy of PDT and reducing its side effects may be possible by combining PSs with nanomaterials, which would also allow for targeted therapy to specific receptors. PDT is continuously being developed to improve its effectiveness, and ongoing studies aim to minimize unwanted side effects and identify contraindications for its use.

Keywords: Photodynamic therapy; Photosensitizers; Pancreas; Esophagus

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1. Introduction

Light has been used in medical therapy since ancient times. The Egyptians, Chinese, and Indians employed light to treat a variety of diseases, including vitiligo, rickets, psoriasis, skin cancer, and even psychosis. One of the earliest known uses of sunlight for medicinal purposes was heliotherapy, which the Greeks introduced around 3000 years ago. Herodotus, a renowned physician of the second century BC, is often considered the father of heliotherapy. The Greeks favored a form of treatment in which the entire body was exposed to sunlight. At the beginning of the 20th century, the first literature reported on the "photodynamic effect" was presented by a medical student Oscar Raab and his professor Herman von Tappeiner. They showed that exposure of certain dyes to sunlight could lead to cell death, as these dyes sensitized microorganisms to light.

In 1948, Figge and Weiland conducted extensive research on the possibility of using various porphyrin derivatives in photodynamic therapy (PDT) and diagnostics.[1] They demonstrated the use of hematoporphyrin (HDP) as a tool for cancer detection. However, at that time, this approach still carried the risk of toxic side effects. They also assessed the effectiveness of other compounds, such as coproporphyrin, protoporphyrin, and zinc HDP. In 1955, scientist Schwartz investigated the nature of HDP and found that it was a mixture of many different porphyrins, each with distinct properties. PDT gained further development when it was discovered that HDP derivatives increased the fluorescence of tumors in patients. This breakthrough was made by a team of doctors from the Mayo Clinic. In a review by Kessel D published in 2019, the historical details of the 1972 first usage of fluorescent in biology were described.[1] This study demonstrated the ability to eliminate glioma cells in tumors in both animal models and established cell cultures.[1,2]

Soon after, this mechanism was extended to the treatment of cancers of the lungs, esophagus, reproductive organs, brain, and head and neck. By the end of 1996, it was estimated that tens of thousands of patients with various types and stages of cancer had been treated with PDT. In Poland, the use of PDT began in 1986. Despite advances in cancer treatment over the past decades, cancer remains a major medical challenge, claiming millions of lives and negatively affecting the quality of life of survivors. The primary treatments for cancer remain chemotherapy and radiotherapy, both of which have serious side effects. Patients undergoing these treatments often report severe pain. Consequently, finding alternative treatment regimens that provide improvement in health status, with the possibility of complete recovery and minimal to no side effects, is a priority for clinicians and researchers.[3]

This review explores various important aspects of PDT, including its mechanism of action, and provides an overview of its historical development. We also highlight the promising results of photosensitizer (PS) use, combination therapies, and the prospects these innovations bring. The current directions of PDT are presented in Figure 1.

2. Mechanism of action of PDT

PDT relies on the concomitant presence of molecular oxygen, a PS, and near-infrared or visible light. Each of these ingredients is non-toxic on its own and does not damage tissues or cells. Ideally, the PS is absorbed and accumulates within the target cells. Since the PS is harmless when not exposed to light, any potential toxicity in PDT can be minimized by selectively illuminating only specific areas. A PS absorbs light energy and transfers it to nearby molecules that do not absorb light. This energy or electron transfer results in the production of highly reactive molecules, enabling PDT to function through three primary mechanisms: Direct destruction of target cells, damage to blood vessels that lead to ischemia and tumor cell death, and activation of the immune response. PSs enhance the activation of immune cells, such as neutrophils, macrophages, and lymphocytes, which, in turn, increase the secretion of interleukin (IL)-6, IL-10, and tumor necrosis factor (TNF), contributing to tumor cell death. Cell death in PDT occurs through two mechanisms: necrosis and apoptosis. Necrosis refers to the local death of tissues, which triggers acute inflammation at the site of injury. The byproducts of necrotic tissue breakdown are toxic to the body. On the other hand, apoptosis is programmed cell death, controlled enzymatically, and does not induce inflammation. PS is a molecule with electrons arranged in specific molecular orbitals. In its ground state, PS contains electron pairs with opposite spins in low-energy orbitals. When exposed to light of a specific wavelength, PS absorbs energy, causing an electron to be excited into a higher-energy orbital without changing its spin. This change creates a short-lived singlet state. The PS may return to its ground state by releasing heat or light. Alternatively, it may transition to a triplet state through intersystem crossing (ISC), where the electron spin is reversed. The longer PS remains in the triplet state, the greater the likelihood that it will encounter other molecules, leading to the formation of chemically reactive compounds. There are two primary processes that occur

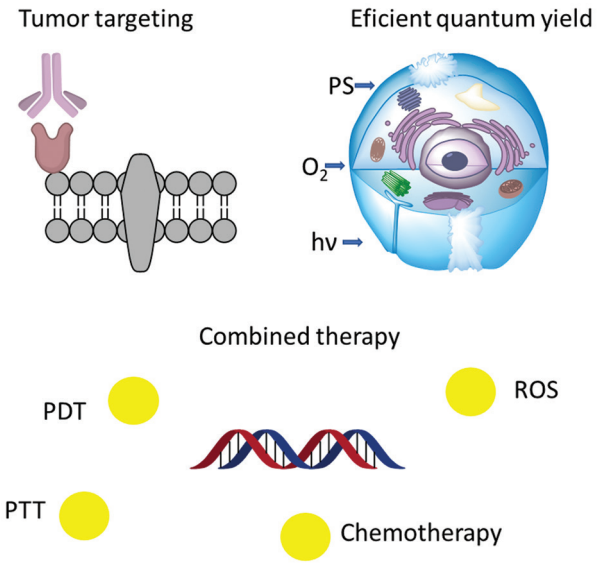

Figure 1. Current directions of photodynamic therapy
Abbreviations: PS: Photosensitizer; PDT: Photodynamic therapy; PTT: Photothermal therapy; ROS: Reactive oxygen species; h: Planck's constant; v: The frequency of light, O_2: Oxygen molecules.

when the PS is in the triplet state. In the first process, the PS removes an electron from a reducing molecule in its vicinity. Molecules that donate electrons include, for example, tyrosine in proteins, guanine in nucleic acids, or tryptophan. This electron transfer creates a pair of radical anions and radical cations. In an oxygen-rich environment, the PS radical anion transfers its electron to molecular oxygen, generating a superoxide anion, which effectively restores the PS to its original state. The superoxide anion can act as a reducing agent or as a monovalent oxidant. Although it does not directly interact with lipids, nucleic acids, or carbohydrates, the superoxide can oxidize small molecules such as leucoflavins, sugar tautomers, sulfates, tetrahydroflavins, and catecholamines. Superoxide radical reaction with other biologically relevant radicals may lead to the formation of potentially toxic, cell-damaging products. In biological systems, the most common phenol is the amino acid tyrosine. When the superoxide reacts with nitric oxide, a strong oxidant, peroxynitrite, is produced, which can react with carbon dioxide and bicarbonates to form nitrosoperoxycarbonate. The carbonate radical anion is a single-electron oxidizer that can remove electrons from tyrosine and tryptophan. In addition, superoxide can oxidize protein clusters, such as enzymes involved in the Krebs cycle and dehydratases. Disruption of these clusters, particularly the iron-sulfur [4Fe-4S] clusters, can have harmful effects. First, it impairs aerobic energy production and biosynthetic pathways by inactivating Krebs cycle enzymes. Second, it generates hydroxyl radicals, that is, strong oxidants, through the Fenton reaction, where released iron from the [4Fe-4S] clusters acts as a catalyst for hydrogen peroxide. The iron ions, in their reduced form, bind to anionic molecules, such as nucleic acids, proteins, lipids, and other cellular components. The stable hydrogen peroxide can diffuse through membranes and react with iron bound to biomolecules, generating highly reactive hydroxyl radicals. The radicals can damage cells at the site where they are formed. In the second process, the energy of the PS is transferred to molecular oxygen, which, in its ground state, is a triplet. The transfer of energy excites one of the two unpaired electrons into a high-energy orbital, causing a spin flip, which converts oxygen into a singlet. Singlet oxygen ($^1O_2$) is considered the main destructive agent in PDT. While biological systems have enzymatic defenses against superoxide, there are no developed antioxidant enzymes to remove $^1O_2$, likely due to its short lifespan.[4]

3. Mechanisms of cancer cell destruction in PDT

PDT targets cancer cells, specifically their microcirculation, as well as the functioning of the host's immune system. To achieve the desired therapeutic effect, certain conditions must be met, influencing each of these aspects. PDT reduces the number of clonogenic cancer cells through direct photodamage; however, this event does not lead to the complete eradication of pathological cells. This limitation arises from serveral factors, one of which is the heterogeneous distribution of the PS within the tumor. Another key factor is the availability of oxygen in the tissue being treated. Limited oxygen availability can restrict the direct killing of cancer cells (Figure 2). These constraints give rise to two primary mechanisms: photochemical oxygen consumption and the direct impact of PDT on tissue microcirculation. The amount of oxygen available during PDT depends on the photobleaching of the PS. As the PS is reduced, the rate of oxygen consumption during therapy also decreases. Fractionating the delivered light is crucial, as it allows the tissue to reoxygenate during breaks in illumination. Furthermore, PDT-induced microcirculation damage, particularly when higher doses of PSs such as Photofrin are used, disrupts oxygen delivery to tissues. Vascular mechanisms of PDT vary significantly with different PSs. For instance, PDT with Photofrin leads to vasoconstriction, leakage of macromolecules from vessels, leukocyte adhesion, and clot formation, all of which are associated with platelet activation and the release of thromboxane. PDT can also induce vasoconstriction by inhibiting the production or release of nitric oxide by the endothelium. Damage to the normal vascular system surrounding the tumor can significantly hinder the effectiveness of PDT. Studies have shown that cancer cannot be effectively treated when the tissue surrounding the tumor is damaged during PDT. Recent research suggests that treatment with a high fluence rate inhibits tumor cure. However, no differences in tumor perfusion or oxygenation were observed between treatments with low or high fluency. These findings indicate that the protection provided by the normal vascular system around the tumor is a crucial factor in the long-term control of cancerous tissue during PDT (Table 1). To optimize the efficacy of PDT, it is important to regularly monitor parameters such as the concentration of the PS in the tissues, the rate of photobleaching, blood oxygen levels, and blood flow.[3]

4. Literature search method

Articles for this review were retrieved from online databases (PubMed/MEDLINE) using the following search terms: "photodynamic therapy," "PDT," and "photosensitizers."

5. Results

The total number of articles identified was 92 and the number included in the current review is 45. In this paper, only studies in English are selected. The first cited here

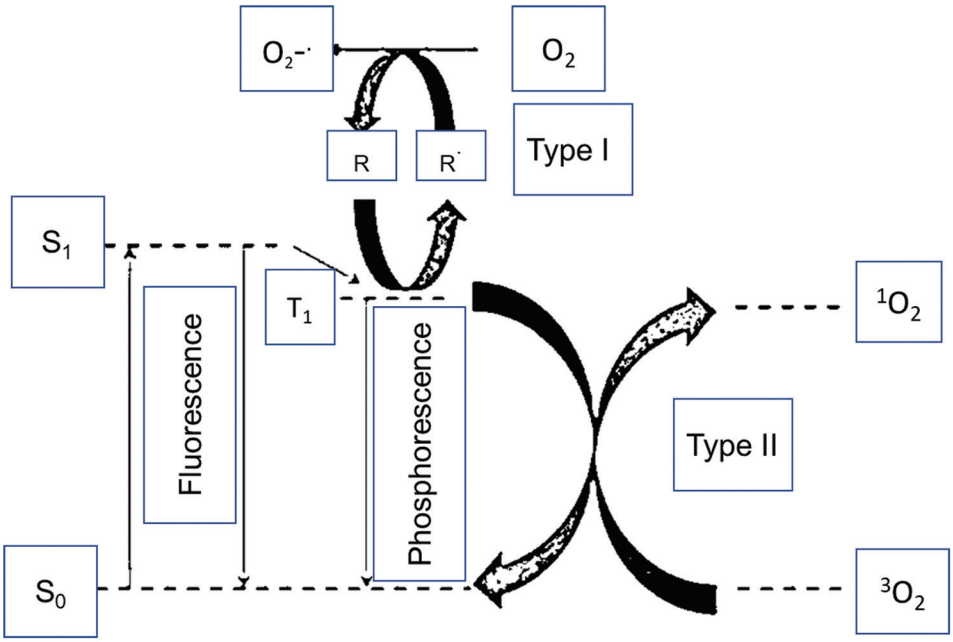

Figure 2. The mechanistic presentation of photodynamic therapy
Abbreviations: $^1O_2$: Singlet oxygen; $^3O_2$: Triplet oxygen; O_2: Oxygen; O_2^-: Superoxide anion; R: Radical; R·: Free radical.

Table 1. Efficacy of photodynamic therapy in the treatment of various cancers

Type of cancer	Photodynamic therapy (%)
Skin	85 – 95
Head and neck	70 – 90
Lung	50 – 70
Esophageal	70 – 80
Bladder	70 – 85
Pancreatic	60 – 75
Prostate	60 – 80

clinical study involved 15 patients with unresectable, high-stage tumors.[5] All patients were allergic to verteporfin at a dose of 0.4 mg/kg, whereas 13 received treatment using single fiber lasers and two with multiple fibers, each 690 nm in length, placed transdermally. The light dose was gradually increased until constant necrosis was achieved, and all procedures were monitored using a computed tomography (CT) scan. Necrotic changes of 12 nm in size were observed in all patients, though the volumes varied. No side effects were reported in patients who underwent chemotherapy. This study concluded that PDT with verteporfin for the treatment of malignant pancreatic cancer (PCa) is safe, and the compound is flexible in terms of administration.

The next study aimed to evaluate PDT in PCa through a similar phase 1 trial.[6] It involved 16 patients with adenomas located near the head of the pancreas that were not amenable to surgery. Patients were administered an oral dose of 0.15 mg/kg mesotetrahydroxyphenyl chlorite, followed by light delivery to the tumor through percutaneous fiber insertion under CT guidance 3 days later. Three patients subsequently received chemotherapy. Significant tumor necrosis was observed in all patients, as evidenced by imaging studies. Fourteen of the 16 were discharged after 10 days. Several adverse events, including gastrointestinal bleeding and duodenal obstruction, were recorded, which may have been related to the treatment. The median survival after PDT was 9.5 months, with seven out of 16 patients surviving for 1 year. This study highlighted that, while pancreatic tumor necrosis was achieved as expected, caution should be exercised, particularly in cases of tumors infiltrating the duodenal wall.

In another study, the feasibility and safety of endoscopic PDT in the treatment of unresectable ampullary cancer were evaluated.[7] Sixteen patients received an intravenous dose of 4 mg/kg of a HDP derivative, followed by red light (630 nm wavelength) administered during duodenoscopy two days later. A constant light energy density of either 50 J or 200 J/cm^2 was used, with three or four exposures during each session. Treatment was repeated up to 5 times at intervals of 3 – 6 months. Skin hypersensitivity was the only complication observed, affecting three patients. Tumor size was assessed every 4 – 8 weeks, and biopsy specimens were taken if the tumor was not visible macroscopically.

In patients with small tumors, a reduction in tumor mass and even remission were observed for several months. However, patients with widespread duodenal involvement showed only a modest response.

Another study focused on patients with colorectal cancer metastases to the liver, a group with a poor prognosis due to the scarcity of patients eligible for curative liver resection.[8] In this phase 1 trial, PDT was used to treat 31 liver metastases in 24 patients with unresectable colorectal cancer metastases. PS 5, 10, 15, 20-tetrakis(m-hydroxyphenyl)bacteriochlorin (mTHPBC) was administered intravenously at doses of 0.6 mg/kg or 0.3 mg/kg. Tumors were illuminated for 300 – 600 s using optical fibers inserted subcutaneously after 120 h, or after 48 h in some subjects. The scattered light dose was 740 nm. Results indicated tumor necrosis in all patients 1 month after PDT. A small proportion of patients reported mild pain, and the majority experienced transient subclinical hepatotoxicity. No serious clinical complications were noted, except for pancreatic damage in one patient and skin damage in another. Harmless side effects included transient phlebitis and mild skin phototoxicity from excessive light during treatment. The study concluded that PDT with mTHPBC is a safe and effective treatment for unresectable colorectal cancer liver metastases.

Meanwhile, one study showed that treatment with PDT for locally advanced pancreatic cancer (LAPC) may be limited due to the unfavorable prognosis associated with the disease.[9] The investigators hypothesized that PDT for LAPC is safe, technically feasible, and can lead to increased tumor necrosis. In this phase 1 study, patients with untreated LAPC were administered intravenous porfimer sodium, followed by endoscopic ultrasound-guided PDT (EUS-PDT) 2 days later. EUS-PDT was performed by puncturing the tumor with a 19-gauge needle, and a 1.0 cm diameter light diffuser emitting 630 nm light was introduced. CT scans were conducted 18 days after PDT to assess pancreatic necrotic changes. Seven days following the CT scan, intravenous Nab-paclitaxel and gemcitabine were administered weekly in a 3-week cycle until disease progression or unacceptable cytotoxicity occurred. In terms of primary outcomes, 12 patients with tumors located in the head, neck, trunk, or tail of the pancreas underwent EUS-PDT. In half of these patients, an increase in tumor volume and a higher percentage of tumor necrosis was observed in comparison to the initial imaging tests. After a follow-up period of 10.5 months, the median time to progression was 2.6 months, and the median overall survival was 11.5 months. During the treatment course, eight serious adverse events were reported, although none of them were directly attributed to the therapy. The study concluded that EUS-PDT for LAPC is safe and results in measurable tumor necrosis. A phase 2 trial is planned to further investigate these findings.

5.1. The use of PDT in the treatment of pancreatic cancer

Pancreatic cancer is a condition associated with a high mortality rate. The primary treatment options are surgery or chemotherapy; however, an increasing number of cases are diagnosed at advanced stages, making surgery infeasible. In addition, chemotherapy does not bring the desired results due to the resistance exhibited by PCa cells. Given its high efficacy against cells resistant to radiotherapy and chemotherapy, PDT has emerged as a viable alternative for patients who are not candidates for surgery. Clinical practice has confirmed the effectiveness of this minimally invasive treatment method. However, PDT faces several limitations when used in clinical settings, including challenges related to insufficient delivery of PSs, dependence on tumor oxygenation, and the ability of malignant tumors to evade treatment. To address these challenges, researchers are increasingly focusing on the development of PS nanoparticles (NPs) and various nanocarrier systems to enhance the cellular uptake and distribution of PSs within the body. Encapsulation of PSs in NPs significantly increases their accumulation in PCa tumors, owing to improved solubility and stability in the bloodstream. Various strategies have been explored to develop multidrug codelivery NPs, which facilitate synergistic PDT-based therapies, thereby enhancing the effectiveness and prolonging the therapeutic response.[10]

While pancreatic surgery is technically challenging, PDT offers a minimally invasive alternative. Despite its lower invasiveness and promising potential for treating PCa, PDT is not without its adverse effects, such as gastrointestinal hemorrhage and duodenal obstruction. These side effects are partly due to the limited selectivity of current PSs, which results from their non-selective distribution. This non-specific distribution creates a risk of photodamage to adjacent organs and prevents adequate accumulation of PS at tumor sites. Furthermore, during follow-up after PDT, some cases have shown liver metastasis and tumor regrowth at the edges of the treated area.[10] It is believed that these phenomena may be attributed to insufficient oxygenation within the tumor. In addition, PDT alone is insufficient to fully combat cancer. As a result, specially modified NPs loaded with PSs are being used to enhance PS solubility, improve oxygen consumption, and facilitate more effective delivery. These NPs are often functionalized with special ligands that enable targeted delivery of PSs to cancerous pancreatic cells, thereby significantly improving the efficacy of PDT for PCa. As mentioned earlier, PDT

is more effective when combined with other therapies, such as photothermal therapy, which generates additional oxidative stress. The effectiveness of PDT in alleviating tumor burden in patients with unresectable localized PCa has been demonstrated in several clinical trials, with relatively few complications. Despite promising pre-clinical data showing enhanced efficacy of NP-based PSs compared to conventional PSs, their clinical application in the treatment of PCa remains challenging. This limitation is due to the ongoing refinement of these compounds' functionalities and the lack of research on clinically relevant models. PDT was the first approved as an endoscopic procedure for esophageal cancer in both the United States and Japan. Initially, it was used as a palliative local treatment for patients with obstructed esophageal cancer. PDT is also indicated as eradication therapy for dysplastic Barrett's esophagus, a precursor to esophageal adenocarcinoma, and is supported by level 1 evidence. In Japan, PDT has been approved as a curative treatment for superficial esophageal cancer lesions that are difficult to manage with endoscopic resection. In addition, PDT using second-generation PSs has been approved for treating early local recurrence following radiotherapy, a condition that is challenging to treat using other methods. PDT has also been evaluated in other gastrointestinal cancers, including gastric cancer, cholangiocarcinoma, and PCa.[11]

5.2. Treatment of esophageal cancer using PDT

Esophageal cancer is associated with a very poor prognosis, with a 5-year survival rate of only 12.5%. The standard treatment for this cancer has traditionally been the resection of part of the esophagus; however, due to the high morbidity associated with surgery, less invasive methods such as endoscopic mucosal resection (EMR), coagulation, and PDT are increasingly used. In PDT, the light source required for this method is provided by diffusers, which are cylindrical and highly flexible. These diffusers are positioned near the tumor site using an endoscope. To protect healthy esophageal tissue, a balloon is inflated next to the diffuser. Initial studies focused on using PDT as a palliative treatment for obstructive tumors, although it has also proven effective for treating superficial lesions. The type of PS used is a key factor determining the treatment's effectiveness. When sodium porfimer was used, the complete response rate was 87%, and similar results were achieved with mTHPBC.[12] Despite the high efficacy of PDT in treating esophageal cancer, the method is not without undesirable side effects. The most frequently recorded symptoms included transient skin photosensitivity, fistula stenosis, and perforation, particularly when red light was used. However, when green light was employed, these side effects were not observed, and the effectiveness of the therapy remained unchanged.

PDT is especially useful in the treatment of Barrett's esophagus, a condition in which the epithelial cells in the lower esophagus are affected, often as a result of chronic reflux of gastric contents into the esophagus. This condition causes damage to the esophageal epithelial cells, leading them to resemble those of the stomach, a process known as metaplasia. While most cases of Barrett's esophagus do not progress to cancer, individuals with this condition have an increased risk of developing esophageal cancer. Treatment typically involves controlling acid reflux symptoms and monitoring the esophagus for signs of malignant changes. However, when cancerous transformations occur in Barrett's esophagus, PDT is considered. PDT can be used to eliminate cancerous or altered cells, but treatment must be tailored to the individual case by a physician specialized in this type of therapy. The suitability of PDT for treating Barrett's esophagus depends on various factors, including the severity of the changes and the patient's overall health.

One study examined the effects of Photofrin in 77 patients with unresectable, obstructive esophageal cancer.[12] Patients were initially administered Photofrin at a dose of 1.5 – 2.0 mg/kg, followed by endoscopic treatment with 630 nm light 48 h later. The most common complications following PDT were minor, including esophageal stenosis, esophagitis, pleural effusion, and sunburn. Twenty-nine patients required more than one cycle of PDT, and an additional seven patients required the implantation of an expandable metal stent due to recurrent dysphagia. The study concluded that PDT is a safe and effective treatment for alleviating obstruction and bleeding in esophageal cancer.

In the subsequent study, patients with advanced esophageal cancer were randomized to receive either PDT using sodium porfimer and an argon laser dye, or laser therapy using a neodymium-doped yttrium aluminum garnet (Nd: YAG) laser.[13] A total of 236 patients were randomized, and 218 were treated at 24 sites. Dysphagia improved equally in both groups, with nine complete tumor responses occurring after PDT and two after Nd: YAG therapy. Better outcomes were observed in patients whose tumors were located in the upper and lower thirds of the esophagus, as well as in patients with longer tumors or those who had previously undergone treatment. Compared to the Nd: YAG, PDT patients experienced more mild-to-moderate complications, including sunburn. In addition, treatment discontinuation occurred in only 3% of patients after PDT, compared to 19% after Nd: YAG, primarily due to side effects. PDT with sodium porfimer demonstrated overall equivalent efficacy in alleviating dysphagia in esophageal cancer compared to Nd: YAG laser thermal ablation, with an objective tumor response

rate that was equal to or better than Nd: YAG. The main limitation of PDT is temporary photosensitivity, but PDT is easier to perform and involves fewer invasive procedures than Nd: YAG laser therapy, which was a key conclusion of the study.

In another study on esophageal cancer, patients were orally administered 60 mg/kg of 5-aminolevulinic acid (ALA). Twenty-four hours after administration, gastroscopy was performed. Initially, tumor localization was done using white light, after which the light source was switched to red light with an intensity of 100 J/cm^2 for 600 s. Gastroscopy was repeated 48 h later and again seven days after the first procedure. The degree of dysphagia was recorded both before treatment and 14 days after treatment. The study included patients with advanced, unresectable tumors – two of whom had squamous cell carcinoma of the middle esophagus and three had terminal esophageal adenocarcinoma – mild, self-limited reactions were observed in all patients. Treatment had no significant effect on liver and kidney function, hemoglobin concentration, or leukocyte count, which helped ensure the accuracy of the test results. Most patients showed improvement in dysphagia. The final conclusion of the study was that PDT, using ALA as a PS and non-laser therapy, may provide an effective approach for treating dysphagia in such patients.[14]

Given the promising results of new PSs, such as ALA, in reducing skin phototoxicity during PDT, Maier *et al.*[15] conducted a study to evaluate the effectiveness of poly hematoporphyrin (Photosan) as a PS in PDT for advanced esophageal cancer. This study aimed to compare the effectiveness of ALA with Photosan in the treatment of advanced esophageal cancer, with a focus on skin phototoxicity and dysphagia reduction. Following diagnosis, 22 patients received therapy using ALA, whereas 27 patients received Photosan. PDT was delivered using a dye laser with a wavelength of 630 nm, and hyperbaric oxygen therapy was used as an adjunct. Both groups demonstrated therapeutic effects, including visible narrowing of the tumor's diameter and length, with slightly greater effectiveness noted for the Photosan treatment. Neither group experienced severe treatment-related complications, and no cases of sunburn were reported. No mortality occurred during the treatment period. The study concluded that Photosan is more effective than ALA in PDT for esophageal cancer.

Another study involved 64 patients with various types of cancer, including five with esophageal cancer.[16] The PS was administered intravenously, and light with a wavelength of 630 nm was delivered through quartz fibers. After treatment, complete remission was achieved in 48 of the 58 treated patients, including those with esophageal cancer. The only significant side effect was skin hypersensitivity. The study showed that PDT is effective in treating superficial tumors.

The greatest risk factor for esophageal cancer is Barrett's esophagus. Kelly *et al.*'s[17] conducted a study to compare argon plasma coagulation and PDT for the ablation of Barrett's esophagus. A total of 65 patients were included, with 34 assigned to argon plasma coagulation and 34 to PDT using ALA. Multiple sessions were conducted over 24 months. After completion of treatment, a macroscopic reduction in the area of Barrett's esophagus was observed in all patients. In the argon plasma coagulation group, better results were noted in terms of esophageal obstruction (97%) and a lower incidence of occult glands (24%) compared to PDT (50%). Both methods were effective in the ablation of Barrett's esophagus.

In a separate study,[18] patients with esophageal cancer were monitored for 5 years and underwent PDT using the same PSs as in the previous study. A biopsy of the primary segment of Barrett's esophagus was taken according to the Seattle protocol, followed by endoscopy. Patients who did not respond to PDT or had recurrent neoplasia underwent additional EMR therapy. Initially, better results were observed in patients treated with ALA, and most patients responded well. Compared to Photofrin, ALA showed greater effectiveness and caused fewer relapses. However, after longer follow-up, there was no significant difference in the long-term complete reversal of intestinal metaplasia and dysplasia. Four patients in each group developed invasive esophageal adenocarcinoma. The likelihood of long-term remission was significantly increased in patients who initially responded to ALA-PDT therapy. As a result, radiofrequency ablation combined with EMR has become the preferred minimally invasive ablative therapy due to its lower therapeutic efficacy compared to PDT.

In Ackroyd *et al.*'s study,[19] 36 patients with dysplastic Barrett's esophagus, who received omeprazole for acid suppression, were assigned to receive oral ALA at a dose of 30 mg/kg or a placebo. Four hours later, laser endoscopy was performed. Follow-up endoscopic examinations were performed at 1, 6, 12, and 24 months. In the ALA group, 16 of 18 patients showed a reduction in the affected area. In the placebo group, a 10% reduction in surface area was observed in two patients, whereas no changes were noted in 16 patients. None of the patients treated with PDT showed columnar epithelial dysplasia in the treated area, whereas 12 patients in the placebo group still exhibited dysplasia. No serious side effects, either short- or long-term, were observed. The therapeutic effects lasted up to 24 months.

The question of whether PDT has long-term effectiveness in patients with high-grade dysplasia in Barrett's esophagus

was addressed by Gray and Fullarton.[20] In their research, 21 patients received Photofrin intravenously 48 h before endoscopy, during which laser light with a wavelength of 630 nm was administered. Biopsies and endoscopies were performed every 6 – 12 weeks. Gray and Fullarton found that 16 patients remained free of high-grade dysplasia for a median of 62 months. Three patients developed an adenoma between the 40th and 50th month. PDT was effective in 84% of cases, making it a viable treatment for patients unable to undergo surgery.

Another question regarding whether Barrett's esophagus can be completely removed was addressed by Hage *et al.*[21] In their study, patients were assigned to three groups, where the first group received ALA-PDT intravenously in one dose, followed by light administration after 4 h. The second group received the same compound in two doses, with light applied after the same interval. The third group was treated with argon plasma coagulation in two sessions. The best result was achieved in the third group, where complete elimination was observed in 100% of patients after 6 weeks. In the first and second groups, complete elimination was observed in 86% and 93% of patients, respectively. In summary, argon plasma coagulation alone or ALA-PDT combined with coagulase can lead to the complete regression of Barrett's epithelium.

5.3. Immunological effects of PDT

The use of PDT also has adverse effects on the human body. PDT-induced changes in cell membranes, as well as in organelle membranes when using most PSs, may lead to side effects. These changes can affect signaling pathways, including increased expression of early immune response stress genes and proteins. Another potential effect is inflammatory cell damage. In such cases, membrane lipids are damaged through the degradation of phospholipids, leading to the release of fragments of arachidonic acid and lipids, which act as mediators of inflammation. While inflammatory signaling after PDT can have detrimental effects, it also has beneficial outcomes, such as the initiation and support of leukocyte recruitment from the blood. A notable consequence is the swift accumulation of neutrophils, which can remain in tumor blood vessels and significantly contribute to endothelial damage. As a result, the disintegration of these neutrophils results in the release of harmful oxygen radicals, myeloperoxidase, and lysosomal enzymes, which break down proteins and damage cancer tissue.

In evaluating the effectiveness of PDT in controlling inflammation, a study involving 28 patients with severe chronic periodontitis is particularly relevant. Patients were randomly assigned to either the PDT treatment group or a control group. Using the Luminex test, the expression of important cytokines – such as IL-1α, IL-1β, IL-8, vascular endothelial growth factor (VEGF), interferon-γ, TNF-α – was quantitatively assessed. Significant differences in cytokine levels were observed between the groups for IL-1α, IL-1β, IL-8, and VEGF, whereas the remaining cytokines showed no statistical differences. This study suggests that PDT may enhance the benefits of periodontal treatment by modulating immune responses.[22]

Increasing research indicates that PDT may also be effective in treating non-cancer conditions, such as genital warts and condyloma. A study was conducted to confirm the high cure rates and low recurrence rates in the treatment of condyloma with ALA-PDT. At various time intervals after treatment, the recruitment and significance of immune cells at the lesion sites were examined using immunohistochemical analysis. The study involved 15 patients who had several cycles of PDT. The infiltration of clusters of differentiation (CD) 3, CD4, CD8, CD1a, and CD68 cells was assessed using immunocytochemical staining, and biopsy samples were collected at baseline and at various times during the study. The best results were achieved in the treatment of anal condyloma. As treatment progressed, there was an increase in the infiltration of CD4+ T lymphocytes and Langerhans cells in the skin. Patients who responded to treatment also exhibited an increase in CD8+ lymphocytes. These results suggest that rapid activation of the immune response (CD4+ lymphocytes, Langerhans cells) may be responsible for healing to confirm previous clinical data.[23]

Wu *et al.*'s[24] assessed the effectiveness of PDT in reducing inflammatory infiltrates in periodontal disease, with a particular focus on how it affects the organization of the collagen network. Two drug delivery systems (DDS) were tested: Liposomes and nanoemulsions. The study involved 16 patients with advanced periodontal disease who were candidates for tooth extraction. The patients were divided into two groups, with one group receiving liposomes and the other receiving nanoemulsions. Seven days before therapy began, each patient had one tooth undergo PDT, whereas another tooth served as a control. After PDT, the density of the collagen fibers increased in the treated tooth compared to the control. PDT affected the immune system in both groups, but the responses differed. In the liposome group, the number of macrophages decreased, while in the nanoemulsion group, the number of Langerhans cells decreased. These results demonstrate that PDT has an impact on the immune system when used as a therapy for gingivitis and chronic periodontitis.

Another study aimed to investigate the effect of PDT as a therapy for multiple plantar warts.[5] The study involved

61 patients with the condition, who were randomly assigned to appropriate treatment groups. One group received PDT, whereas the other, the control group, was treated with cryotherapy. Several parameters were evaluated during the treatment: the assessment of skin lesions, pain levels, relapse rates, and side effects. Both groups showed improvement in skin lesions, but the PDT group demonstrated a slightly higher improvement (13.39 ± 3.88% before treatment to 1.48 ± 2.50% after the final treatment). After 3 months of therapy, the recurrence rate was significantly lower in the PDT group, whereas cryotherapy showed a recurrence rate of 20 – 53%. The study concluded that PDT had a more positive effect on the recurrence rate of multiple plantar warts compared to the traditional cryotherapy method.

Meanwhile, Theodoraki *et al.*[25] examined the effectiveness of PDT in treating head and neck squamous cell carcinoma (HNSCC). For an anti-tumor response to occur, PDT must trigger a local inflammatory reaction within the tumor cells. This study involved nine patients with HNSCC. Blood samples were collected before, during, and after PDT to assess the effects on the immune system. The frequency and number of T and B lymphocytes, CD4+CD39+ T regulatory cells (Tregs), and natural killer (NK) cells were evaluated using flow cytometry. The results showed an increase in the number of Tregs and NK cells in patients treated with PDT. In addition, serum concentrations of IL-6 and IL-10 were elevated, whereas the level of perforin decreased. Despite being a topical therapy, PDT produced a systemic response. The increased number of immune cells observed supports the effectiveness of PDT in HNSCC patients.

Immunosuppression may reduce the effectiveness of PDT. Niacinamide, that is, Vitamin B3, has been shown to prevent immunosuppression caused by UV radiation. However, the cause of immunosuppression during PDT is not well understood. Therefore, Thanos *et al.*[26] investigated the effect of niacinamide on immunosuppression induced by PDT. The study involved 22 healthy participants who tested positive in a Mantoux test. The second group received niacinamide orally at a dose of 500 mg, or a placebo, once daily for one week. Specific sites were then irradiated to assess immunosuppression at both irradiated and control sites. The study reported that PDT with the vehicle or placebo caused significant immunosuppression, whereas oral niacinamide therapy reduced it. Contrarily, low-frequency PDT did not induce immunosuppression in patients treated with topical niacinamide. The conclusion was that niacinamide could potentially prevent PDT-induced immunosuppression as a low-cost, effective method.

Meanwhile, de Moraes *et al.*[27] explored histomorphological changes and immunodetection following PDT using chloroaluminum phthalocyanine (AlClPc). The study involved eight participants who were scheduled for tooth extraction. Seven days before the extraction, 40 μL of AlClPc nanoemulsion was injected into their gums, followed by radiation exposure. The opposite side was used as the control. Tissue samples were collected 7 days after exposure and analyzed. The results showed edema in the treated tissues. No side effects were reported, and the therapy was well tolerated. These findings indicate that AlClPc is safe for clinical use in gingival tissue.

Evangelou *et al.*[28] investigated the effect of PDT on leukocyte migration following clinical treatment of basal cell carcinoma. The lesions were treated with methylaminolevulinate (MAL), and samples were collected before and after treatment. The study found that MAL caused rapid infiltration of neutrophils after PDT. In addition, a decrease in the number of Langerhans cells was observed just 1 h after PDT. The decrease in Langerhans cells could potentially impair the antitumor response, suggesting the need for further research in this area.

Finally, a study explored the use of PDT in the treatment of periodontitis. The clinical material used was gingival fluid. Two groups were studied: One group received scaling and root planning alone, whereas the other group received PDT with a 638-nm laser and toluidine blue. Bleeding was assessed over a 3-month period, and a mouth rinse test was performed to evaluate the level of polymorphonuclear cells. Improvements were observed in all outcomes compared to baseline, with the only significant difference noted in TNF-α. The study concluded that a single PDT exposure did not produce significant results in the treatment of periodontitis.[29]

6. Discussion of commonly used PSs in PDT

Porphyrins are a group of organic chemical compounds that contain a porphyrin ring. These compounds are activated by light in the wavelength range of 600 to 700 nm. The porphyrin ring consists of four (five-membered) rings connected by methine bridges (–CH=), forming a planar structure. The central metal ion, typically a magnesium ion, is coordinated with the ring and plays a key role in many of the biological functions of porphyrins. The most well-known porphyrin is heme, a key component of hemoglobin, which is responsible for transporting oxygen in the blood. Other porphyrins are involved in various biological processes, such as cellular respiration, electron transport in mitochondria, and metabolism. One example of a porphyrin-based PS is HDP, which is used in PDT. When exposed to light, HDP undergoes a photodynamic

reaction that leads to cellular destruction. HDP is activated by light in the wavelength range of 763 nm. Chlorophyll, another porphyrin, plays a vital role in photosynthesis in plants. It absorbs sunlight and converts it into chemical energy, which is then used to synthesize organic compounds.

Chlorophyllins are chemical compounds derived from chlorophyll. More specifically, they are chlorophyll derivatives in which the magnesium ion is replaced by another metal, most commonly copper. These compounds are known for their characteristic green color, similar to chlorophyll, and have various medical and industrial applications. The activation range for this PS is 690 nm.

Phthalocyanines are a group of organic dyes containing a phthalocyanine ring, whose structure is similar to that of chlorophyll. However, phthalocyanines contain nitrogen atoms in a place where the magnesium is found in chlorophyll. The most important and well-known member of this group is copper phthalocyanine. Phthalocyanines are activated by light in the range of 650 – 800 nm.

Bacterioporphyrins are chemical compounds that, like other porphyrins, contain the characteristic porphyrin ring. They differ from typical porphyrins such that they are found in bacteria, and their structure may vary from those present in eukaryotic organisms. The activation range for bacterioporphyrins is 600 – 700 nm.

7. Distribution of PSs to cancer cells

The mechanisms underlying the distribution of PSs used in PDT to cancer cells are not fully understood. However, it is believed that their selective permeability through cancer tissue plays a key role in this process. As the hydrophobicity of a given chemical compound increases, the affinity of the PS for cancer tissue also increases. This notion is supported by studies showing that PSs bound to liposomes exhibit greater effectiveness and selectivity in targeting cancer cells. The distribution of these sensitizers is enhanced by the inclusion of amphiphilic systems, which facilitate their delivery to the cancer cells. These systems are stable in aqueous environments, yet possess non-polar compartments that can accommodate substrates. One effective strategy involves combining PSs with low-density lipoproteins (LDLs), as various types of cancer cells secrete a significant number of membrane receptors for LDLs. In cancer cells, PSs localize in the plasma membrane, Golgi apparatus, rough endoplasmic reticulum, and mitochondrial membrane. The mode of PS transport into the cell determines the type of cellular response in the target tissue. When PSs are transported by albumin, extensive impairment of the vascular system occurs, leading to tumor ischemia and hypoxia (Table 2). Conversely, when PSs are transported by LDLs, early and significant damage to cancer cells takes place, primarily through necrotic and apoptotic processes.

8. Side effects and contraindications in PDT

PDT has relatively few contraindications. These include hypersensitivity to the ingredients of topical preparations used in the treatment of dermatological conditions and diseases associated with photosensitivity (e.g., lupus and porphyrias). Patients with porphyrias, a group of rare metabolic diseases linked to impaired porphyrin synthesis, may be at increased risk of photosensitization reactions. As such, the use of PDT may be limited in these individuals. People with advanced kidney or liver disease may have a reduced ability to eliminate photosensitizing agents, which could increase the risk of side effects. The safety of PDT during pregnancy has not been fully investigated, and as a precaution, it is generally recommended to avoid PDT in pregnant women, particularly during the first trimester. It is worth emphasizing that each case should be considered individually, with decisions regarding the use of PDT made by the attending physician, who must take into account the patient's overall health and the specifics of their condition. Regardless, side effects manifest as a modulation of the inflammatory response and local immunosuppression, which may result in pain, burning, swelling, and redness at the irradiation site. These symptoms typically resolve within a few hours after the procedure, although swelling and erythema may persist for several days. A late side effect may include hyper- or hypo-pigmentation at the treatment site, particularly in patients with darker skin types. Skin peeling is also a normal reaction to PDT. In addition, PDT may increase the expression of antigens on the surface of cancer cells, which can enhance the immune response. General reactions such as fatigue or headache have also been reported in some patients.

It is important to note that PDT has shown promising results as a treatment for Barrett's esophagus, though its use may be limited due to certain undesirable effects. With ongoing studies in modern laboratories and clinical facilities, efforts are underway to improve the efficacy of PDT while minimizing side effects. Furthermore, research is exploring the potential impact of genetic abnormalities on PDT effectiveness. By integrating the knowledge gained so far, PDT has the potential to become a breakthrough treatment in medicine.[30]

9. New perspectives in PDT

The heavy atom effect and chemical configuration play crucial roles in enabling boron-dipyrromethene (BODIPY) derivatives to generate $^1O_2$ for PDT. BODIPY and its

Table 2. The most commonly used photosensitizers in photodynamic therapy

Photosensitizer	Generic name	Wavelength (nm)	Application
Porphyrins	Photofrin, 5-aminolevulinic acid	600 – 700	Skin (superficial), head and neck, internal tumors (sometimes)
Chlorophyllins	Verteporfin	690	Eye diseases, mainly macular degeneration
Phthalocyanines	Chlorine aluminum sulfonate	650 – 800	Surface and internal
Bacterioporphyrins	Fotoditazin	600 – 700	Bacterial skin infections
Hematoporphyrin	Tookad	763	Prostate cancer

derivatives are synthesized by reacting 2,2'-dipyrromethene derivatives with boron trifluoride-diethyl ether complex ($BF_3(C_2H_5)_2O$) in the presence of triethylamine or 1,8-diazabicyclo5.4.0]undec-7-ene. Recent advancements have led to the design and synthesis of BODIPY-based platinum complexes for PDT, where the introduction of platinum atoms has been shown to improve both solubility and $^1O_2$ quantum yield.[31] BODIPY-hetero[5]helicene compounds, which combine the structures of BODIPY and hetero [5]helicene, have also been synthesized. These compounds exhibit excellent optical properties and high ISC efficiency, with ISC efficiency correlating to the torsion angles of the compounds. Studies have explored their potential as PDT agents, and one BODIPY-hetero[5] helicene compound was found to effectively kill cancer cells upon light exposure. The anticancer efficacy of this compound was verified using clonogenic and MTT assays, showing that they can effectively target and kill cancer cells from various tissue origins, including U2-OS, MCF-7, and MDA-MB-231 cell lines after photo exposure [B]. PDT using aza-BODIPY has also been shown to induce apoptosis in MCF-7 breast cancer cells by activating p53 and caspase 3. Flow cytometry analysis revealed that 28% of the cells underwent apoptosis. Gene expression analysis post-PDT exhibited downregulation of epidermal growth factor, lymphoid enhancer-binding factor 1, WNT family member 1, transcription factor 7, and transforming growth factor beta receptor II genes, alongside upregulation of caspase 3 and tumor protein P53. PDT also impairs cell connectivity and affects the cell cycle. Notably, these effects were not observed in control cells and MCF-7 cells under dark field conditions, indicating that aza-BODIPY possesses potent antitumor photodynamic activity.[32,33]

Boron-dipyrromethene compounds are widely utilized in various fields, particularly in biomedicine and technology. In biomedicine, BODIPYs are used as fluorescent probes for bioimaging and as $^1O_2$ generators in PDT. They also serve as fluorescent sensors, bioconjugate components, laser dyes, and switches. In technology, BODIPYs are applied in solar fuel generation, photovoltaic devices, antenna systems, photoredox catalysis, photooxidation of organic pollutants, and the photoinitiation of polymerization. A key challenge in the development of BODIPY compounds is improving their solubility in water and physiological media while preventing the formation of non-fluorescent aggregates. One promising approach is the incorporation of hydrophilic fragments, such as polyethylene glycol (PEG), N,N-bis(2-hydroxyethyl)amine, sugars, nucleotides, or ionic groups (carboxylic, sulfuric, and ammonium acids) into the BODIPY structure, which enhances their solubility in water and prevents aggregation. The integration of PEG into drugs improves their solubility in water, facilitates cellular uptake, and increases the overall therapeutic efficacy.[34]

The amphipathic polymer PS PEG-BODIPY was developed as a carrier for DDS with real-time tracking properties. Composed of a hydrophilic PEG segment and a hydrophobic BODIPY segment, PEG-BODIPY enables the encapsulation of anticancer drugs, such as doxorubicin (DOX), into micelle spaces. These micelles spontaneously self-assemble in aqueous environments into bilayer amphiphilic polyethylene glycol-grafted (PEGylated) BODIPY polymers. A cellular uptake study of PEGylated BODIPY and DOX@PEGylated BODIPY nanoformulations showed that MDA-MB-231 cells endocytosed these nanoformulations within 24 h.[35] The fluorescence intensity of PEGylated BODIPY significantly increased with the concentration of nanoformulations, and both types of nanoformulations were primarily localized in lysosomes.

The results of studies on the anticancer effects of PEGylated BODIPY and DOX@PEGylated BODIPY nanopreparations demonstrated high phototoxicity and low toxicity in dark conditions.[36] Upon irradiation, PEGylated BODIPYs exhibited cytotoxicity with an IC50 of approximately 25 nM, whereas DOX@PEGylated BODIPYs showed even stronger phototoxicity, with an IC50 of around 10 nM. The higher IC50 for DOX@PEGylated BODIPY can be attributed to the combination of BODIPY-mediated PDT and DOX chemotherapy.

In DOX@PEGylated BODIPY nanopreparations, BODIPY compounds generate $^1O_2$ during light irradiation, not only inducing phototoxicity in cancer cells but also

promoting the degradation of the nanopreparations. This degradation facilitates the release of DOX into the cell nucleus, thereby enabling the combined action of PDT and chemotherapy. In conclusion, these micelles can serve as nanocarriers for chemotherapeutic drugs such as DOX, thus enabling a dual treatment approach of photodynamic and chemotherapeutic anticancer treatment. The synthesized water-soluble BODIPY dyes, designated 24 and 25, modified with PEG, showed improved solubility and fluorescence quantum yield in water while minimizing the tendency to aggregate.[37]

BODIPY conjugates 24 and 25 demonstrated no toxicity to MCF-7 cells at low concentrations for 24 h. These dyes were permeable to MCF-7 cells and accumulated in the cytoplasm, as confirmed by confocal microscopy. These findings highlight the promising potential of these conjugates for use in fluorescence bioimaging.[38]

By combining the modified BODIPY with hydrophilic PEG, the amphipathic polymer PEG-BODIPY 26 was synthesized, exhibiting effective PDT capabilities with "favorable" phototoxicity against HepG2 and 4T1 cell lines. Amphiphilic macromolecules 26 can self-assemble into micelles of appropriate size, allowing for long-term circulation in the body and targeted accumulation in tumor sites. Polymer PEG-BODIPY 26 functions both as a PS and as a fluorescent probe, presenting excellent therapeutic and imaging properties *in vitro* and *in vivo*.

Aza-BODIPYs are structural analogs of organic dyes from the group of 4,4-difluoro-4-bora-3a,4a-diaza-s-indacenes (i.e., BODIPY), known for their high fluorescence quantum yield, photostability, and absorption and emission properties. These compounds exhibit absorption and emission across the visible light to near-infrared spectrum. Some aza-BODIPY derivatives also contain photosensitizing groups. Photosensitization is a process in which a PS absorbs light and transfers energy to another substance, inducing a chemical or physical reaction. These derivatives are employed in PDT for cancer treatment, using their ability to generate reactive oxygen species under light exposure. In medicine, photosensitization is most commonly associated with PDT. Tetraaryl aza-BODIPY, a derivative that serves as the basis for constructing a PS, exhibits absorption properties within the therapeutic window, ensuring effective treatment while minimizing toxicity and side effects. Due to the presence of a heavy atom, this compound can efficiently generate $^1O_2$. Another effective PS used in PDT is the biotin-conjugated aza-BODIPY derivative, obtained through Sonogashira coupling. This modification increases the compound's affinity for cancer cells, making it more effective compared to other PSs.[39]

Zinc(III)-dipicolylamine di-iodo-BODIPY is a chemical complex that has attracted significant interest due to its unique properties and potential applications in various scientific and technological fields. This compound, which consists of a zinc atom, dipicolylamine, and di-iodine BODIPY, represents an innovative combination in coordination chemistry.

These properties make zinc(III)-dipicolylamine di-iodo-BODIPY highly useful in nanotechnology, where it can be employed to construct nanomaterials with specific properties. Moreover, the compound's fluorescent capabilities open up applications in biochemistry, enabling the tracking of cells and chemicals in living organisms. In medical diagnostics, this complex can be utilized for tissue imaging, contributing to the advancement of modern diagnostic techniques. Its unique properties make it valuable across emerging fields of science, advancing progress in optical materials, nanotechnology, cell biology, and medical diagnostics. Furthermore, its ability to interact with metal ions allows for use in ion recognition and separation processes, which are applied in various branches of chemistry. Its fluorescence also enables precise tracking of biological processes at the cellular level, which is critical for analyzing disease mechanisms and molecular interactions. The compound's ability to enhance contrast can help identify pathological changes at early stages, a crucial factor for effective diagnosis and treatment. Moreover, this compound has potential applications in optical sensors, where its responsiveness to specific chemicals or ions can facilitate the detection and monitoring of various chemical processes.[40] Recent advancements in PDT utilizing nanotechnology, including the use of quantum dots as innovative PSs or energy donors, along with the combination of PDT with radiotherapy and immunotherapy, represent promising future approaches for cancer treatment.[41] The incorporation of PSs into conjugated polymer NPs has proven beneficial by improving $^1O_2$ formation through effective energy transfer. The evolution of nanotechnology has emerged as a promising avenue for enhancing the performance of existing PSs and overcoming significant challenges in cancer PDT.[42] In addition, silicon phthalocyanines are recognized as suitable PSs for PDT due to their ability to generate $^1O_2$ with a long triplet lifetime.[43]

10. Future clinical prospects of PDT

Convincing clinicians and experienced doctors to adopt PDT in clinical practice remains challenging due to the ongoing development of this treatment in cancer therapy. Treatment regimens still require optimization and standardization to ensure improved therapeutic efficacy. Moreover, unfavorable side effects have been

reported during PDT, but these were primarily due to the use of inappropriate treatment protocols. For this reason, researchers emphasize that, with the appropriate selection of PSs and continuously refined therapy techniques, undesirable effects can be minimized or avoided. In the development of PDT, a key focus should be on the potential for combining PDT with other treatment modalities, such as immunotherapy or gene therapy. Such combinations could significantly enhance the efficiency and effectiveness of PDT in cancer treatment, leading to improved clinical outcomes. Furthermore, ongoing advancements in PDT involve the development of more advanced phototherapy technologies and the refinement of personalized treatment approaches, which tailor therapy to individual patients based on their specific needs and symptoms. For PDT to be a more effective tool in cancer therapy, efforts must focus on improving its selectivity and precision, that is, further research into better methods of delivering PSs to target areas and increasing the selectivity of PDT in killing pathological cells while sparing healthy tissues. In addition, optimizing treatment protocols, including determining the ideal PS doses, light exposure durations, and overall therapy length, could significantly increase the effectiveness of PDT.

11. Conclusion

Despite its current successes, PDT continues to evolve and requires further research and innovation to fully realize its potential in treating various conditions. Both pre-clinical and clinical studies have yielded promising results regarding PDT as an effective method for treating cancers of various locations and stages. For PDT to be widely accepted by medical professionals as a legitimate treatment option, its evolving mechanism and the positive outcomes observed in past research must be acknowledged. To successfully incorporate PDT into clinical practice, physicians need comprehensive training not only in the technique itself but also in the proper use of PSs, which are critical to the therapy's effectiveness. Unfortunately, mastering PDT for cancer treatment requires an extended learning process, partly due to the risk of side effects if the light activates the PS in normal tissues, potentially causing harm. Despite these challenges, the promising results suggest that PDT could become a key therapeutic approach in the fight against cancer.

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The authors declare that they have no competing interests.

Author contributions

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REVIEW ARTICLE

Emerging biomarkers in major depressive disorder: Diagnostic, prognostic, and therapeutic implications

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Abstract

Recent advancements in biomarker research for the diagnosis, prognosis, and treatment strategies of major depressive disorder (MDD) have yielded significant findings that warrant documentation. The clinical demand for biomarkers persists due to the limited accuracy and efficiency of subjective diagnostic approaches. This review scrutinized research papers related to MDD biomarkers published between January 2011 and till July 2024, focusing exclusively on human studies with statistically significant results. The compiled biomarkers encompass cellular membrane receptors, cytoplasmic organelles, and genomic and epigenomic intranuclear markers. Cell surface molecular receptors implicated in MDD pathogenesis include brain-derived neurotrophic factor (BDNF) receptors, N-methyl D-aspartate receptors (NMDAR), and interleukin (IL) receptors. Endogenous compounds with diagnostic and prognostic potential, such as L-carnitine and alpha-L-carnitine, have also been identified. Transcriptomic biomarkers, including mRNA expression levels of the BDNF, IL-1β, macrophage migration inhibitory factor, and tumor necrosis factor-alpha (TNF-α), have demonstrated utility in MDD management. MicroRNAs (miRNAs), the endogenous molecules that alter the structure of mRNA, show potential for diagnosis and treatment outcome prediction, with miR-221-3p, miR-129-5p, miR-134, and miR-184 emerging as key candidates for MDD monitoring. Long non-coding RNAs (lncRNAs), such as GSK3βAS1, GSK3βAS2, and GSK3βAS3 have been investigated for the evaluation of disease severity and treatment response. Most recently, the pathological role of circular (circRNA) and DNA methylation in MDD has also been documented. The rs155979 polymorphism in the lncRNA NOTHSAT102891 was significantly associated with depression and risk of suicide. The data compiled in this review aim to guide further research in the quest for biomarkers that will improve the management of MDD.

Keywords: Biomarkers; Cell signaling markers; Intranuclear markers; Major depressive disorder

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1. Introduction

Major depressive disorder (MDD), also known as clinical depression, is characterized by a persistent feeling of sadness and a loss of interest in daily activities, significantly impairing social and physical functioning. It is associated with an increased risk of self-harm and substance abuse.[1] It has a remitting and relapsing course and each episode lasts longer than 2 weeks. Due to poor treatment response, the majority of patients progress to chronic or treatment-resistant depression (TRD).[2] Worldwide, approximately 300 million people suffer from this debilitating disease accounting for 3.4% of the global population, with prevalence rates varying from across countries.[3] In our part of the world specifically in South Asia, this percentage is relatively low (3.0%) which may be attributed to insufficient reporting, lack of awareness about mental health disorders, and hesitancy in seeking medical care due to social barriers.[4] According to global health metric data, the years lived with disability due to depressive disorders increased by 47% during the last three decades for all ages and both sexes and marked MDD as the third leading cause of disability globally.[5] Mental health disorders impose an alarming burden on national and international health budgets but even then MDD has not received as much research attention as much as other diseases, e.g., cardiovascular disease and cerebrovascular accidents.[6] Despite extensive research, scientists could not reveal much about MDD pathophysiology which led to a rise in its prevalence and chronicity.

A biomarker is a computable biological measure that directly correlates with the detection or absence of a specific disease process, its severity, and/or its risk of evolving. Biomarkers are the essential guide and presently the keystone of disease management.[7] Biomarkers can serve as trait markers when they define disease pathogenesis or as state markers when they define the clinical progression of a disease. Due to the lack of differentiating markers that distinguish MDD from other disorders of this category, such as bipolar disorder and generalized anxiety disorder, diagnosis often takes months or even years and still relies on decades-old patient-centered and clinician-centered assessment interviews. Efficient disease management demands biomarkers for screening purposes, prediction, diagnosis, prognosis, and treatment response. However, presently there is no officially approved biomarker for highly prevalent MDD. Recently, various research projects explored valid and reliable biomarkers for predicting and diagnosing various forms of MDD, including early-onset MDD, late-onset MDD, and TRD. In parallel, scientists have probed biomarkers for predicting treatment responses to various antidepressants, aiming to personalized treatment and prevent TRD. Biomarker discovery has the potential to save time and money while benefiting both patients and physicians. Here, we have compiled potential biomarkers for MDD authenticated by published literature.

1.1. Pathophysiology of MDD

MDD is believed to arise from complex interactions between genetic, environmental, and biological factors. The pathophysiology of MDD is multifaceted, involving dysregulation in neurotransmitter systems, inflammation, and neuroplasticity changes, collectively contributing to the manifestation of depressive symptoms. It has been reported that genetic predisposition plays a significant role in the development of MDD. Family and twin studies have consistently shown that MDD is heritable, with heritability estimates ranging from 30% to 40%.[8] Several genetic variants have been implicated in MDD, including those affecting the serotonin transporter gene (5-HTT) and the brain-derived neurotrophic factor (BDNF) gene. Polymorphisms in these genes can influence neurotransmitter function and neuroplasticity, thereby increasing the risk of developing MDD.[9] Apart from genetic variations, environmental stressors are also critical in triggering depressive episodes, particularly in individuals with a genetic predisposition. Adverse life events such as childhood trauma, loss of a loved one, or chronic stress can precipitate the onset of MDD. These stressors can activate the hypothalamic-pituitary-adrenal (HPA) axis, leading to increased production of cortisol, a stress hormone that has been associated with depression.[10] Chronic stress can also result in epigenetic changes, altering gene expression and further contributing to the risk of MDD.[11]

The monoamine hypothesis of depression posits that dysregulation of neurotransmitters, such as serotonin, norepinephrine, and dopamine, is central to the pathophysiology of MDD. Reduced levels of these neurotransmitters in the synaptic cleft can lead to depressive symptoms. Antidepressant medications, which increase the availability of these neurotransmitters, provide indirect support for this hypothesis.[11,12] The single most potent neurotransmitter of the brain is glutamate as it plays a major role in learning, cognition, and mood stabilization by enhancing synaptogenesis and neuronal plasticity. Stress induces neurons to secrete glutamate at synaptic junctions, which strongly binds to the N-methyl D-aspartate receptor (NMDAR), initiating downstream signaling pathways. Blocking NMDAR or reducing glutamate levels exerts a profound antidepressant effect, as classically observed with ketamine administration in patients with suicidal thoughts.[13]

Inflammation is increasingly recognized as a critical component in the pathophysiology of MDD. Elevated levels of pro-inflammatory cytokines, such as interleukin-6

(IL-6) and tumor necrosis factor-alpha (TNF-α), have been observed in individuals with MDD.[11,13] These cytokines can influence brain function by altering neurotransmitter metabolism, reducing neurogenesis, and disrupting neuroplasticity. Inflammatory markers have also been linked to treatment-resistant depression, highlighting their potential role in MDD pathology.[14]

Neuroplasticity, the brain's ability to reorganize and form new neural connections, is impaired in MDD. Reduced neuroplasticity in regions, such as the hippocampus and prefrontal cortex, is associated with depressive symptoms. The neurotrophic hypothesis suggests that decreased levels of BDNF contribute to impaired neuroplasticity and neurogenesis in MDD.[15] Antidepressant treatments have been shown to increase BDNF levels and enhance neuroplasticity, further supporting this hypothesis.[16] The HPA axis, which regulates the body's response to stress, is often dysregulated in individuals with MDD. Hyperactivity of the HPA axis leads to elevated cortisol levels, which can have deleterious effects on the brain, including hippocampal atrophy and impaired neurogenesis.[17] These changes can contribute to the cognitive and emotional symptoms of MDD. Normalizing HPA axis function is a target of some antidepressant therapies, with the aim of reducing cortisol levels and mitigating their negative impact on the brain. The pathophysiology of MDD is not attributable to a single factor but rather to the integration of various biological systems. Dysregulation in neurotransmitter systems, inflammation, and impaired neuroplasticity interact in a complex manner to produce the symptoms of MDD. Understanding these interactions is crucial for developing more effective treatments. For example, combining anti-inflammatory agents with traditional antidepressants may enhance treatment efficacy for some patients.[18] Figure 1 demonstrates the overall pathology of MDD.

2. Material and methods

2.1. Selection criteria

Research articles published from January 2011 till July 2024 were selected according to the following criteria: (a) Studies in the English language, (b) studies in adult humans diagnosed with MDD and compared with age-matched healthy controls, (c) studies that applied valid statistical tools to explore association, (d) Studies with a sample size not <10, (e) all animal studies were excluded, and (f) all randomized controlled trials and clinical trials were selected.

2.2. Search strategy

Research papers were identified by searching PubMed (https://pubmed.ncbi.nlm.nih.gov/) and Cochrane Library (https://www.cochranelibrary.com) using the following mesh words: Major depression, diagnostic biomarkers, prognostic biomarkers, and genetic biomarkers. After

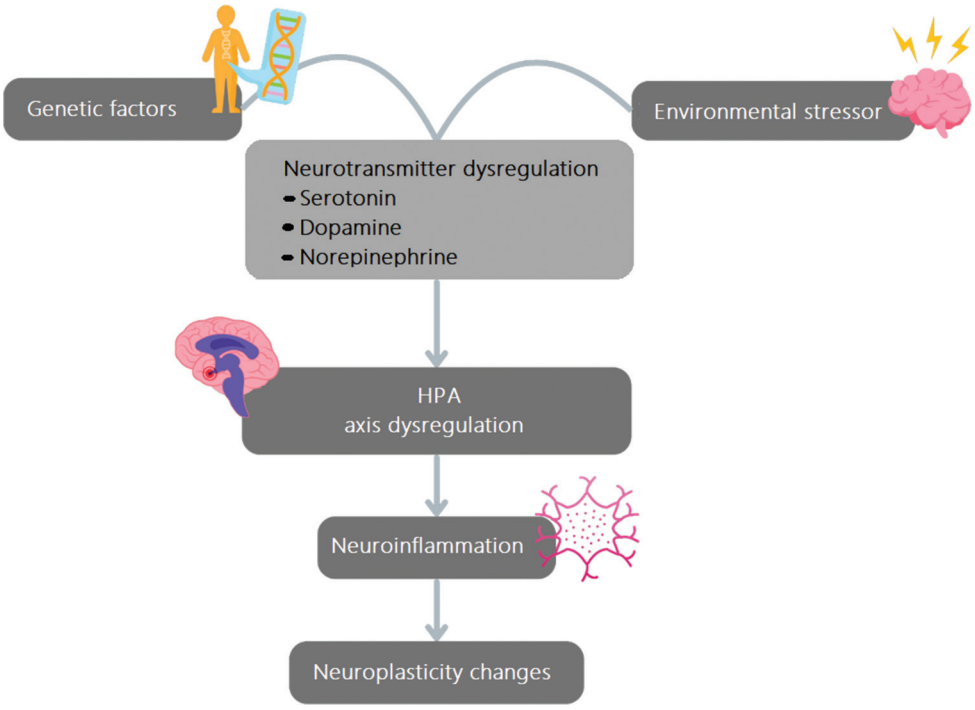

Figure 1. Pathophysiology of MDD. Figure created by the authors.
Abbreviations: HPA: Hypothalamic-pituitary-adrenal; MDD: Major depressive disorder.

removing duplicates, 568 articles in English were acquired. Based on the title and abstract, only 80 articles met the selection criteria. The search strategy is summarized in Figure 2.

3. Findings and discussion

3.1. Cell surface signaling biomarkers

MDD has been linked to various pathological processes activated by abnormal cellular signaling such as inflammation and immune mediation. These signaling pathways play a pivotal role in MDD pathogenesis and their components may provide clues for diagnosing MDD, predicting treatment response, or understanding treatment resistance. Toll-like interacting protein and vascular endothelial growth factor a (VEGFa) interact with numerous signaling components through their receptors. Both factors may serve as biomarkers for distinguishing patients with MDD, especially those who suffered from early childhood abuse.[19] Homocysteine acts as an agonist over the N-methyl D-aspartate (NMDA) subtype of glutamate receptor. Homocysteine can be a potential biomarker for registering MDD among patients with acute coronary syndrome.[20] Dehydroepiandrosterone sulfate (DHEAS) is a well-known neurosteroid and is vital for neuronal function through multiple cellular pathways. Its plasma concentration has been declared as a biomarker for treatment response.[21] With an animal model of depression, Blugeot *et al.* demonstrated that decreased serum BDNF levels along with normal corticosterone concentration may serve as a predictive biomarker for MDD vulnerability.[22] They also showed that the agonist of tropomyosin kinase B (TrkB), a BDNF receptor, will lead to the alleviation of MDD symptoms. Through multiplex bead-based assay analysis, TNF-α levels significantly differentiated cases of high-risk TRD.[23] In the genome-based therapeutic drugs for depression (GENDEP) study, participants were divided into two groups to determine drug response in relation to the baseline C reactive protein (CRP) levels, which proved its role as a biomarker for treatment response.[24] *Druzhkova et al.* pointed out the significant role of IL-6 and ciliary neurotrophic factor in the diagnosis of MDD and observed an acute stress-induced rise in TNF-α and glucose levels, suggesting the involvement of inflammatory and metabolic pathways.[25] Tolahunase *et al.* utilized elevated serum BDNF levels to validate MDD treatment response and demonstrated that elevated sirtuin 1 levels and decreased cortisol levels may also serve the same purpose.[21] Gadad *et al.* declared inflammatory proteins, i.e., interferon-gamma and eotaxin/CCL1, as predictors of treatment response in MDD patients.[26]

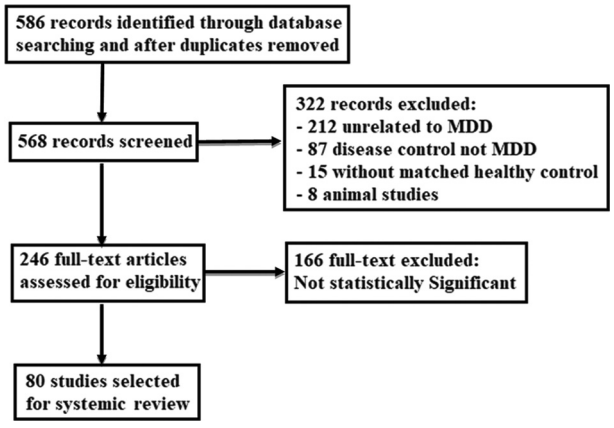

Figure 2. Flow chart for article selection
Abbreviation: MDD: Major depressive disorder.

Bot *et al.* worked on signal transduction, cell communication, and immune pathways to explore biomarkers for active MDD and declared pancreatic polypeptide, macrophage migration inhibitory factor (MIF), ENRAGE, IL-1 receptor antagonist, tenascin-C growth regulated alpha protein and von Willebrand factor as useful biomarkers.[27] On the other hand, Ramsey *et al.* worked on inflammatory pathway profile and declared CRP, trefoil factor 3, cystatin C, fetuin-A, β2-microglobulin, CD5L, FASLG receptor, and TNF receptor 2 with sufficient sensitivity and specificity (area under the curve [AUC] =0.63) for male gender.[28] Carboni *et al.* worked on treatment response predictors and established baseline cutoff values with significant accuracy and specificity for IL-6, IL-10, and TNF-α when using paroxetine, and for CRP when using venlafaxine as an antidepressant.[29] Most recently, Park *et al.* and Han *et al.* explored the role of a novel brain-specific chemokine, family with sequence similarity 19 member A5 (FAM19A5), as a biomarker for the pathogenesis of MDD. The researcher guaranteed authenticity with a significant area under the curve (AUC = 0.785), 60% sensitivity, and 100% specificity.[30,31] Yang *et al.* showed an excitatory relationship between proBDNF/p75 neurotropic receptors (p75NTR) signaling and inflammatory cytokines (IL-1β and IL-10) in the peripheral blood mononuclear cells of subjects diagnosed with MDD.[32] A summary of the cell surface signaling biomarkers in MDD is shown in Table 1.

3.2. Organelle-based or cytoplasmic biomarkers

L-carnitine and alpha L-carnitine are endogenous compounds that promote the β-oxidation of long-chain fatty acids in the mitochondria. Li- Juan *et al.* and Nasca *et al.* utilized human samples and established these compounds for diagnosing MDD, grading severity, and assessing treatment response, with acceptable sensitivity and specificity (AUC = 0.694 to 0.849).[33,34] Du *et al.* demonstrated that glucocorticoid (GC) toxicity results in

Table 1. Summary of cell surface signaling biomarkers in MDD

Biomarker	Source/Type	Role in MDD	Key findings	References
TOLLIP	Immune cells	Inflammation	May distinguish MDD patients with childhood abuse	19
VEGF	Blood	Neurogenesis	Potential diagnostic marker for MDD	30
Homocysteine	Blood	NMDAR agonist	Potential biomarker for MDD in acute coronary syndrome patients	20
DHEAS	Blood	Neurosteroid	Treatment response biomarker	21
BDNF	Serum	Neuroplasticity	Predictive biomarker for MDD vulnerability	22

Abbreviations: BDNF: Brain-derived neurotrophic factor; DHEAS: Dehydroepiandrosterone sulfate; MDD: Major depressive disorder; NMDAR: N-methyl D-aspartate receptors; TOLLIP: Toll-like interacting protein; VEGF: Vascular endothelial growth factor.

depression by stimulating the opening of mitochondrial permeability transition pores through transcriptional upregulation of cyclophilin D.[35] Cyclophilin D inhibition using a mitochondria-targeted compound, mito-apocynin, and a GC receptor antagonist, mifepristone, protects against mitochondrial dysfunction, synaptic loss, and behavioral deficits induced by GC.[35]

4. Intranuclear biomarkers

Intranuclear biomarkers (Figure 3) are the product of genetic machinery that plays a crucial cellular role including neurogenesis, neuro-inflammation, receptor toxicity, synaptogenesis, aging apoptosis, and mitochondrial respiratory chain.[36] The various intranuclear biomarkers and their clinical relevance are shown in below Table 2.

4.1. Transcriptional biomarkers for MDD

Transcriptional biomarkers are the members of the transcriptome family produced by DNA. They are broadly classified into the transcriptome, encompassing protein-coding RNAs (messenger RNAs; mRNAs) involved in protein synthesis, and the epitranscriptome, encompassing non-coding RNAs, such as microRNAs (miRs), long non-coding RNAs (lncRNAs) and circular RNAs (circRNAs), involved in the regulation of protein synthesis. Recent advances in medical science have pointed out the fundamental role of these genomic and epigenomic components in the pathogenesis of highly intractable diseases, especially cancers and neurological disorders.[42,43]

4.1.1. mRNA expression as biomarkers for MDD

The dissociation between "predictors" and "targets" of antidepressant responders was first reported by Cattaneo *et al.* who extensively explored mRNA-based biomarkers for MDD management. MDD non-responders had higher baseline mRNA levels of IL-1β, MIF, and TNF-α. Antidepressants reduced these levels significantly but there was no effect on disease severity. MDD remission was associated with a significant rise in mRNA levels of BDNF and vascular growth factor (VGF) and a decrease in mRNA levels of IL-6 and GC receptor function-related

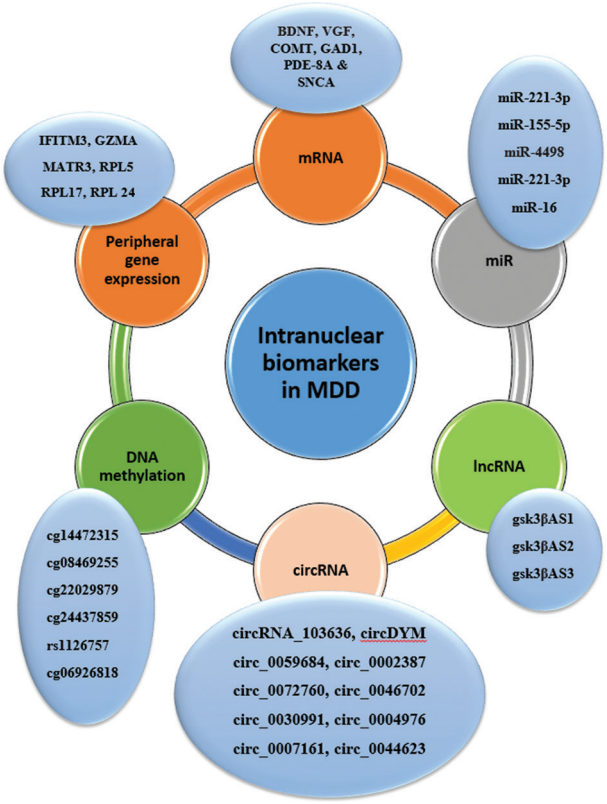

Figure 3. Intranuclear biomarkers associated with MDD. Figure created by the authors.
Abbreviations: BDNF: Brain-derived neurotrophic factor; circRNA: Circular RNA; lncRNA: Long non-coding RNAs; MDD: Major depressive disorder; mRNA: Messenger RNA; miR: microRNA; PDE-8A: phosphodiesterase 8A; SNCA: Alpha-synuclein.

FKBP-5.[44] Cattaneo *et al.* determined highly specific cutoff values for MIF and IL-1β mRNA levels from peripheral leukocytes that predict treatment response among MDD patients from a registered cohort as well as an independent cohort.[37] Lin *et at.* demonstrated a significant role of the mRNA expression levels of NMDAR genes (SRR, PSAT1, GCAT, GAD1, NRG1, and COMT) in white blood cells by plotting receiver operating characteristics curve for the accurate diagnosis of drug naïve MDD patients.[45] Most recently, significant differences in the phosphodiesterase

Table 2. Intranuclear biomarkers and their clinical relevance in MDD

Biomarker	Type	Role in MDD	Clinical utility	References
mRNA of IL-1β	Transcriptomic	Inflammation	Predicts treatment response	37
miR-221-3p	Epitranscriptomic	Post-transcriptional modification	Diagnostic and prognostic marker	38
gsk3βAS1 lncRNA	Epitranscriptomic	Synaptic plasticity	Diagnostic and therapeutic marker	39
circRNA_103636	Epitranscriptomic	Development stage-specific expression	Diagnostic marker with 73% sensitivity and 65% specificity	40
DNA methylation of GSK3β gene	Epigenetic	Gene regulation	Diagnostic marker with 100% sensitivity and specificity	41

Abbreviations: circRNA: Circular RNA; IL: Interleukin; lncRNA: Long non-coding RNAs; MDD: Major depressive disorder; mRNA: Messenger RNA; miR: microRNA.

8A (PDE-8A) mRNA editing profile have been observed in brain areas, including the dorsolateral prefrontal cortex and anterior cingulate gyrus, between depressed suicidal victims and healthy controls. They further suggested that suicidal ideation results from immune response-mediated brain damage due to the involvement of PDE-8A, and that in the future, this may act as a predictive biomarker for suicide.[46] In the same year, Salvetat *et al.* invented an application for estimating the mRNA editing profile of PDE8A from blood, saliva, and urine samples, demonstrating its potential for identifying mood disorders, including suicidal behavior.[47] Rotter *et al.* discovered a positive correlation between the RNA expression level of alpha-synuclein (SNCA) and the severity of MDD – characterized by the Hamilton Depression Rating Scale (HAM-D 17) and Beck's Depression Inventory (BDI-II) score.[48] The study showed that mRNA expression levels of SNCA, a presynaptic membrane protein, could serve as a potential biomarker for both diagnosis and grading MDD.[48] Most recently, it has been documented that mRNA expression of serotonin transporter (SERT) on the surface of peripheral blood mononuclear cells (PBMCs) is significantly reduced in subjects with MDD.[49]

4.1.2. miRs expression as biomarkers for MDD

miRs are small endogenous molecules consisting of approximately 20 nucleotides that play a role in post-transcriptional modification of mRNA. Several neurobiological processes including neurogenesis, neuronal proliferation, and synaptic plasticity have been linked to this epitranscriptome. As miR expression in the peripheral circulating cells changes both during MDD progression and remission, their levels could serve as potential biomarkers and may facilitate the management of intricate MDD.[50] Differential expression (DE) of miR can be determined through tissues (e.g., brain), cells (e.g., lymphocytes and monocytes), and body fluid exosomes (e.g., blood, urine, and saliva).[51,52] A summary of significant miR expression patterns for MDD diagnosis and treatment response is given in Table 3 and Table 4.

Torres-Berrio *et al.* documented that miR-218 expression in the prefrontal cortex significantly correlates with susceptibility to depression and its expression can be detected in the blood as well.[68] Feng *et al.* identified miR-221-3p expression in human serum and suggested that it can predict depressed mood.[38] Their work revealed that IFN-α-induced activation of nuclear factor kappa B (NF-κB) in astrocytes, mediated by miR-221-3p targeting of IRF2, may be one of the potential mechanisms underlying MDD.

4.1.3. lncRNA expression as biomarkers for MDD

Non-coding RNAs are pivotal for many cellular functions, such as splicing, gene regulation, chromosome structure regulation, and hormone-like activity. lncRNAs, which range from 200 to several hundred nucleotides, have recently been explored as genetic biomarkers for various diseases. While protein and DNA constitute the majority of chromatin, an increasing number of studies have revealed that lncRNAs occupy a large portion of chromatin and act as regulators of nuclear architecture and the expression of nuclear chromatin as coding RNA (genomic role) or non-coding RNA (epigenomic role).[69]

Liu *et al.* were the first to link co-expression of lncRNA and mRNA with MDD pathogenesis. Chromosomal regions chr10:874695-874794, chr10:75873456-75873642, and chr3:47048304-47048512 were identified as major sites co-expressions associated with MDD. Gene ontology (GO) and pathway analyses revealed metabolic pathway and neurodevelopment disease (Alzheimer's disease and Parkinson's disease) pathways.[70] Two studies examined the DE of lncRNAs in PBMCs among patients of schizophrenia (SZ), MDD, and generalized anxiety disorder (GAD).[71,72] After cross-validation, six downregulated lncRNAs (TCONS_00019174, ENST00000566208,

Table 3. miR expression for MDD diagnosis

Tissue	miR Expression	Associated signaling pathways	References
PBMCs (human)	Upregulated miR-26b, miR-4743, miR-4498, miR-4485 and miR-1972	GO, KEGG, and BioCarta	53
PBMCs (human)	Upregulated miR-26b, miR-1972, miR-4485, miR-4498, and miR-4743 Downregulated miR-338	Wnt/VEGF/mTOR/ErbB pathways	51
CSF (human)	Upregulated miR-125a-5p, miR-30a-5p, let-7d-3p, miR-34a-5p, miR-221-3p, miR-29b-3p, miR-10a-5p, miR-375, miR-155-5p, miR-33a-5p, miR-139-5p. Downregulated miR-185-5p, miR-590-5p miR-106b-5p, miR-15Bb-5p, miR-451a	Serine protein kinases 5HT/CRH/Glu/Wnt/VEGF/mTOR/ ErbB/LTD pathways	54
Serum (human)	Upregulated miR-221-3p, miR-34a-5p, let-7d-3p Downregulated miR-451		
CSF and serum (human and rat)	Downregulated miR-16	Serotonin pathway	55
PBMCs	Upregulated miR-199a-5p, miR-24-3p and miR-425-3p Downregulated miR-1915-3p, let-7a-5p, let-7d-5p and let-7f-5p	Wnt/mTOR/ErbB/insulin/Jak-STAT pathway	56
Blood (human)	Upregulated pmiR-chr11, miR-3158-3p, miR-433, miR-3944-5p Downregulated miR-1275, miR-4516, miR-30e-3p, miR-148b-3p	BRPF1 (required for mouse hippocampus development)	57
Hippocampus (mouse)	Downregulated miR-124	NO and cytokines inflammatory pathway	58
PBMCs, monocytes (human)	Downregulated PBMC: let-7e, miR-146a, and miR-155 Monocytes: miR-146a and miR-155 (Both upregulated after treatment)	TLR4 signaling pathway	59
Plasma (human)	Downregulated miR-184		60
PBMCs (human)	Upregulated ENSG00000238243.3_1, ENSG00000126353.3_2 ENSG00000198034.10_1, ENSG00000254996.5_2 ENSG00000184613.10_2, ENSG00000139193.3_2 ENSG00000101224.17_1, ENSG00000197153.4_2 ENSG00000103064.13_2, ENSG00000256235.1_1 Downregulated ENSG00000150991.14_2, ENSG00000172057.9_2	CC chemokine receptor activity, T cell receptor pathways.	47
Plasma (human) Hippocampus, PFC (rat)	Upregulated miR-134	Microglial activation pathway	61
Extracellular vesicles	Downregulated miR-92a-3p miR-129-5p	Microglial activation pathway	62
PFC	miR-194-5p, miR-25-3p, miR-125-5p	Th17 differentiation in the immune system	63

Abbreviations: BRPF1: Bromodomain and PHD finger-containing protein 1; CSF: Cerebrospinal fluid; CRH: Corticotropin releasing hormone; Glu: Glutamate; lncRNA: Long non-coding RNAs; LTD: Long-term depression; MDD: Major depressive disorder; miR: microRNA; mTOR: Mammalian target of rapamycin; NO: Nitric oxide; PBMC: Peripheral blood mononuclear cells; PFC: Prefrontal cortex; VEGF: Vascular endothelial growth factor; 5HT: 5 – hydroxyl tryptan.

Table 4. MiR expression for MDD treatment response

Tissue/Sample	miR Expression	Associated signaling pathways	References
PBMCs (Human)	Upregulated miR-4743, miR-4498, miR-4485 and miR-1972 Downregulated miR-4485	GO, KEGG and BioCarta	53
Plasma and PFC (human) Plasma (mouse)	Downregulated miR-146a-5p, miR-146b-5p, miR-24-3p and miR-425-3p	MAPK/Wnt	52
Plasma, exosome EDP, PNBCs	Upregulated miR-26a (in EDP – exosomes depleted plasma) miR-494 (in exosomes and EDP) Among MDD-treated patients	MAPK and Wnt	64
Plasma (human)	Upregulated miR-16-5p, miR-146a-5p and miR-21-5p	Morphogenesis, COPII vesicle coating, IP3 metabolic process, apoptotic process, cytoplasmic stress granule, NO metabolic process, NO synthase, and virion assembly	65
PBMCs (human)	Upregulated miR-27a-3p, miR-197-3p, miR-22-5p, miR-221-3p, miR-126-3p, miR-128-1-5p, miR-30b-5p, miR-339-3p, miR-301a-3p, miR-345-5p, miR-505-3p, miR-1249, miR-132-3p, miR-550a-5p, miR-589-5p, miR-769-5p, miR-10b-5p, miR-210-3p, miR-628-3p, let-7d-3p, miR-148a-5p, miR-155-5p, miR-140-3p, miR-150-3p, miR-181a-5p, miR-24-3p, miR-629-5p, let-7a-3p, miR-194-5p, miR-28-3p, miR-378a-3p, miR-6852-5p, miR-7706	Prion diseases (TGFβ) and morphine addiction signaling pathways	66
Plasma (human)	Upregulated miR-135a-5p (the higher the miR-135a-5p expression, the faster the remission.)	MAPK and Wnt	67

Abbreviations: EDP: Exosomes depleted plasma; lncRNA: Long non-coding RNAs; MDD: Major depressive disorder; miR: microRNA; MAPK: Mitogen-activated protein kinase; NO: Nitric oxide; PBMC: Peripheral blood mononuclear cells; PFC: Prefrontal cortex; PNBC: Peripheral nucleated blood cells; TGFβ: Transforming growth factor beta.

NONHSAG045500, ENST00000517573, NONHSAT034045, and NONHSAT142707) were found highly sensitive and specific for the diagnosis of MDD (AUC = 0.719). Seki *et al.* discovered RMRP, a nuclear DNA-encoded lncRNA and a component of nuclear RNase mitochondrial RNA processing (MRP) complex, as a potential biomarker for the diagnosis and severity assessment of MDD in both human and animal research models.[73] Most recently, Liu *et al.* conducted *in vitro* and *in vivo* studies on regulatory antisense lncRNAs of GSK3β, a serine-threonine kinase involved in synaptic plasticity, neurogenesis, and resilience to neuronal injury.[39] The study identified gsk3βAS1 [ENST00000482027], gsk3βAS2 [ENST00000491262], and gsk3βAS3 [BC035247] as novel diagnostic and therapeutic biomarkers for MDD. A summary of lncRNAs that play a significant role in the diagnosis and treatment response of MDD is presented in Table 5.

4.1.4. circRNA expression as biomarkers for MDD

Previously considered just a byproduct of genetic malfunctioning, circRNAs have now been assigned multiple regulatory roles in physiological and pathological processes at the nuclear and cellular levels. The mysterious circRNAs are formed from pre-mRNA through back-splicing of introns, exons, or both, while the canonical splicing of the same pre-mRNA results in the formation of protein-coding mRNA.[74,75]

Cui *et al.* were the first to recommend the expression of circRNAs (circRNA_103636) in PBMCs as a biomarker for MDD diagnosis and treatment response, with 73% sensitivity and 65% specificity as determined by receiver operating characteristic (ROC) curve analysis.[40] Zhang *et al.* conducted experiments to validate the ameliorative effects of circRNA DYM (circDYM) expression in depressive-like mice models. *In vitro* studies on BV-2 cells revealed that circDYM inhibits miR-9 which increases target-HECT domain E3 ubiquitin protein ligase 1 (HECTD1) and depresses microglial activation.[61] Following Zhang *et al.*, Song *et al.* sorted out the correlation between downregulated plasma circDYM and MDD up to 94% sensitivity.[55]

Table 5. Significant lncRNA related to MDD

Expression	Utility	Signaling pathway	References
Downregulated gsk3βAS1	Diagnostic marker (upregulated post-treatment)	Synaptic plasticity	39
Downregulated TCONS_00019174 ENST00000566208 NONHSAG045500 ENST00000517573 NONHSAT034045 NONHSAT142707	Diagnosis and treatment response	Ribosome activities, Alzheimer's disease, RNA degradation, pancreatic cancer, Parkinson's disease, cell cycle, DNA replication, prostate cancer, Huntington's disease and long-term depression	71
Upregulated Y5, MER11C, PCAT1, PCAT29	Diagnosis and severity	Gene regulation	73
Downregulated gsk3βAS1 [ENST00000482027], gsk3βAS2 [ENST00000491262] gsk3βAS3 [BC035247]	Diagnosis, severity, and treatment response (upregulated post-treatment)	Wnt signaling pathway	39
Downregulated RMRP	Diagnostic and severity assessment	Gene regulation	73
Downregulated TCONS_00019174	Diagnosis	Cellular pathways	71
Upregulated ENSG00000229807.1_2 ENSG00000234449.2_1 ENSG00000205663.5_1 ENSG00000271964.1_2 ENSG00000279995.1_2 ENSG00000271109.1_1 ENSG00000205662.2_1 ENSG00000244620.1_1 ENSG00000204282.4_2 ENSG00000225938.1_1 Downregulated ENSG00000273295.1_1 ENSG00000218537.1_1 ENSG00000271869.1_1	Diagnosis	CC chemokine receptor activity and cytokine-cytokine receptor interactions and T cell receptor	47

Abbreviation: MDD: Major depressive disorder.

An *et al*. investigated DE of circRNAs among patients of MDD and type II diabetes and reported that the following circRNAs were exclusively and significantly expressed in MDD, summarized in Table 6.[76] Yu *et al.* documented that circHIPK2 expression was significantly enhanced (AUC = 0.796) in MDD individuals and it is highly specific for diagnosing and predicting MDD l.[77]

4.1.5. DNA methylation in MDD

Numata *et al.* explored the role of DNA methylation in diagnosing MDD using blood samples.[41] A multiplex DNA methylation profile, predominantly from gene promoter regions within CpG islands, demonstrated 100% sensitivity and specificity as a diagnostic biomarker for MDD. Among 313 differentially methylated CpG sites in the CGIs in the gene promoter regions, several genes, such as DGKH (cg00109274), GSK3B (cg14472315), and SGK1 (cg06642177), have been implicated in MDD. DGKH and SGK1 are specific for bipolar disorder whereas the GSK3B is specific for MDD.[41] Clive *et al.* worked on validating their previously identified biomarker – spindle and kinetochore-associated protein (SKA2) – which predicts suicide risk and post-traumatic stress disorder (PTSD).[78] DNA methylation of SKA2 revealed three probes (cg08469255, cg22029879, and cg24437859) that were significantly correlated with the biosignature for DNA methylation-based suicidal behavior prediction. Powel *et al.* investigated IL-11 gene-related DNA methylation across the CpG island and its role in predicting MDD treatment response in the GENDEP clinical trial.[79] In the same clinical trial, the role of IL-11 transcriptional differences and the associated single nucleotide polymorphism (SNP), rs1126757, in predicting MDD treatment response was also determined. DNA methylation in the IL-11 gene has thus proved to

Table 6. circRNA expressed in MDD

Upregulated		Downregulated	
circRNA ID	**Gene name**	**circRNA ID**	**Gene name**
circ_0046702	YES1	circ_0009024	TXLNG2P
circ_0059684	ZNF337-AS1	circ_0008297	DDX3Y
circ_0002387	TNIK	circ_0001953	ZFY
chr5:162909647-162911251	HMMR	chr5:178043882-178044435	CLK4
circ_0072760	CCNB1	circ_0006660	CHPT1
chr3:195781950-195782172	TFRC	chr19:11759172-11759299	ZNF833P
circ_0030991	CUL4A	circ_0003068	SYNE1
circ_0004976	ASXL2	chr11:66372959-66373063	CCS
circ_0007161	YAF2	circ_0028904	RNF10
circ_0044623	LUC7L3	chr1:41474465-41474562	CTPS1 9

Abbreviations: YES1: YES Proto-Oncogene 1, Src Family Tyrosine Kinase), ZNF337-AS1: ZNF337 Antisense RNA 1, TNIK: TRAF2 and NCK-Interacting Protein Kinase, HMMR: Hyaluronan Mediated Motility Receptor, CCNB1: Cyclin B1, TFRC: Transferrin Receptor, CUL4A: Cullin 4A, ASXL-2: ASXL Transcriptional Regulator 2, YAF2: YY1 Associated Factor 2, LUC7L3: LUC7 Like 3 Pre-MRNA Splicing Factor, TXLNG2P: Taxilin Gamma Y-Linked, DDX3Y: DEAD-Box Helicase 3 Y-Linked, ZFY: Zinc Finger Protein Y-Linked, CLK4 - CDC Like Kinase 4, CHPT1: Choline Phosphotransferase 1, ZNF833P: Zinc Finger Protein 833, Pseudogene, SYNE1: Spectrin Repeat Containing Nuclear Envelope Protein 1, CCS: Copper Chaperone For Superoxide Dismutase, RNF10: Ring Finger Protein 10, CTPS19: CTP Synthase 1.

Table 7. Genes involved in MDD remission

Drug	Clinical variable	Genetic variable
Escitalopram	Appetite, sleep change, somatic symptoms, and interest activity	rs1392611, rs10812099, rs1891943, rs151139256, rs11002001, rs62182022, rs28373080, rs7757702, rs76557116, rs9557363, rs2704022
Nortriptyline	Not applicable	rs6794400, rs79693177, rs12874087, rs2345113, rs17091959, rs10792321, rs199561596, rs144829540, rs149619279, rs34319049, rs151132095, rs37596, rs8053632, rs111685823, rs4279984, rs17057129, rs5889536, rs34841556, rs4773117, rs8082631

Abbreviation: MDD: Major depressive disorder.

be an authentic biomarker for predicting MDD treatment response and may guide the selection of best-suited treatment in the future.[79] Kang *et al.* worked on the DNA methylation profile of the BDNF gene and revealed it could precisely predict suicidal ideation status and also the treatment outcome.[80] Ju *et al.* identified differentially methylated positions (DMP) in peripheral DNA samples for predicting MDD treatment response.[81] After validations, DMP in the CHN2 gene (cg06926818) was successfully replicated, demonstrating 100% specificity and sensitivity.[81]

4.1.6. Peripheral gene expression in MDD

Guilloux *et al.* identified and validated a set of six peripheral genes with 76% accuracy and 86% sensitivity for the prediction of MDD drug responses. These double-validated genes include IFITM3 (INF-induced transmembrane protein 3), GZMA (granzyme A), MATR3 (matrin 3), and ribosomal proteins RPL5, RPL17, and RPL24.[82] Iniesta *et al.* documented a validated set of genes for drug-specific treatment response among MDD subjects. For escitalopram response, both genetic and clinical variables predict remission with high accuracy, while for nortriptyline response, only genetic variables serve as predictors (AUC = 0.77) (summarized in Table 7).[83]

Pajer *et al.* worked for authenticated genetic peripheral markers for diagnosing MDD at earlier onset.[84] A set of 11 candidate transcriptomic genes (ATP11C, CD59, IGSF4/CADM1, MAF, RAPH1, AMFR, CAT, CDR2, CMAS, PSME1, and PTP4A3) could differentiate MDD cases from healthy subjects with sufficient accuracy. Similarly, Redei *et al.* explored diagnostic genetic markers from peripheral blood samples and reported that RAPH1, KIAA1539, and DGKA gene expression demonstrated the highest specificity and sensitivity for differentiating MDD cases, regardless of treatment status, as determined by ROC curve analysis.[85] Most recently, Wang *et al.* documented differentially expressed hub genes, including MRPS2, MRPS7, MRPS11, MRPS18, MRPL2, MRPL9, MRPL15, MRPL16, MRPL27, MRPL36, RPS3, RPS19, RPS19, RPL6, RPL11, RPL19, RPL26L1, RPL29, NAS2, NHP2, and RPP38, which were highly specific and sensitive for MDD diagnosis (ROC AUC >0.8).[86] Furthermore, Liang *et al.*

and Li *et al.* have recently conducted case–control studies in a Chinese cohort and established that the rs155979 polymorphism in the lncRNA NONHSAT102891 was significantly associated with depression susceptibility and risk of suicide.[87,88]

5. Conclusion

This review highlights the progress made in identifying cellular and nuclear biomarkers for MDD. These biomarkers hold promise for enhancing diagnostic accuracy, predicting treatment response, and personalizing therapeutic approaches. However, further research is required to validate these biomarkers in clinical settings and to explore their application in routine clinical practice. Despite extensive and advanced research, the cure for MDD is partial and inadequate. Major challenges persist in screening, diagnosis, effective treatment, and prognosis. Identifying specific genetic factors could enable personalized treatment strategies, potentially improving outcomes.

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Conflict of interest

The authors declare they have no competing interests.

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ACCSCIENCE PUBLISHING

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REVIEW ARTICLE

Vericiguat for heart failure with reduced ejection fraction (HFrEF): A review of its potential benefits in Pakistan

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Abstract

Heart failure (HF) is categorized by left ventricular ejection fraction (LVEF) into three groups. HF with reduced ejection fraction (HFrEF) is one of these groups characterized by the heart's inability to pump sufficient blood to meet the body's needs, resulting from the left ventricle's impaired ability to contract effectively. The Canadian Cardiovascular Society (CCS) guidelines recommend vericiguat for hospitalized patients experiencing worsening symptoms of HFrEF. This article reviews vericiguat's efficacy and potential benefits in Pakistani patients with HFrEF. A literature search from 2013 to 2024 was conducted using PubMed, ScienceDirect, and Google Scholar, employing keywords such as guidelines, heart failure, Pakistan, and reduced ejection fraction. Soluble guanylate cyclase (sGC) stimulators, like vericiguat, have shown benefits in patients with left ventricular hypertrophy and fibrosis by reducing afterload through vasodilation. Vericiguat (2.5 – 10 mg taken orally once daily) shows promise in reducing cardiovascular mortality and hospitalization in adults with LVEF ≤45%. Vericiguat may alleviate Pakistan's growing cardiovascular disease burden. Expedited access to this innovative therapy can be achieved through collaborative efforts among policymakers, healthcare authorities, and international research centers, potentially reducing hospitalization rates in Pakistani HFrEF patients.

Keywords: Vericiguat; Heart failure with reduced ejection fraction; Pakistan; Soluble guanylate cyclase stimulator; Cardiovascular disease

1. Introduction

Heart failure (HF) is a multifaceted clinical condition characterized by dyspnea or exertional limitation due to impaired ventricular filling, impaired blood ejection, or a

combination of both.[1] HF affects an average of 64.3 million people worldwide, making it a significant global cardiac pathology.[2] As the population ages and the outcomes of acute cardiovascular events improve, the prevalence of HF has increased.[2] Despite new therapies and management strategies, individuals with HF still face a poor prognosis, with a 5-year survival rate estimated at around 50% following the initial diagnosis.[2] Recurrent hospital stays and the need for additional parenteral therapy during exacerbations further indicate worse prognoses and lower quality of life.[2] HF with reduced ejection fraction (HFrEF) is a condition where the heart is unable to pump enough blood to meet the body's needs due to an inability of the left ventricle to contract sufficiently.[1] This leads to clinical manifestations such as shortness of breath and fatigue. Ejection fraction (EF), which measures the amount of blood pumped out with each contraction, is normally 55% and above, whereas HFrEF is defined by an ejection fraction of 40% or less.[1,3] The HF is categorized into HF with preserved ejection fraction (HFpEF, ejection fraction ≥50%),[4] mid-range ejection fraction (HFmrEF, EF: 40 – 49%),[5] and reduced ejection fraction (HFrEF, ejection fraction ≤40%).[1,3] HFrEF poses a significant health challenge globally and is particularly prevalent in Pakistan, where approximately 2.8 million people are affected by congestive heart failure (CHF).[5] The prevalence of CHF is higher in urban areas and among older adults, driven by high rates of diabetes, hypertension, and obesity.[5] In Pakistan, HFrEF management involves a combination of lifestyle modifications – such as quitting smoking, exercising regularly, and adhering to a healthy diet – alongside pharmacological treatments including diuretics, angiotensin-converting enzyme inhibitor (ACEi), angiotensin II receptor blocker (ARBs), beta-blockers, and mineralocorticoid receptor antagonists (MRAs) In severe cases, surgical interventions such as implantable cardioverter-defibrillators (ICDs) or cardiac resynchronization therapy (CRT) are considered.[6] Recent advancements in HFrEF management, such as vericiguat, provide hope for improving outcomes in these patients. Vericiguat is an oral soluble guanylate cyclase stimulator that enhances the activity of cyclic guanosine monophosphate (cGMP), which plays a critical role in regulating cardiovascular, renal, and metabolic functions.[1] The VICTORIA trial specifically targeted a higher-risk population with HFrEF, characterized by elevated N-terminal pro-B-type natriuretic peptide (NT-proBNP) levels, lower EFs, and recent hospitalizations for HF.[1] The trial demonstrated that vericiguat significantly reduced the composite outcome of cardiovascular death or first HF hospitalization in these patients (35.5% vs. 38.5%; hazard ratio, 0.90 [95% confidence interval, 0.82 – 0.98]), primarily driven by a reduction in HF hospitalization, over a median follow-up of 10.8 months.[1] Table 1 summarizes the key outcomes of the VICTORIA trial, comparing Vericiguat and placebo groups in terms of cardiovascular events, mortality, and adverse effects. However, the reduction in cardiovascular death was not statistically significant (16.4% vs. 17.5%; hazard ratio, 0.93 [95% confidence interval, 0.81–1.06]).[1] This suggests that vericiguat is particularly beneficial for HFrEF patients with worsening symptoms who remain clinically unstable despite being on guideline-directed medical therapy (GDMT), as evidenced by high NT-proBNP levels and frequent hospital admissions. The findings of the VICTORIA study can be contrasted with those of the EMPEROR-Reduced study,[7] which focused on a slightly different patient population. The EMPEROR-Reduced study included patients with a broader range of baseline ejection fractions and NT-proBNP levels, which may explain the different outcomes observed between the studies.[7] In addition, the treatment protocols in the EMPEROR-reduced study emphasized the comprehensive use of GDMT, whereas, in the VICTORIA trial, a significant portion of the cohort was not optimally managed according to current guidelines. This discrepancy highlights the need for precise patient selection and optimization of background therapy when evaluating the efficacy of novel treatments like vericiguat in real-world settings.[7] Evidence from the VICTORIA study, supported by additional analyses, suggests that the ideal candidates for vericiguat are HFrEF patients with an ejection fraction of 40% or less, elevated NT-proBNP levels, and a history of recent HF hospitalizations or outpatient intravenous diuretic use. These patients typically exhibit worsening symptoms despite being on maximally tolerated doses of standard therapies, including ACE inhibitors, ARBs, beta-blockers, and MRAs.[7] As such, vericiguat should be considered an adjunctive therapy for patients at high risk of adverse outcomes to reduce the likelihood of further hospitalizations and potentially improve survival, in line with European and American HF management guidelines.[7] This review evaluates the efficacy of vericiguat and its potential benefits for Pakistani patients with HFrEF.

2. Methodology

For this review, relevant articles were searched on electronic databases including PubMed, Science Direct, and Google Scholar from 2013 to 2024 using keywords such as guidelines, HF, Pakistan, and reduced ejection fraction. Inclusion criteria include article types such as cross-sectional studies, narrative reviews, systematic reviews, meta-analyses, and case reports that met the study's objectives, written in the English language, conducted

Table 1. Outcomes of cardiovascular events, mortality, and adverse effects in heart failure patients (vericiguat-treated group versus placebo) based on VICTORIA Trials

Outcome	Vericiguat group (n=2526)	Placebo group (n=2524)	HR	*P*-value	References
Primary outcome (CV death or HF hospitalization)	897 (35.5%)	972 (38.5%)	0.90	0.02	7
Cardiovascular death	414 (16.4%)	442 (17.5%)	0.92	0.27	7
All-cause death	513 (20.3%)	535 (21.2%)	0.95	0.38	7
Heart failure hospitalization	691 (27.4%)	747 (29.6%)	0.90	0.048	7
Serious adverse events	828 (32.8%)	878 (34.8%)	-	-	7
New anemia occurrence	344 (13.6%)	266 (10.5%)	-	<0.001	7

Abbreviations: CV: Cardiovascular; HF: Heart failure; HFrEF: Heart failure with reduced ejection fraction; HR: Hazard ratio.

in Pakistan, and focused on the Pakistani population diagnosed with HF. However, we excluded article types such as editorials, commentaries, and perspectives that did not meet the study's objectives, were written from other countries, and focused on other populations diagnosed with HF.

3. Results

3.1. Preclinical evidence about therapeutic benefits of vericiguat

The nitric oxide (NO)-soluble guanylate cyclase (sGC)-cyclic guanosine monophosphate (cGMP) (NO-sGC-cGMP) pathway is crucial for cardiovascular function but becomes impaired in HF, diminishing the heart's natural protective mechanisms.[8] This disruption is due to reduced NO availability and changes in the redox state of sGC, making it less responsive to NO.[8] Vericiguat, an sGC stimulator, enhances sGC sensitivity to endogenous NO, offering a more physiological effect compared to sGC activators like cinaciguat, which bypass NO but elevate risk of hypotension.[8] In preclinical studies, vericiguat demonstrated efficacy in restoring NO/sGC signaling across various animal models of HF, leading to improved cardiac function and hemodynamic.[9] The effects of vericiguat were dose-dependent, with higher doses leading to increased cGMP levels and enhanced heart function, emphasizing the importance of determining optimal dosing for clinical applications.[9] In addition, preclinical safety data indicated that vericiguat was well-tolerated in animal models, showing no significant adverse effects at therapeutic doses.[9] These promising results laid the groundwork for human trials, including the VICTORIA Phase 3 trial, where vericiguat showed potential in improving clinical outcomes for HF patients.[9] In summary, preclinical studies highlight vericiguat's mechanism of action, efficacy in restoring NO-sGC-cGMP signaling, dose-dependent effects, and favorable safety profile, strongly supporting its therapeutic role in chronic HF and possibly other cardiovascular conditions.[9]

3.2. Global trials of vericiguat in patients with heart failure and reduced ejection fraction

Various potential benefits of sGC stimulators have been recognized, including the capacity to prevent or even reverse left ventricular hypertrophy and fibrosis, as well as decrease left ventricular afterload due to systemic and pulmonary vasodilation.[10] Consequently, restoring adequate NO-sGC-cGMP signaling is and recognized as an essential treatment objective for HF, with vericiguat enhancing sGC sensitivity to endogenous NO.[10] A study that explored the potential of vericiguat as the fifth cornerstone in the treatment of HFrEF, found that the drug yielded promising treatment outcomes.[11] The promising results demonstrated by vericiguat in high-risk HF patients indicate its potential as a valuable therapeutic option for HFrEF treatment, possibly leading to the recommendation of quintuple therapy, in addition to standard optimal medical therapy.[11] A study on eligibility for vericiguat in a real-world HF population included patients from the Sweden HF registry, who were aged 18 or older, with their ejection fraction and HF duration recorded between May 2000 and December 2018.[12] Using the VICTORIA trial eligibility criteria, only 21% of patients with chronic HFrEF were eligible for vericiguat, while 47% met the criteria based on guidelines and regulatory labelling.[12] Prior hospitalization for HF was the most restrictive factor in determining eligibility, resulting in the exclusion of 49% of patients with chronic HFrEF. The eligibility criteria for vericiguat set by the VICTORIA trial, guidelines, and regulatory labeling were designed to target a high-risk population with HFrEF in a real-world setting.[12] A consensus statement from India, based on expert opinions regarding the identification and pharmacological management of worsening HF, concluded that vericiguat effectively reduces the risk of cardiovascular death and hospitalization for adults with symptomatic chronic HF and an ejection fraction of <45%.[13] This medication is recommended for individuals who have been recently hospitalized for HF or require outpatient intravenous diuretics.[13] The initial dose of

vericiguat is 2.5 mg, taken orally once daily with food; this dose should be increased approximately every 2 weeks until it reaches the target maintenance dose of 10 mg once daily, as tolerated by the patient.[13] African Americans were significantly underrepresented in the VICTORIA trial, accounting for only 4.9% of the vericiguat arm and 5% of the placebo arm.[14] However, this underrepresentation should be considered in the context of the trial's global nature, with the majority of trial sites located in Eastern Europe and the Asia Pacific region – have low populations of the African diaspora.[14] It is worth noting that other recent positive HF trials, such as PARADIGM-HF and DAPA-HF, also had low representation of African Americans, with percentages ranging between 4.4% and 5.1%.[14] Table 2 shows the eligibility criteria for inclusion of HFrEF patients in vericiguat clinical trials.

At present, clinical trial data on vericiguat in Pakistan are scarce, raising concerns given the increasing prevalence of cardiovascular diseases in the country. Conducting a clinical trial of vericiguat in Pakistan would be a significant step forward in addressing the management of HFrEF among the Pakistani population. Such a trial would not only benefit the local population but also contribute to enhancing global HF care. Figure 1 shows the role of vericiguat in HFrEF.

3.3. Potential benefits of vericiguat for HF management in Pakistan

Among FDA-approved medications between 2021 and 2022, vericiguat was notable, with the most commonly reported side effects of hypotension and anemia – both exceeding 5% of reported incidents.[14] Using sacubitril/valsartan in the Pakistani population with HFrEF is generally safe and well-tolerated, as indicated by stable hemodynamic parameters and a notable improvement in left ventricular function and functional class.[15] Thus, this therapy can be considered as a primary treatment option for patients with HFrEF, with a gradual dose escalation over a 6-week timeframe.[15]

The benefit of vericiguat in heart failure with reduced ejection fraction (HFrEF) was studied in patients experiencing worsening heart failure events.[13] This population includes those with a history of decompensation events leading to emergency department visits and hospitalizations. The focus is on optimizing treatment for high-risk patients, particularly those who have not responded adequately to standard therapies.[13] In Pakistan, HF is a major public health issue, and further research is needed to explore the use and role of vericiguat in patients with HFrEF.

3.4. Safety profile of vericiguat and its adverse effects

A study has shown that vericiguat has proven effective in reducing cardiovascular mortality and hospitalization rates for HF patients with reduced ejection fraction (HFrEF), but did not demonstrate a therapeutic benefit for those with preserved ejection fraction (HFpEF).[16] The treatment showed a favorable safety profile, with common adverse events including hypotension, syncope, and anemia.[17] Consequently, vericiguat is indicated for HFrEF patients with a minimum systolic blood pressure of 100 mmHg, particularly those experiencing symptom worsening despite optimal medical therapy, including ACE inhibitors, beta-blockers, spironolactone, and SGLT2 inhibitors.[16]

Another study found that vericiguat significantly lowered the risk of cardiovascular death or hospitalization due to HF in high-risk patients, showcasing its positive impact on clinical outcomes.[16]

3.5. Flaws and limitations of VICTORIA'S trial

The VICTORIA trial demonstrated that vericiguat significantly reduced the risk of cardiovascular death and hospitalization for heart failure compared to placebo, with a hazard ratio of 0.90 for the primary composite outcome (Table 1). Hospitalization rates were slightly lower in the Vericiguat group (35.5%) versus placebo (38.5%), indicating a modest benefit (Table 1). However, this marginal reduction may reflect suboptimal baseline therapy among participants, suggesting Vericiguat's full potential could be greater under optimized conditions. While generally well-tolerated, Vericiguat was associated with a higher incidence of new anemia (Table 1). The

Table 2. Eligibility criteria for inclusion of heart failure with reduced ejection fraction patients in vericiguat clinical trials

Eligibility Criteria	Description	Reference (s)
Symptoms despite optimal medical care	HFrEF patients experiencing symptoms despite receiving optimal medical care.	12-14
Worsening heart failure	HFrEF patients with worsening condition despite receiving treatment.	12-14
Usage of nitrates	HF patients using nitrates.	12-14
Increased NT-proBNP levels	Patients with elevated levels of N-terminal pro-B-type natriuretic peptide (NT-proBNP), indicating HF severity.	12-14 12-14

Abbreviations: HF: Heart failure; HFrEF: Heart failure with reduced ejection fraction; NT-proBNP: N-terminal pro-B-type natriuretic peptide.

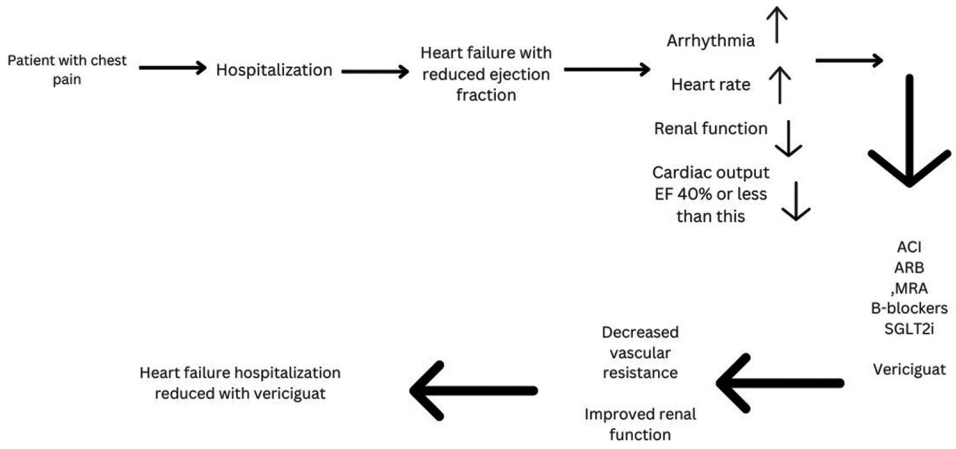

Figure 1. The role of vericiguat in heart failure with reduced ejection fraction. Image created by authors.
Abbreviations: ACEi: Angiotensin-converting enzyme inhibitor; ARB: Angiotensin II receptor blocker; B-blockers: Beta-blockers; cGMP: Cyclic guanosine monophosphate; EF: Ejection fraction; GTP: Guanosine triphosphate; HFrEF: Heart failure with reduced ejection fraction; MRA: Mineralocorticoid receptor antagonists; NO: Nitric oxide; sGC: Soluble guanylate cyclase; SGLT2i: Sodium-glucose cotransporter-2 inhibitors.

inclusion of the *post hoc* analysis by Senni *et al.* and other relevant studies adds depth to the discussion of these flaws.[7] Senni's analysis, for example, underscores the importance of patient stratification and tailored therapy in HF management, particularly in assessing new treatments like vericiguat.[7] The analysis found significant variations in treatment efficacy based on baseline risk factors, which aligns with the concerns raised about the mixed population and subgroup differences in the current study.[7] In addition, studies focusing on the importance of adhering to GDMT in clinical trials highlight the need for rigorous application of existing guidelines to ensure new therapies are evaluated against a standard of care that reflects best practices in HF management.[7]

3.5.1. Mixed population

The inclusion of a diverse patient population in the study, characterized by variations in age, NT-proBNP levels, ejection fraction, and other clinical parameters, presents both strengths and limitations for the study's generalizability and interpretation.[7] While a heterogeneous cohort can offer a broad perspective on the potential benefits of vericiguat across different subgroups, it also complicates the ability to generalize findings to specific patient populations with HFrEF.[7] For instance, the variation in NT-proBNP levels, a biomarker for HF severity, could lead to differing responses to therapy and skewed outcomes.[7] Patients with higher NT-proBNP levels typically have a worse prognosis; if these patients were unevenly distributed across the study groups, it might impact the findings regarding the vericiguat's effectiveness.[7]

Moreover, age and ejection fraction differences among the participants can significantly affect the outcomes, as older patients or those with lower ejection fractions often have different comorbidities and treatment responses.[7] This diversity makes it challenging to draw robust conclusions about the efficacy of vericiguat for the general HFrEF population without a more nuanced stratification of results by these variables.[7] Previous studies, including a *post hoc* analysis by Senni *et al.*, have highlighted the importance of patient selection and stratification in evaluating HF therapies, suggesting that more uniform populations may provide clearer insights into drug efficacy and safety profiles in clinical practice.[7]

3.5.2. Inadequate guideline-directed medical therapy

A study[18] failed to ensure that a substantial portion of the cohort received GDMT, specifically the recommended triple therapy comprising an ACEi or ARB, a beta-blocker, and an MRA, which undermines its claims about the effectiveness of vericiguat. GDMT is the cornerstone of HFrEF management, and its omission or underuse could confound the study results, making it difficult to isolate the true impact of vericiguat.[18] Patients not receiving full GDMT may have had worse baseline characteristics or more severe disease, leading to poorer outcomes regardless of additional therapies like vericiguat.[18]

The lack of adherence to GDMT also limits the study's[18] applicability to real-world settings, where adherence to guidelines is crucial for improving patient outcomes.[18] Previous research has shown that patients who receive comprehensive GDMT have significantly better outcomes, including reduced mortality and hospitalization rates, compared to those who do not.[18] Thus, the observed benefits of vericiguat treatment in this study[18] may reflect an effect on a population that is not optimally managed, rather than

the additive benefit of the drug itself.[18] To better assess the efficacy of new drugs, future studies should ensure that all participants are on full GDMT before introducing additional therapies.[18]

3.5.3. Subgroup differences between quartiles

The presence of notable differences between quartiles (e.g., Q1 – Q3 vs. Q4) in terms of baseline characteristics and outcomes suggests potential biases in the study design[7] that may limit its ability to deliver robust conclusions about the general HFrEF population.[7] For example, if patients in Q4 had higher baseline NT-proBNP levels or worse kidney function compared to those in Q1 – Q3, the overall outcomes could be skewed by these more severe cases, making the treatment appear less effective than it might be in a more homogenous population.[7]

This variability highlights the need for more precise stratification in the study[7] design to ensure comparability across different patient subgroups.[7] The *post hoc* analysis by Senni *et al.* emphasizes that subgroup analysis is critical in interpreting clinical trial results for HF treatments.[7] Their findings suggest that patients with high-risk profiles or more advanced disease stages may not benefit as much from certain therapies, which could explain the differences observed between quartiles in the current study.[7] Thus, while the study provides valuable insights on the use of vericiguat, its design limits the applicability of its findings to the broader HFrEF population, necessitating further research with more homogeneous patient groups.

3.6. Challenges faced in the treatment of HFrEF

Studies have shown that patients with HFrEF in Pakistan are not receiving the optimal medication dosages, resulting in suboptimal treatment outcomes.[3] This is likely due to a variety of factors, including a lack of awareness about optimal dosages, a lack of access to medications, and poor adherence to the treatment regimen. The main challenges faced in the treatment of HFrEF are poor adherence to the treatment plan and side effects from the medications. Adherence can be a challenge because some of the medications need to be taken multiple times a day and must be continued for life.[6] Side effects of the medications can include dizziness, fatigue, cough, and headaches. Some people may also experience low blood pressure and kidney problems. Another challenge can be the cost, as some HF medications are expensive.

Depression is a common comorbidity in patients with CHF in general, and it is associated with increased morbidity and mortality.[19] While this has been studied in high-income countries, the prevalence and impact of depression in CHF patients in low- and middle-income countries like Pakistan is less well-understood.[19] A study showed that the clinical assessment of CHF symptoms, as determined by the NYHA classification, did not accurately reflect the severity of the disease.[17] It was also found that many CHF patients with reduced ejection fraction were not being prescribed the appropriate medications, such as ACE inhibitors and beta blockers, as recommended by clinical guidelines. This indicates a need for improved assessment and treatment of CHF in Pakistan.[20]

3.7. Consideration when administering or using medications for HFrEF

There are a few considerations when using the above medications to treat HFrEF; the patient's other health conditions and risk factors should be considered before administering the drugs. For example, if a patient has diabetes or kidney problems, some of these medications may not be suitable for them.[21] Another consideration is the patient's age and overall health. Beta-blockers, for example, may not be suitable for elderly patients or those with a history of heart block or asthma.[21] It is also important to consider whether the patient is pregnant or breastfeeding, as some of these medications can be harmful to the fetus or baby.[21] Table 3 presents potential challenges and considerations for vericiguat implementation in Pakistan.

3.8. Current guidelines of vericiguat and its implications in Pakistan

In patients with HFrEF, vericiguat, a new oral sGC activator, has demonstrated promise in lowering the risk of cardiovascular death and HF hospitalizations.[22,23] For instance, vericiguat stimulates the biochemical pathways of cGMP and NO, which are impaired in patients with HF.[24] Therefore, patients with HFrEF who are experiencing symptoms even after receiving the best medical care and whose HF is worsening may consider vericiguat as a new treatment option. Patients with HFrEF who fit specific criteria might consider receiving vericiguat medication. Based on the guidelines and trial conditions, the eligibility to receive vericiguat varies; about 21.4% of patients with HFrEF would be eligible for vericiguat treatment based on the VICTORIA trial's criteria.[12] The most significant factors influencing this eligibility include the usage of nitrates, increased NT-proBNP levels, and recent hospitalization for HF.[24] The VICTORIA study, a prospective, randomized, double-blind, placebo-controlled study, evaluated the efficacy and safety of vericiguat in patients with HF.[24] The study found that vericiguat supplementation, at a target dose of 10 mg twice a day, reduced cardiovascular death and hospitalization for HF in patients with clinical manifestations of chronic HF and reduced ejection fraction.[12] In Pakistan, the introduction of vericiguat may have an impact on the management of

Table 3. Challenges and considerations in implementing vericiguat therapy for heart failure with reduced ejection fraction in Pakistan: accessibility, education for healthcare providers, and regulatory approval

Challenges and Considerations	Description	References
Accessibility and affordability	Ensuring that vericiguat is readily available and affordable to patients in Pakistan by taking into account the economic factors and accessibility of healthcare infrastructures.	21
Education for healthcare providers	Providing information and training to healthcare professionals in Pakistan on the appropriate use of vericiguat as a treatment for heart failure.	21
Regulatory approval process	Navigating the regulatory approval process in Pakistan to ensure that vericiguat meets safety and efficacy standards for use in heart failure treatment.	21

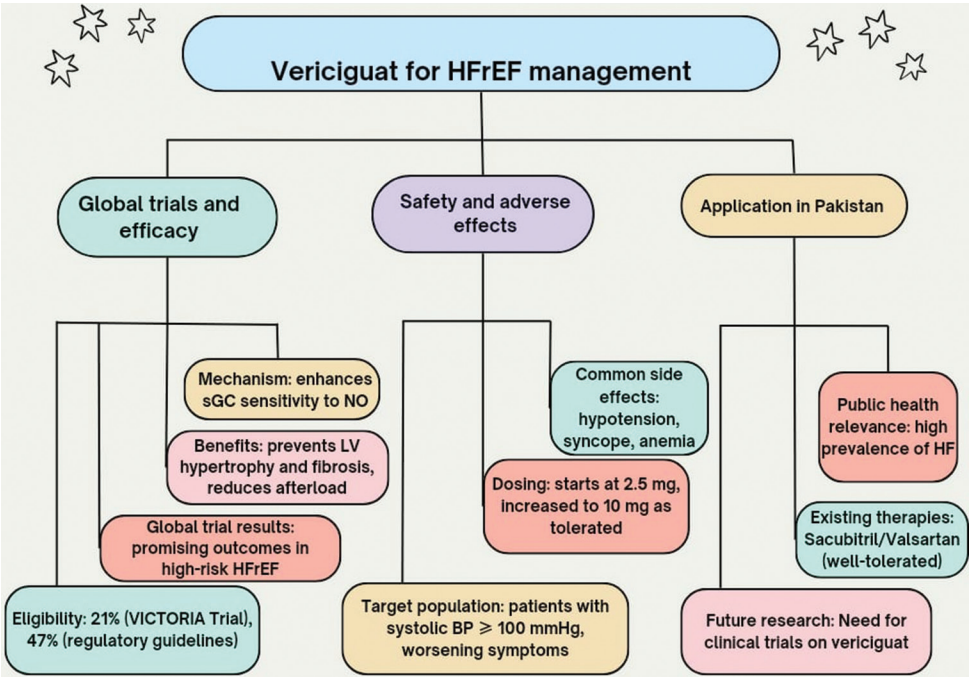

Figure 2. Summary flowchart for the efficacy, safety, and application of vericiguat for the management of heart failure with reduced ejection fraction. Image created by authors.
Abbreviations: BP: Blood pressure; HF: Heart failure; HFrEF: Heart failure with reduced ejection fraction; LF: Left ventricle; NO: Nitric oxide; sGC: Soluble guanylate cyclase.

HFrEF.[24] In individual patients with HFrEF, particularly those at higher risk for HF hospitalization, the addition of vericiguat to the treatment regimen may be considered.[25] However, the effectiveness of the combination of vericiguat with sacubitril and valsartan in HFrEF is currently unknown.[26] In Pakistan, the use of vericiguat could enhance cardiac disease management by exerting a targeted pharmacological effect, specifically by increasing the sensitivity of soluble guanylate cyclase (sGC) to endogenous nitric oxide (NO), thereby improving heart function.[23] Vericiguat has the potential to improve outcomes for individuals with HFrEF, especially in terms of lowering hospitalizations and cardiovascular deaths.[1] Its implementation in Pakistan might improve patient outcomes and the management of heart disease. However, to fully comprehend its effectiveness when used with other medications such as valsartan and sacubitril, more research is needed.

4. Future perspective for vericiguat in Pakistan

Considering Pakistan's high rate of HF, integrating vericiguat into clinical practice may have a significant impact on patient outcomes and healthcare systems. HF and other cardiovascular diseases are becoming more common in Pakistan, a situation that calls for creative and practical solutions.[27] Due to its distinct mode of action and encouraging outcomes from clinical trials, vericiguat is a solid contender for inclusion in national HF management

protocols.[27] It is recommended that Pakistani policymakers and healthcare authorities keep a close eye on the ongoing Phase III trials of vericiguat.[27] By incorporating this innovative therapy into existing treatment plans, hospitalization rates may be decreased, and patients with HF may have a higher quality of life.[27] To ensure optimal use and maximize patient benefits, extensive training programs for healthcare providers should be introduced alongside the adoption of vericiguat.[27] Given its demonstrated efficacy and safety in preliminary clinical trials, Pakistan's regulatory bodies ought to think about expediting vericiguat's approval process. Partnerships with foreign pharmaceutical firms and research centers may make this drug more accessible, guaranteeing that Pakistani patients will be among the first to take advantage of this innovative therapy.[27]

In Pakistan, a study showed vericiguat's effectiveness in reducing all-cause mortality, cardiovascular mortality, and HF-related hospitalizations, regardless of atrial fibrillation (AF) status.[28] In 2019, the prevalence and disability rates for HF in Pakistan were 405.12 and 35.80 per 100,000 populations, respectively, with the highest burden in Islamabad and the lowest in Khyber Pakhtunkhwa.[29] Females saw a higher increase in HF prevalence from 1990 to 2019 compared to males, and the burden of HF increased in the 10 – 49 age group.[29] The integration of GDMT and recent pharmacological advancements in Pakistan reflects an effort to align with international standards, although resource constraints pose significant challenges.[30] Access to educational resources, patient education, and improvements in healthcare policy are crucial to enhancing HF outcomes in such settings.[30] Figure 2 shows the flowchart for the adoption of vericiguat in HFrEF management in Pakistan.

5. Conclusion

The use of vericiguat in the treatment of HF in Pakistan is an important topic of discussion that warrants significant attention. The use of this drug would reduce the burden of HF in Pakistan. It is crucial for policymakers and healthcare authorities to demonstrate strong political will and implement effective research and policy strategies to facilitate the adoption of vericiguat in Pakistan.

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The authors declare that they have no competing interests.

Author contributions

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Writing – original draft: All the authors
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ORIGINAL RESEARCH ARTICLE

Preclinical evaluation reveals comparable toxicology and pharmacology of the erythropoietin biosimilar GB*poietin*® and Eprex®

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Abstract

Erythropoietin (EPO) is an essential growth factor for erythropoiesis. We report the results of the preclinical safety evaluation of GB*poietin*®, a recombinant human EPO (rhEPO), through a comparative acute toxicity study with the reference product, Eprex®. The products were administered subcutaneously into Wistar rats for both the single-dose and repeated-dose toxicity studies. Hematological and biochemical parameters were measured for all test subjects before the first dose and the day after the last dose in both studies. Necropsy and histopathology of representative subjects from each group were also performed to find any pathological changes, such as degeneration or cellular necrosis in internal organs such as the kidney, liver, lung, and spleen. Both GB*poietin*® and Eprex® comparative toxicology studies, which were not significantly different ($P > 0.05$), revealed similar pharmacologically driven mechanisms of toxicity. Although hematological parameters stayed within the normal range throughout the study, improved profiles of hemoglobin and hematocrit ($P < 0.05$) confirmed the therapeutic effect of rhEPO in both studies. Moreover, the initial and final values of aspartate aminotransferase, alanine aminotransferase, and blood urea nitrogen were comparable ($P > 0.05$) for both experimental products. The study established that the toxicological profiles of GB*poietin*® and Eprex® were similar and aligned with the known pharmacology of EPO alfa, demonstrating proof of "totality" and "no residual uncertainty."

Keywords: Erythropoietin; Preclinical study; Single-dose toxicity; Repeat-dose toxicity; GB*poietin*®; Drug safety profile

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1. Introduction

The hormone erythropoietin (EPO), a glycoprotein, is essential for the production of red blood cells (RBCs). In adults, EPO is mostly produced in the kidney's peritubular cells and released into the bloodstream.[1] The EPO receptor (EPOR) on bone marrow erythroid progenitors is bound to circulating EPO, initiating a number of signaling pathways that promote the development of mature RBCs.[2] It was originally isolated from

the urine of aplastic anemia patients and characterized as a 34000 Da protein.[3] The expression of this endogenous glycoprotein is controlled by the transcription factor hypoxia-inducible factor.[4] Carbohydrates constitute 40% of the molecule, primarily in the form of sialic acid, which is distributed across three N-linked and one O-linked glycosylation sites. These carbohydrates are necessary for the biological function of EPO.[5] The terminal sialic acid residues prevent rapid hepatic clearance, preserving its activity in the bone marrow. Conversely, the biological activity of EPO *in vivo* is lost when sialic acid is enzymatically removed by neuraminidases.[6] The kidneys are the primary physiological site of EPO synthesis, and its production increases in response to a localized reduction in renal oxygen supply or systemic anemia. EPO interacts with its target cells through EPORs on the cell surface. The receptor undergoes homodimerization upon EPO binding, activating Janus kinase 2 (JAK2) through transphosphorylation. Phosphorylated receptor tyrosines recruit intracellular proteins like STAT5, which, upon activation, enter the nucleus to induce erythroid gene transcription. Phosphatases dephosphorylate JAK2 to downregulate the receptor. EPO can bind to the tissue-protective receptor, specifically EPOR/CD131 heterodimer, to play a critical role in tissue protection and immune regulation in addition to its function in erythropoiesis. These receptors are expressed mostly on erythroid progenitor cells, but they are also present in neuronal, endothelial, multipotent hematopoietic, and embryonic stem cells.[7]

As a therapeutic agent, EPO is made using recombinant DNA technology in mammalian cell cultures that were transfected with the human *EPO* gene.[8] Recombinant DNA technology has enabled the production of recombinant human EPO (rhEPO), allowing its extensive therapeutic application in clinical settings.[9] Non-clinical tests should be conducted following manufacturing changes in a biologic product and for products claimed to be similar to an already approved one.[10,11] A series of *in vitro* receptor binding studies and *in vivo* investigations were required under the guidelines "if there are specific uncertainties or concerns regarding safety."[12] Clinical evidence suggests that rhEPO can be used to treat anemia associated with conditions such as chronic renal insufficiencies, rheumatoid arthritis, premature birth, chemotherapy, transfusions, and hematological diseases.[13] When rhEPO is administered, no allergies have been reported; nevertheless, a small number of patients have had arthralgias and local cutaneous responses.[14] Preclinical trials involving rhEPO are, therefore, important for establishing its safety, and efficacy, and understanding its potential therapeutic applications across various medical conditions.[15]

The purpose of this study is to provide a comprehensive overview of the preclinical studies conducted on rhEPO, with a focus on its safety, efficacy, and potential as a drug product. As a prelude to clinical trials and subsequent regulatory approval, preclinical investigations play a pivotal role in elucidating the pharmacological properties, toxicological profiles, and mechanisms of action of novel therapeutic agents.[16,17] The exploration of rhEPO begins with an examination of the protein's structure and the underlying genetic modifications that render it a recombinant biopharmaceutical.[18] By delving into the molecular intricacies of rhEPO, we aim to establish a foundational understanding of its design and functionality. In addition, this study will highlight the significance of rhEPO in the context of endogenous EPO while emphasizing the potential advantages and limitations of using the recombinant form.

The first-ever alfa epoetin product, Eprex®, is a prescription medication with a track record of safety and effectiveness.[19] Eprex® was manufactured by Johnson & Johnson and it was the first EPO formulation to receive regulatory approval in Europe in 1988. In the early 1990s, physicians outside of the United States adopted the subcutaneous route of administration of EPO for hemodialysis patients due to the socioeconomic benefit for the patients.[20] GB*poietin*® is a biosimilar of Eprex®, which was developed by Globe Biotech Limited and synthesized in genetically engineered Chinese hamster ovary cells. Upstream and downstream process development and validation were done for large-scale production.[1] Step-by-step analytical results confirmed the biosimilarity of GB*poietin*® to Eprex® in terms of molecular characterization.[21]

This study investigated the preclinical safety assessments, encompassing acute and chronic toxicity studies in relevant animal models. Comprehensive evaluations of potential adverse effects, dose-dependent responses, and organ-specific toxicity are crucial in establishing a safety profile for rhEPO, guiding subsequent clinical trial designs, and mitigating risks associated with human administration.[22] This study, designed for the preclinical safety assessment of GB*poietin*®, includes the evaluation of single- and repeated-dose toxicity. With this goal, the product was compared to a commercial homolog (Eprex®) in Wister rats to assess the toxicity of GB*poietin*®.

2. Materials and methods

2.1. Materials

The drug product, GB*poietin*® (code name: GBPD002), was obtained from Globe Biotech Limited (Bangladesh) while the reference product, Eprex®, was purchased from

Janssen (USA). Ethylenediaminetetraacetic acid (EDTA) was purchased from Merck (USA).

2.2. Formulation of GB*poietin*®

The sample was formulated using formulation buffer (L-glycine, sodium chloride, sodium phosphate, Polysorbate 80, water for injection; pH: 6.8 – 7.2) and AKTA Flux S (GE Healthcare, USA). After sterile filtration through a 0.22-micron PES Sartopore 2 filter (Sartorius Stedim, Germany), the pre-formulated bulk sample was transferred to quality control (QC) for testing as per the specification. The sterile, pre-formulated bulk drug substance was then transferred to a fill-finish facility in a 2D single-use bag (Sartorius Stedim, France) for filling and packaging while maintaining a temperature of 4 – 8°C. The sterile 1 mL empty long syringes (Schott, Switzerland) were filled using automatic combo filling and closing machine (Tofflon, China) at 1 mL volume for dose preparation. The pre-filled syringes were packaged (blistered) using the HM-AV Plus blister machine (Hoonga, The Republic of Korea). After blistering completion, the drug product was stored at 4 – 8°C. Finally, the pre-filled syringes were tested for QC as per the specification and transferred to the pre-clinical (animal) study center, maintaining a temperature of 4 – 8°C, for the toxicology study.

2.3. Animal selection

Wistar rats (*Rattus norvegicus*) were used for the single- and repeated-dose safety and toxicity studies. A total of 75 Wistar male and female rats (10 – 15 weeks old) were selected and isolated 5 days before the dosing. After attentive monitoring and conditioning, 36 rats (18 males and 18 females) were subjected to single-dose toxicity analysis and 24 rats (12 males and 12 females) were subjected to repeated-dose toxicity analysis. The temperature of the experimental animal room was 26 ± 2°C and the relative humidity was 60 ± 5%. The room was an HVAC-controlled ISO class 7 room with 70% fresh air intake and full exhaust. The rats were individually housed in a polypropylene cage with proper water and feed and kept under 12 h of the day-night cycle. The target weight of male animals was 185 ± 20 g and female animals were 175 ± 20 g. In the entire experiment, 20% of additional animals (males and females) were used as substitutes for the excluded animals. Healthy young adult animals were used. Females were nulliparous and non-pregnant. At the beginning of the study, each animal was between 10 and 15 weeks old. The weight difference of animals used was minimal, not exceeding ± 20% of the mean weight for each sex. These animals were used to replace any individuals that were excluded during the study periods. The study plan and procedures were approved by the internal ethical review board of Globe Biotech Limited, complying with local ethical regulations. No treatment randomization and blinding methods were used in the study and sample sizes were determined by the resource equation method.

2.4. Single-dose toxicity assay

A total of 36 Wister rats, including both male and female, were used for the study comprising four treatment groups, a placebo group, and a control group – each group consists of three male and three female rats. The four treatment groups were assigned to either normal (500 IU/kg) or toxic dose (1500 IU/kg) to assess the toxicological similarity of GB*poietin*® to Eprex®. Treatment-1 and Treatment-2 were injected with normal and toxic doses of GB*poietin*®, respectively, while Treatment-3 and Treatment-4 were injected with normal and toxic doses of Eprex®, respectively. The placebo group was injected with 100 µL of formulation buffer used for GB*poietin*® and Eprex®. The control group was not given any injection, serving as a negative control of the study. Two days before dosing, whole blood (approximately 200 µL) was collected in 2% EDTA from each rat for the complete blood count (CBC) analysis. Similarly, whole blood was also collected after the last dosing at day 14 for the CBC analysis. Pharmacodynamic (PD) endpoints including RBC count, white blood cell (WBC) count, hemoglobin (HGB) level, platelet (PLT) count, hematocrit (HCT) level, mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), and mean corpuscular hemoglobin concentration (MCHC) were measured for all test subjects before injection and on the last day of the 14-day study using BK-6190-Vet auto hematology analyzer (Biobase, Germany). Similarly, alanine aminotransferase (ALT)/glutamic pyruvic transaminase (GPT), aspartate aminotransferase (AST)/serum glutamic-oxaloacetic transaminase (sGOT), and blood urea nitrogen (BUN) assays were performed on blood serum using a semi-automatic chemistry analyzer (Biobase, Germany) to assess the toxic effect of GB*poietin*® and Eprex® on the liver and kidney. Body temperature and weight were also measured during the whole study period. A necropsy of representative subjects from each treatment, placebo, and control groups was done to check for abnormalities in the kidney, lung, liver, spleen, and heart. During the necropsy, external surfaces, all orifices, cranial cavities, external surfaces of the brain and spinal cord, thoracic, abdominal, and pelvic cavities, cervical tissues, and organs were also examined. Finally, histopathological evaluation was carried out to find any pathological significance like degeneration or cellular necrosis in the rat internal organs such as the kidney, liver, lung, and spleen.

2.5. Repeated-dose toxicity assay

A total number of 24 rats were separated into four different groups consisting of 6 rats (3 males and 3 females) in each group. There were four different treatment groups (1 and 2), one placebo group, and one control group. Each rat of the two treatment groups was subcutaneously (SC) injected with sterile 125 IU/kg, 250 IU/kg, 500 IU/kg, 750 IU/kg, 1000 IU/kg, 1250 IU/kg, and 1500 IU/kg of GB*poietin*® (Group 1) and Eprex® (Group 2) on 7 consecutive days. The placebo and the control groups were similar to those in the single-dose toxicity assay. For CBC analysis, whole blood was collected 2 days before dosing as well as after the last dosing on day 7 using similar protocols described in Section 2.4. PD endpoints including RBC, WBC, HGB, HCT, MCV, MCH, MCHC, and PLT count were measured for all test subjects before the first dose injection and the day after the last dose. ALT, AST, and BUN assays as well as necropsy tests and histopathological evaluations were performed as described in Section 2.4**.** Body temperature and weight were also measured during the whole study period.

2.6. Data evaluation and statistical analysis

The variables used for statistical processing are body weight, body temperature, hematological and biochemical parameters, as well as microscopy findings. Central tendency and dispersion statistics, including mean, standard deviation, maximum, and minimum values, were calculated. The variable FD (difference between the final and initial value) was calculated to assess the treatment effects on hematological and biochemical parameters. The assumptions of normal distribution and homogeneity of variance were verified using the Kolmogorov-Smirnov and Shapiro-Wilk tests, and the Levene test, respectively, before the analysis of body weight and body temperature variables for each evaluation time point. A parametric analysis of variance (ANOVA) or a non-parametric alternative (Kruskall–Wallis test) was used, depending on whether the data followed a normal distribution. Paired comparisons were performed in consecutive intervals, using either the paired t-test or the Wilcoxon test, depending on whether the data followed a normal distribution. The results from the histopathological studies were analyzed using cross-tabulated classification tables and the test for association of independence (Fisher's exact test). The data were processed with Microsoft Excel, 2010, running on the Windows operating system. The mean difference between the test and the comparator product was calculated using linear mixed-effect analysis of variance, along with the corresponding *P*-value. Statistical significance was defined as a $P < 0.05$. A $P > 0.05$ for the PD parameters of the sample formulations was considered similar and non-significant.

3. Results

3.1. Body weight assessment

Body weight increased steadily and significantly during the single-dose toxicity study, as shown in Figure 1A. The differences in body weight for all study groups at different evaluation time points (day 0, 7, and 14) suggested that the body weight gains were significant ($P < 0.05$) for Treatment-2 ($P = 0.01$), Treatment-4 ($P = 0.006$), Control ($P = 0.001$), and Placebo ($P = 0.017$) groups but were not significant in Treatment-1 ($P = 0.09$) and Treatment-3 ($P = 0.07$) groups. On the other hand, during the repeated-dose toxicity study, body weight increased steadily but not significantly, as shown in Figure 1C; the differences in body weight across all study groups at different evaluation time points (day 0, 3, and 7) were not significant ($P > 0.05$). The results remained consistent when the data were analyzed independently per gender or evaluation time points. An increase of body weight for all the groups suggests a normal evolution of body weight which is an indicator of sound health for the animals, substantiating the non-toxicity of the GB*poietin*® under both single- and repeated-dose toxicity studies. The absence of negative effects on body weight gain is favorable for the evaluation of the substance under the study, as a decrease in body weight is one of the primary clinical symptoms of stress or illness in this rat strain. Therefore, the increase in body weight serves as indirect evidence of the non-toxicity of the substance being analyzed.

3.2. Body temperature assessment

During the single-dose toxicity test, the changes in body temperature were not significant ($P > 0.05$) for all four treatment groups (Treatment-1, Treatment-2, Treatment-3, Treatment-4) and Placebo group ($P = 0.009$), while nearly significant in Control group ($P = 0.07$), as shown in Figure 1B. On the other hand, the changes in body temperature were not significant ($P > 0.05$) for all study groups (Treatment-1, Treatment-2, Placebo, and Control) in the repeated-dose toxicity study, as shown in Figure 1D. These results remained consistent when the data were analyzed independently per gender or evaluation time points, indicating normal health for the animals and substantiating the non-toxicity of the GB*poietin*®. In addition, the absence of changes in body temperature outside the normal range serves as an indirect indicator of non-toxicity for the test animals.

3.3. Hematological assessments

When analyzing the results of the hematological tests, differences were detected among treatment groups of both doses of GB*poietin*® and the reference drug, Eprex®.

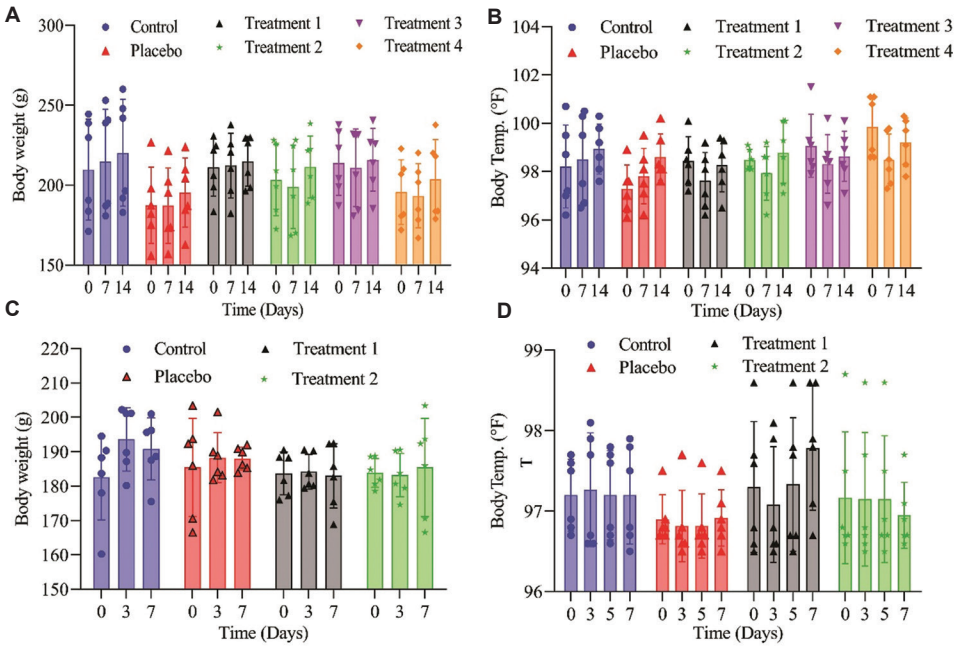

Figure 1. Changes in the body weight and body temperature of the subjects. Body weight (A) and temperature (B) changes in single-dose toxicity studies. Changes in body weight (C) and temperature (D) in repeated-dose toxicity studies. Data are presented as mean ± standard deviation.

3.3.1. Single-dose toxicity study

The change in RBC count was significant ($P < 0.05$) in Treatment-1 ($P = 0.02$), Treatment-3 ($P = 0.02$), and Placebo ($P = 0.00$) groups but not significant ($P > 0.05$) in Treatment-2 ($P = 0.16$), Treatment-4 ($P = 0.14$), and Control groups ($P = 0.43$) as shown in Figure 2A. A significant ($P < 0.05$) change in WBC count in Treatment-1 ($P = 0.05$), Treatment-4 ($P = 0.038$), and Control ($P = 0.001$) groups was observed; however, the changes found in Treatment-2 ($P = 0.80$), Treatment-3 ($P = 0.80$), and Placebo groups ($P = 0.86$) were insignificant ($P > 0.05$), as shown in Figure 2B. The change in HGB level was significant ($P < 0.05$) in Treatment-1 ($P = 0.01$), Treatment-3 ($P = 0.00$), and Placebo ($P = 0.001$) groups but not significant ($P > 0.05$) in Treatment-2 ($P = 0.11$), Treatment-4 ($P = 0.05$), and Control ($P = 0.27$) groups, as shown in Figure 2C. For the PLT count, we did not find any significant ($P < 0.05$) differences in all the groups; Figure 2D. The percentage of HCT was also checked, where the changes in HCT percentage were significant ($P < 0.05$) in Treatment-1 ($P = 0.01$), Treatment-3 ($P = 0.02$), and Placebo groups ($P = 0.00$) but not significant ($P > 0.05$) in Treatment-2 ($P = 0.218$), Control ($P = 0.55$), and Treatment-4 ($P = 0.08$) groups, as shown in Figure 2E. The changes in MCV values were significant ($P < 0.05$) in Treatment-1 ($P = 0.02$), Treatment-4 ($P = 0.01$), and Placebo ($P = 0.02$) groups but not significant ($P > 0.05$) in Treatment-2 ($P = 0.20$), Control ($P = 0.86$), and Treatment-3 ($P = 0.07$) groups, as shown in Figure 2F. The amount of MCH was also measured, and the changes were found to be significant ($P < 0.05$) in Placebo ($P = 0.03$) group but not significant ($P > 0.05$) in Treatment-1 ($P = 0.54$), Treatment-2 ($P = 0.91$), Treatment-3 ($P = 0.37$), Treatment-4 ($P = 0.97$), and Control groups ($P = 0.91$) as shown in Figure 2G. The changes in MCHC count were significant ($P < 0.05$) in Treatment-1 ($P = 0.03$) and Placebo ($P = 0.06$) groups; however, it was not significant ($P > 0.05$) in Treatment-2 ($P = 0.47$), Treatment-3 ($P = 0.28$), Treatment-4 ($P = 0.40$), and Control ($P = 0.90$) groups, where the initial and final values were very close to each other as shown in Figure 2H.

3.3.2. Repeated-dose toxicity study

For the analysis of one-way ANOVA and t-test, the hematology parameters were compared between the measurements on day 0 and day 7 in all the study groups. The RBC count increased in all study groups but Treatment-1 and Treatment-2 groups showed the highest count compared with Control and Placebo groups, as shown in Figure 3A. The differences in RBC count between the two treatment groups were very significant ($P = 0.0002$). We also compared the differences between the groups by doing a t-test, which showed significant differences between Treatment-1 and Control ($P = 0.002$), Treatment-2 and Control ($P = 0.002$), Treatment-1 and Placebo ($P = 0.002$), Treatment-2 and Placebo ($P = 0.002$)

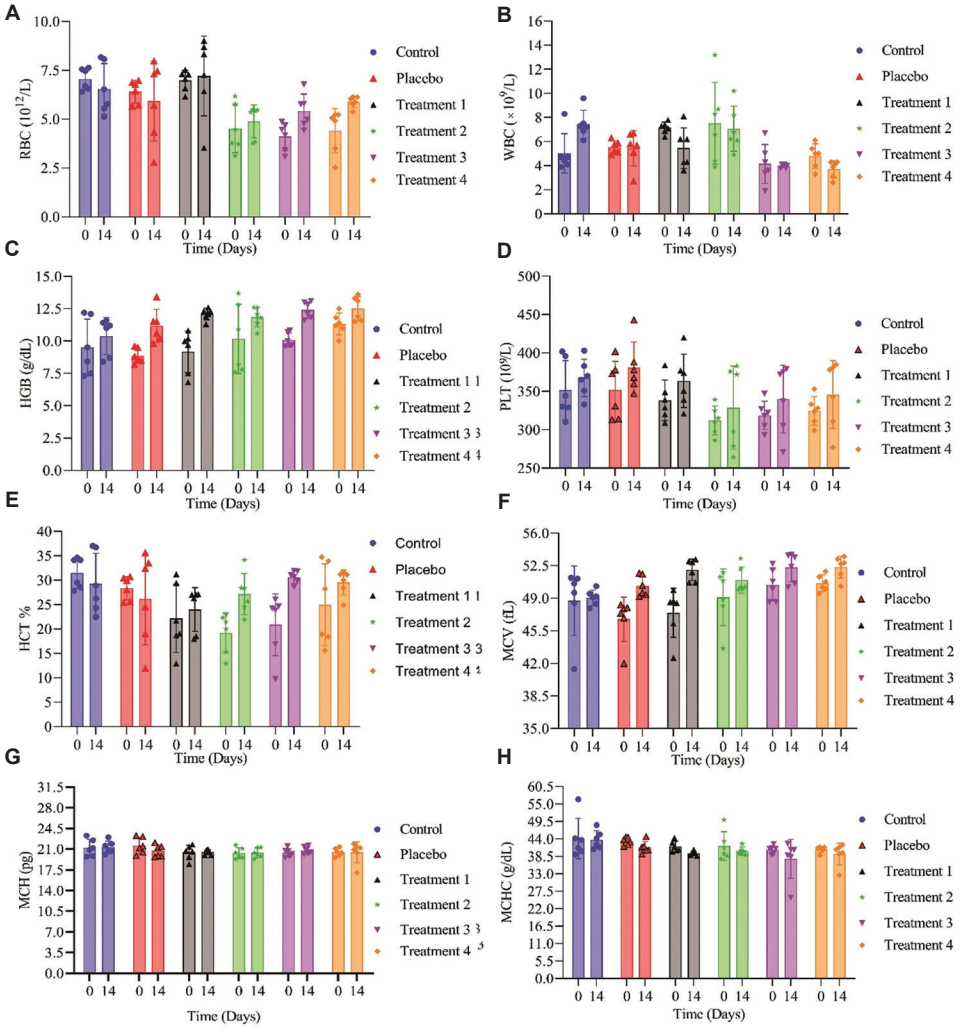

Figure 2. Hematological test findings of single-dose toxicity study. (A) Red blood cell count; (B) White blood cell count; (C) Hemoglobin count; (D) Platelet count; (E) Hematocrit; (F) Mean corpuscular volume; (G) Mean corpuscular hemoglobin; (H) Mean corpuscular hemoglobin concentration. Data are presented as mean ± standard deviation.

but no significant differences between Treatment-1 and Treatment-2 ($P = 0.83$) and Placebo and Control ($P = 0.64$). WBC count decreased within the normal range in all study groups, including Control group, and their results were almost similar, as shown in Figure 3B. However, the differences in WBC count among the groups were not significant ($P = 0.80$). HGB levels increased in all study groups but Treatment-1 and Treatment-2 groups showed the highest levels compared with Control and Placebo groups, as shown in Figure 3C. The differences in HGB levels on day 0 and day 7 among the different study groups were very significant ($P = 0.0004$). HCT also increased in Treatment-1 and Treatment-2 groups but decreased in Control and Placebo groups, as shown in Figure 3E. The differences in HCT percentage among the different study groups were very significant ($P = 0.0001$). We also found significant differences between Treatment-1 and Control ($P = 0.01$), Treatment-2 and Control ($P = 0.000$), Treatment-1 and Placebo ($P = 0.04$), Treatment-2 and Placebo ($P = 0.000$). The MCV increased within the normal range in all study groups, including Control, where Treatment-1 group showed the highest level compared with Treatment-2, Placebo, and Control groups, as shown in Figure 3F. When the differences in HCV count among the groups were analyzed, we did not find any significant ($P = 0.64$) differences. MCH level decreased within the normal range in all study groups except Treatment-2 group where the results were insignificant ($P = 0.46$), as shown in Figure 3G. MCHC decreased in Treatment-2 and Control groups but increased in Treatment-1 and Placebo groups, as shown in Figure 3H. The differences in MCH levels among the groups were not significant ($P = 0.27$).

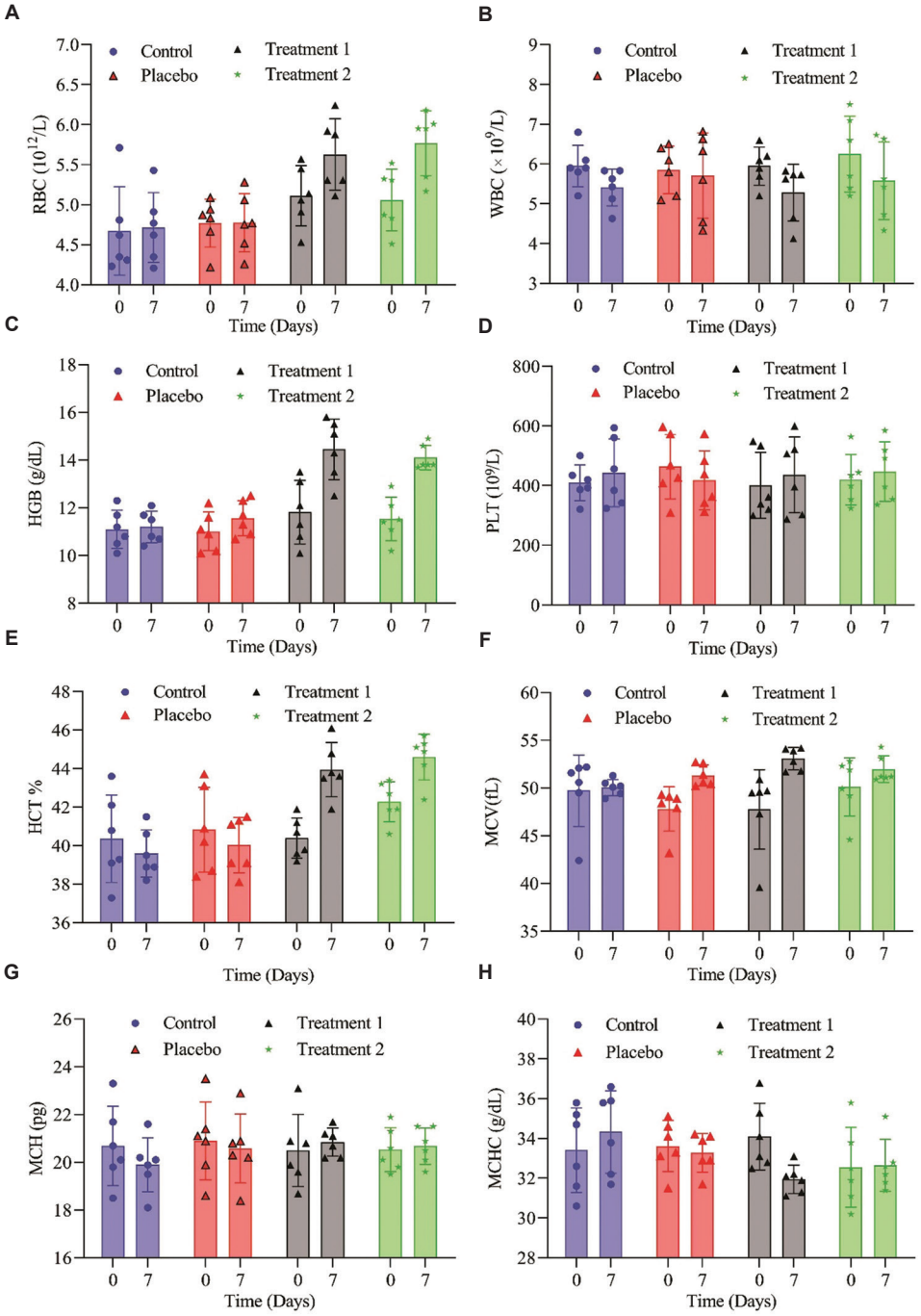

Figure 3. Hematological test findings of repeated-dose toxicity study. (A) Red blood cell count; (B) White blood cell count; (C) Hemoglobin count; (D) Platelet count; (E) Hematocrit; (F) Mean corpuscular volume; (G) Mean corpuscular hemoglobin; (H) Mean corpuscular hemoglobin concentration. Data are presented as mean ± standard deviation.

Finally, PLT count increased within the normal range in all study groups, including Control group, and their results were almost similar, as shown in Figure 3D. However, the differences in PLT count among the groups were significant ($P = 0.44$).

3.4. Biochemical tests

3.4.1. ALT/GPT assay

Increased ALT was observed in all animal groups of the toxicity study. In the single-dose toxicity study, the

Treatment-4 group showed the highest positive value among all the study groups, whereas Treatment-1 showed the lowest value, as shown in Figure 4A. The change in ALT levels was significant ($P < 0.05$) in Treatment-2 ($P = 0.03$), Treatment-4 ($P = 0.00$), Control ($P = 0.01$), and Placebo ($P = 0.06$) groups but not significant ($P > 0.05$) in Treatment-1 ($P = 0.14$) and Treatment-3 ($P = 0.10$) groups. On the other hand, in the repeated-dose toxicity study, ALT levels also increased in all study groups, where Treatment-2 and Control groups showed higher values compared to Treatment-1 and Placebo groups, as shown in Figure 4D. However, the difference in ALT levels between the two treatment groups was not significant ($P < 0.05$).

3.4.2. AST assay

AST level increased in all animal groups under study, where Treatment-4 group showed higher values compared to all other groups in the single-dose toxicity study, as shown in Figure 4B. The change in AST levels was significant ($P < 0.05$) in Treatment-1 ($P = 0.02$), Treatment-2 ($P = 0.02$), Treatment-4 ($P = 0.01$), Control ($P = 0.01$), and Placebo ($P = 0.06$) groups but not significant ($P > 0.05$) in Treatment-3 ($P = 0.28$) group. The AST level increased within the normal range in all study groups, including Control group, and their results were almost similar, as shown in Figure 4E. However, the analysis of AST levels among the groups showed no significant ($P = 0.76$) differences.

3.4.3. BUN assay

BUN level also increased in all groups under toxicity study, except Treatment-3 group in the single-dose toxicity test, as shown in Figure 4C. The change in BUN levels was significant ($P < 0.05$) in Treatment-1 ($P = 0.03$), Treatment-2 ($P = 0.00$), and Treatment-3 ($P = 0.00$) groups. However, it was not significant ($P > 0.05$) in Treatment-4 ($P = 0.80$), Control ($P = 0.31$), and Placebo ($P = 0.12$) groups, in which the initial and final values were very close. The BUN level also increased in Treatment-1, Treatment-2, and slightly in Control groups but decreased in Placebo group in

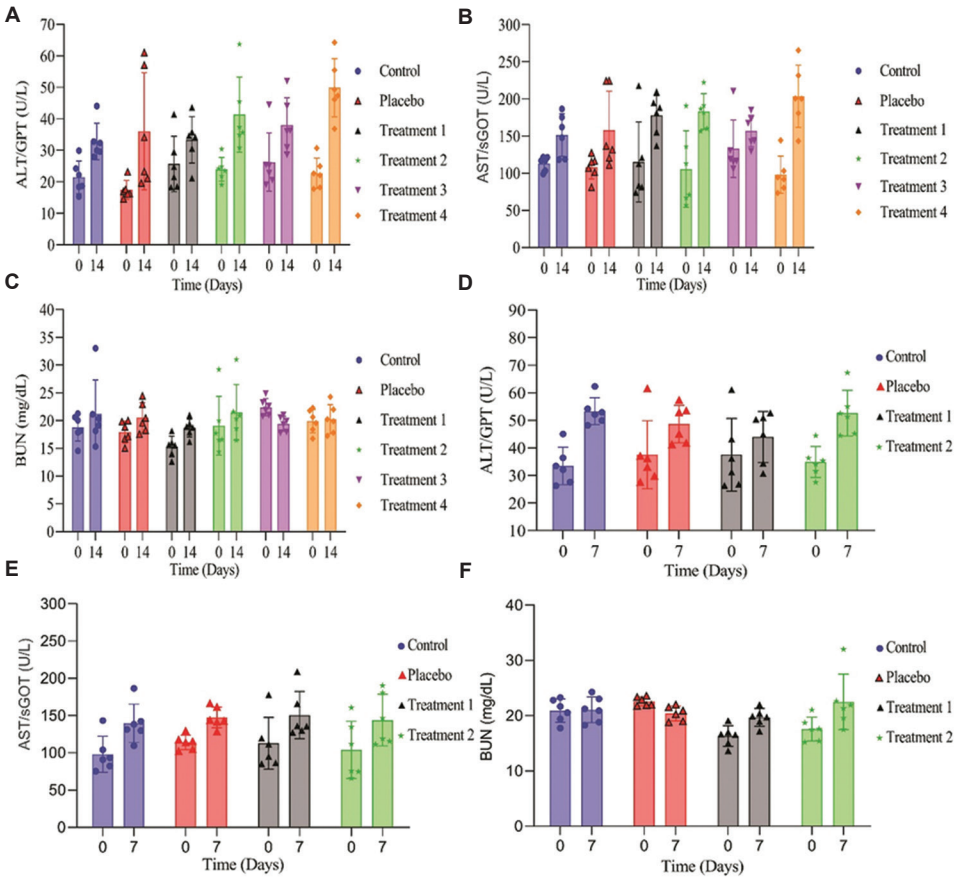

Figure 4. Analysis of biochemical parameters of the study subjects. (A-C) show the findings of alanine aminotransferase (ALT)/glutamic pyruvic transaminase (GPT), aspartate aminotransferase (AST)/serum glutamic-oxaloacetic transaminase (sGOT), and blood urea nitrogen (BUN) assays, respectively, for single-dose toxicity tests. (D-F) show the findings of ALT/GPT, AST/sGOT, and BUN assays, respectively, for repeated-dose toxicity tests. Data are presented as mean ± standard deviation.

the repeated-dose toxicity study, as shown in Figure 4F. However, the differences in BUN levels among the groups were not significant ($P = 0.02$).

3.5. Necropsy findings

Single representative subjects from all Treatment, Placebo, and Control groups were euthanized for necropsy findings. External surfaces, all orifices, cranial cavities, external surfaces of the brain and spinal cord, thoracic, abdominal, and pelvic cavities, cervical tissues, and organs were observed for any abnormalities. However, no abnormal lesions were found during necropsy examination, as shown a Figure 5.

3.6. Histopathology study

No morphological signs of toxicity were observed in internal organs such as the kidney, liver, lung, and spleen of experimental animals from the single- and repeated-dose toxicity studies (Figure 6). No lesions of pathological significance like degeneration or cellular necrosis were found on these internal organs in all treatment groups, including the group that received Eprex®.

4. Discussion

This study presents the overall results of the preclinical safety evaluation of GB*poietin*® in rats through a comparative acute toxicity study with Eprex®, following subcutaneous administration. The findings demonstrated that the toxicological profiles of GB*poietin*® and Eprex® were similar and aligned with the known pharmacology of EPO alfa, previously studied for a comprehensive safety and toxicity assessment of rhEPO.[23] No side effects or signs of toxicity were observed during daily observations of the animals inoculated with GB*poietin*® or Eprex® in the comparative acute toxicity assay. There were no changes in fur or pigmentation, and the eyes and mucosal surfaces appeared normal, as did the somatomotor activity and behavior. Proper responses to stimulation were obtained, and no deaths were reported during the study. Upon evaluation of the inoculation site, no signs of damage attributable to the administration of rhEPO.[24-26] were evidenced. The only abnormalities detected were hemorrhagic areas observed in the repeated-dose toxicity study, but these were attributed

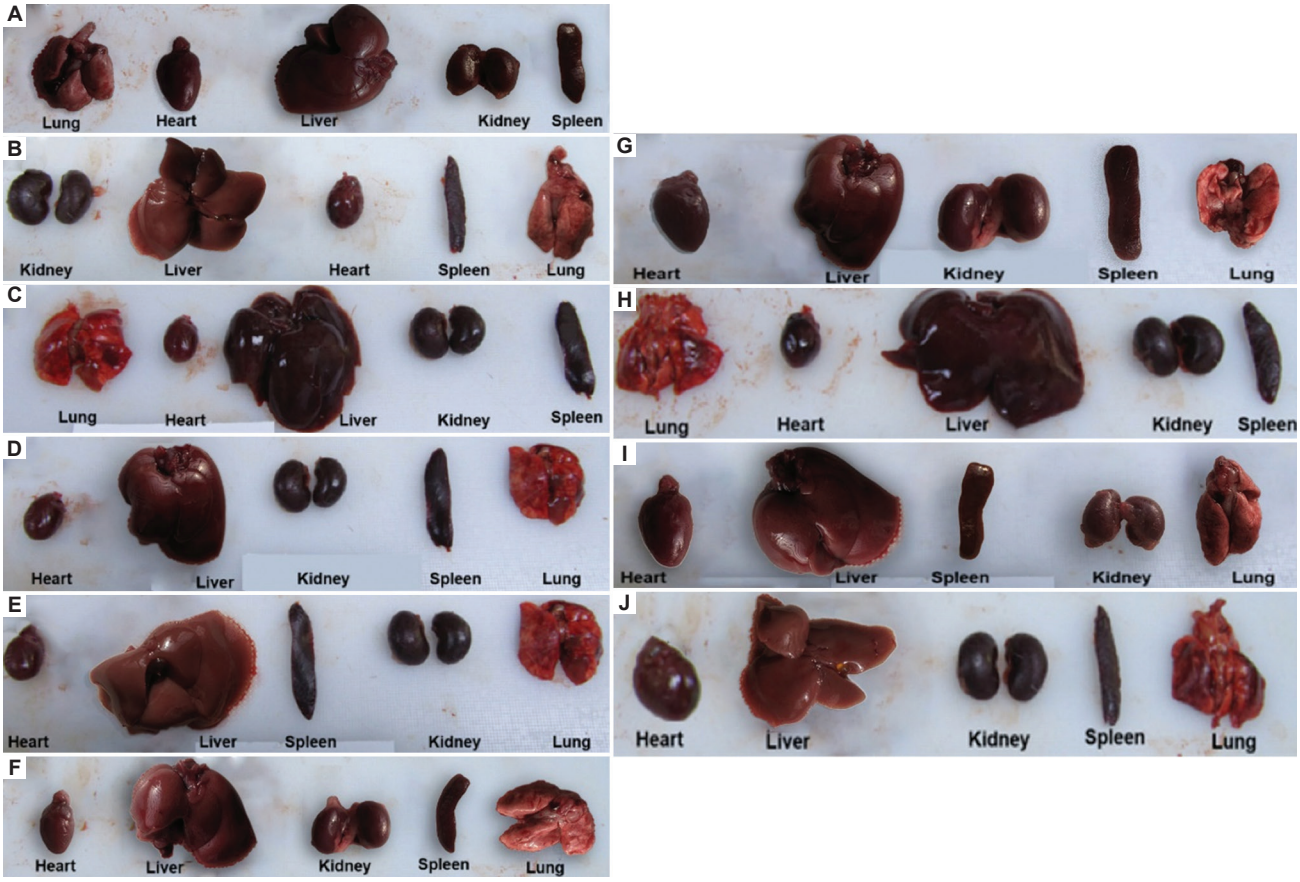

Figure 5. Representative necropsy findings of both single- and repeated-dose toxicity studies. (A) Control, (B) Placebo, (C) Treatment 1, (D) Treatment 2, (E) Treatment 3, and (F) Treatment 4 groups of single-dose toxicity study. (G) Control, (H) Placebo, (I) Treatment 1, and (J) Treatment 2 groups of repeated dose toxicity study.

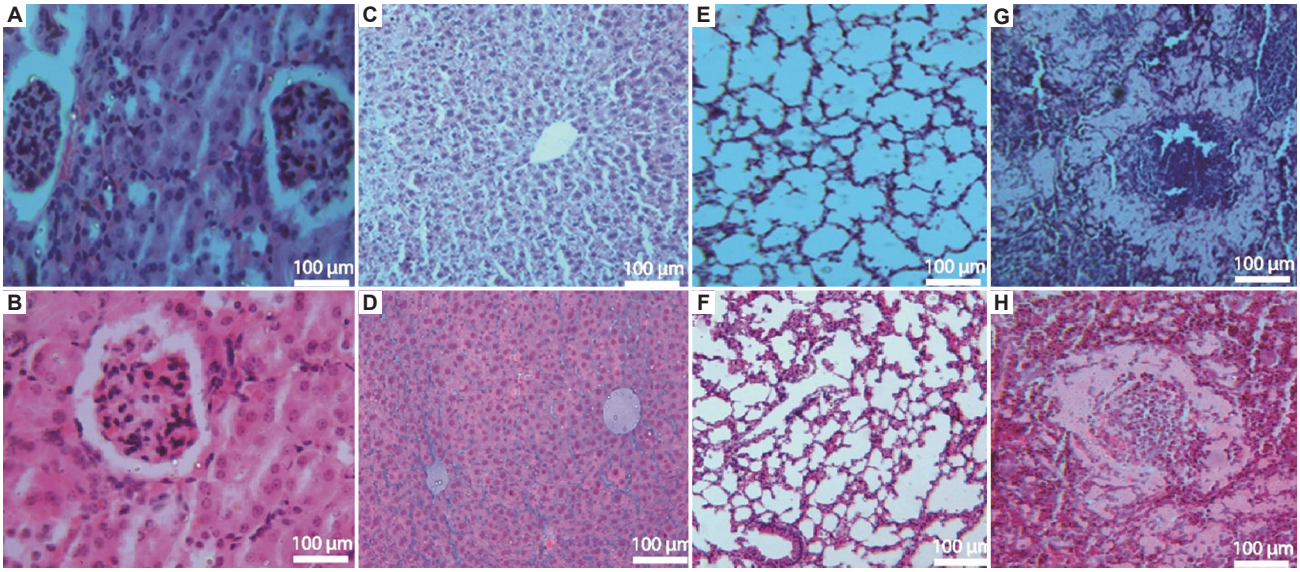

Figure 6. Representative histopathological findings. (A and B) kidney, (C and D) liver, (E and F) lung, (G and H) spleen, for both single- and repeated-dose toxicity studies, respectively.

to the method and the repetitive nature of the inoculation rather than to rhEPO itself,[27-29] as they appeared with the same intensity in the placebo group. The results of the hematological tests were normal when compared to their values before the start of the study.

Body weight increased steadily and significantly ($P < 0.05$) during the single-dose toxicity study; we found similar differences in body weight in all study groups at different evaluation time points (days 0, 3, and 7). The results were insignificant ($P > 0.05$) for the repeated-dose toxicity study as well. The increase of this parameter in all the study groups translates into a normal evolution of body weight for both genders indicating good health for the animals, further substantiating the non-toxicity of the GB*poietin*® in both studies. The absence of negative effects on body weight gain is favorable for evaluating the substance under study, as a decrease in body weight is one of the primary clinical symptoms of stress or illness in this rat strain.[30,31] Therefore, the increase in body weight is indirect evidence of non-toxicity for the substance under analysis. No significant changes in body temperature were observed among the treatment groups. This result remained similar when the same data were analyzed independently by gender or evaluation time points, which indicated normal health for the animals, further supporting the non-toxicity of the GB*poietin*®. Abnormal changes in body temperature under test conditions have been shown to indicate induced toxicity.[32,33] Accordingly, the absence of significant changes in body temperature outside the normal range suggests that severe toxicity was unlikely in the test animals during the experiment.

The results of the hematological tests were normal when compared to their values before the start of the study. Differences were found in the groups inoculated with GB*poietin*®, Eprex®, or the placebo in both single- and repeated-dose studies. The values stayed within the normal range for the species used in the study.[27,34] WBC levels decreased within the normal range across all study groups, including the control, with results being largely similar. The absence of WBC count changes beyond the normal range serves as evidence of the non-toxicity of the test item under analysis.[35] The high RBC count, HGB level, and HCT, together with the slight increase in PLT, confirm the therapeutic effect of EPO. These high counts of hematological parameters also reveal the therapeutical similarity of GB*poietin*® compared with Eprex®. These results are attributed to the mobilization of hematopoietic progenitor cells to the peripheral blood, which is the reported mechanism of action of the product under study.[36] On the other hand, MCV increased in all groups under study with Treatment-1 showing higher value than all other groups in the toxicity study. The differences in MCV levels among the groups were insignificant ($P < 0.05$). MCH levels increased in Treatment-2, Treatment-3, and Control groups, while they decreased in Treatment-1, Treatment-4, and Placebo groups, though the values were within the normal range ($P = 0.46$) in all study groups. In single-dose toxicity study, MCHC levels decreased in all animal groups, while they decreased in Treatment-2 and Control groups and increased in Treatment-1 and Placebo groups in the repeat-dose toxicity study. The absence of change in MCV, MCH, and MCHC levels outside the normal range demonstrates the non-toxicity of the test item under

analysis.[37] Collectively, these results also demonstrate the similarity between the two experimental EPO preparations, namely GB*poietin*® and Eprex®.

Our result is in agreement with the findings of other studies, where similar levels of biochemical parameters were reported (ALT/GPT, AST, and BUN).[38] In our study, we reported that serum ATL/GPT increased in all the treatment groups. Serum AST and BUN also increased beyond the normal level but the increases were less than that of the ALT/GPT levels in both single- and repeated-dose toxicity tests. Similar studies suggest that the upper level within the normal range of biochemical parameters indicates the uniformity of liver function.[39] It also serves as evidence for non-toxicity of the test item under analysis.[40] The observation suggests that GB*poietin*® is safe, with a toxicity profile similar to that of the reference product Eprex®.

The results of the necropsies for both experiments showed no indication of any abnormalities or anatomical changes. After two doses, the hemorrhagic regions were observed for single animal from each group (control, placebo, and treatment groups). Presumably, the trauma from repeated subcutaneous injections at the same location is associated with this symptom.[6] Thus, the macroscopic results support the clinical findings, indicating no changes attributable to EPO and no signs of local irritation or injury. Regardless of the dosage or volume of administration, the lack of macroscopic damage in the organs and tissues of the experimental animals is a crucial factor in assessing the safety of the tested product in both trials. One of the observed histopathological findings was the absence of lesions of pathological significance like degeneration or cellular necrosis in internal organs. These reactions have been described in the literature as a consequence of the intense metabolic activity of the organ, and their spontaneous appearance has been reported for this animal species.[41] The presence of these findings in the control groups indicates that their occurrence is not dependent on the effect of the different rhEPO doses in any of the assayed formulations. Notably, the histopathological results further support the safety of the test product GB*poietin*®, demonstrating similarity to the reference product Eprex®.

5. Conclusion

This study establishes that the toxicological profiles of GB*poietin*® and Eprex® are similar and align with the known pharmacology of EPO alfa. It is also important to note that the histopathological results preclude any noticeable toxicity effect of the test product, GB*poietin*®, in major organs and injection sites. Based on the experimental results, we conclude that the biological response of GB*poietin*® and Eprex® is essentially equivalent. Therefore, they can be considered biosimilar in pre-clinical settings, with a clinical study in humans potentially providing further insights.

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Conflict of interest

Kakon Nag is the CEO of Globe Biotech Limited, Dhaka, Bangladesh and R&D Management Solution Inc., Ontario, Canada. However, this has not influenced the content of the manuscript. The other authors declare that they have no competing interests.

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Ethics approval and consent to participate

The study plan and procedures for experiments were approved by the internal ethical review board (IECB-PCS: Internal Ethical Clearance Board for Pre-Clinical Study) of Globe Biotech Limited, which complies with local and international regulations. The ethical approval reference number is GB/EC/19/001.

Consent for publication

Not applicable.

Availability of data and materials

The data that support the findings of this study are available within the article or are available from the corresponding author upon reasonable request.

Further disclosure

The study was published in the preprint journal BioRxiv under the title "Preclinical study of the human recombinant Erythropoietin (GBPD002) compared with Eprex®" with a DOI number 10.1101/2024.06.05.597513.

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ACCSCIENCE PUBLISHING

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ORIGINAL RESEARCH ARTICLE

Repeated ketamine doses elevate superoxide dismutase activity in a pharmacological model of schizophrenia-like phenotypes in mice

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Abstract

This study evaluated behavioral phenotypes and superoxide dismutase (SOD) enzymatic activity in a repeated sub-anesthetic dose of ketamine (KET) administered to model schizophrenia in an animal study. The animals were divided into three (3) experimental groups. The KET alone group received sub-anesthetic dose of KET (20 mg/kg) for 14 consecutive days. The control group vehicle (VEH) received distilled water (10 mL/kg) as a VEH, while the KET and risperidone (RISP) group (KET + RISP) received a sub-anesthetic dose of KET (20 mg/kg) alone for 7 consecutive days, followed by RISP (0.5 mg/kg) administered 1-h post-KET treatment from days 8 to 14. All treatments were administered intraperitoneally (i.p.). Twenty-four hours after the last treatment, behavioral phenotypes (locomotor activity and cognition) were assessed using the locomotor activity cage and the elevated plus maze (EPM). Thereafter, SOD enzymatic activity was evaluated in homogenized brain tissue from each mouse using spectrophotometric analysis. Animals that received KET (20 mg/kg i.p) alone showed a significant ($P < 0.05$) increase in movement counts and rearing events in the locomotor activity test. It also prolonged the latency to enter the open arms during the anxiety-induced cognitive assessment in the EPM, compared to animals that received distilled water or those that received KET and RISP. SOD enzymatic activity was significantly elevated in the KET group compared to the VEH and KET + RISP groups. The elevated SOD enzymatic activity may represent a compensatory response to the oxidative stress induced by repeated sub-anesthetic doses of KET.

Keywords: Superoxide dismutase; Ketamine; Schizophrenia; Phenotypes; Mice; Model

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1. Introduction

Superoxide dismutase (SOD) is an endogenous antioxidant enzyme that plays a critical role in converting superoxide radicals into less toxic hydrogen peroxide and oxygen molecules, thus protecting cells from oxidative damage.[1] In schizophrenia, altered SOD enzymatic activity has been observed, with studies showing both increased and decreased activities, depending on the stage of the disease and treatment status, with increased levels in first-episode schizophrenic patients.[2] Changes in endogenous

antioxidant enzyme activity are implicated in the pathogenesis of schizophrenia.[3] Increased SOD enzymatic activity has been reported in schizophrenic patients.[4] In severe schizophrenia, changes in SOD enzymatic activity have been experimentally shown to be associated with the general pathology of the disorder and the exacerbation of its symptoms.[2]

Ketamine (KET), an N-Methyl-D-Aspartate (NMDA) receptor antagonist, is commonly used in preclinical research to model schizophrenia in animals. Its sub-anesthetic doses have been shown to induce a variety of schizophrenia-like symptoms, including hyperactivity, cognitive deficits, social withdrawal, anxiety and behavioral changes, which mimic positive, negative, and cognitive symptoms of the disorder as seen in humans.[5] Acute or repeated KET administration has been used to induce schizophrenia-like phenotypes and to study the underlying neurobiological mechanisms in animal studies.[6,7] The drug's action on glutamatergic neurotransmission, specifically its antagonism of NMDA receptors, leads to an increase in dopamine release, which is thought to be the underlying mechanism of some of these effects.[8] The antagonistic effect of KET on NMDA receptors alters cognitive functions, as demonstrated in assessments of working memory using alternation tasks,[9] which could be associated with anxiety overload. In human studies, administration of sub-anesthetic doses of KET in healthy volunteers blocks NMDA receptors, thereby interfering with sensory information processing.[10] A single low dose of KET in the range of 10 – 30 mg/kg induces impaired working memory in a dose-dependent manner; the impaired working memory can be reversed by atypical antipsychotic drugs such as risperidone (RISP).[11] Behavioral paradigms such as hyperactivity and space exploration in the open field test (OFT) are used to assess psychotic-like behaviors in rodents.[7] The simplicity, reproducibility, and versatility of these paradigms make them essential tools for evaluating schizophrenia-like behaviors in animal models. Schizophrenia, a complex psychiatric disorder, is phenotypically characterized by hyperactivity as a positive symptom in rodents.[6] The OFT is particularly effective in evaluating hyperactivity, which is integral to schizophrenia pathophysiology.[12] Behavioral tests, including the elevated plus maze (EPM), have been extensively used to assess anxiety, risk-taking behavior, and cognitive impairments, which are often altered in schizophrenia.[13] The validity of these tests in assessing cognitive impairments lies in their ability to evaluate excessive and chronic anxiety, which may be interpreted in the context of thinking, memory, concentration, and decision-making.[13] Brain cognitive processing is a function of dopaminergic transmission in the prefrontal cortex (PFC) and is critically important for working memory.[8] *In vivo* microdialysis study have shown that acute KET administration impairs working memory, which is accompanied by increased dopamine release in the PFC.[14] In patients with schizophrenia, KET exacerbates both positive and negative symptoms, indicating that NMDA receptor antagonists may act on a neural system already compromised in psychosis.[15]

In animal models, KET induces behavioral and electrophysiological changes that parallel psychosis-like features observed in humans.[7] Late adolescence and early adulthood stages reflect the developmental periods when schizophrenia symptoms typically emerge in humans.[7,16] Behavioral, biochemical, and cognitive deficits induced at this stage closely mimic human schizophrenia phenotypes, including hyperactivity, sensorimotor gating deficits, and cognitive impairments.[7,16] Studies using pharmacological interventions, such as glutamate receptor antagonists like KET, demonstrate that developmental manipulations during late adolescence or early adulthood effectively reproduce schizophrenia-like behaviors. This allows for the evaluation of therapeutic interventions, such as antipsychotics or antioxidants, during critical neurodevelopmental windows in rodents.[7] Repeated administrations of low doses of KET (10, 20, and 30 mg/kg) have been shown to increase glutamate release in the PFC, thus non-NMDA receptors activation triggers dysregulation of dopaminergic neurotransmission in the PFC, thereby contributing to cognitive impairments.[17] Psychotic-like phenotype induction by KET suggests that endogenous dysfunction or dysregulation of NMDA receptor-mediated transmission may play a role in schizophrenia pathogenesis, potentially in conjunction with altered dopamine transmission. Furthermore, NMDA receptor antagonism affects serotonergic and GABAergic transmission in the PFC.[16] Most animal studies on NMDA receptor blockade focus on single-dose drug administration to induce schizophrenia-like phenotypes and mimic behavioral deficits. However, the effects of prolonged NMDA receptor antagonism in exploring the behavioral and neurobiological abnormalities of schizophrenia remain underexplored.[7]

Chronic exposure to sub-anesthetic doses of KET alters oxidative stress markers, including changes in SOD enzymatic activity, potentially driven by the activation of nicotinamide adenine dinucleotide phosphate oxidase in neurons.[18,19] These changes may also reflect a compensatory response to oxidative damage.[19,20] KET-induced schizophrenia models not only mimic behavioral symptoms but also replicate underlying biochemical abnormalities, such as the dysregulation of the endogenous antioxidant system.[21,22] While SOD is critical

in the pathogenesis of schizophrenia, limited research has explored its association with psychopathological symptoms. Notably, studies have revealed a significant positive correlation between SOD enzymatic activity and symptom severity across the positive, negative, and general psychopathology subscales of the positive and negative syndrome scale in chronic schizophrenia patients.[23] Elevated SOD enzymatic activity has also been linked to impaired cognitive performance, particularly in memory and decision-making tasks, as observed in the Y-Maze and locomotor behavior in an OFT.[24]

Neuroprotective effects of atypical antipsychotics in addressing the underlying pathophysiological mechanisms of the disorder have been reported. These therapeutic effects could be attributed to their role in microglial activation or maintenance of antioxidant mechanisms in the cerebral cortex and hippocampus.[25,26] This study examines the effects of repeated sub-anesthetic doses of KET administration on SOD enzymatic activity and schizophrenia-like behaviors in mice. Hyperactivity was measured in the OFT, while spatial working memory, as an indicator of cognitive dysfunction, was assessed with the EPM. These assessments provide insights into hyperactivity and anxiety-induced poor exploratory performance as representations of positive and cognitive symptoms of schizophrenia in an animal model, respectively.

2. Materials and methods

2.1. Animals

Male and female Swiss mice at postnatal days 56 – 70 (8 – 10 weeks) used for this study were obtained from the Department of Pharmacology and Therapeutics Animal House, Faculty of Basic Medical Sciences, University of Ibadan (Ibadan, Nigeria). They were housed in plastic cages under standard laboratory conditions (12 h light/dark cycle, temperature range of 25 ± 2°C, and relative humidity of 60 ± 5). The mice were provided with a standard diet and water ad libitum. They were acclimatized in the laboratory for 2 weeks before the experimental procedure. The animals were handled according to the institutional-based research ethics committee and National Institutes of Health (NIH) guidelines for the use and care of laboratory animals.

2.2. Drugs and chemicals

KET hydrochloride (Ranbaxy Pharm. Nigeria), RISP (Afrab Chem, Nigeria), carbonate buffer, pH 9.5, 200 mM (G-Biosciences, St Louis, USA), phosphate buffer solution, 1.0 M, pH 7.4 (Sigma-Aldrich, USA), and adrenaline (Sigma-Aldrich, USA) were used in this study. All chemicals and drugs were of analytical and pharmaceutical grades, respectively.

2.3. Dose selection

Doses administered were based on previous studies that demonstrated the induction of schizophrenia-like behaviors and altered oxidative stress in murine models.[6,26] KET (20 mg/kg) was administered to the KET group and RISP (0.5 mg/kg) was given along with KET to the KET + RISP group, and distilled water (10 mL/kg) was administered to the Vehicle (VEH) group as the VEH, based on previously established protocols.[6] KET was diluted in distilled water (VEH) and administered intraperitoneally (i.p.) as previously described.[26] Similarly, RISP was dissolved in the VEH before intraperitoneal (i.p.) administration.[6,26]

2.4. Experimental design

Male and female mice were randomly assigned to one of three groups: control (VEH), KET, or KET and RISP (KET + RISP), with no sex-specific ratio, as previously used.[6] The KET group received sub-anesthetic dose of KET (20 mg/kg, i.p.) alone once daily for 14 consecutive days. The control group (VEH) was given distilled water (10 mL/kg, i.p.) for the duration of the experiment. Animals in the KET and RISP (KET + RISP) group were pre-treated with KET (20 mg/kg, i.p.) from day 1 to day 7 and received RISP (0.5 mg/kg, i.p.) from the day 8 to day 14, 1 h after KET administration.[22]

2.4.1. Behavioral assessments

Twenty-four hours after the last treatment, each mouse was subjected to behavioral tests to assess schizophrenia-like symptoms. Hyperactivity was evaluated using the OFT, while cognitive deficits were evaluated using the EPM, as previously described with some modifications.[25-27] Each animal was tested individually, beginning with the OFT, followed by the EPM.

2.4.2. OFT

Hyperactivity was evaluated in an open field apparatus for 5 min, as previously described,[26] with minor modifications. Briefly, spontaneous locomotor activity was measured in an activity cage (Ugo Basile, Varese, Italy) with dimensions of 39 × 28 × 26 cm, featuring a front-view glass wall. The movement activity of each mouse was automatically recorded by the activity cage for 5 min. Data were presented as the number of horizontal and vertical motor activities measured by the apparatus in response to animal movements, and the number of times each mouse raises the forelimbs (rearing), respectively. The apparatus was thoroughly cleaned with 70% ethanol before introducing subsequent mouse. Before the locomotor activity test, each animal was habituated to the activity cage (Ugo Basile, Varese, Italy) for 10 min daily over 3 consecutive days after chronic KET administration.

2.4.3. EPM

Cognitive function was assessed using an EPM apparatus with 2 open arms (25 × 5 cm) and 2 closed arms (25 × 5 × 20 cm) extending from the cross-center platform (5 × 5 cm). The entire apparatus was elevated to 50 cm above the floor. At the start of the test, each mouse was placed on the cross-center platform facing an open arm. The latency period to enter the open arms was observed and recorded for 5 min using a stopwatch as a paradigm for assessing memory impairment.[13,27] A prolonged delay in entering the open arms was considered an indicator of altered exploratory performance, representing anxiety-induced decision-making deficits in working memory. Before testing, each mouse was exposed to the EPM apparatus for 5 min daily over 3 consecutive days to ensure familiarity.

2.5. Biochemical analysis

Immediately after the behavioral tests, each mouse was sacrificed by cervical dislocation, performed by trained and experienced personnel, in accordance with the institution and national guidelines on animal welfare. Then, the animals were decapitated, and their brain tissues were carefully removed. The whole brain tissue of each animal was homogenized for 60 s in 5 mL of 10% w/v cold phosphate buffer (0.1 M, pH 7.4, 4°C) using a homogenizer. The homogenates were stored in a refrigerator until biochemical analysis was carried out. Subsequently, the homogenates were centrifuged at 10,000 g for 10 min at 4°C. The resulting homogenized supernatant was separated from the pellets for SOD activity assay. The SOD activity in each brain homogenate was determined as described.[28] Briefly, 0.5 mL of the homogenate was diluted with 4.5 mL of distilled water to make a 1 in 10 dilution. Anhydrous sodium bicarbonate (6.897 g) was added to the carbonate buffer solution to achieve a pH of 10.2. An aliquot of 0.2 mL of the diluted sample was added to 2.5 mL of 0.05 M carbonate buffer (pH 10.2) and allowed to equilibrate in a spectrophotometer. The reaction was initiated by adding 0.3 mL of freshly prepared 0.3 mM adrenaline (epinephrine) dissolved in distilled water, which was quickly mixed by inversion. The reference cuvette (blank) contained 2.5 mL of carbonate buffer, 0.3 mL of substrate (adrenaline), and 0.2 mL of distilled water. The increase in absorbance at 480 nm was monitored for 60, 120, and 180 s.

Calculation: Increase in absorbance per minute $= \frac{A3\text{-}A0}{2.5}$; [28]

Where A_0 = absorbance after 60 s, A_3 = absorbance after 180 s

$$\%\,\text{inhibition} = 100 - \frac{\text{Increase in absorbance for substrate}}{\text{Increase in absorbance for blank}} \times 100$$

One unit of SOD activity is defined as the amount of SOD necessary to cause 50% inhibition of the oxidation of adrenaline.[28]

2.6. Statistical analysis

The data (behavioral and biochemical) were expressed as mean ± S.E.M and analyzed using one-way analysis of variance and *post hoc* tests (Student's Newman-Keuls) for the multiple comparisons where appropriate using GraphPad InStat® Biostatistics software (GraphPad Software Inc. USA). A level of $P < 0.05$ was considered statistically significant for all tests.

3. Results

3.1. KET-induced hyperactivity

Locomotor activity, assessed by measuring movement counts over 5 min (in seconds) and the number of rearing events, showed that the KET group exhibited a significant ($P < 0.05$) increase in movement counts and rearing events in the OFT compared to the VEH and KET + RISP groups. Specifically, the KET group showed an average movement of 549.0 ± 19.9 and 16.40 ± 0.93 rearing events. The control group (VEH) showed an average movement of 377.4 ± 14.6 and 9 ± 0.71 rearing events, while the RISP-treated group (KET + RISP) showed an average movement of 446.0 ± 10.2 and 10.40 ± 0.93 rearing events, respectively (Table 1). The significant increase in both movement counts and rearing events in the KET group suggests that KET induces hyperactivity. The control group showed the lowest activity levels, while the KET + RISP group exhibited intermediate activity (Table 1), indicating that RISP may mitigate some of the hyperactive behavior induced by KET (20 mg/kg, i.p.) administration.

3.2. Exploratory performance in the EPM

The KET group showed poor exploratory performance in the EPM test, with significant increase ($P < 0.05$) in latency to enter the open arms. The mean latency value for the KET group was 34.30 ± 1.77 s, in contrast to 18.79 ± 0.47 and 24.36 ± 1.75 s for the VEH and KET + RISP groups, respectively (Table 2). The increased latency in the KET group suggests that KET impaired spatial memory, as evidenced by the poor performance after prior exposure and the increased time spent to enter the open arms. The control group showed the shortest latency, while the KET + RISP group showed an intermediate latency (Table 2), suggesting that RISP may have cognitive-enhancing effect, reducing the altered decision-making and exploratory performance deficits induced by KET administration.

3.3. KET increases SOD activity

Animals in the KET group, which received KET alone for the duration of the experiment, showed a significant

Table 1. Locomotor activity assessment

S/N	Treatments	Movement counts/5 min in activity cage (secs)	Numbers of rearing
1.	VEH	377.4±14.6**	9±0.71**
2.	KET	549.0±19.9	16.40±0.93
3.	KET+RISP	446.0±10.2**	10.40±0.93**

Notes: Ketamine-induced hyperactivity (spontaneous movement and rearing events) in mice.
Values represent mean±S.E.M of 5 animals per group. In one-way analysis of variance test (ANOVA), ($F^{[2}.000, 9.552] = 31.43$, $P<0.0001$; $F^{[2}.000, 11.^{40} = 20.88$, $P<0.0001$), VEH and KET+RISP groups showed significant difference when compared with KET group. **$P<0.05$ as compared with KET group.
Abbreviations: VEH: Vehicle; KET: Ketamine; RISP: Risperidone.

Table 2. Evaluation of anxiety-induced poor exploratory performance

S/N	Treatments	Latency period in open arms entries in EPM/5 min) (sec)
1	VEH	18.79±0.47**
2	KET	34.30±1.77
3	KET+RISP	24.36±1.75**

Notes: Ketamine-induced altered spatial working memory (cognitive dysfunction) in mice.
Values represent mean±S.E.M of 5 animals per group. In one-way analysis of variance test (ANOVA), ($F^{[2}.000, 8.551] = 28.95$, $P<0.0001$), VEH and KET+RISP groups showed significant difference when compared with KET group. **$P<0.05$ as compared with KET group.
Abbreviations: VEH: Vehicle; KET: Ketamine; RISP: Risperidone; EPM: Elevated plus maze.

($P < 0.05$) increase in SOD activity, with a mean value of 49.98 ± 0.51 unit/mg protein, compared to the VEH and KET + RISP groups, which showed mean values of 34.84 ± 1.06 and 36.52 ± 1.30 unit/mg protein, respectively (Figure 1).

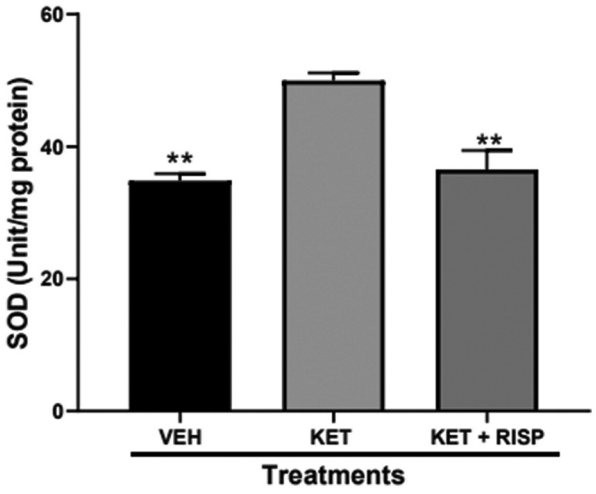

Figure 1. Superoxide dismutase activity in brain samples. Bars represent mean ± S.E.M of 5 animals per group. In one-way analysis of variance test (ANOVA), ($F^{[2}.000, 9.055] = 67.55$, $P < 0.0001$), VEH and KET + RISP groups showed significant differences when compared with KET group. **$P < 0.05$ as compared with KET group.
Abbreviations: VEH: Vehicle; KET: Ketamine; RISP: Risperidone; SOD: Superoxide dismutase.

4. Discussion

This study investigated the behavioral phenotypes and alterations in SOD enzymatic activity in a mouse model of schizophrenia induced by repeated sub-anesthetic doses of KET. Rodent models are widely utilized to study the biochemical and behavioral aspects of schizophrenia, providing insights into its underlying pathophysiology.[7,16] Behavioral paradigms such as the OFT and the EPM are particularly effective in evaluating hyperactivity and anxiety, respectively.[7] The EPM is also employed to assess altered exploratory behavior, which serves as a measure of cognitive impairments[13,27] integral to schizophrenia pathophysiology.[16] In these models, EPM assesses anxiety-induced decision-making processes during exploratory performance as spatial working memory by evaluating rodents' ability to adapt their exploratory behavior in response to environmental cues as evaluated by latency period to enter the open arms.[13,27] Frequent entries and preference for the closed arms, resulting in prolonged latency time to enter the open arms, suggest deficits in decision-making processes during exploration of the habituated environment. This behavior serves as a measure of anxiety-associated poor exploratory performance.[27] The OFT is particularly effective in evaluating hyperactivity.[12] Notably, alterations in the endogenous antioxidant system, marked by changes in antioxidant enzyme activity, have linked oxidative stress to behavioral deficits in schizophrenia.[2] Activities of endogenous antioxidant enzymes underscore the critical role of oxidative stress in schizophrenia pathophysiology. Alterations in brain biochemical activities, including induced oxidative stress and compensatory enzymatic activities of SOD, have been reported following the administration of psychotomimetic drugs like KET.[29,30] often coupled with schizophrenia-like behavioral manifestations.[6] Single and repeated administrations of KET are commonly employed to alter glutamate neurotransmission, serving as a model for psychosis-like effects. By acting as an NMDA receptor antagonist, KET induces a dissociative state characterized by disorganization and sensory perception.[14] In this study, findings from the OFT demonstrated that repeated sub-anesthetic doses of KET induced significant hyperactivity in a mouse model of schizophrenia. Increased horizontal and vertical movement, as well as rearing events, in the

KET-treated mice suggests heightened motor activity, which is the characteristic of KET's psychostimulant properties.[31,32] The enhanced locomotor activity observed in experimental animals following the administration of KET is often considered a model for the positive symptoms of schizophrenia, including arousal and disorganized behavior. This behavioral response may be attributed to heightened dopaminergic activity, particularly in the nucleus accumbens,[33] and disrupted cortical connectivity induced by the hypofunction of glutamatergic activity.[8] As such, deficit in behavior that followed repeated sub-anesthetic doses of KET administration may be triggered by the combined effects of direct or indirect dopamine agonists and NMDA receptor antagonists.[34] Cognitive function, as inferred from the EPM performance, was also impaired in the mice that received KET alone. The KET group demonstrated a prolonged latency period to enter the open arms ($P < 0.05$), which may reflect high level of anxiety-induced poor task performance due to deficits in decision-making processes.[13,27,30] Altered exploratory performance and frequent entries into the closed arms as indicatives of phenotypic anxiety were observed in KET group during the EPM test. This finding is consistent with previous findings linking anxiety overload to impaired normal cognitive processes in schizophrenia models.[27,35] This behavioral change might be due to the disruption of normal glutamatergic neurotransmission caused by KET, leading to impaired cognitive and memory functions.

Schizophrenia pathophysiology is intricately linked to oxidative stress, marked by a disruption between the production of reactive oxygen species (ROS) and antioxidant defense mechanisms. Elevated ROS activity, primarily resulting from NMDA receptor dysfunction, disrupts glutamate homeostasis, contributing to excitotoxicity and synaptic damage. These biochemical alterations are closely associated with the development of schizophrenia's positive, negative, and cognitive symptoms. Among the key components of the antioxidant defense system, SOD plays a pivotal role by converting superoxide radicals into hydrogen peroxide (H_2O_2) and oxygen, thereby preventing the formation of peroxynitrite through the interaction of nitric oxide (NO) with superoxide radicals.[29] The altered SOD enzymatic activity observed in schizophrenia is of particular interest, as it provides insights into the oxidative mechanisms underlying the disorder and the potential neuroprotective or neurotoxic implications of SOD enzymatic activity. While heightened SOD enzymatic activity may reflect a compensatory mechanism against excessive ROS production,[19,20] it also suggests an overwhelmed antioxidant defense system, which can lead to persistent oxidative damage.[17] In this study, the impact of repeated sub-anesthetic doses of KET administration on SOD enzymatic activity in a mouse model of schizophrenia was investigated. The findings revealed that repeated KET administration significantly elevated SOD enzymatic activity, which may indicate an enhanced endogenous antioxidant system resulting from an increased oxidative stress response. These results align with existing literature emphasizing oxidative stress as a central component of schizophrenia pathology and the potential of KET to exacerbate this condition under certain dosing regimens.[22] Also, the observed increase in SOD enzymatic activity may represent a compensatory response to counteract elevated ROS levels induced by KET's pharmacological actions.[19,35] Elevated SOD enzymatic activity may reflect attempts to preserve overall redox balance, although it can simultaneously exacerbate the neurobiological and behavioral symptoms of schizophrenia.[36] Moreover, KET's effect on NMDA receptors may lead to increase NO generation via the NO/Ras/extracellular-regulated kinase 1/2 pathway,[37] and it may also interfere with mitochondrial function, particularly complex I activity, which further contributes to oxidative stress.[29,36] This disruption impairs oxidative phosphorylation, increases mitochondrial NO synthase activity, and generates superoxide radicals and NO.[37-39] These alterations likely trigger heightened SOD enzymatic activity as a compensatory response. Interestingly, preconditioning studies involving volatile anesthetics have demonstrated that transient inhibition of complex I, accompanied by a moderate increase in NO or superoxide radicals, activates SOD, resulting in a subsequent increase in its activity.[38,39] However, in schizophrenia models, KET's persistent effects may overwhelm this protective mechanism.[38,39] Furthermore, heightened SOD enzymatic activity may signify increased neuroinflammation, as oxidative stress activates pro-inflammatory pathways, leading to cytokine release and neuronal damage.[3,36] Neuroinflammation exacerbates schizophrenia symptoms, creating a cycle of oxidative damage and inflammation.[36]

The relationship between oxidative stress and schizophrenia symptoms was further underscored by behavioral correlations observed in the study. Elevated SOD enzymatic activity may contribute to phenotypic hyperactivity and cognitive impairments, which could be associated with the reinforcing role of oxidative stress and antioxidant dysregulation in schizophrenia. These findings highlight the potential of antioxidant therapies to alleviate schizophrenia-related symptoms.

Treatment with the atypical antipsychotic drug RISP (0.5 mg/kg, intraperitoneally) provided significant therapeutic benefits in the study. RISP mitigated KET-induced hyperactivity in the OFT and improved cognitive performance in the EPM test. Importantly, RISP

administration significantly ($P < 0.05$) reduced elevated SOD activity in KET-treated mice. This reduction was accompanied by behavioral symptom amelioration, consistent with clinical findings in schizophrenic patients receiving RISP.[40] The ability of antipsychotic medications to modulate the oxidative stress defense system may contribute to their therapeutic efficacy.

5. Conclusion

This study confirms that KET-induced NMDA receptor blockade leads to hyperactivity, cognitive impairment, and elevated SOD enzymatic activity, mirroring key schizophrenia-like phenotypes. Elevated SOD enzymatic activity was observed as a response to repeated sub-anesthetic doses of KET administration. The heightened SOD enzymatic activity likely represents a compensatory mechanism aimed at mitigating the effects of oxidative stress induced by KET administration. However, this response may also contribute to the persistence or exacerbation of neurobiological and behavioral symptoms associated with schizophrenia.

The findings suggest that oxidative stress plays a central role in the pathophysiology of KET-induced schizophrenia models. Elevated SOD enzymatic activity highlights the dysregulation of antioxidant defenses in response to excessive ROS production. Targeting this oxidative imbalance with antioxidant therapies holds potential for alleviating neurobiological and behavioral symptoms. Further investigations could focus on assessing oxidative and antioxidant markers, inflammatory cytokines, and neurotransmitter parameters to explore their modulation by therapeutic interventions.

These results emphasize the interplay between SOD enzymatic activity and NMDA receptor dysfunction in schizophrenia and underscore the importance of antioxidant approaches that restore redox balance. Such approaches may also improve behavioral and cognitive outcomes in schizophrenia models.

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Conflict of interest

The authors declare that they have no competing interests.

Author contributions

Conceptualization: All authors
Formal analysis: Yusuf Usman, Adegbuyi Oladele Aderibigbe
Investigation: Yusuf Usman
Methodology: All authors
Writing-original draft: Yusuf Usman
Writing-review & editing: Adegbuyi Oladele Aderibigbe, Fatai Adewale Fehintola

Ethics approval and consent to participate

All experimental procedures were approved by the Animal Care and Use Research Committee (ACURC), Faculty of Basic Medical Sciences, Federal University of Health Sciences Ila-Orangun (FUHSI/BMS/AREC/2401). All the procedures were in-line with the guidelines set forth by the NIH for the care and use of laboratory animals.

Consent for publication

Not applicable.

Availability of data

Data supporting the findings of this study are available from the corresponding author upon reasonable request.

Further disclosure

The paper has been uploaded to or deposited in a preprint (bioRxiv repository, with MS ID#: BIORXIV/2024/610179. doi: https://doi.org/10.1101/2024.08.28.610179 https://biorxiv.org/cgi/content/short/2024.08.28.610179v1).

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ACCSCIENCE PUBLISHING

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ORIGINAL RESEARCH ARTICLE

Antioxidant effects of curcumin in unilateral spinal cord injury model in adult male rats

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Abstract

Inflammatory responses and oxidative stress (OS) play a significant role in the development of spinal cord injury (SCI), as evidenced by both pre-clinical and clinical studies. This research aimed to assess the potential antioxidant and anti-inflammatory properties of curcumin (CuC) as a therapeutic approach in a unilateral SCI model using male rats. We used 40 adult male Wistar rats (each weighing 220 – 250 g) that were randomly assigned to one of the five experimental groups: (1) Control (Con), (2) Model (SCI animals), (3) Model+CuC20, (4) Model+CuC40, and (5) Model+CuC80. Accordingly, the SCI animals in Model+CuC20, Model+CuC40, and Model+CuC80 groups received 20, 40, and 80 mg/kg/day of CuC through the intraperitoneal route, respectively. We assessed functional recovery, measured OS indicators, including malondialdehyde (MDA), glutathione (GSH), superoxide dismutase (SOD), and total antioxidant capacity (TAC) in the blood, and evaluated protein levels of caspase 1, NOD-like receptor family pyrin domain-containing 3 (NLRP3), and apoptosis-associated speck-like protein containing a CARD (ASC) in the spinal cord tissue. The CuC treatment groups showed a significant enhancement in functional recovery, a marked decrease in MDA levels, and a notable elevation in SOD activity relative to the SCI animals. Model+CuC40 and Model+CuC80 animals exhibited a significant improvement in GSH activity and TAC level as compared to the SCI animals. The results also showed a dramatic decrease in the protein concentration of NLRP3, ASC, and Casp1 in the Model+CuC40 and Model+CuC80 groups relative to the Model group ($P < 0.0001$). In conclusion, the treatment with CuC significantly enhanced functional recovery in SCI rats by effectively mitigating OS and reducing inflammatory markers.

Keywords: Curcumin; Spinal cord injury; Neuroinflammation; Oxidative stress

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1. Introduction

The pathogenesis of spinal cord injury (SCI) entails two distinct phases: primary injury, which refers to the immediate impact resulting from the initial trauma, and secondary injury, characterized by a cascade of pathological processes that ensue post-trauma.[1] The ensuing complications of SCI include oxidative stress (OS), inflammation, and

neurotoxicity, with OS being a significant contributor to secondary SCI.[2-4] OS occurs when there is an excess of free radicals in the body's cells, overwhelming the antioxidant defenses that typically neutralize these harmful molecules.[5] Free radicals, primarily oxygen-based molecules such as superoxide and hydroxyl radicals, are naturally produced during cellular processes. However, after SCI, there is a significant increase in free radical production due to factors like ischemia (lack of blood flow) and hypoxia (lack of oxygen).[6,7]

In addition to OS, neuroinflammation is a fundamental factor in the onset and progression of SCI. One of the key components of this inflammatory mechanism is the activation of the NOD-like receptor family pyrin domain-containing 3 (NLRP3) inflammasome, which recruits pathways that enhance the secretion of inflammatory cytokines.[8] This activation results in pyroptosis, a specific type of programmed cell death, occurring at the site of injury, thereby worsening tissue damage and inflammation.[9] Furthermore, previous research has shown NLRP3 activation following SCI in animal and clinical studies.[3,10]

The body's antioxidant mechanisms are unable to cope with the substantial surge in free radicals, resulting in the irremeable devastation of neuronal cells. This damage leads to neuronal death and tissue destruction.[11,12] Research indicates that preventing free radical production after SCI can preserve nerve cells from oxidative damage, potentially mitigating the pathological processes associated with SCI and promoting nerve repair and regeneration.[13,14] Pro-oxidants are often linked to the onset of various diseases, while antioxidants are typically employed to prevent or mitigate these conditions.[15,16]

Commonly found as a spice in the Indian kitchen and used in traditional Chinese medicine, curcumin (CuC) is a natural compound that constitutes 60 – 70% of the curcuminoids derived from the rhizomes of *Curcuma longa*.[17] CuC has been well-researched and is known for its diverse bioactive properties applied in traditional medicine.[18] It acts as a potent antioxidant through two primary mechanisms: first, by directly scavenging free radicals such as superoxide anions and hydroxyl radicals to prevent oxidative damage, and second, by enhancing cellular defenses against OS. This enhancement occurs through the reduction of lipid peroxidation, an increase in glutathione (GSH) levels, and the stimulation of key proteins such as superoxide dismutase (SOD) and catalase (CAT), which collectively contribute to its protective effects against OS in several pathological conditions beyond SCI.[3,19-22] Despite the aforementioned beneficial effects, the ingestion of high doses of CuC can lead to adverse gastrointestinal reactions, including stomach upset, nausea, gastroesophageal reflux disease, diarrhea, and dizziness.[23,24]

This current research aimed to investigate the antioxidant and anti-inflammatory impacts of CuC in a unilateral SCI model using male rats. Recognizing the detrimental roles of free radicals and inflammatory cytokines in SCI, we explored how different doses of CuC could modulate OS and inflammation. Specifically, we assessed the impacts of CuC on OS indicators and components of the NLRP3 inflammasome, which are critical in mediating inflammatory responses.

2. Materials and methods

2.1. Animals

A total of 40 adult male Wistar rats, each weighing 220 – 250 g, were procured from the animal room at the Tehran University of Medical Sciences (TUMS) in Tehran, Iran. These rats were housed under carefully controlled standard laboratory conditions, with a consistent ambient temperature maintained at 22 – 24°C. They were provided with unlimited access to both food and fresh water, ensuring their well-being throughout the study. The study protocol received approval from the Institutional Research Ethics Committee at TUMS (Approval No.: IR.TUMS.SPH.REC.1400.160), and the ethical treatment of animals was adhered to in the present study.

2.2. Study design

The animals were divided into separate experimental groups (n = 8 in each group) to facilitate comparative analyses of the treatment effects: (1) Control group, in which the animals underwent laminectomy without any induction of SCI, serving as a baseline for evaluating the effects of the treatments. (2) Model group, in which the animals underwent the unilateral SCI procedure and received a vehicle treatment, allowing researchers to assess the effects of SCI without any therapeutic intervention. (3) Model + CuC20, Model + CuC40, and Model + CuC80 groups, in which the animals that had been induced with unilateral SCI received CuC at 20, 40, and 80 mg/kg/day, administered through intraperitoneal injection, which is pivotal in evaluating the potential therapeutic effects of CuC on recovery following SCI. Each of these groups was carefully monitored throughout the study to assess behavioral, functional, and histological outcomes, contributing vital information to the understanding of SCI treatments and the role of CuC in neuroprotection and recovery.

2.3. Surgery procedure

The animals underwent anesthesia using a carefully calibrated mixture administered through intraperitoneal

injection consisting of 80 mg/kg of ketamine and 15 mg/kg of xylazine. This combination was selected for its efficacy in providing both sedation and analgesia, ensuring the animals remained unconscious and free from pain throughout the surgical procedure. Following anesthesia, the fur over the surgical area was shaved to ensure a sterile environment, minimizing the risk of infection. A laminectomy was then performed at the T8 – T9 vertebral levels using a precision tool known as a micro rongeur (Fine Science Tools, USA). This step involved removing a section of the vertebral bone to access the spinal cord directly. To induce SCI, an extradural clip was employed for compression, requiring the placement of an aneurysm clip on the right side of the spinal cord. This clip exerted a controlled closing force of 0.90 N for a duration of 1 min, simulating the impact of a traumatic injury. After the surgical procedure was completed, the incision site was meticulously suture-closed to promote healing and reduce the risk of complications during the recovery phase. This careful approach ensures both the integrity of the surgical site and the welfare of the animals involved in the study.

2.4. Motor behavior analysis

The progression of functional recovery in each animal was meticulously evaluated using the Basso, Beattie, and Bresnahan (BBB) test, a widely recognized and validated behavioral assessment tool for measuring locomotor function following SCI in rodent models. The BBB test scores range from 0 to 21, with higher scores indicating better locomotor function. Each rat was observed for specific behaviors such as movement, coordination, and weight-bearing ability during a series of standardized tasks, which included walking on a flat surface and navigating obstacles. Assessments began 24 h post-SCI and continued at 48 h and 72 h, with additional evaluations conducted weekly throughout the duration of the study (4 weeks).

2.5. Sample preparation

After the completion of the experimental procedures (after 4 weeks), the animals were humanely sacrificed in accordance with ethical guidelines to minimize suffering. This process typically involved administering an overdose of anesthetic, ensuring that the animals were unconscious and pain-free before euthanasia. Following sacrifice, spinal cord tissue and blood samples were collected for further analysis. The spinal cord tissues were carefully excised and stored in sterile containers, whereas blood samples were obtained through cardiac puncture, a method that allows for the collection of blood directly from the heart, ensuring high-quality samples. Once collected, the blood samples were centrifuged at 6000 rpm for 20 min to separate the plasma from the cellular components. The supernatants, which contain biochemical markers, were carefully gathered for subsequent assays. To preserve the integrity of the samples, both tissue and blood supernatants were stored at –80°C.

2.6. Measurement of OS

The present study focused on several key parameters, including malondialdehyde (MDA), GSH, SOD, and total antioxidant capacity (TAC). These parameters were measured using specific kits from ZellBio (Germany), and quantitation of OS and antioxidant levels in biological samples was conducted according to the manufacturer's protocols. The MDA assay (CAT No. ZB-MDA-96A) was used to assess lipid peroxidation, indicating oxidative damage. The GSH assay (CAT No. ZB-GSH-96A) measures the levels of this critical antioxidant, whereas the SOD assay (CAT No. ZB-SOD-96A) evaluates the activity of this enzyme that protects against OS. Finally, the TAC assay (CAT No. ZB-TAC-96A) provides a comprehensive measure of the overall antioxidant capacity of the samples. Following the manufacturer's instructions for each assay ensured accurate and reproducible results, contributing to the understanding of the biochemical changes associated with SCI and the potential therapeutic effects of the treatments administered.

2.7. Enzyme-linked immunosorbent assays (ELISA)

To measure the amounts of NLRP3, apoptosis-associated speck-like protein containing a CARD (ASC), and caspase 1 (Casp-1) proteins, ELISA assays were employed to analyze freshly extracted spinal cord tissue samples. Upon collection, the spinal cord tissues were carefully homogenized in a suitable lysis buffer to extract the proteins. Following homogenization, the samples were centrifuged to remove cellular debris, leaving a clear supernatant that contained the soluble proteins for analysis. ELISA kits for NLRP3 (MyBiosource, CA, United States), ASC (MyBiosource, CA, United States), and Casp-1 (Cusabio, Wuhan, China) that are specific for rats were used to measure the protein levels of these three proteins according to the manufacturers' protocol.

2.8. Statistical analysis

We used IBM® Statistical Package for the Social Sciences Statistics software (ver. 20) to analyze data, and the results are expressed as mean ± standard deviation. A one-way analysis of variance was conducted followed by Tukey's *post hoc* test to calculate the differences between groups. A $P < 0.05$ was considered to indicate statistical significance.

3. Results

3.1. Functional recovery

Our results showed that animals with SCI had significantly lower BBB scores relative to the Control group animals

($P < 0.05$). No meaningful difference in the BBB scores was reported between the Model+CuC20 animals and the Model group animals ($P > 0.05$). Model+CuC40 and Model+CuC80 animals demonstrated improvement in functional recovery compared to the Model group animals (Figure 1, $P < 0.0001$ for comparisons). These results indicate that higher doses of CuC may promote functional recovery in SCI, highlighting its potential therapeutic benefits.

3.2. Blood levels of OS indicators

3.2.1. MDA level

Our findings indicated a significant rise in the level of MDA in the Model group animals relative to the Control group animals ($P < 0.0001$). In contrast, a notable reduction in MDA concentrations was observed in the Model+CuC20 group animals relative to the Model group animals ($P = 0.01$). In addition, both the Model+CuC40 and Model+CuC80 groups exhibited significant reductions in MDA levels when compared to the Model animals (Figure 2, $P < 0.0001$ for both treatments). These results imply that CuC treatment may effectively reduce oxidative damage in SCI, contributing to improved outcomes in cellular health.

3.2.2. SOD activity

Our results also indicated a significant rise in SOD activity in the Model group animals compared to the Control group animals ($P < 0.0001$). No statistically significant differences in SOD activity were reported in the Model+CuC20 group relative to the Model group ($P > 0.05$). Notably, following the administration of 40 and 80 mg/kg of CuC, there was a significant modulation in SOD levels relative to the SCI animals (Figure 3, $P < 0.0001$ for both treatments). These findings suggest that the intervention not only mitigated OS but also likely enhanced overall cellular health.

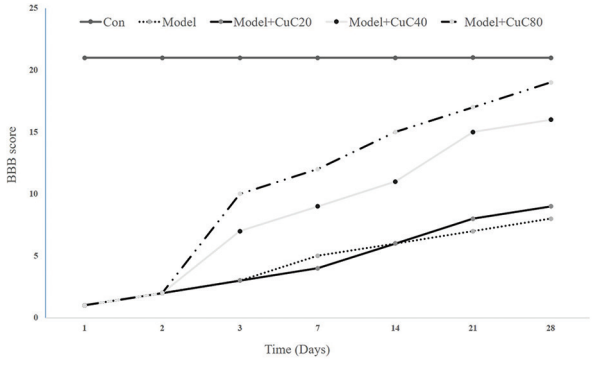

Figure 1. CuC effects on the BBB scores in experimental animals
Abbreviations: BBB score: Basso, Beattie, and Bresnahan score; Con: Control; CuC: Curcumin.

3.2.3. GSH activity

The activity of GSH was raised in the SCI-induced animals relative to the Control group animals ($P < 0.0001$). Mean GSH activity was similar between the Model+CuC20 and the Model groups ($P > 0.05$). Furthermore, both the Model+CuC40 and Model+CuC80 animals demonstrated a substantial increase in GSH activity relative to the Model group (Figure 4, $P < 0.0001$ for both treatments). These findings suggest that the intervention may have effectively alleviated OS and contributed to improved cellular health.

3.2.4. TAC level

In comparison to the Control group animals, TAC was diminished in the SCI animals ($P < 0.0001$). No significant differences in TAC levels were observed between the

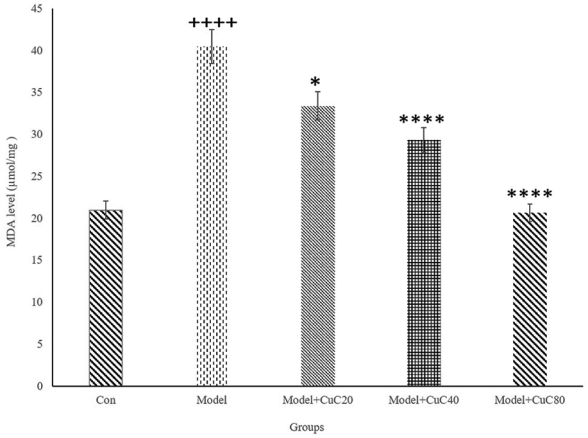

Figure 2. CuC effects on the blood concentration of MDA in the SCI-induced animals. $^{++++}P<0.0001$ compared to Control group; $^{*}P<0.05$, $^{****}P<0.0001$ compared to Model group.
Abbreviations: Con: Control; CuC: Curcumin; MDA: Malondialdehyde; SCI: Spinal cord injury.

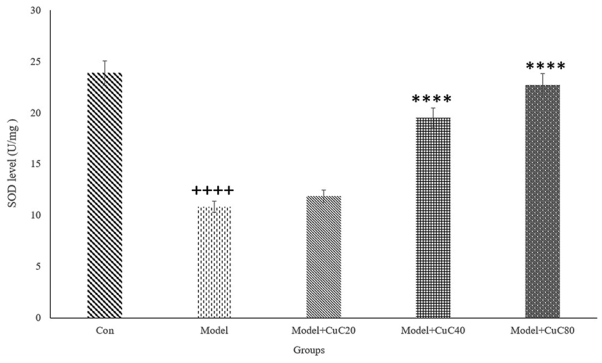

Figure 3. CuC effects on the blood concentration of SOD in the SCI-induced animals. $^{++++}P<0.0001$ compared to Control groups; $^{****}P<0.0001$ compared to Model group.
Abbreviations: Con: Control; CuC: Curcumin; SCI: Spinal cord injury; SOD: Superoxide dismutase.

Model+CuC20 and the Model groups ($P > 0.05$). However, both the Model+CuC40 and Model+CuC 80 groups demonstrated a significant elevation in TAC levels relative to the Model group (Figure 5, $P < 0.0001$ for both treatments). These results regarding TAC levels suggest that the intervention enhances the body's natural defense mechanisms, thereby improving resilience against oxidative damage.

3.3. Protein concentrations of inflammatory mediators

3.3.1. NLRP3

In comparison to the control animals, the protein concentrations of NLRP3 were significantly elevated in the tissue samples of Model group animals ($P < 0.0001$). No statistically significant difference was observed in the NLRP3 protein levels of the Model+CuC20 group animals relative to the Model group animals ($P > 0.05$). However, both the Model+CuC40 and Model+CuC80 groups demonstrated a prominent decrease in NLRP3 protein levels relative to the Model group (Figure 6, $P < 0.0001$ for both treatments).

3.3.2. ASC

Significant differences in the tissue concentration of ASC protein were observed between the Model groups and the Control group ($P < 0.0001$). In contrast, no meaningful differences in the ASC protein level were found between the Model+CuC20 and the Model groups ($P > 0.05$). Following treatment with 40 and 80 mg/kg of CuC, there was a significant reduction in the protein concentration of ASC relative to the Model group animals (Figure 7, $P < 0.0001$ for both treatments).

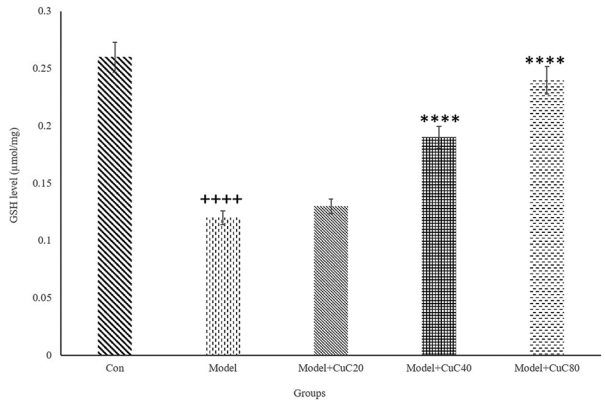

Figure 4. CuC effects on the blood concentration of GSH in the SCI-induced animals. $^{++++}P<0.0001$ compared to Control group; $^{****}P<0.0001$ compared to Model group.
Abbreviations: Con: Control; CuC: Curcumin; GSH: Glutathione; SCI: Spinal cord injury.

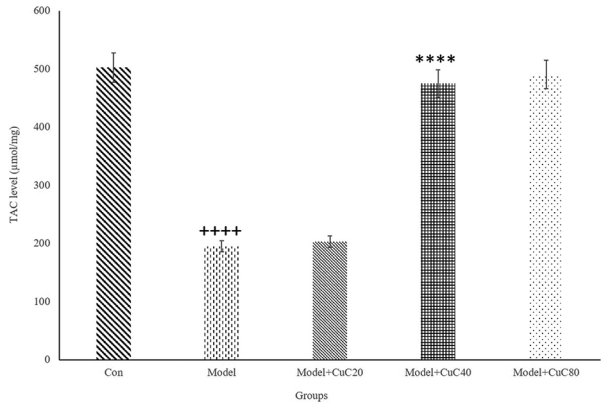

Figure 5. CuC effects on the blood concentration of TAC in the SCI-induced animals. $^{++++}P<0.0001$ compared to Control group; $^{****}P<0.0001$ compared to Model group.
Abbreviations: Con: Control; CuC: Curcumin; SCI: Spinal cord injury; TAC: Total antioxidant capacity.

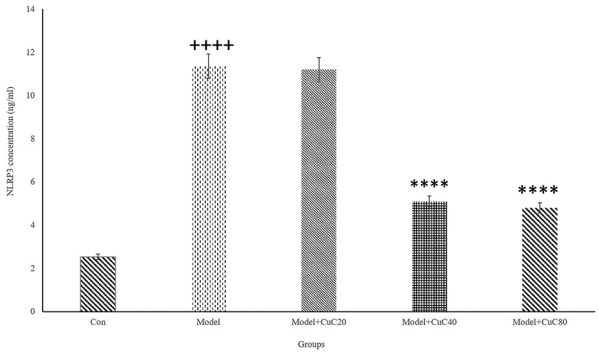

Figure 6. CuC effects on the protein concentration of NLRP3 in the SCI-induced animals. $^{++++}P<0.0001$ compared to Control group; $^{****}P<0.0001$ compared to Model group.
Abbreviations: Con: Control; CuC: Curcumin; NLRP3: NOD-like receptor family pyrin domain-containing 3; SCI: Spinal cord injury.

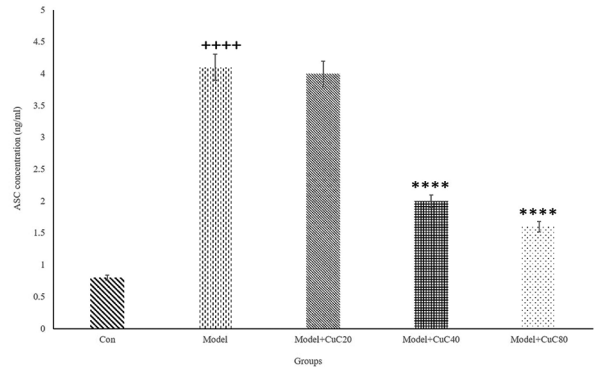

Figure 7. CuC effects on the protein concentration of ASC in the SCI-induced animals. $^{++++}P<0.0001$ compared to Control group; $^{****}P<0.0001$ compared to Model group.
Abbreviations: ASC: Apoptosis-associated speck-like protein containing a CARD; Con: Control; CuC: Curcumin; SCI: Spinal cord injury.

3.3.3. Casp-1

The outcomes demonstrated a prominent increase in the protein concentration of Casp-1 in the tissue samples of Model group animals relative to those of Control group animals ($P < 0.0001$). Meanwhile, no significant differences in the protein concentration of ASC were found between the Model+CuC20 and the Model groups ($P > 0.05$). Both Model+CuC40 and Model+CuC80 groups showed significant reductions in the protein concentration of Casp-1 when compared to the Model group (Figure 8, $P < 0.0001$ for both treatments).

4. Discussion

In this research, we investigated the antioxidant effects of varying doses of CuC in a SCI model utilizing male Wistar rats. Our findings revealed that SCI significantly elevates the levels of MDA, a well-established marker of OS, indicating increased lipid peroxidation and cellular damage. Concurrently, we observed a marked reduction in the body's antioxidant defenses, evidenced by decreased levels SOD, GSH, and TAC. These results underscore the oxidative imbalance that occurs following SCI, highlighting the detrimental impact of OS on recovery. Furthermore, our study revealed that SCI led to the upregulation of the NLRP3 inflammasome, characterized by increased protein levels of NLRP3, ASC, and Casp-1. This upregulation reflects an enhanced neuroinflammatory response following SCI, contributing to the overall pathological process.

SCI poses a significant global health challenge, with thousands of new cases diagnosed annually, frequently resulting in profound and debilitating effects on individuals' quality of life.[25] The intricate pathophysiological mechanisms that underlie SCI, especially the complex interactions between primary and secondary injury processes, are not yet fully understood. This knowledge gap presents considerable obstacles to the development of effective therapeutic strategies aimed at improving patient outcomes.[26,27] While the initial trauma and primary injury mechanisms are often unpredictable, there is an urgent need for targeted interventions that can mitigate the detrimental effects of secondary injury, particularly those related to OS and neuroinflammation.[28] Pre-clinical research has provided compelling evidence for the role of the NLRP3 inflammasome in the pathogenesis of SCI. The NLRP3 inflammasome is formed by the assembly of NLRP3, ASC, and Casp-1 in response to both external infections and internal signaling stimuli. This assembly leads to the activation of Casp-1, resulting in the release of pro-inflammatory cytokines such as interleukin (IL)-1β and IL-18.[29] Moreover, prior studies have demonstrated that inhibiting NLRP3 inflammasome activity can effectively reduce neuroinflammation and enhance functional recovery in animal models of SCI.[30] While the initial trauma and primary injury mechanisms are often unpredictable, our research emphasizes the urgent need for targeted interventions that can mitigate the detrimental effects of secondary injury, particularly those related to OS and neuroinflammation. These findings suggest that targeting the NLRP3 inflammasome may represent a promising therapeutic approach to mitigate the adverse effects of neuroinflammation and improve recovery following spinal cord injuries.

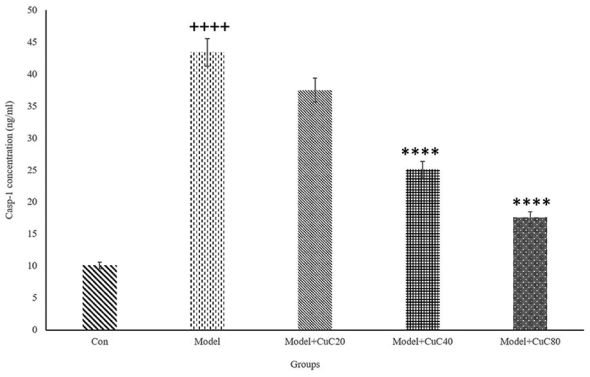

Figure 8. CuC effects on the protein concentration of Casp-1 in the SCI-induced animals. $^{++++}P<0.001$ compared to Control group; $^{****}P<0.001$ compared to Model group.
Abbreviations: Casp-1: Caspase 1; Con: Control; CuC: Curcumin; SCI: Spinal cord injury.

In this study, SCI was induced in rats by applying an aneurysm clip to the right side of the spinal cord for a duration of 1 min. In the treatment groups, CuC was administered through intraperitoneal injection at three different doses: 20, 40, and 80 mg/kg. These doses were selected to evaluate the dose-dependent effects of CuC on recovery and neuroprotection following the SCI induced. The findings of this study demonstrated that by induction of SCI, the MDA level was increased, and the SOD and GSH activities and TAC levels were decreased in the animals. Consistent with our findings, Kim *et al.*[31] demonstrated that following the SCI, the amount of oxidant parameters was increased, and the levels of antioxidant parameters were decreased.

CuC ($C_{21}H_{20}O_6$) is a polyphenol extracted from turmeric, a common spice and traditional Chinese medicine. CuC exhibits promising effects against inflammation, OS, apoptosis, and neurodegeneration.[22] Our findings revealed that CuC-treated groups exhibited a significant reduction in the level of MDA relative to the untreated SCI animals. Model+CuC40 and Model+CuC80 animals demonstrated a significant increase in the levels of SOD, GSH, and TAC in comparison to the SCI model animals. Gao *et al.*[32] showed

that CuC treatment can decrease the levels of MDA, induce the activation of GSH peroxidase, and ameliorate OS in rat models. Dong *et al.*[33] investigated the effects of CuC in a mouse wound model by administering it into the abdominal cavity. Their findings indicated that CuC exhibited a protective effect on nerve tissue. This effect was related to the upregulation of nuclear factor erythroid 2-related factor 2 (Nrf2), a key regulator of the antioxidant response. The study also noted the upregulation of downstream antioxidant enzymes, suggesting that CuC may enhance the body's ability to combat OS and promote nerve protection and recovery following injury. In 2018, Caillaud *et al.*[34] conducted research to explore the impact of continuous topical treatment with low doses of CuC on nerve function and regeneration after sciatic nerve injury in mice. These findings indicate that CuC has a protective effect against OS by reducing the secretion of ROS generated by macrophages. In addition, it lowers lipid peroxidation levels and boosts the expression of the transcription factor Nrf2, which plays a crucial role in regulating antioxidant responses. Furthermore, applying low doses of CuC locally shows potential as an effective treatment for peripheral nerve regeneration.[34] These findings are consistent with the results of our current research.

In addition to the assessment of OS parameters, functional recovery was evaluated in the present research. The BBB test outcomes showed a notable reduction in scores of BBB for animals with SCI in comparison to the Control group animals. Among the animals treated with 40 and 80 mg/kg CuC, we observed a meaningful elevation in the BBB scores relative to the untreated SCI animals. Kim *et al.*[31] assessed the effects of CuC on the development of SCI in a rat model. They found that CuC (200 mg/kg) significantly improved functional recovery in the early stages following SCI. Furthermore, they presented that CuC decreased the MDA levels, increased the activity of SOD, and reduced the inflammation. The results of this study are in agreement with the findings of Kim *et al.*'s research.[31] The findings of the mentioned studies are in line with the outcomes of the present study. Research conducted by Alvarado-Sanchez *et al.*[35] demonstrated that CuC effectively reduced concentrations of hydroxyl radicals, nitric oxide, and lipid peroxidation following SCI, although it did not significantly affect SOD activity. Our results align with these findings; however, we observed significant effects of CuC on SOD activity, which contrast with the outcomes reported by Alvarado-Sanchez *et al.*[35] This discrepancy may be attributed to the timing of the OS parameter measurements. In our study, we assessed the levels of OS parameters 4 weeks post-SCI and treatment, whereas their study evaluated these parameters just 24 h after SCI.

We also investigated the protein concentrations of inflammatory mediators, specifically ASC, NLRP3, and Casp-1, at the injury site. Our findings revealed a significant increase in these markers after SCI. The results also showed a dramatic reduction in the concentrations of NLRP3, ASC, and Casp1 proteins in the animals receiving 40 and 80 mg/kg of CuC, in comparison to the Model group animals. This suggests a noteworthy decrease in the levels of these inflammatory proteins. Prior studies have demonstrated that CuC can substantially enhance recovery following SCI by fostering the development of new nerve cells (neurogenesis) and modulating inflammatory pathways. For example, research conducted by Lee *et al.* revealed that administering CuC orally at a dosage of 0.4 mg/kg for 2 weeks led to an increase in neurogenesis by affecting brain-derived neurotrophic factor concentrations in the hippocampus, a crucial area of the brain involved in learning and memory.[36] The results of another study revealed that administering a low dose of CuC (exceeding 0.2 mg/kg) resulted in an increase in the generation of new cells within the hippocampus, thereby enhancing neurogenesis in adult mice.[37] Barati *et al.*[38] investigated the CuC effects on rats with SCI. They found that CuC might help improve movement in the early stages of the injury by promoting nerve repair and reducing the inflammation caused by astrocyte activity. The results of the mentioned studies were in accordance with the results of this study related to the inflammasome complex.

Numerous studies have consistently shown that CuC effectively mitigates OS. This protective effect is achieved through multiple mechanisms including: (1) reducing lipid peroxidation products such as MDA, 4-hydroxynonenal, and protein carbonyls, (2) enhancing the activity of GSH peroxidase and SOD, which are essential antioxidant enzymes, and (3) activating the Nrf2, a crucial regulator of the body's antioxidant response.[39-41] Despite the potential health benefits CuC can offer, taking large amounts can lead to unpleasant side effects such as stomach upset, nausea, dizziness, gastroesophageal reflux disease, and diarrhea, and our findings proved that consuming CuC at moderate dose could mitigate SCI development.

5. Conclusion

This study found that moderate and high doses of CuC effectively reduced OS and inflammation caused by SCI. Since a marked decrease was not noted between animals receiving varying CuC doses, a moderate dose of CuC is recommended.

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Conflict of interest

The authors declare that they have no competing interests.

Author contributions

Conceptualization: Gholamreza Hassanzadeh
Formal analysis: Babak Ebrahimi
Investigation: Babak Ebrahimi, Gholamreza Hassanzadeh
Methodology: Babak Ebrahimi, Atousa Yarahmadi, Neda Ghaffari
Writing–original draft: Atousa Yarahmadi, Neda Ghaffari
Writing–review & editing: Gholamreza Hassanzadeh

Ethics approval and consent to participate

This research project was approved by the Ethical Committee of Tehran University of Medical Sciences (IR.TUMS.SPH. REC.1400.160).

Consent for publication

Not applicable.

Availability of data

Data are available from the corresponding author upon reasonable request.

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ACCSCIENCE PUBLISHING

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MINI-REVIEW

Transformative natural product-drug combinations: Advancing techniques to enhance efficacy against drug-resistant pathogens

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Abstract

This review explores the development, modification, and optimization of potent active compounds using combinatorial synthetic methods that incorporate phytoconstituents from plant extracts, aiming to enhance efficacy and mitigate resistance. Attenuated total reflectance-Fourier transform infrared spectroscopy, gas chromatography-mass spectrometry (GC-MS), and antibacterial data from the literature were employed to validate these strategies. The methodology emphasized precise harvesting, pre-treatment, and extraction processes to ensure the quality and efficacy of the plant extracts. Various extraction methods and solvents were used to isolate specific phytoconstituents, followed by further purification through chromatography. The review proposes three strategies: (i) reacting single or multiple plant extracts with reagents such as acids or catalysts, (ii) combining plant extracts with ineffective drugs to induce structural changes that enhance antibacterial efficacy, and (iii) integrating plant extracts with drugs not originally intended for the target disease to explore new structural functionalities. Significant findings include synergistic effects observed when *Psidium guajava* and *Calotropis procera* extracts were combined with antibiotics, leading to substantially increased zones of inhibition against resistant bacteria. GC-MS analysis identified numerous bioactive compounds, some of which with known anticancer properties, suggesting potential applications beyond antibacterial effects. These innovative combinatorial approaches demonstrate the potential to yield new compounds with enhanced pharmacological properties, highlighting the critical role of plant extracts in drug discovery and development. This review underscores the promise of harnessing natural products to combat multi-drug resistance, paving the way for advanced research and development in pharmaceutical applications.

Keywords: Combinatorial synthetic methods; Phytoconstituents; Drug resistance; Plant extracts; Antimicrobial activity; Synergistic effects; Drug discovery and development

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1. Introduction

Drug resistance represents a pressing global challenge that impacts clinical, community, and environmental health, as well as food security and development. The emergence

of antimicrobial resistance (AMR) complicates treatment efficacy by enabling pathogens to evolve mechanisms that resist drug therapy, leading to persistent infections and increased transmission rates. According to Cesur and Demiröz[1] and Olga *et al*.,[2] this phenomenon not only endangers health outcomes but also amplifies the burden on healthcare systems worldwide. The World Health Organization[3] underscores the severity of the issue, reporting that AMR causes approximately 700,000 deaths annually. Projections indicate that this figure could escalate to 10 million deaths per year by 2050 if effective interventions are not implemented. Several interrelated factors, such as overuse and misuse of antibiotics in medicine, agriculture, and animal husbandry, drive the escalation of AMR. Practices such as over-prescription, self-medication, and the use of antibiotics as growth promoters in livestock exacerbate the problem.[4] The pipeline for new antibiotics is worryingly sparse due to significant financial and scientific barriers faced by pharmaceutical companies.[5] Moreover, environmental pollution and climate change contribute to the spread and mutation of resistant pathogens, further complicating efforts to control AMR.[4,6]

1.1. Addressing AMR with medicinal plant compounds

The production of bioactive compounds from medicinal plants necessitates the use of sophisticated techniques to ensure scalability and efficiency. Direct extraction can be impractical, prompting the need for semi-synthesis, total synthesis, and biotechnological approaches as viable alternatives. Semi-synthesis involves modifying natural compounds to enhance their efficacy or ease of production. A notable example is the production of the critical anticancer drug paclitaxel (Taxol). Advances in synthetic chemistry have enabled the development of total synthesis routes for compounds like paclitaxel and its derivatives, improving availability and reducing the costs associated with natural extraction methods.[7,8] Biotechnological methods, including genetic engineering and microbial fermentation, have revolutionized the production of complex natural products. For instance, genes responsible for paclitaxel biosynthesis have been successfully integrated into microorganisms such as *Escherichia coli* and *Saccharomyces cerevisiae*, enabling these microbes to produce paclitaxel precursors.[7] In addition, plant cell culture techniques have been employed to cultivate paclitaxel from *Taxus* cell cultures.[8,9] The semi-synthesis of bioactive terpenoids, steroids, and polyketides from uncultivated bacterial symbionts demonstrates the potential of these approaches.[10,11] Biotechnological advancements, including plant tissue culture and microbial fermentation, highlight the feasibility of producing these compounds at scale.[12] Genetic engineering has also been utilized to produce complex natural products like artemisinin through yeast, addressing challenges associated with traditional extraction methods from *Artemisia annua*.[11]

1.2. The role of medicinal plants in combating AMR

Medicinal plants are rich sources of diverse phytochemicals, the composition of which varies depending on the plant species, its geographical location, and the extraction solvent used.[13-16] Studies by Khosravi,[17] Alzohairy,[18] and Jafarnejad *et al.*[19] suggest that aqueous plant extracts may effectively treat bacterial infections, sometimes showing comparable or even superior efficacy to certain antibiotics while potentially reducing the development of bacterial resistance. Drug repurposing also offers a promising strategy in the fight against drug-resistant pathogens. This approach involves using known compounds or existing drugs to treat recalcitrant infections and diseases.[20] Moreover, two potent bioactive compounds with diverse pharmacological activities present new opportunities for treating resistant infections. For example, the triple synergistic inhibitory activities of riboprine and forodesine against the enzymes RNA-dependent RNA polymerase, exonuclease, and adenosine kinase in the replication of severe acute respiratory syndrome coronavirus 2.[21,22]

This review explores strategies to develop, modify, or optimize potent active compounds using a combinatorial synthetic method that utilizes phytoconstituents present in plant extracts. The aim is to enhance their efficacy and reduce the potential for pathogen resistance to existing or new compounds. Evidence from attenuated total reflectance-Fourier transform infrared spectroscopy (ATR-FTIR), gas chromatography-mass spectrometry (GC-MS), and antibacterial studies in the literature is presented to support these approaches.

2. Development and testing of antimicrobial strategies incorporating medicinal plant

Before plant extracts can be utilized, a meticulous process involving harvesting, pre-treatment, and various extraction methods must be followed to ensure the quality and efficacy of the final product. The initial step, harvesting, must be timed appropriately to capture the peak concentration of desired phytochemicals. Pre-treatment processes such as drying, grinding, and sometimes fermentation prepare the plant material for extraction, thereby enhancing the efficiency and yield of the subsequent steps. Different extraction methods, such as maceration, infusion, decoction, and modern techniques like supercritical fluid

extraction, are selected based on the chemical nature of the target compounds and their intended applications. The choice of solvent – whether organic, inorganic, or a combination of solvents – plays a critical role in selectively isolating specific phytoconstituents. Once extracted, these compounds often require further purification through techniques like chromatography to isolate the compound of interest, ensuring that the final product is potent and suitable for its intended use. This comprehensive approach ensures that plant extracts used in various applications, from pharmaceuticals to nutraceuticals, are both effective and safe.[23-25]

2.1. Strategy 1

A single plant extract or a combination of plant extracts can undergo chemical reactions by adding reagents such as organic or inorganic acids, alkalis, or catalysts while modifying the reaction conditions (e.g., temperature, pressure). A study by Gideon[26] exemplified this synergistic strategy by combining plant extracts with antibiotics to combat AMR. In the experiment, 0.4 mL of concentrated sulfuric acid was added to 10 mL of aqueous *Psidium guajava* (guava) leaf extract. The mixture was boiled at 100–110°C for 30 min, followed by centrifugation. The antimicrobial analysis revealed that untreated guava extract (at 5.0 µg/mL) had no inhibition zones against *Salmonella* spp., *E. coli*, *Streptococcus* spp., and *Staphylococcus aureus*. However, the 0.4 mL acid-treated guava extract (at 0.1 µg/mL) showed inhibition zones for all four bacterial strains, ranging from 7 mm to 12 mm, as depicted in Figure 1. The clear zones of inhibition in Figure 1 were primarily observed in the acid-treated plant extract combined with the antibiotic.

ATR-FTIR analysis, as depicted in Figure 2 and Table 1, revealed significant changes in the peaks present in each sample. The unreacted extract displayed 14 peaks, while the reacted extract showed 10 peaks. Shift in peaks, the formation of new peaks, and a decrease in transmittance were observed. A decrease in transmittance is attributed to higher concentrations of compounds with certain functional groups, while the emergence of new peaks may be due to the formation of new or analogous compounds.[26] On centrifugation and drying at room temperature, the unreacted guava extract yielded a total mass of 13.7 mg, whereas the reacted guava extract yielded 177.2 mg, which is 1,293.4 times heavier than the unreacted guava extract. This significant difference in mass is attributed to the formation of larger and more complex molecules, which typically have higher molar masses.[27]

Further exploration of this approach could involve isolating specific phytoconstituents and then initiating reactions between non-bioactive constituents of one plant extract and bioactive constituents of another, or between different solvent extracts (e.g., chloroform or n-hexane) from various plants. Multiple combinatorial reactions can be designed and executed based on expected outcomes.

Plant extracts also play a key role as capping and stabilizing agents in nanoparticle synthesis.[28] Saddaf *et al.*[29] synthesized silver nanoparticles (AgNPs) using aqueous root extracts from four medicinal plants and tested their antimicrobial efficacy on six different bacteria strains.

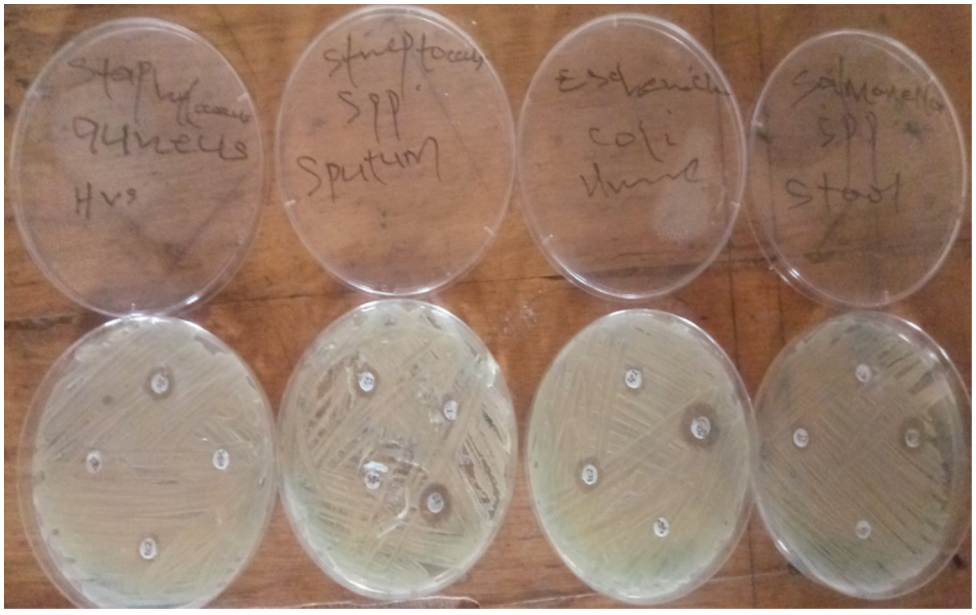

Figure 1. Antibacterial activities of the prepared samples against resistant bacteria. Image reproduced with permission from Gideon.[26]

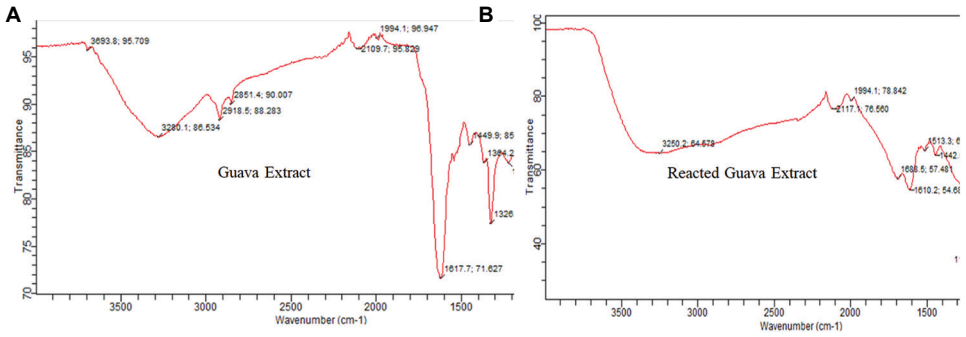

Figure 2. Attenuated total reflectance-Fourier transform infrared spectroscopy spectra of guava extract (A) and reacted guava extract (B). Image reproduced with permission from Gideon.[26]

Table 1. Fourier transform infrared spectroscopy analysis of guava extract and reacted guava extract, including attributed functional groups. Reproduced with permission from Gideon[26]

FTIR functional group region	Wavenumber range (cm^{-1})	Guava extract peaks (cm^{-1})	Reacted guava peaks (cm^{-1})	Associated phytochemicals
O-H/N-H Stretch	3,200–3,600	3,693.8; 3,280.1	3,250.2	Alcohols, phenols, amines, or carboxylic acids
C-H Stretch	2,850–3,000	2,918.5; 2,851.4	-	Alkanes
Clkanes Stretch	2,100–2,260	2,109.7; 1,994.1	2,117.1; 1,994.1	Alkynes or nitriles
C=O Stretch	1,630–1,820	1,617.7	1,688.5; 1,610.2	Ketones, aldehydes, esters, or carboxylic acids
N-H Bending	1,500–1,600	-	1,513.3	Primary and secondary amines
C-H Bending	1,400–1,500	1,449.9	1,442.5	Aromatic compounds

Abbreviation: FTIR: Fourier transform infrared spectroscopy.

Saddaf *et al.*'s results showed that the root extracts of *Bergenia ciliata* and *Bergenia stracheyi* (at 1200 µg/well) and their synthesized AgNPs (at 150 µg/well) did not inhibit the growth of *E. coli*, *Staphylococcus haemolyticus*, or *Bacillus cereus*. In contrast, the root extracts of *Rumex dentatus* and *Rumex hastatus* (at 1200 µg/well) were unable to inhibit these bacteria, but their synthesized AgNPs (at 150 µg/well) successfully inhibited bacterial growth. According to Saddaf *et al.*[29] (p321), "the antibacterial activity of each type of AgNPs depends on the surface modification by plant extract, which makes them effective against specific bacteria." In response to this statement, it can be argued that if the modification occurred on AgNPs themselves, rather than on the plant extract, lower levels of inhibition would have been observed in *B. ciliata* and *B. stracheyi* due to the contributory effect of silver's inherent antimicrobial activity. Instead, it is the modification of the phytoconstituents in these aqueous root extracts that promotes antimicrobial functionality, as demonstrated by Gideon.[26]

2.2. Strategy 2

This strategy involves initiating a reaction between a single plant extract and an ineffective drug, known for treating the disease of interest. The process includes adding a reagent – such as an inorganic/organic acid, alkali, or catalyst – and modifying the reaction conditions. For antibacterial applications, multiple plant extracts can be reacted with different inexpensive antibiotics. The antibiotic must be confirmed to be ineffective against the target bacteria, as using an antibiotic that is fully effective against the bacteria of interest could reduce the effectiveness of the final product if structural changes are made. For example, Gideon and Ladan[30] achieved consistent synergistic effects preparing a mixture of (i) 1 mg/mL amoxicillin with aqueous leaf extract of *Calotropis procera*; (ii) 1 mg/mL ampicillin with aqueous leaf extract of *C. procera*; (iii) 100 µg/mL azithromycin with aqueous leaf extract of *C. procera*; and (iv) 100 µg/mL ampicillin with aqueous leaf extract of *C. procera*, and reacting each with concentrated sulfuric acid at 110°C for 20 min. Greater zones of inhibition were observed, as shown in Figure 3. The clear zones of inhibitions in Figure 2 predominantly reflect the acid-treated plant extract in combination with the antibiotics. This method increased the synergism of the plant extract-antibiotic combinations, enhancing their effectiveness from 33% (as reported by Eze *et al.*,[31] Moussaoui and Alaoui,[32] and Haq *et al.*[33]) to 100% at 1 mg/mL.

In a follow-up study by Gideon *et al.*,[34] GC-MS analysis of combination (i) from Gideon and Ladan's study[30] was

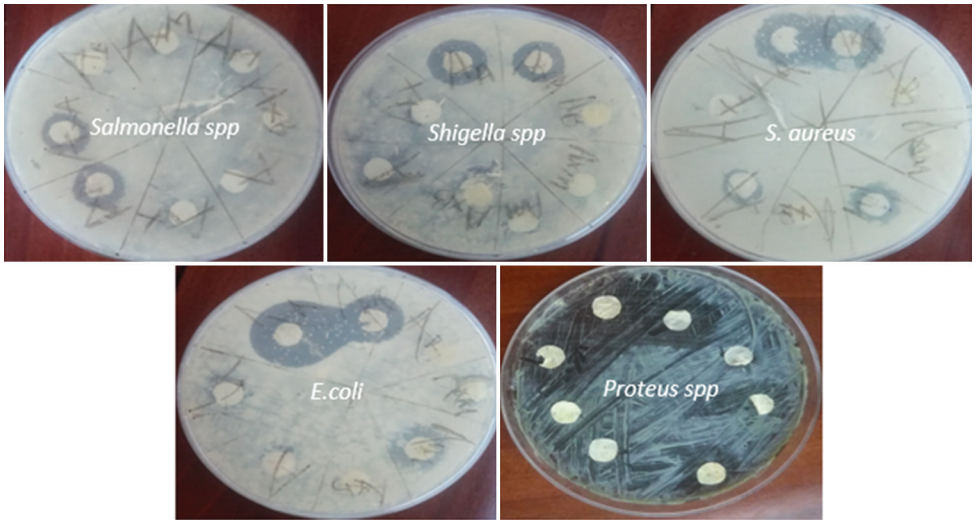

Figure 3. Antibacterial activities of prepared samples against resistant *Salmonella* spp., *Shigella* spp., *Staphylococcus aureus*, *Escherichia coli*, and *Proteus* spp. Image reproduced with permission from Gideon and Ladan.[30]

carried out to investigate the transformations in both the phytoconstituents and the antibiotic that contributed to the observed synergistic activity. The clear inhibition zones in Figure 3 primarily represent the acid-treated plant extract combined with the antibiotics.

The GC-MS analysis revealed 53 phytoconstituents, of which 18 were known for their biological activity, as shown in Table 2. Three of these constituents – farnesol,[35] 4-amino-1-pentanol,[36] and an imidazole derivative resembling the drug ribavirin[37,38] – were reported to have anticancer properties.

Further exploration can involve reactions between non-bioactive constituents from different plant extracts, the bioactive constituents of one plant combined with the non-bioactive constituents of another, chloroform extracts of various plants, or isolated compounds with n-hexane extracts. Multiple combinatorial reactions can be designed and carried out based on predicted outcomes.

2.3. Strategy 3

This strategy involves initiating a reaction between a plant extract and a drug not originally intended for treating the disease of interest, or any reagent suspected to possess good structural functionality that might enhance antimicrobial activities. Various methods can be employed while modifying the reaction conditions.

Gideon *et al.*[39] developed a regimen by making 24 different combinations of guava leaves and clove aqueous extracts with aspirin, tetracycline, and co-trimoxazole, each underwent different reaction stages. Antimicrobial susceptibility tests revealed that a single-stage combination of co-trimoxazole with clove extract (Scl), a two-stage combination of aspirin and clove extract (Ac2), and a two-stage combination of aspirin and guava extract (Ag2) were all effective against the resistant isolates of *Salmonella* spp.

The antibacterial activity optimization of aqueous leaf extract of *P. guajava* against resistant clinical isolates of *S. aureus*, *E. coli*, *Streptococcus* spp., and *Salmonella* spp. was carried out by Gideon.[26] He added 4 mL of guava extract to 4 mL of aspirin solution, boiled the mixture in a water bath, then added 0.4 mL of sodium hydroxide and continued boil for 5 min. A fresh 2 mL portion of guava extract was added, followed by 0.3 mL of sulfuric acid, and the mixture was boiled for another 10 min before centrifugation. The antimicrobial activity is shown in Figure 4.

The ATR-FTIR analysis revealed significant findings regarding the interaction between aspirin and *P. guajava*. From the ATR-FTIR results depicted in Figure 5 and Table 3, new peaks emerged in the combined product, including 3,235.3 cm^{-1} (O-H stretching from guava extract), 2,918.5 cm^{-1} (C-H stretching, alkanes from guava extract), 2,105.9 cm^{-1} (C5. stretching, alkynes from aspirin solution), 1,455.7 cm-1 (C-H bending, alkanes from guava extract), 1,748.1 cm^{-1} (C=O stretching, esters from aspirin solution), 1,677.3 cm^{-1} (C=C stretching, alkenes from aspirin solution), 1,602.8 cm^{-1} (C=C stretching, aromatics from aspirin solution), and 1,453.7 cm^{-1} (C-H bending, alkanes from aspirin solution). These changes in peak positions and transmittance levels suggest significant structural modifications, with reduced transmittance indicating higher concentrations of compounds with specific functional groups.[26] The combined presence of peaks from both guava extract and aspirin solution

Table 2. Bioactive phytoconstituents from *Calotropis procera* leaf extract combined with ampicillin. Reproduced with permission from Gideon *et al.*[34]

Pk#	RT	Area%	Compound name	Molecular weight
1	5.318	3.35	Cystamine	152.0
3	5.528	2.46	4-amino-1-Pentanol	103.0
5	5.959	1.42	N-methoxy-1-ribofuranosyl-4-imidazolecarboxylic amide	155.0
6	6.275	0.73	1,2,5-oxadiazol-3-carboxamide, 4,4'-azobis-, 2,2'-dioxide	284.0
9	7.361	1.94	3,3-dimethyl-4-(1-aminoethyl)-Azetidin-2-one	142.0
11	7.891	1.37	5-methyl-2-Heptanamine	128.0
20	12.065	1.36	dl-phenylephrine	167.0
23	14.598	1.16	Cystine	240.0
32	21.074	2.03	1,2,5-oxadiazol-3-carboxamide, 4,4'-azobis-, 2,2'-dioxide	284.0
34	22.127	0.73	Metaraminol	167.0
38	25.796	2.83	3-propoxyamphetamine	193.0
43	29.445	0.12	Hexadecanoic acid, methyl ester	270.0
49	30.437	2.68	1-docosene	308
50	31.083	0.16	9-octadecenoic acid (Z)-, methyl ester	296.0
52	36.087	0.78	3,7,11-trimethyl-2,6,10-dodecatrien-1-ol	222.0
53	36.620	13.04	Oleic acid	264.0

Abbreviations: Pk#: Number of peaks; RT: Retention time.

Table 3. Fourier transform infrared spectroscopy analysis of guava extract, aspirin, and the guava-aspirin mixture, with attributed functional groups. Reproduced with permission from Gideon[26]

FTIR functional group region	Wave number range (cm^{-1})	Guava extract peaks (cm^{-1})	Aspirin peaks (cm^{-1})	Reacted guava peaks (cm^{-1})	Associated phytochemicals
O-H/N-H stretch	3,200 – 3,600	3,693.8; 3,280.1	3,488.8	3,235.3	Alcohols, phenols, amines, or carboxylic acids
C-H stretch	2,850 – 3,000	2,918.5; 2,851.4	2,870.1; 2,959.5; 2,698.6; 2,586.8; 2,545.8	2,918.5	Alkanes
Clkanes stretch	2,100 – 2,260	2,109.7; 1,994.1	2,091.0	2,105.9	Alkynes or nitriles
C=O stretch	1,630 – 1,820	1,617.7	1,804.0; 1,837.6; 1,751.8; 1,684.3; 1,602.8	1,748.1, 1,677.3, 1,602.8	Ketones, aldehydes, esters, or carboxylic acids
N-H bending	1,500 – 1,600	-	-	-	Primary and secondary amines
C-H bending	1,400 – 1,500	1449.9	1483.5; 1457.4	1453.7	Aromatic compounds

Abbreviation: FTIR: Fourier transform infrared spectroscopy.

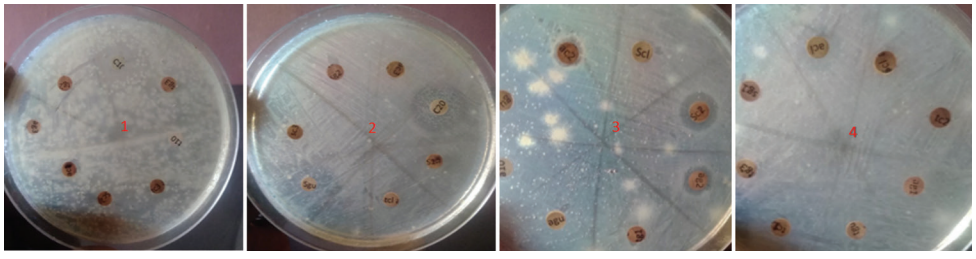

Figure 4. Antimicrobial susceptibility test for 31 prepared disks against *Salmonella spp*. Image reproduced with permission from Gideon *et al.*[39]

confirmed the formation of a new compound with modified structural characteristics, suggesting that the combination of aspirin with guava extract may lead to a novel product with enhanced antibacterial properties. This

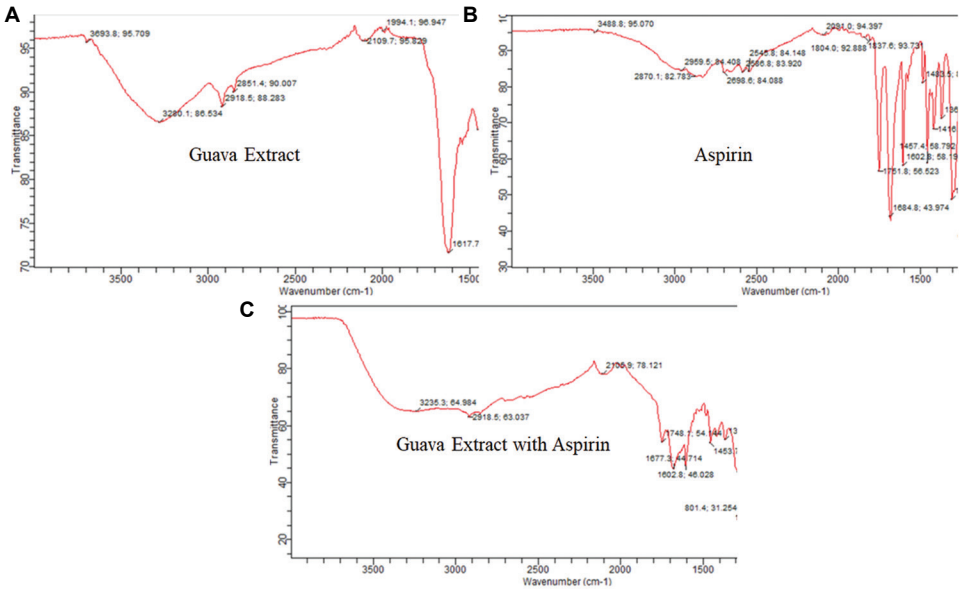

Figure 5. Attenuated total reflectance-Fourier transform infrared spectroscopy spectra of guava extract (A), aspirin (B), and guava-aspirin mixture (C). Image reproduced with permission from Gideon.[26]

method can be further explored by reacting non-bioactive constituents of one plant extract with those of another, bioactive constituents of one plant with non-bioactive constituents of another, or using different solvents and isolated compounds. Various combinatorial reactions can be designed to achieve the desired outcomes.

3. Conclusion

This review outlines strategies for leveraging plant extracts in the fight against drug resistance. Functional group analysis, GC-MS data, and antimicrobial screenings from various sources demonstrate that combining plant extracts with drugs or other structurally functional reagents can induce significant structural and functional changes, resulting in the formation of intermediate or new compounds. These compounds may act individually or synergistically to combat multi-drug-resistant bacteria. For instance, the GC-MS analysis identified potent anticancer properties, suggesting that these combinations may also offer therapeutic potential in antifungal, anti-inflammatory, antiviral, and anticancer applications, potentially yielding positive outcomes similar to the observed antibacterial effects. Therefore, this approach holds significant promise and warrants further exploration using advanced synthetic methods and tools to optimize drug discovery and development. By harnessing the potential of plant extracts and employing sophisticated techniques, we can develop novel treatments to effectively address the growing challenge of drug resistance.

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CASE SERIES

Evaluation of galactomannan and 1,3-β-d-glucan assays as diagnostic tools for intracranial fungal infection: A case series

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Abstract

Fungal infections of the central nervous system have dramatically surged over the past decade, particularly in semitropical regions such as Karachi. Herein, we retrospectively evaluated the diagnostic potential of galactomannan (GM) and 1,3-β-d-glucan (BDG) assays for intracranial fungal infections (IFI) at a tertiary care facility in Karachi. A total of 12 patients (3 immunocompromised and 9 immunocompetent) aged 18 – 60 years underwent serum fungal biomarker testing, imaging studies, and cerebrospinal fluid (CSF) analysis. Suboptimal GM and high BDG titers indicated invasive mycoses. Computed tomography scan revealed fungal sinusitis, and magnetic resonance imaging revealed brain parenchyma involvement. Fungal biomarkers helped rule out a neoplastic etiology. Normal GM and high BDG titers negated the probability of aspergillosis. Further, culture tests helped identify the causative organisms and tailor the treatment. Our findings emphasize the diagnostic value of GM and BDG assays in IFIs. Furthermore, we recommend the use of CSF specimens for fungal biomarker assays in future diagnostic protocols.

Keywords: Intracranial fungal infection; Galactomannan; Fungal sinusitis; d-glucan; 1,3-β-d-glucan

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1. Background

The incidence of fungal infections in the central nervous system (CNS) has increased over the past decade and is primarily attributed to the growing population of immunocompromised individuals. The fungal species predominantly responsible for these infections include yeasts (e.g., *Candida* spp.), molds, and filamentous fungi (e.g., *Aspergillus* spp.).[1] *Candida albicans*, which typically spreads through the hematogenous route, is a common cause of CNS infections that often present as meningitis. In addition, it sometimes manifests as chronic meningitis. It can lead to brain abscesses, spinal infections, ventriculitis, or mycotic aneurysms and vasculitis, ultimately leading to cerebral infarctions.[2] The underlying mechanism for *Candida* meningitis is the invasion of fungi through the blood–brain barrier (BBB).[3]

Aspergillus fumigatus and *Aspergillus flavus* are strongly associated with invasive aspergillosis (IA) and fungal sinusitis, respectively.[4] *Aspergillus* infections are primarily transmitted through spores, and the lungs, head, and neck – particularly the paranasal

sinuses (PNSs), orbit, and cranial areas – are the primary disease sites.[5] Disease progression in these regions is accompanied by pain, fever, ophthalmic complications, epistaxis, and cough.[6] The symptoms progress gradually and often include proptosis, monocular blindness, congestion, classic meningitis symptoms, and hemorrhage.[7] Owing to its angioinvasive properties, *Aspergillus* can affect the branches of the internal carotid artery (ICA) in the ethmoidal or orbital regions, leading to microaneurysms and cerebral emboli.[8] Furthermore, bone destruction is observed in 30 – 50% of patients with sinus infections.[9]

The European Organization for Research and Treatment of Cancer and the Mycoses Study Group have included galactomannan (GM) and 1,3-β-d-glucan (BDG) assays as microbiological criteria for diagnosing fungal infections.[10] GM, a polysaccharide antigen primarily found in the cell walls of *Aspergillus* spp., can be detected using the Platelia *Aspergillus* enzyme immunoassay (Bio-Rad Laboratories, Inc., headquartered in Hercules, California, USA). This method utilizes serum or bronchoalveolar lavage fluid samples. However, cerebrospinal fluid (CSF) can be used to treat cerebral aspergillosis.[10-12]

BDG, a cell wall component of most fungi (excluding *Cryptococci*, *Zygomycetes*, and *Blastomyces dermatitidis*), is a panfungal serological marker. Although BDG is not specific to IA, it can be used to diagnose invasive candidiasis.[10-12] Of the four available BDG detection assays (Fungitell, Wako, Fungitec-G, and Maruha), only Fungitell (Associates of Cape Cod, Inc., 124 Bernard E. Saint Jean Drive, East Falmouth, Massachusetts 02536-4445, USA) has been FDA-approved for use with serum.[10]

Pakistan faces unique challenges because of its humid climate, which fosters fungal growth and widespread immunosuppressive therapy use. Furthermore, owing to limited data on intracranial fungal infections (IFIs) and restricted availability and affordability of neuroimaging or CSF analysis, diagnoses are delayed or missed. Consequently, the timely detection of fungal infections must be improved by assessing specific diagnostic parameters such as GM and BDG assays, which can enhance the diagnostic accuracy and facilitate prompt treatment. Given the low socioeconomic status of patients in our setup, we must focus on targeted diagnostic markers to streamline patient and healthcare provider processes.

This study contributes to the existing literature on IFIs by presenting a case series of patients with IFIs who were admitted to the Jinnah Postgraduate Medical Center, a tertiary care hospital in Karachi, Pakistan, between November 2022 and May 2023. We aimed to evaluate the potential role of serum fungal biomarkers as an adjunct to early diagnostic tools in resource-limited low- and middle-income countries such as Pakistan.

2. Case series

We retrospectively reviewed the medical records of patients with clinically suspected IFI. Only patients with fungal assay data (GM and BDG levels), neuroimaging studies (computed tomography [CT] of the PNS and magnetic resonance imaging [MRI]), and CSF analysis results were included in this study. We solely focused on the diagnostic approach for suspected cases of IFI from a single center and did not address the treatment regimens or management outcomes. The interpretation of the BDG and GM index values is provided in Table 1.

2.1. Case 1

A 48-year-old man presented with headaches, vertigo, left-sided visual loss, and hearing loss 2 months after surgical excision of a parasellar space-occupying lesion. CSF analysis revealed an elevated protein level, a decreased glucose level, and an increased white blood cell (WBC) count (lymphocytes, 95%). The serum was positive for BDG, whereas Gram and acid–fast bacteria staining yielded negative results.

Contrast-enhanced MRI of the brain revealed an intracranial extra-axial mass in the right parasellar region. The mass extended into both cavernous sinuses and involved the right supraclinoid ICA, cerebellar peduncle, and brainstem.

2.2. Case 2

A 51-year-old man with fungal sinusitis presented with swelling in the left maxillary region 8 months after a craniotomy. GM and BDG fungal biomarker assays were positive. Contrast-enhanced MRI of the brain revealed changes in the left maxillary sinus with ipsilateral intraorbital and intracranial extensions. The CT PNS confirmed the findings of chronic left maxillary sinusitis with osteomyelitis.

2.3. Case 3

A 61-year-old man with hepatitis C and uncontrolled type-2 diabetes presented with proptosis, pain, lacrimation, and

Table 1. Cutoffs for the serum fungal diagnostic assays and their interpretation

Tests	Cutoff	Interpretation
β-d-glucan	<60 pg/mL	Negative
	60–80 pg/dL	Intermediate*
	>80 pg/dL	Positive
Galactomannan	<0.5	Negative

Notes: *Repeat test-recommended; **GMI >0.5 in two consecutive samples.

rhinorrhea of the right eye, which progressed to painless visual loss. CT PNS exhibited thickening of the soft tissues along the right optic canal, further compressing the optic nerve. The serum was positive for BDG.

2.4. Case 4

A 30-year-old woman presented with a 3-month history of headaches and vomiting. A plain CT PNS revealed a complete opacification of the sphenoid sinus, right posterior ethmoid air cells, and right frontal sinus, indicating superadded fungal colonization. The serum BDG titers were elevated.

2.5. Case 5

A 40-year-old woman presented with headaches. The fungal assay was positive, revealing elevated BDG levels. CSF analysis revealed a normal glucose, an elevated protein level, and 20% polymorphs. The gram staining and cryptococcal antigen test yielded negative results.

2.6. Case 6

A 32-year-old man presented with headache, right eyelid drooping, diplopia that progressed to complete ophthalmoplegia and visual loss. A plain CT PNS revealed mucosal thickening in the right maxillary sinus and right posterior ethmoidal air cells, indicating a fungal sinus disease with intracranial extension. The fungal biomarkers were negative. CSF analysis revealed a normal glucose, an increased protein level, and 90% lymphocytes.

2.7. Case 7

A 35-year-old man presented with headaches and left eye pain. Serum fungal biomarkers were negative. Gadolinium-enhanced brain MRI revealed sinonasal fungal colonization with intraorbital and intracranial extensions.

2.8. Case 8

A 40-year-old man presented with chronic headaches. CSF analysis revealed a markedly elevated protein level, normal glucose level, and 95% lymphocytes. The fungal potassium hydroxide prep yielded a negative result, and the serum was positive for BDG.

2.9. Case 9

An 18-year-old woman presented with headache, eye pain, blurred vision, nasal blockage, and shortness of breath. The serum was positive for BDG. CT PNS revealed sinus opacification and cribriform plate erosion, indicating intracranial extension. Brain MRI confirmed the presence of pansinusitis with fungal colonization and cortical erosion.

2.10. Case 10

A 36-year-old woman presented with a headache and left maxillary swelling. The fungal assays revealed positive GM and BDG titers. Consistent with the clinical features, magnetic resonance angiography, and venography revealed abnormal signal intensity of fungal colonization, infiltrative soft tissue thickening, optic nerve encasement, and ICA attenuation.

2.11. Case 11

A 35-year-old man presented with chronic right-sided headaches. The serum was positive for BDG. CSF analysis revealed an elevated protein level, normal glucose level, and 95% lymphocytes. Brain MRI revealed fungal sinusitis with cribriform plate erosion and orbital apex extension.

2.12. Case 12

A 27-year-old man presented with a chronic history of headaches, facial numbness, burning sensation over the upper right side of the face, diplopia, and fever. CT PNS revealed sphenoid sinus opacification and cavernous sinus extension. Brain MRI revealed sinonasal polyposis with fungal colonization and skull base involvement. The serum was positive for GM and BDG. CSF analysis revealed a turbid fluid, high protein content, 90% neutrophils, and markedly low glucose level.

(The clinical profile and fungal assays of the patients are listed in Table 2).

3. Discussion

The CNS is an immunologically privileged site with a specialized comparatively impermeable BBB. However, immunocompromised states predispose individuals to opportunistic and pathogenic mycosis. CNS manifestations typically arise from hematogenous or disseminated invasion from pulmonary, intestinal, or cardiac sites. Nevertheless, direct intracranial spread can occur from the paranasal sinuses, intraorbital extension, petromastoid area, and retropharyngeal spaces. Furthermore, the colonization of ventricular drains, shunts, and central venous lines placed during trauma management, as well as the colonization during craniotomies, facilitate direct mycosis implantation.[13]

In 2017, Jabeen *et al*. reported that approximately 1.78% of individuals suffer from serious fungal infections, excluding oral candidiasis, allergic fungal sinusitis, and cutaneous infections.[14] However, the actual burden of fungal infections, particularly IFIs, remains unknown because of the lack of centralized surveillance. Compared with fungal infections, which are usually rare, IFIs are rarer. However, they are relatively common in

Table 2. Characteristics and serum fungal biomarker levels of the included patients

Case	Age (years)	Sex	Comorbidities	Headache	Serum fungal biomarker		CSF analysis		
					GM index (OD)	BDG levels (pg/mL)	Glucose (mg/dL)	Protein (mg/dL)	WBCs (×10E9/L)
1	48	M	Parasellar space-occupying lesion (postsurgical status)	Yes (acute)	0.098	408.442	24	109	0.012
2	51	M	Fungal sinusitis (postsurgical status)	No	3.016	523.438	23	189	0.315
3	61	M	Type-2 diabetes mellitus and hepatitis C	No	0.269	170.833	-	-	-
4	30	F	None	Yes (chronic)	0.533	523.438	-	-	-
5	40	F	None	Yes (acute)	0.361	159.899	62	62	0.012
6	32	M	None	Yes (acute)	0.119	10.708	61	47	0.008
7	35	M	None	Yes (acute)	0.22	7.812	-	-	-
8	40	M	None	Yes (chronic)	0.488	298.994	60	408	0.085
9	18	F	None	Yes (acute)	0.212	236.771	-	-	-
10	36	F	None	Yes (chronic)	1.324	500	-	-	-
11	35	M	None	Yes (chronic)	0.160	500	51	99	0.340
12	27	M	None	Yes (chronic)	0.781	500	11	97	0.071

Notes: Green: Indicates positive result; Red: Indicates negative result.
Abbreviations: BDG: 1,3-β-d-glucan; F: Female; GM: Galactomannan; M: Male; OD: Optic density.

immunocompromised individuals. If such infections go unnoticed or the management is delayed, they can be fatal. In our case series, all patients were healthy, except for three patients. During presentation, our patients had no history of fungal, pulmonary, or dermatological mycotic infections.

Sarwar *et al.* (2020) highlighted the diagnostic benefits and efficacy of fungal markers (GM and BDG) in the early identification of IA and concluded that BDG is sensitive while GM is specific for IA.[15] Our study focused on using the same serological markers to timely detect IFIs. Among the three immunocompromised patients, one exhibited positivity for GM and BDG and two exhibited positivity for BDG. In addition, among the immunocompetent patients, two yielded negative results for serum fungal biomarkers, five exhibited positivity for BDG, and two exhibited positivity for GM and BDG.

Although CSF fungal culture was not performed in all patients, fungal infection was evident in those who underwent the test. In most patients, normal GM levels and high BDG levels negated the risk of aspergillosis.

In patients clinically suspected to have IVI but without typical meningitis signs, the combination of CSF analysis and other investigations can help establish a provisional diagnosis of fungal infection even when fungal assays are negative; this indicates that with further studies on this approach, a single CSF specimen could be sufficient for fungal biomarker analyses, general analysis, and culture tests. The combination of serological and CSF fungal biomarker assays and the clinical interpretation of CSF analysis and culture tests may be a promising noninvasive diagnostic approach for patients, especially those with an atypical presentation or any contraindication to radiological imaging.

Although biopsy is the gold standard for making a final diagnosis, imaging modalities such as CT and MRI are invaluable for diagnosing IFIs. CT PNS is the preferred noninvasive investigation for making a presumptive diagnosis. At this stage, fungal biomarkers may help differentiate neoplastic lesions from infectious lesions. On CT, fungal sinusitis with intracranial extension appears as near-complete opacification of the infected sinus, expansion of the involved sinus, and remodeling and thinning or erosion of the sinus wall.[16] MRI is more specific for CNS parenchymal mycoses, exhibiting hypo-to-iso-intense signals on T1-weighted images, extremely hypo-intense signals on T2-weighted images, and bright homogenous enhancement on postgadolinium images.[17,18]

Radiographic imaging remains the preferred diagnostic tool for IFIs. However, serological fungal markers (GM and BDG) and CSF analysis are crucial adjuncts for prompt diagnosis and early onset of treatment, providing morbidity- and mortality-related benefits.

This study has certain limitations. Our institution is a government tertiary care hospital with limited research facilities, which limit long-term patient follow-up. Thus,

only the importance of fungal markers in early diagnosis could be assessed and not treatment efficacy.

4. Conclusion

Given the limited data on fungal infections in Pakistan and the lack of IFI-centered studies, the epidemiology is largely unknown. Consequently, the diagnostic approach is suboptimal. Although imaging studies and biopsy are the gold standards for IFI diagnosis, the gap in noninvasive modalities must be assessed. Our findings highlight the importance of serological fungal biomarkers (GM and BDG) and CSF analysis as adjuncts to the gold standard diagnostic tools. Furthermore, we propose the inclusion of fungal biomarker assays using CSF specimens in future diagnostic protocols.

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Conflict of interest

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Author contributions

Conceptualization: Suneel Kumar, Zuhaa Rehman
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Ethics approval and consent to participate

This study was approved by the Institutional Review Board Committee of JPMC under the IRB number NO.F.2-81/2023-GENL/92/JPMC.

Consent for publication

The retrospective case series was conducted using deidentified data from the department registry. Hence, consent for publication was not required.

Availability of data

The data can be shared on request.

Further disclosure

Part of or the entire set of findings has been presented in a conference – 9th Annual Neuroscience Conference. The paper has been uploaded to ResearchGate (doi: 10.13140/RG.2.2.33961.61287).

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CASE REPORT

Drug-induced hypoglycemia in a patient with Type 2 diabetes and renal impairment: A case report

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Abstract

Hypoglycemia is a serious adverse effect in the pharmacological treatment of Type 2 diabetes mellitus (T2DM), exacerbated by comorbidities that affect drug metabolism and clearance. This report describes a case involving a 61-year-old man with hypertension and T2DM. The patient experienced hypoglycemia while taking a combination of voglibose and metformin hydrochloride. His condition was further complicated by urosepsis, acute-on-chronic kidney disease, and hydroureteronephrosis calculi, which likely altered drug clearance and pharmacokinetics. This condition increased his risk of hypoglycemia. Effective management required close monitoring of blood glucose levels and adjustments to the treatment regimen, considering his renal impairment. This case highlights the risks associated with combination therapy in patients with renal issues and underscores the importance of personalized treatment plans, vigilant glucose monitoring, and consideration of renal function to reduce the risk of drug-induced hypoglycemia in patients with T2DM and comorbidities.

Keywords: Hypoglycemia; Combination therapy; Renal impairment; Glycemic control; Type 2 diabetes mellitus

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1. Introduction

Diabetes mellitus is a chronic metabolic disorder characterized by high blood glucose levels due to insufficient insulin production or ineffective insulin action. It is a global health concern, affecting approximately 463 million adults worldwide in 2019, and the prevalence projected to increase to 700 million by 2045.[1] In managing Type 2 diabetes mellitus (T2DM), optimal glycemic control must be maintained to prevent complications such as cardiovascular disease, nephropathy, retinopathy, and neuropathy.[2]

Pharmacotherapy is essential for achieving glycemic control in patients with T2DM. Classes of antidiabetic agents, including biguanides, sulfonylureas, dipeptidyl peptidase-4 (DPP-4) inhibitors, and alpha-glucosidase inhibitors, target different pathways involved in glucose metabolism.[3] Combination therapy, involving the concurrent use of multiple antidiabetic agents with complementary mechanisms of action, is frequently employed to achieve better glycemic control in patients who do not respond adequately to monotherapy.[4,5]

However, the use of combination therapy in T2DM comes with risks. Hypoglycemia, one of the most concerning complications associated with antidiabetic agents, is characterized by abnormally low blood glucose levels. Hypoglycemia can have serious consequences, ranging from mild symptoms such as confusion and shakiness to severe neurological impairment, coma, or death if left untreated.[6,7] The risk of hypoglycemia is further increased in patients with renal impairment due to alterations in drug clearance and pharmacokinetics.[8,9]

Renal impairment is a common comorbidity in patients with T2DM, affecting approximately 40% of this patient population.[10,11] The kidneys are crucial in the elimination of drugs from the body, and any impairment in renal function can lead to the accumulation of drugs and their metabolites, potentially increasing the risk of adverse effects.[12] Moreover, in patients with renal impairment, dose adjustments and careful selection of antidiabetic agents must be considered to prevent drug-induced hypoglycemia or other adverse events.

Voglibose, an alpha-glucosidase inhibitor, delays the absorption of carbohydrates in the intestine, thereby reducing postprandial hyperglycemia.[13,14] Metformin, a biguanide, is commonly used as a first-line therapy in T2DM management owing to its ability to reduce hepatic glucose production and improve insulin sensitivity.[10,15] Both voglibose and metformin have shown efficacy in improving glycemic control; however, their use in combination therapy and the potential associated risks, particularly in patients with renal impairment, have not been extensively studied.[14,15]

This case report describes the clinical presentation of a 61-year-old male patient with a medical history of hypertension and T2DM. He developed hypoglycemia while taking a combination of voglibose and metformin hydrochloride for glycemic control. The patient also had a history of urosepsis, acute-on-chronic kidney disease (CKD), and hydroureteronephrosis calculi, which may have contributed to altered drug clearance and pharmacokinetics, thereby increasing the risk of hypoglycemia.

This case report primarily aimed to highlight the potential risks associated with combination therapy for diabetes, particularly in patients with renal impairment. This case underscores the importance of close monitoring of blood glucose levels and renal function in patients with T2DM and renal impairment to prevent drug-induced hypoglycemia. Another aim is to raise awareness among healthcare providers regarding the need for individualized treatment plans and cautious selection of antidiabetic agents in this patient population.

To the best of our knowledge, this is one of the few reported cases of hypoglycemia associated with the combined intake of voglibose and metformin hydrochloride in a patient with T2DM and renal impairment. The case highlights the challenges faced in achieving optimal glycemic control in patients with multiple comorbidities, in the presence of renal impairment. The findings from this case report contribute to the growing body of literature on the risks and considerations associated with combination therapy in patients with T2DM and renal impairment.

2. Case presentation

A 61-year-old man with a history of type 2 diabetes mellitus (T2DM) and hypertension presented to the hospital with diminished consciousness, slurred speech, and profuse sweating. His antidiabetic regimen included glimepiride (2 mg), metformin (500 mg), and voglibose (0.2 mg). Two months prior, the patient had experienced hydroureteronephrosis calculi, acute-on-CKD, and urosepsis (Tables 1 and 2).

Table 1. Patient characteristics and baseline parameters

Parameter	At presentation	At discharge/follow-up
Age	61 years	-
Sex	Male	-
HbA1c	9.4%	7.6%
Fasting glucose	50 – 70 mg/dL	110 – 130 mg/dL
Postprandial glucose	Not assessed	140 – 180 mg/dL
Serum creatinine	2.6 mg/dL	2.4 mg/dL
eGFR	26 mL/min/1.73 m² (Stage 3b CKD)	33 mL/min/1.73 m²
Blood urea nitrogen	60 mg/dL	Not reassessed
Comorbidities	Hypertension, CKD, and nephrolithiasis	-

Abbreviations: CKD: Chronic kidney disease; eGFR: Estimated glomerular filtration rate; HbA1c: Hemoglobin A1c.

Table 2. Medication regimens and adjustments

Therapy	Dosage	Indication	Outcome
Glimepiride	2 mg/day	T2DM	Discontinued due to hypoglycemia
Metformin	500 mg/day	T2DM	Discontinued due to CKD
Voglibose	0.2 mg/day	Postprandial glucose control	Discontinued
Linagliptin	5 mg/day	T2DM with CKD	Initiated, improved glycemic control
Losartan	50 mg/day	Hypertension	Continued
Amlodipine	5 mg/day	Hypertension	Continued
Atorvastatin	10 mg/day	Dyslipidemia	Continued

Abbreviations: CKD: Chronic kidney disease; T2DN: Type 2 diabetes mellitus.

2.1. Pathophysiological and clinical considerations

The combination of glimepiride, metformin, and voglibose increased the risk of hypoglycemia in this patient due to several factors (Table 3). The presence of renal impairment resulted in reduced clearance of glimepiride, thereby prolonging its hypoglycemic action. In addition, CKD diminishes gluconeogenesis, a key counterregulatory mechanism. While metformin and voglibose are not typically associated with hypoglycemia, their use in combination with sulfonylureas, particularly in patients with CKD, can potentiate this risk. Furthermore, the patient's renal impairment was likely exacerbated by his history of urosepsis and nephrolithiasis, which contributed to altered drug clearance and pharmacokinetics.

2.2. Post-intervention management and alternative therapy

After discontinuing the combination therapy, linagliptin, a DPP-4 inhibitor, was prescribed. In CKD, linagliptin is metabolized primarily by the liver and does not require dose adjustments, making it an appropriate choice for this patient.

On examination, the patient's vital signs were stable, and abdominal and systemic evaluations were unremarkable. Laboratory findings included high glycated hemoglobin (HbA1c) levels, indicating poor glycemic control, and renal function tests showed elevated creatinine and urea levels, indicative of renal impairment (Table 4).

The patient was diagnosed with hypoglycemia, with blood glucose levels ranging from 50 to 70 mg/dL. Immediate management involved a 25% dextrose infusion, which improved his consciousness and speech. Antacids led to symptomatic relief. Continuous glucose monitoring revealed improvement over the subsequent days.

The hypoglycemic episodes were attributed to an adverse drug reaction from the combination of glimepiride, metformin, and voglibose, compounded by the patient's impaired renal function. This therapy was discontinued, and an alternative regimen (linagliptin) was initiated.

The patient was diagnosed with hypoglycemia, with blood glucose levels ranging from 50 to 70 mg/dL. A 25% dextrose infusion was administered, which rapidly improved consciousness and speech. Antacids were also provided for symptomatic relief.

2.3. Renal function and glycemic outcomes (Tables 4 and 5)

2.3.1. Renal parameters

At presentation: Serum creatinine of 2.6 mg/dL, blood urea nitrogen of 60 mg/dL, and estimated glomerular filtration rate (eGFR) of 26 mL/min/1.73 m², consistent with acute worsening of stage 3b CKD.

At discharge: eGFR improved to 33 mL/min/1.73 m², and serum creatinine decreased to 2.4 mg/dL, reflecting partial renal recovery.

Table 3. Pathophysiological considerations of hypoglycemia

Factor	Mechanism	Effect on hypoglycemia
Reduced drug clearance	Impaired renal excretion of glimepiride	Prolonged hypoglycemic action
Decreased gluconeogenesis	CKD-related metabolic changes	Reduced endogenous glucose production
Combination therapy	Overlapping mechanisms of action	Potentiated risk of hypoglycemia

Abbreviation: CKD: Chronic kidney disease.

Table 4. Laboratory investigations

Test	Result at presentation	Normal range
Serum creatinine	2.6 mg/dL	0.6 – 1.2 mg/dL
eGFR	26 mL/min/1.73 m²	≥90 mL/min/1.73 m²
Blood urea nitrogen	60 mg/dL	7 – 20 mg/dL
HbA1c	9.4%	<7.0%
Hemoglobin	10.5 g/dL	13.5 – 17.5 g/dL (male)
Urine protein	+1	Negative
Fasting glucose	50 – 70 mg/dL	70 – 100 mg/dL

Abbreviations: eGFR: Estimated glomerular filtration rate; HbA1c: Hemoglobin A1c.

Table 5. Glycemic and renal function outcomes post-intervention

Parameter	Baseline (before change)	Follow-up (post-intervention)
HbA1c	9.4%	7.6%
Fasting glucose	50 – 70 mg/dL	110 – 130 mg/dL
Postprandial glucose	Not assessed	140 – 180 mg/dL
eGFR	26 mL/min/1.73 m²	33 mL/min/1.73 m²

Abbreviations: eGFR: Estimated glomerular filtration rate; HbA1c: Hemoglobin A1c.

2.4. Glycemic control (Tables 4 and 5)

Initial: HbA1c was 9.4%, and fasting glucose levels ranged from 50 to 70 mg/dL.

Post-intervention: HbA1c improved to 7.6% within 3 months. Fasting glucose levels stabilized at 110 – 130 mg/dL, and postprandial levels ranged from 140 to 180 mg/dL.

2.5. Additional clinical findings

2.5.1. Urine examination

Urine analysis showed mild proteinuria (+1) without microscopic hematuria. No growth was found on the urine culture, ruling out active infections. The history of nephrolithiasis and prior urosepsis was considered relevant to the baseline of patient with CKD.

2.5.2. Comorbidities and therapies

The patient had hypertension, which was managed with losartan and amlodipine, and dyslipidemia treated with atorvastatin. His HbA1c level was 10.5 g/dL, indicative of CKD-induced anemia.

2.5.3. Outcome and clinical implications

Glycemic control improved significantly without further hypoglycemic episodes. Linagliptin proved to be effective and safe, stabilizing blood glucose levels and preventing further renal compromise.

This case underscores the importance of individualized DM management in patients with renal impairment. Healthcare providers should carefully assess pharmacokinetics and comorbidities when selecting antidiabetic therapies to balance glycemic control with minimized adverse effects.

3. Discussion

This case report highlights the clinical challenges of managing a 61-year-old male patient with T2DM, hypertension, and CKD, emphasizing the recurrent hypoglycemic episodes associated with the combination therapy of glimepiride, metformin, and voglibose. The report underscores the need for tailoring antidiabetic therapy to individual patient factors such as renal impairment and comorbidities.

The combination of glimepiride, metformin, and voglibose is a widely used regimen for managing T2DM. Glimepiride, a sulfonylurea, stimulates pancreatic beta cells to release insulin, metformin, a biguanide, reduces hepatic glucose production and enhances insulin sensitivity, and voglibose, an alpha-glucosidase inhibitor, delays carbohydrate absorption, reducing postprandial glucose spikes. Despite their efficacy, these agents are associated with an increased risk of hypoglycemia, particularly in vulnerable populations such as those with CKD.

In this case, substituting linagliptin for the previous combination therapy effectively improved glycemic control and minimized the risk of hypoglycemia. Linagliptin is safe for the kidneys and aligns with current clinical guidelines for managing diabetes in patients with CKD. Post-intervention outcomes showed stable blood glucose levels and improved renal function, reinforcing the importance of selecting antidiabetic agents based on the patient's renal status and overall health condition.

The patient's medical history, including urosepsis, nephrolithiasis, and acute-on-CKD, complicated the challenges of drug clearance and pharmacokinetics. CKD can impair the excretion of medications, leading to drug accumulation and an increased risk of adverse effects. In this case, the combination therapy likely exacerbated the risk of hypoglycemia due to the reduced renal clearance of glimepiride and the impaired gluconeogenic capacity associated with CKD.

3.1. Pathophysiological mechanisms for increased hypoglycemia risk in CKD

Patients with CKD are particularly susceptible to hypoglycemia owing to the following mechanisms:

1. Reduced renal clearance of insulin: Impaired kidney function prolongs insulin half-life, increasing systemic levels.
2. Diminished gluconeogenesis: CKD compromises renal gluconeogenesis, a key counter-regulatory process in hypoglycemia prevention.
3. Coexistent malnutrition and anemia: CKD-associated anemia and reduced caloric intake lower glycogen stores and weaken hypoglycemia responses.
4. Enhanced sensitivity to hypoglycemic agents: Drugs such as sulfonylureas and insulin are cleared more slowly in CKD, amplifying their effects.

3.2. Assessing the cause of hypoglycemia

The patient had multifactorial hypoglycemic episodes, driven by both CKD-related alterations in metabolism and the pharmacodynamics of glimepiride, metformin, and voglibose. Glimepiride's prolonged action due to reduced renal clearance, coupled with impaired compensatory mechanisms such as gluconeogenesis, significantly contributed to the hypoglycemia observed.

3.3. Implications for clinical practice

T2DM management in CKD requires careful consideration of drug pharmacokinetics and potential adverse effects. Adjusting or discontinuing agents with a high hypoglycemia risk is essential to avoid complications. In this case, replacing the combination therapy with linagliptin improved glycemic control without inducing further hypoglycemia.

Regular monitoring of renal function and glycemic control, along with close patient–provider collaboration, is critical to ensure safe and effective diabetes and CKD

management. This case underscores the importance of individualized treatment plans that account for the interplay between diabetes and comorbid conditions.

3.4. Limitations

As a single-patient case report, these results cannot be generalized to all individuals with similar conditions. Differences in comorbidities, renal function, and pharmacogenetics may affect outcomes, emphasizing the need for a personalized approach to managing T2DM and associated comorbidities. This report contributes to the understanding of T2DM management in patients with CKD, offering valuable insights into optimizing therapeutic strategies while minimizing adverse effects.

4. Conclusion

This case report highlights the complexities of managing T2DM in patients with renal impairment and comorbidities, particularly the heightened risk of hypoglycemia associated with certain antidiabetic regimens. The recurrent hypoglycemic episodes experienced by the patient while taking a combination of glimepiride, metformin, and voglibose were attributed to altered drug clearance and pharmacokinetics resulting from renal dysfunction. Switching to linagliptin, a renal-friendly alternative antidiabetic agent, resulted in improved glycemic control and resolution of hypoglycemia. This case emphasizes the need to tailor diabetes management to individual patient needs, with careful consideration of comorbidities, regular monitoring of blood glucose levels, and assessment of renal function to prevent adverse outcomes and ensure safe and effective therapy.

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Conflict of interest

The authors declare that they have no competing interests.

Author contributions

Conceptualization: All authors
Investigation: All authors
Writing–original draft: All authors
Writing–review & editing: All authors

Ethics approval and consent to participate

Verbal and written consent was obtained from the patient before his participation.

Consent for publication

A verbal and written formed consent was obtained from the patient.

Availability of data

Not applicable.

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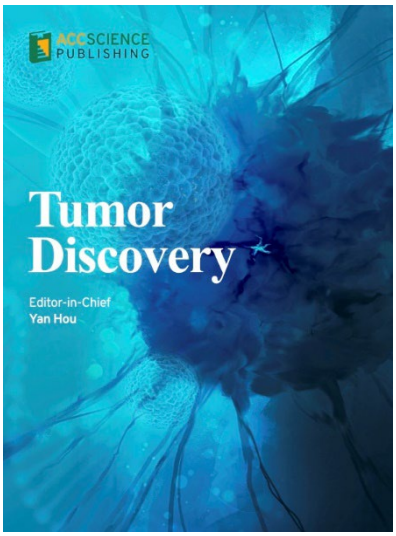

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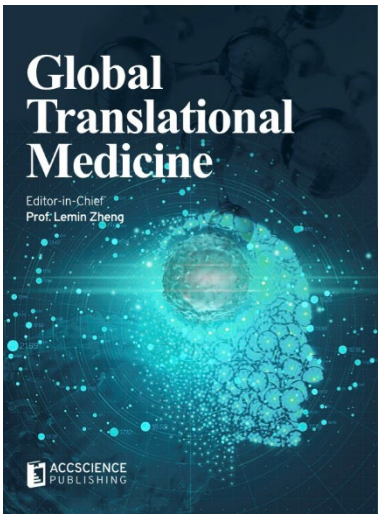

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