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Anti-inflammatory, Antioxidant, and Wound-Healing Effects of Photobiomodulation on Type-2 Diabetic Rats

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Abstract

Introduction: In the current study, the effects of photobiomodulation (PBM) treatments were examined based on biomechanical and histological criteria and mRNA levels of catalase (CAT), superoxide dismutase (SOD), and NADPH oxidase (NOX) 1 and 4 in a postponed, ischemic, and infected wound repair model (DIIWHM) in rats with type 2 diabetes (DM2) during the inflammation (day 4) and proliferation (day 8) stages.

Methods: To study ischemic wound repair in a diabetic rat model (DIIWHM), 24 rats with type-2 diabetes were randomly divided into four groups and infected with methicillin-resistant *Staphylococcus aureus* (MRSA). The control groups consisted of CG4 (control group on day 4) and CG8 (control group on day 8), while the PBM groups comprised PBM_4 (PBM treatment group on day 4) and PBM_8 (PBM treatment group on day 8). These group assignments allowed for comparisons between the control groups and the PBM-treated groups at their respective time points during the study.

Results: On days 4 and 8 of wound restoration, the PBM_4 and PBM_8 groups showed substantially modulated inflammatory responses and improved formation of fibroblast tissue compared with the CG groups ($P<0.05$). Concurrently, the effects of PBM_8 were significantly superior to those of PBM_4 ($P<0.05$). The antioxidant results on days 4 and 8 revealed substantial increases in CAT and SOD in the PBM groups compared with the CGs ($P<0.05$). Substantial decreases were observed in the antioxidant agents NOX1 and NOX4 of the PBM_4 and PBM_8 groups compared with both CG groups ($P<0.05$).

Conclusion: PBM treatments significantly sped up the inflammatory and proliferating processes in a DHIIWM in DM2 animals by modifying the inflammatory reaction and boosting fibroblast proliferation. Overall, the current findings indicated substantially better results in the PBM groups than in the CG groups.

Keywords: Diabetes mellitus; Wound healing; Inflammatory phase; Proliferation phase; Superoxide dismutase.

Introduction

Type 2 diabetes mellitus (DM2) is a chronic and metabolic disorder arising from defects in both insulin action and secretion, and it is characterized by persistent hyperglycemia. Individuals with DM2 exhibit an impaired cellular sensitivity to insulin, known as insulin resistance, a condition that hampers the ability of cells to efficiently take up glucose from the bloodstream. As a result, glucose accumulates in the blood, leading to elevated blood sugar levels. The impaired cellular response to insulin contributes to the characteristic metabolic disturbances observed in DM2.[1] Patients with DM2 comprise more than 85% of all diabetic cases worldwide. The most recent studies have demonstrated that the global prevalence of DM2 was 425 million in 2017 and is projected to reach 629 million by 2045.[2] Moreover, nearly 49.7% of cases of DM remain undetected.[3] Hence, incorrect management of DM may result in chronic problems.[4]

The persistence of DM critically raises the possibility of peripheral artery disease (PAD), that is, atherosclerosis of the arteries of the lower extremities. DM hastens the course of PAD in diabetic patients, makes them more

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prone to ischemic events, and reduces their functional status compared with individuals without DM.[5,6] Another possible result of DM is diabetic foot disease, defined as a group of syndromes in which such problems as neuropathy, ischemia, and infection may cause tissue breakdown, leading to complications and amputation.[7] *Diabetic foot ulcers (*DFUs) are full-thickness sores that appear below the ankle in DM patients, regardless of duration.[8]

DFUs, the most common form of chronic ulcers, occur under conditions of infection, ischemia, metabolic, and neuropathy diseases and result in insignificant injury repair and management effects. [9] Fifteen percent of diabetic patients suffer from DFU, and amputations (usually of lower extremities) are seen in 14%-24% of DFU patients. The mortality rate of patients with lower extremity amputation approaches 50–59% five years after amputation.[10] DFUs impose a heavy financial burden on patients and healthcare settings.[11] At least half of all amputations occur in people with DM, most commonly because of an infected DFU.[12] Methicillin-resistant *Staphylococcus aureus* (MRSA) is the most frequently seen pathogen in DFUs, where its prevalence is approximately 15%-30%.[13]

Wound repair refers to the physiological response to a distraction in the regular skin architecture. During this process, multiple cell types and cytokines need to be coordinated. This intricate process is vulnerable to abnormal regulation secondary to DM and ischemia.[14] Oxidative stress plays a central role in the progress of both microvascular and cardiovascular disorders in diabetes patients. The metabolic defects of DM cause the excessive secretion of mitochondrial superoxide in vascular endothelial cells, which activates the five key pathways implicated in the pathogenesis of complications. Through these pathways, high intracellular reactive oxygen species (ROS) levels lead to abnormal neoangiogenesis in reaction to ischemia conditions and the activation of numerous pro-inflammatory pathways.[15] The ability to quickly restore the integrity of broken skin barriers is a significant goal of therapies for chronic wounds.[16] However correct the principles directing the standard of care for DFUs are, a considerable gap remains between current results and optimal wound healing.[17]

Despite current insights, major barriers to achieving suitable therapeutic effects still exist.

Fortunately, one strategy to overcome poor wound healing may be the use of photobiomodulation (PBM), which stimulates restoration, relieves pain, and reduces inflammation. PBM activates an extensive variety of transcription factors that enhance cell endurance, migration, proliferation, as well as new protein formation. It can also improve antioxidant defenses and decrease oxidative stress.[18-20]

In a recent preclinical test, Moradi et al established that PBM alone or in combination with allograft cells of adipose-derived stem cells significantly enhanced the injury healing maturation phase of a delayed healing MRSA-infected wound in type 2 diabetic rats. This study, however, did not examine the antioxidant and oxidative stress levels or stereological factors of repairing injuries under impacts of PBM alone at proliferation and inflammation periods of the injury repair course.[21]

Catalase (CAT)[22] and superoxide dismutase (SOD)[23] are antioxidant agents. SOD is the initial detoxifying enzyme and the cell's top ROS-fighting antioxidant. CAT is the most common antioxidant found in almost all living organs that use oxygen, and its cofactors are iron and manganese, which complete the detoxification process imitated by SOD.[22] NADPH oxidase (NOX) 1 and NOX 4 are main regulators of cell proliferation, differentiation, and growth[24] and are considered primary indicators of oxidative damage. The oxidases of NADPH are ROS-generating enzymes that regulate numerous redox signaling reliant pathways.

The histological and gene expression parameters involved in wound restoration provide key information about repairing wounds, deliver vital information on repairing tissue biology, and reveal novel possible targets for investigation.[25] Such information can help scientists and clinicians choose management strategies for wound restoration in diabetic patients.[26]

In the present work, it was hypothesized that the use of PBM treatments can hasten injury restoration through the enhanced transition from the inflammatory response to the proliferation step, which may be explained by the alteration of antioxidants and oxidative stress parameters as well as the inflammatory response in the injury restoration process of diabetic animals. The current experimental test aimed to assess the effects of PBM on the stereological, antioxidant, and oxidative stress biomarkers of repair tissue at the inflammation and proliferation steps of a delayed healing, ischemic, and infected model (DIIHWM) in DM2 rats.

Materials and Methods

Animals and Study Design

A total of 24 male Wistar rats, each three months old and weighing approximately 270 g, were obtained from the experimental animal center of the Pasteur Institute of Iran. These rats were kept in the laboratory for a period of 2 weeks for acclimatization. Prior to allocation into four groups (Gs) with n = 6 each, all rats underwent the DHIIWM procedure. The untreated rats (CG4 and CG8) in the initial Gs did not receive any medication. The rats in groups 3 and 4 (PBM4 and PBM8) were exposed to PBM and euthanized on days 4 and 8, respectively. The surgical interventions were conducted in a sterile environment, utilizing intramuscular injections of ketamine (50 mg/kg) and xylazine (5 mg/kg).[5,27]

On days 4 and 8, the animals were euthanized using inhalation anesthetic agents such as carbon dioxide or isoflurane gas, and specimens containing healing tissue were collected. Days 4 and 8 were designated as inflammatory and proliferating, accordingly.

Induction of Type 2 Diabetes Mellitus

The rats were given drinking water containing 10% fructose (Biobasic, Canada) for 14 consecutive days.[28] Next, each rat received an injection of streptozotocin (STZ, 40 mg/kg Santa Cruz Biotechnology, Inc., USA, i.p.). After seven days, the level of blood glucose was evaluated in all rats, and those with a blood glucose level higher than 250 mg/dL were considered to have diabetes (DM2).[29] According to the protocol, the injected rats were reserved for three weeks to confirm the presence of DM2.[28]

Clinical Examinations

During the experiment, the body weight and the blood glucose level of each rat were monitored.

Surgery

The surgeries were conducted in a clean setting, using injections of ketamine (50 mg/kg) and xylazine (5 mg/kg) into the muscles to induce anesthesia. While the rats were under general anesthesia and standard conditions, a flap of skin measuring 10 × 3.5 cm was made on their back. In the center of the flap, a circular full-thickness injury with a 12 mm diameter was created. After that, a silicone ring retainer was fixed all over the injured place by silk sutures. Prior to the operation and for 5 days following the operation, all animals received treatment with 20 mg/kg ibuprofen every 8 to 12 hours.[21]

MRSA Inoculation Into the Wounds

MRSA strain (ATCC 25923) was used to infect the wound.

Briefly, a colony of MRSA was used with a concentration of 2×10^8 in 1 cc. Immediately after the operation, 100 µL of MRSA containing 2×10^7 MRSA was added to the injury area.[21]

Photobiomodulation

The wounds created in groups 3 and 4 were exposed to PBM using the following parameters: wavelength of 890 nm, average peak power of P 1.08 W, peak power (P) of 80 W, power density of 0.001, spot size of 1 $cm^2 W/cm^2$, pulse reputation of 80 Hz, duration of each pulse180 ns, time of each exposure 300 seconds, energy density of 0.32 J/cm^2 (MUSTANG 2000 LO7 pen, Technica Co., Russia). After the wounds were created, the rats underwent PBM therapy for 8 weeks, 6 days per week. During this process, the rats were sedated using anesthetic drugs.[21]

Stereological Examination by the Physical Dissector Method

For histopathological analysis, specimens with sections in series, each 5 mm thick, were produced. The following formula was used to determine the numerical densities (Nv) of neutrophils and macrophages on ten slides that had been stained by the hematoxylin and eosin (H&E) technique:

$$Nv = \frac{\Sigma Q}{h \times a/f \times \Sigma p},$$

Where ΣQ is the total amount of cells (nuclei), a/f is the number of frame areas, h is the height of the dissector, and p is the total number of frames that have been tallied throughout all fields. Additionally, N = NvV, where N is the overall number of cells per rat, Nv is the numerical density, and V is the overall volume.[21]

Total RNA Isolation and qRT-PCR Analysis

The TissueLyser (High Purity RNA Isolation Kit, Roche, Germany) was used to extract the whole RNA. The Revert Aid first-strand cDNA synthesis kit was employed for cDNA syntheses. The gene expression of CAT, SOD, NOX1, and NOX4 was measured by QRT-PCR (SYBR Green PCR Master Mix). REST software and the Ct technique were employed to evaluate the GE data, which were subsequently normalized in reference to GAPDH. The primers are listed in Table 1. A total of three replications were conducted for every desired gene.[30]

Statistical Tests

The data are presented as the mean ± standard deviation (SD). Paired sample t-tests were used to measure weight and blood glucose levels before and after diabetic induction, while independent-sample t-tests were used to measure histological parameters and gene expression levels between groups. Additionally, the least significant difference (LSD) tests were utilized to assess the data collected from the different groups. A *P* value of less than 5% was used to determine any statistically significant differences.

Results

Clinical Observations

In comparison to the day following STZ injections, all research groups experienced weight loss on sampling days (Table 2).

Stereological Results

Briefly, during the proliferation and inflammatory stages of injury restoration, macrophages, neutrophils, and modulated inflammatory response significantly decreased in the PBM_4 and PBM_8 groups compared with CG_4 and CG_8 ($P < 0.05$). Concurrently, the PBM_8 group

Table 1. Primer Sequences of the Studied Genes

Target Gene	Forward (F) and Reverse (R) primers	Primer Sequence	Tm (°C)	Amplicon Length (bp)	Accession Number
SOD1	F	TGGGTTCCATGTCCATCAATAC	58.10	95	NM_017050
	R	CTGGACCGCCATGTTTCTTA	57.89		
CAT	F	CTTTGAGGTCACCCACGATATTA	57.98	91	NM_012520
	R	GGAGAATCGGACGGCAATAG	58.22		
NOX1	F	AGGCTCCAGACCTCCATTTGAC	62.01	86	NM_053683.2
	R	CAGCCCCAACCAAGAAACCAGA	63.00		
NOX4	F	GAAGATTTGCCTGGAAGAACC	57.14	124	NM_053524.1
	R	AGGTTTGTTGCTCCTGATGC	58.75		
GAPDH	F	CCATGGAGAAGGCTGGGG	60	195	NM_001394060.2
	R	CAAAGTTGTCATGGATGACC	60		

Reverse (R) and forward (F) sequences, melting temperature (Tm), Amplicon length (bp), and Accession number for *SOD1*, *CAT*, *NOX*1, *NOX4,* and *GAPDH* genes.

Table 2. The blood sugar and body weight of the studied groups

Groups/Factors	Control	PBM
Blood Sugar After STZ injection (mg/dL)	501.4 ± 45.13	504 ± 52.61
Blood Sugar on day 8 (mg/dL)	514 ± 63.4	4613 ± 102.2
Initial weight	256 ± 6.4	258.1 ± 6.55
Secondary weight after STZ injection (g)	322.88 ± 32.44 ($P<0.001$) (in comparison with initial weight)	25.82 ± 37.02 ($P<0.01$) (in comparison with initial weight)
Weight on day 8	218 ± 30.34 ($P<0.01$) (in comparison with initial weight)	325 ± 48.01 ($P<0.001$) (in comparison with initial weight)

Abbreviations: STZ, streptozotocin; PBM, photobiomodulation.

exhibited substantially better consequences than the PBM_4 group ($P<0.05$).

Neutrophils Count

On day 4, there were significantly fewer neutrophils in the group of PBM_4 than in CG_4 ($P<0.001$). On day 8, there were substantially fewer neutrophils in the PBM_8 group compared with CG_8 ($P<0.01$). Moreover, there were substantially smaller numbers of neutrophils in the PBM_8 group compared to PBM4 ($P<0.05$) (Figure 1A).

Macrophages Count

In comparison to CG_4, there were significantly fewer macrophages in the treatment group ($P<0.001$). On day 8, macrophages were a substantially smaller number in the PBM_8 group compared with CG_8 ($P<0.05$). Simultaneously, there were substantially fewer macrophages in the PBM8 group than in the PBM4 group ($P<0.01$) (Figure 1B).

Inflammatory Cells Count

The study observed significantly lower levels of inflammatory cells in all treated groups compared to the control group (CG) on days 4 and 8 ($P<0.001$). Additionally, there were significantly fewer inflammatory cells in the PBM8 group compared to the PBM_4 group ($P<0.01$), indicating a more pronounced reduction in inflammation with the longer duration of PBM treatment (Figure 1C). These findings suggest that both PBM treatments effectively reduced the presence of inflammatory cells, and the extended PBM treatment duration had an additional beneficial effect on suppressing inflammation.

qRT-PCR Expressed Gene Analysis Findings

In summary, on day 4 of wound restoration, the gene expression of the antioxidant agent CAT was substantially higher in the PBM group than in CG ($P<0.05$). The results on day 8 showed substantial increases in CAT in the treatment group compared with CG ($P<0.01$). Concurrently, in comparison with CG, SOD levels significantly increased in the PBM group ($P<0.01$). In terms of antioxidant agents, there were less NOX1 and NOX4 expiration in the PBM_8 groups than in the CG_8 groups ($P<0.01$ for NOX1 and $P<0.05$ for NOX4) (Figure 2).

Catalase

The findings on day 4 revealed substantially less CAT gene expression in the PBM group than in CG ($P<0.05$). On day 8, the outcomes displayed substantially less CAT in the PBM group than in the CG group ($P<0.01$) (Figure 2A).

Superoxide Dismutase

Day 4 data revealed no appreciable changes between

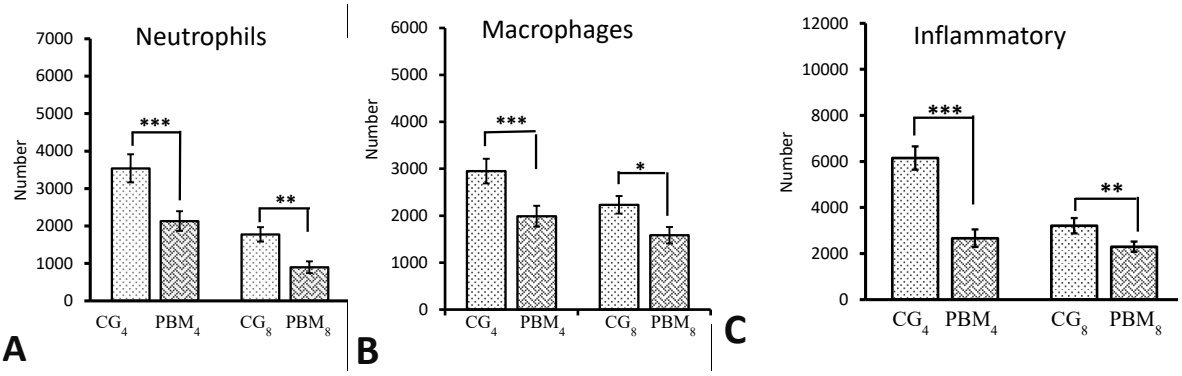

Figure 1. The numbers of neutrophils, macrophages, and inflammatory cells in the wound beds of the studied groups. The significance levels were denoted as follows: *$P<0.05$, **$P<0.01$, and ***$P<0.001$. These tests were conducted to analyze and compare the levels of these specific cell types among the different studied groups. CG_4=control group day_4, CG4, control group day4; CG_8, control group day8; PBM_4, photobiomodulation day4; PBM_8, photobiomodulation day8

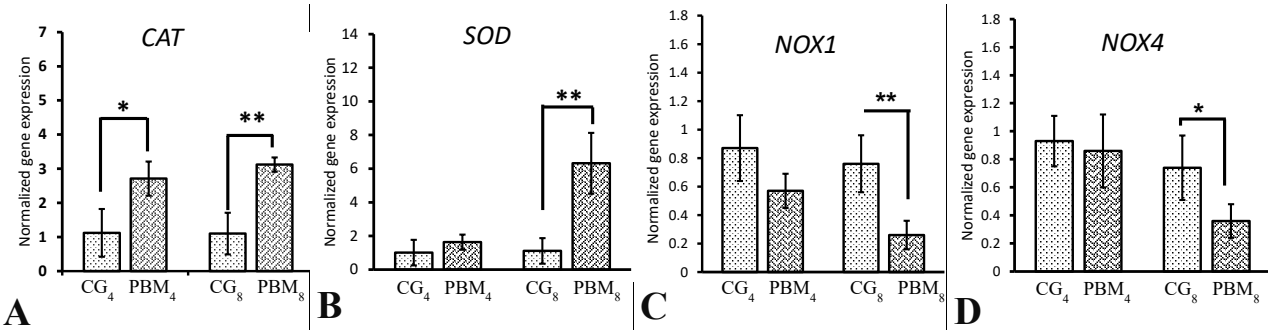

Figure 2. The gene expression levels of catalase (CAT), superoxide dismutase (SOD), and oxidative stress biomarkers, specifically NADPH oxidase (NOX) 1 and 4. The significance levels were denoted as follows: *$P<0.05$, **$P<0.01$, and ***$P<0.001$. CG4, control group day4; CG_8, control group day8; PBM_4, photobiomodulation day4; PBM_8, photobiomodulation day8

the examined groups. On day 8, however, the findings showed that SOD levels were higher in the PBM group than in the CG group ($P<0.01$) (Figure 2B).

NOX1

On day 4, the results showed no significant differences between the groups under investigation. On day 8, however, the findings revealed that NOX1 was significantly lower in the PBM group compared with the CG group ($P<0.01$) (Figure 2C).

NOX4

On day 4, there were no significant differences between the groups; nevertheless, the findings on day 8 revealed that the NOX4 of the PBM group was significantly lower compared to that of the CG group ($P<0.05$) (Figure 2D).

Discussion

Increases in physical diseases that occur during DM are significantly mediated through the secretion of oxidative stress and ROS markers. Patients with DM2 exhibit blood levels of ischemia-modified albumin and oxidative stress-related indicators. Both acute and persistent elevations of blood glucose can cause the production of ROS throughout DM.[31] Accordingly, DM cell culture models have shown that increased glucose levels cause a rise in the mRNA level of oxidative stress biomarkers and cell injuries in cardiomyocytes,[32] endothelial cells,[33] and neurons.[34]

Wound renovation is a dynamic and compound procedure; however, the healing of most diabetic wounds is difficult and long-term. Markers such as hematopoiesis, matrix circulation, neuropathy, hyperglycemia, wound contraction, and microbiomes play main roles in the intricate symphony of diabetic injury repair.

Oxidative stress biomarkers manage the wound healing process through numerous signal pathways. As a result, they could be the main reason why antioxidants and antioxidative enzyme systems actively regulate the release of ROS, which decreases the damage caused by oxidative stress during wound healing.[35]

Earlier in vitro experiments revealed that high concentrations of glucose lead to ROS secretion and, consequently, cell apoptosis.[36] The present study found that a DM2 environment increases inflammatory reaction and oxidative stress biomarkers in injury sites.

The current study also determined that the treatment including PBM significantly controlled the inflammatory reaction and elevated the antioxidant factors in comparison to CG; nonetheless, the PBM_8 group

achieved far superior results compared with the PBM_4 group ($P < 0.05$).

The current findings are consistent with those of Ebrahimpour-Malekshah et al and Bagheri Tadi et al, who discovered that PBM alone and especially in combination with adipose-derived stem cells (ADSs) was more effective than the control group in a DHIIWM in both DM2 and type 1 diabetic rats.[27,37]

Gene expression analysis of antioxidant agents revealed that CAT (on day 4) and SOD (on day 8) were substantially better in the PBM groups than in the CG groups ($P < 0.05$). Furthermore, the PBM groups exhibited substantially greater decreases in NOX1 and NOX4 than the control groups ($P < 0.05$).

An increasing body of evidence supports the role of PBM in promoting wound healing by activating the AKT signaling pathway, which assists in elevating antioxidant levels.[38] PBM has also been found to inhibit the FOXO1 signaling pathway, thereby reducing oxidative stress biomarkers in diabetic wound fibroblasts.[39] Moreover, a previous study reported that diabetic hypoxic wound models reacted positively to PBM.[40] PBM does not injure those fibroblasts exposed to stress, and it has a positive outcome on cell survival and proliferation to increase healing and injury restoration.[40] In a recent review article, Leyane et al concluded that PBM results in photochemical reactions and the creation of ATPs, and it triggers a flow of cellular reactions which cause biological alterations and downstream impacts. These special impacts contain, but are not restricted to, the renovation of cellular action, modification of pain and inflammation, increased tissue renewal and injury restoration, decreased oxidative stress, neuronal regulation, immunomodulation, enhanced cell migration and proliferation, triggered secretion of growth factors, and increased ECM production.[41] In agreement with these studies, the current research determined that the gene expression of CAT (on day 4) and SOD (on day 8) was substantially better in the PBM groups than in the CG groups ($P < 0.05$). Moreover, NOX1 and NOX4 mRNA levels were noticeably reduced in the PBM groups compared with the CG groups ($P < 0.05$).

More than 100 physiologic factors related to wound restoration deficiencies in DM patients have been recognized[42]; thus, the effective management of agents seems to help wound repair.[21,43] To the best of our understanding, no studies have been published on the impact of PBM on the inflammation-related and proliferation phases of healing of contaminated lesions in DM2 rats (or people).

Recently, Xu et al described that the greater expression of HIF-1α in ADS accelerates the healing of diabetic wounds and increases patient survival by improving their paracrine action and inhibiting the DNA impairment induced by ROS secretion.[44] The present researchers previously investigated the effects of PBM on the stereological factors and mRNA levels of some growth factors during the repair process of ulcers in an STZ-induced type 1 diabetic rat in non-infected[45] and infected[20] wound models. Our outcomes displayed that stereological parameters were significantly improved in PBM groups compared with CGs. The RT-PCR findings also revealed greater mRNA levels of HIF-1α in the wound area (including fibroblasts and intrinsic stem cells) in the PBM groups compared with the CG groups.[20,45] Therefore, in the present experiment, it was assumed that PBM may enhance the activity of stem cells and induce HIF1α overexpression in allograft ADS in DM2 rats. HIF1 upregulation in ADSs under the effect of PBM increases the wound-healing benefits of ADS on wounds associated with diabetes by enhancing ADS paracrine activity, thereby reducing oxidative stress and consequent DNA damage caused by ROS release and increasing their lifespan efficiency.

The present experimental work aimed to assess the effects of PMB on histological, antioxidant, and oxidative stress markers in a DIIWHM in DM2 rats. The results showed that the application of PBM significantly improved the wound healing of the DIIWHM in DM2 animals.

Conclusion

PBM treatments substantially enhanced proliferative and inflammatory stages in a DHIIWM in DM2 rats by controlling the inflammatory reaction and increasing granulation tissue creation. All treatment groups showed significantly increased antioxidant biomarkers (SOD, CAT) in the proliferation step. Similarly, the PBM groups showed meaningfully reduced NOX1 and NOX4 markers. Overall, our findings show that the PBM groups achieved significantly better results than the control groups. Additional research is required to provide information on the molecular processes of these therapies in the healing of wounds caused by diabetes.

Authors' Contribution

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Resources: Atarodalsadat Mostafavinia and Abdollah Amini.
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Writing–original draft: Mohammad Bayat, Abdollah Amini.
Writing–review & editing: Sufan Chien, Abdollah Amini.

Competing Interests

There are no competing interests.

Ethical Approval

The current research was authorized by the IRB Faculty of Medicine, Tehran Medical Sciences, Islamic Azad University (File no: IR.IAU.TMU.REC.1400.089).

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