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45S5 Bioactive Glass-Ointment Positively Effects on Wound Healing in Rats by Regulating TNFα, Il-10, VEGF, and TGFβ

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ABSTRACT

Aim: This study aimed to investigate the effects of 45S5 bioactive glass-ointment (BG) on cutaneous wound healing in rats at the molecular, biochemical, and histopathological levels.

Materials and Methods: Thirty-two rats were divided into four groups ($n = 8$): Control, Sham, BG, and DEX (Dexpanthenol). While no wound treatment was applied to the CONTROL, a wound model was created in the Sham, and no treatment was applied. A wound model was created for other groups, and BG and DEX were applied locally for 21 days. During the 21-day experiment period, feed and water consumption and weight changes were observed. Wound areas were calculated on days 0, 3, 7, 4, and 21. Following treatment, the rats were euthanized and tissues from the wound area and blood samples were collected. While the expression levels of tumor necrosis factor-alpha (TNFα), Interleukin 6 (IL6), Interleukin 10 (IL10), transforming growth factor-beta (TGFβ), and vascular endothelial growth factor (VEGF) genes were determined by qPCR, the levels of TNFα, IL6, and IL10 proteins were measured by ELISA.

Results: It was observed that the BG group showed anti-inflammatory activity by suppressing TNFα levels and stimulating IL-10. In addition, it was determined that BG increased fibroblast activity and vascularization.

Conclusion: Current findings showed that topical application of BG has anti-inflammatory effects, while also accelerating healing by increasing vascularity and making positive contributions to tissue healing.

1 | Introduction

The repair process of wounds encompasses a complex biological process that requires activation and coordination of various intracellular and intercellular pathways to reduce the risk of infection and increase healing [1]. Considering the large number of patients with chronic wounds, acute traumas, congenital abnormalities, diabetic wounds, and complicated wounds after surgical operation, wound healing remains a controversial issue in modern medicine [2].

There are many treatments and methods on wound healing. Despite a large number of studies involving treatment methods such as topical applications on the wound, wound dressings, laser

treatments, electrical stimulations, and surgical procedures, topical applications are more widely applied because they are advantageous both in terms of time and financially [3]. With the development of tissue engineering, it has led to the development of various treatment methods based on biomaterials for skin repair and regeneration [4]. BG is in the class of bioceramics. Its structural composition (45S5) consists of naturally occurring silicon dioxide (45%), calcium oxide (24.5%), sodium dioxide (24.5%), and phosphorus oxide (6%). Although it is produced for use in bone and tooth mineralization repair, it has been reported limited study to be effective in wound healing as it interacts with living tissue [5, 6]. Antibacterial, anti-inflammatory, and angiogenic effects are the most important properties of bioactive glasses [7, 8]. Due to its high surface reactivity, it has been reported to bind to soft tissues, similar to the way it binds to bone tissue [9]. In this context, it has been evaluated that bioactive glass has the potential to be used in various tissues in cases of inflammation [8, 9].

The wound healing process consists of four sequential stages including hemostasis, inflammation, proliferation, and tissue remodeling. Wound healing requires a series of organized processes such as fibroblast activity, angiogenesis, reduction of inflammation, non-forming of microbial activity, synthesis of healing-inducing cytokines [1]. Although BG plays an active role in all four steps of wound healing, which has been reported to accelerate skin repair and regeneration, to date, there has been no comprehensive report on its specific mechanisms of action in accelerating wound healing processes [10, 11].

This study aimed to evaluate the wound healing activity of 45S5 BG in excisional full thickness skin wound model in rats in terms of wound contraction and healing at the selected mRNA and protein levels as well as the histopathological changes. The suitability of 45S5 bioactive glass-ointment in wound treatments (in vivo and also at the molecular level) was investigated. While TNFα (Tumor Necrosis Factor-alpha), IL6 (Interleukin 6), IL10 (Interleukin 10), VEGF (Vascular Endothelial Growth Factor), TGFβ (Transforming Growth Factor Beta) expression levels were determined by qPCR, TNFα, IL-6, and IL-10 protein levels were measured with ELISA application.

2 | Materials and Methods

The study approval was obtained from the Local Ethics Board of Animal Experiments of Hatay Mustafa Kemal University (Decision no: 2020/03-1). This study was carried out at Hatay Mustafa Kemal University Experimental Research Application and Research Center. The research was conducted in accordance with the internationally accepted principles for laboratory animal use and care as found in the European Community guidelines (EEC Directive of 1986; 86/609/EEC).

2.1 | Preparation of Bioactive Glass-Vaseline Ointment

45S5 bioactive glass (BONE-G ACTIVE; REF: BGA025.0.5/2), supplied from Meta Bioengineering and R&D Services Inc., was used to produce bioactive glass-Vaseline based on composite wound ointments. Vaseline was used as a base material to prepare BG ointments. As it is known, Vaseline is a material that has been widely used as an ointment substrate in the clinic for decades. Although it does not have a positive or negative effect on wound healing, it keeps the wound moist and prevents it from drying out [12]. The bioactive glass-Vaseline composite ointments were produced according to earlier studies [13, 14]. Briefly, each BG powder with a mean particle size of $<50\,\mu m$ (16 wt.%) was gradually dispersed in melted (at 65°C) Vaseline (84 wt.%) until the composite ointment reached room temperature while being stirred constantly, and then, the cooled bioactive glass-Vaseline composite ointments were stored to use in vivo experiments.

2.2 | Experimental Design

Thirty-two male Wistar albino rats weighing 200–300 g, and 4 months old were used in the study. One week before the beginning of the study, the rats were kept in 23 ± 2°C room temperature, 12 h night and 12 h day conditions. Animals had free access to water and food and was given standard pellet feed. Rats were randomly divided into four groups and housed in individual cages. CONTROL group: No surgical procedure was applied to the animals in this group. SHAM group: This group was untreated after the wound was created. BG group: After the wound model was created on the animals, BG ointment was applied to the wound area for 21 days. DEX group: After the wound model was created in the animals, dexpanthenol ointment (Bephantene Plus, Bayer, Germany) was applied to the wound area for 21 days.

2.3 | Anesthesia and Surgery

Rats were anesthetized with 10 mg/kg xylazine (Alfazyne, EGE-VET, Türkiye) and 50 mg/kg ketamine (Alfamine, EGE-VET, Türkiye). Their dorsal hair was shaved and disinfected with iodine. 2 cm^2 full-thickness wound was created in the interscapular region of the upper back of each rat. Treatments were made according to the aims of the groups and they were placed in individual cages. Soft bandage was applied to the rats on the first day after the wound was created. At the end of the 21st day, full-thickness tissue samples were taken from the injured area under anesthesia, and then, blood was collected from the heart. Tissue samples were divided into three parts. While first part was used for histopathological examination, the other parts were frozen in liquid nitrogen for mRNA and protein analysis.

2.4 | Wound Area Measurements

Wound areas of all animals were photographed individually by a digital camera at 0, 3, 7, 14, and 21 days after wound creation. The wound surface area (cm^2) was measured using Image Jx2 software.

2.5 | RNA Isolation and cDNA Synthesis

Total RNA was isolated from tissue samples according to the modified Trizol method [15]. Approximately 50 mg of tissue

from each sample was homogenized in 1 mL of Trizol Reagent (Thermo Fisher Scientific, USA). Total RNAs obtained after trizol-chloroform-isopropyl alcohol and ethyl alcohol steps were diluted with 20–50 μL of nuclease-free water according to pellet size. The purity (A260/A280 ratio) and concentration values of the isolated RNA were measured with a nucleic acid meter (Merinton SMA-1000, Spectrophotometry, China). In addition, RNA quality was checked electrophoretically (100 V and 30 min).

Genomic DNA digestion was applied to the samples via DNase I kit (DNase I, RNase free, ThermoFisher Scientific, USA) in order to eliminate the genomic DNA contamination that may occur in the samples. Then, cDNA synthesis was performed according to the relevant kit protocol (High Capacity cDNA Synthesis Kit, Thermo Fisher Scientific, USA). The reaction was carried out in a thermal cycler for 60 min at 42°C, 5 min at 25°C, and 5 min at 70°C. After the reaction, the final volumes of the samples were made up to 200 μL with nuclease-free water and samples were stored at −20°C until qPCR application.

2.6 | qPCR Application

Expression levels of target (TNFα, IL6, IL10, VEGF, TGFβ) genes in samples were determined by qPCR (Rotor-Gene Qiagen, USA). After 10 min of denaturation, the reaction protocol was applied in the device for 40 cycles at 95°C for 15 s and at 60°C for 60 s. Amplification was performed using a kit containing SYBR Green dye (Power SYBR Green PCR Master Mix, Thermo Fisher Scientific, Cat no: 4367659, USA). While using the PPIA housekeeping gene as internal control, each sample was run in duplicate. Forward and reverse sequences of primers used for amplification of genes are shown in Table 1.

2.7 | ELISA Application

The levels of TNFα, IL6, IL10 proteins in tissue and plasma samples were determined by ELISA method. First, the tissues stored at −80°C were homogenized in PBS (Phosphate Buffered Saline). Then, total protein analysis in tissue samples was performed according to the Lowry method [18]. The levels of TNFα, IL-6, and IL-10 proteins in plasma and tissue were determined by rat-specific ELISA kits (Bioassay Technology Laboratory, E0764Ra, E0135Ra, E0108Ra, respectively, China) with ELISA Reader (AMR-100; Allsheng, China) at 450 nm wavelength. Target protein levels in tissues were calculated as ng/mg protein.

2.8 | Histopathological Analysis

Tissue samples were fixed in 10% neutral formaldehyde solution for 24–48 h. Afterward, paraffin blocks were obtained by going through routine tissue follow-up processes. Sections of 4–5 μm were taken from paraffin blocks and stained with Hematoxylin-eosin (H-E). Sections were examined under a light microscope (Olympus BX51, Japan). In microscopic examination, histopathological changes such as ulcer and re-epithelialization, fibroblast proliferation, inflammatory cell infiltration, neovascularization, and collagen deposition were evaluated [19]. Re-epithelialization and ulcer; If it has just started from the wound edge or is not present at all (+++); If there is a small opening adjacent to the epithelialization (++); Completed re-epithelialization was scored as (+). Other histopathological changes were scored semiquantitatively by a blinded pathologist as absent (0), mild (+, <30% in area appearance), moderate (++, 31%–60% in area appearance), and severe (+++, >61% in area appearance).

2.9 | Statistical Analysis

In the study, one-way ANOVA and Tukey test, one of the post hoc tests, were performed via SPSS (Version 26.0) program. Data were expressed as mean ± SE or mean ± SD. $p < 0.05$ was considered statistically significant. The expression levels of target genes were normalized according to the housekeeping gene and calculated as fold change [20]. The comparison of the detected protein levels between the groups was made with the one-way ANOVA using the SPSS (Version 26.0) program and the Tukey test, one of the post hoc tests. $p < 0.05$ was considered statistically significant.

TABLE 1 | Forward and reverse sequences of primers.

Genes	Forward and reverse sequences	Product length (bp)	References
PPIA	F: 5′-CAGACAAAGTTCCAAAGACAGCA-3′ R: 5′-CACCCTGGCACATGAATCCT-3′	117	[16]
TNFα	F: 5′-ACTGAACTTCGGGGTGATCG-3′ R: 5′-GCTTGGTGGTTTGCTACGAC-3′	153	Designed in this study
IL6	F: 5′-CTCTCCGCAAGAGACTTCCA-3′ R: 5′-TCTCCTCTCCGGACTTGTGAA-3′	92	Designed in this study
IL10	F: 5′-TTGAACCACCCGGCATCTAC-3′ R: 5′-CCAAGGAGTTGCTCCCGTTA-3′	91	Designed in this study
TGFβ	F: 5′-TGACGTCACTGGAGTTGTCC-3′ R: 5′-CCTCGACGTTTGGGACTGAT-3′	141	Designed in this study
VEGF	F: 5′-TCTCCCAGATCGGTGACAGT-3′ R: 5′-GGCAGAGCTGAGTGTTAGCA-3′	71	[17]

3 | Results

3.1 | Gross Observations of Full-Thickness Wound Healing

Body weight, feed consumption, and water consumption of rats in all groups at the specified time intervals are given in Figure 1. There were no statistically significant differences between the groups in terms of feed consumption (Figure 1a) and body weight averages (Figure 1b). The SHAM group consumed the lowest water and the CONTROL group consumed the highest water throughout the process ($p < 0.05$). When the water consumption data in the Day 7 period were examined, it was determined that the water consumption amount of the BG group was less than the CONTROL group and more than the SHAM group ($p < 0.05$) Considering the water consumption statistical data in the Day 14 and Day 21 period, it was determined that the BG group showed similar results with the CONTROL group, while it consumed statistically more water than the SHAM group ($p < 0.05$).

3.2 | Wound Area Results

The closure in the wound areas was calculated at the specified time intervals and given in Figure 2. Wound areas were recorded at the specified time intervals and area calculations were made. The wound area on days 3, 7, 14, and 21 was significantly less in the BG and DEX groups than in the SHAM group ($p < 0.05$). On days 14 and 21, the wound area of the SHAM group was almost twice the size of the BG and DEX groups. The wound area results of the BG group were almost the same as the DEX group. All results are consistent with the wound healing visuals in Figure 3.

3.3 | Biochemical Parameters

AST, ALT, GGT, TP, UREA, and CRE values were examined and no statistically significant difference was observed between the groups ($p > 0.05$). Changes between groups are given in Figure 4.

3.4 | Gene Expression Levels

Compared to CONTROL group, TNF-α was upregulated in both SHAM and BG groups more than four folds ($p < 0.05$). But its levels were similar between CONTROL and DEX groups. Moreover, IL-6 expression levels were significantly higher in the SHAM and DEX groups than in the CONTROL group ($p < 0.05$). In the DEX group, statistically significant upregulation was determined in DEX group ($p < 0.05$). Furthermore, VEGF and TGFβ were found to be significantly upregulated in the BG and DEX groups compared to the CONTROL group (Figure 5).

3.5 | ELISA Results

Considering the protein levels in tissue and plasma, similar results were observed in the BG group with the CONTROL group in terms of TNFα, IL-6, and IL-10. Plasma TNFα levels were found to be significantly lower in the BG group compared to the SHAM group. The levels of TNFα, IL-6, and IL-10 proteins in tissue and plasma samples between groups were given in Figure 6.

3.6 | Histopathological Results

Skin layers (epidermis, dermis, hypodermis) in CONTROL group rats showed normal histology (Figure 8A). Significant histopathological changes such as ulcer and re-epithelialization, fibroblast proliferation, inflammatory cell infiltration, neovascularization, and collagen deposition were observed in the experimental groups (Figure 8B–D). The highest statistical score at which re-epithelialization started in the experimental groups was found in the SHAM group, followed by the DEX and BG groups, respectively. DEX and BG groups and BG and CONTROL groups were found to be statistically similar (Figures 7 and 8A–C). Fibroblast proliferation, inflammatory cell infiltration, and neovascularization were found to be higher in the SHAM group compared to the CONTROL group. It was observed that related scores decreased in the DEX and BG groups (Figures 7 and 8C,D). In addition, DEX and BG groups in inflammatory cell infiltration, neovascularization and fibroblast proliferation, and CONTROL and DEX groups in fibroblast proliferation were statistically similar. Although BG group decreased scores in collagen deposition, they were statistically similar to the SHAM group.

4 | Discussion

Wound healing is still a clinical problem today. Alternative treatments that promote healing are needed [21, 22]. Scientific research reports published by the World Health Organization (WHO) and independent researchers show that the technological development and use of medical treatments and care materials continues to increase [23]. In the presented study, the effects of BG on the wound on the full-thickness skin wound we created in the dorsal scapular region of rats were examined at molecular, biochemical, and histopathological levels.

In vivo studies examining the effects of 45S5 bioactive glass on wound healing are limited [13, 24], and findings regarding wound healing mechanisms are insufficient in the literature. Although similar markers were evaluated in this study with other studies, markers effective on the inflammatory process and tissue healing were examined in detail, and remarkable and new findings were detected regarding the mechanism of action of 45S5 bioactive glass on wound healing time.

Feed and water consumption in experimental animals is an indicator for welfare and stress assessment [25]. There was no difference between the groups in terms of feed consumption and body weight averages. The fact that BG was the group with the highest water intake among the wound groups was thought to reduce stress factors such as itching or pain on the wound. The significantly lower water consumption of the SHAM group may be associated with a negative effect on the stress caused by the wound or the general clinical appearance of the animals [25].

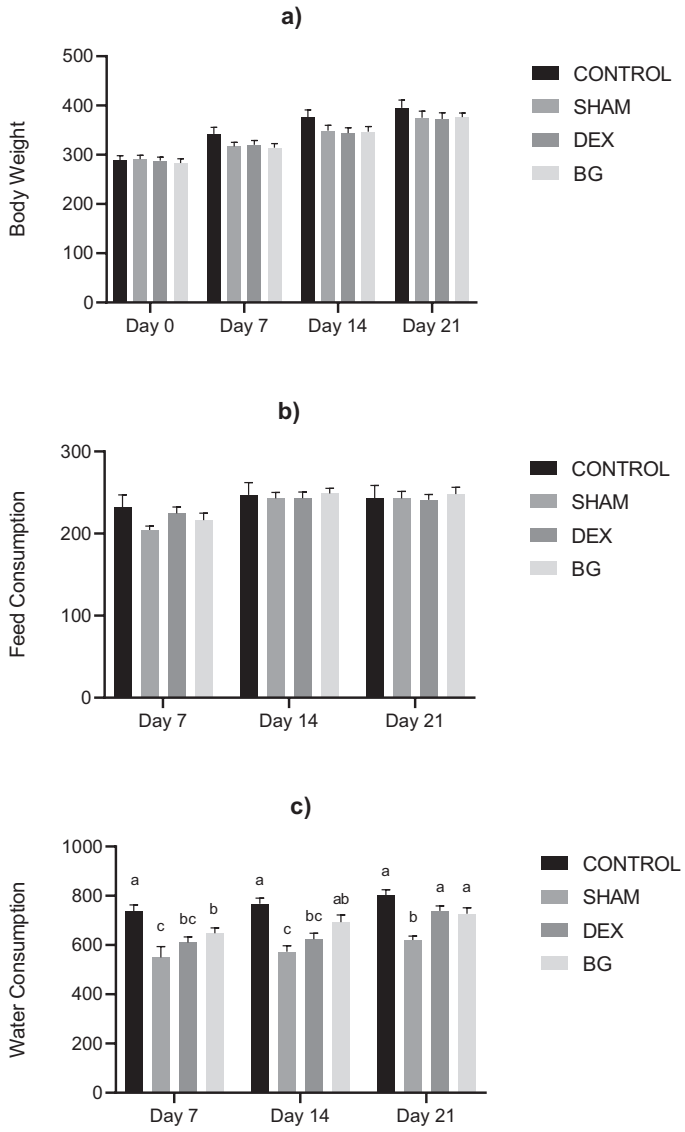

FIGURE 1 | (a) Results of weekly body weight changes by groups (Mean ± SD). (b) Results of feed consumption in groups (Mean ± SE). (c) Results of water consumption in groups (Mean ± SE). $p < 0.05$. a–c: Different letters in same line indicate the difference between groups ($p < 0.05$).

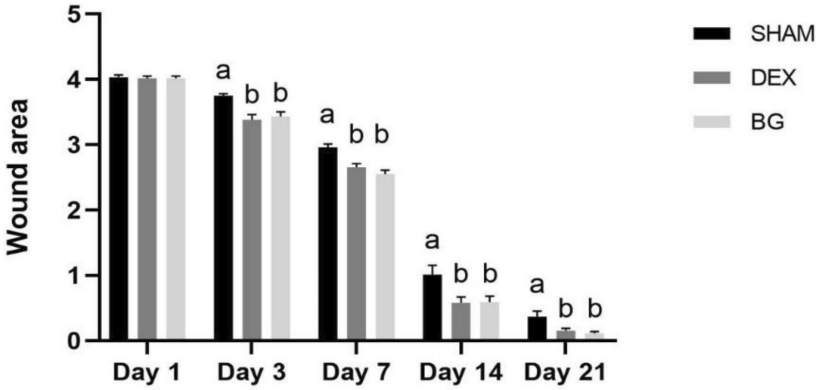

FIGURE 2 | Comparison of wound areas (cm^2) between groups (Mean ± SE). $p < 0.05$. a,b: Different letters in the same line indicate the difference between groups.

Studies have reported that the most effective method to clinically evaluate wound healing is to measure wound areas [24, 26, 27]. In our study, based on significant differences in wound area measurements, time group comparison results of wound areas showed that BG gave better results in topical use than the SHAM group. In addition, similar results were seen in the DEX group

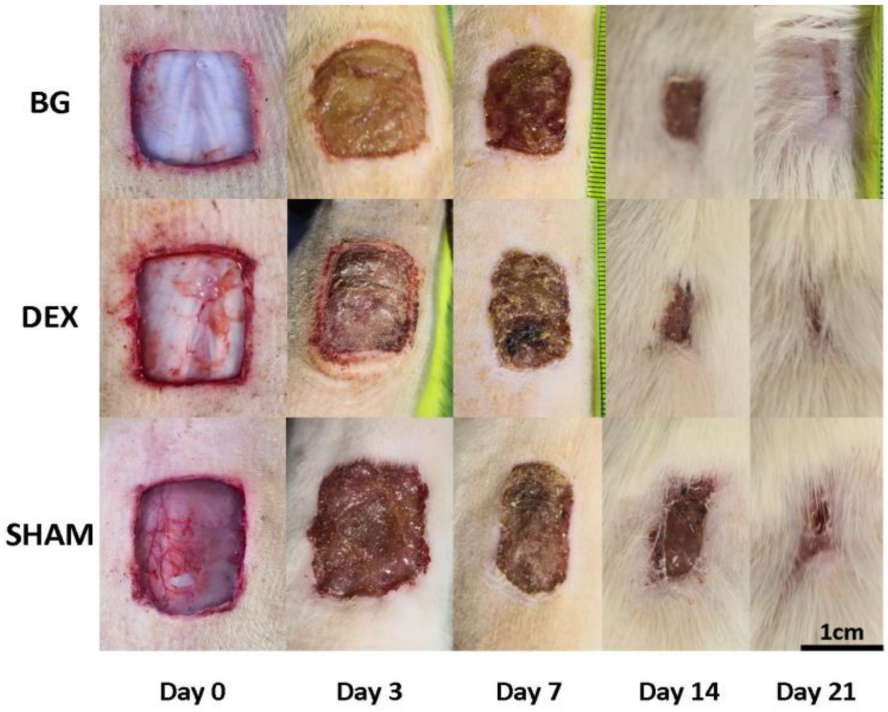

FIGURE 3 | Representative images of the wound healing process on different days in treated rats.

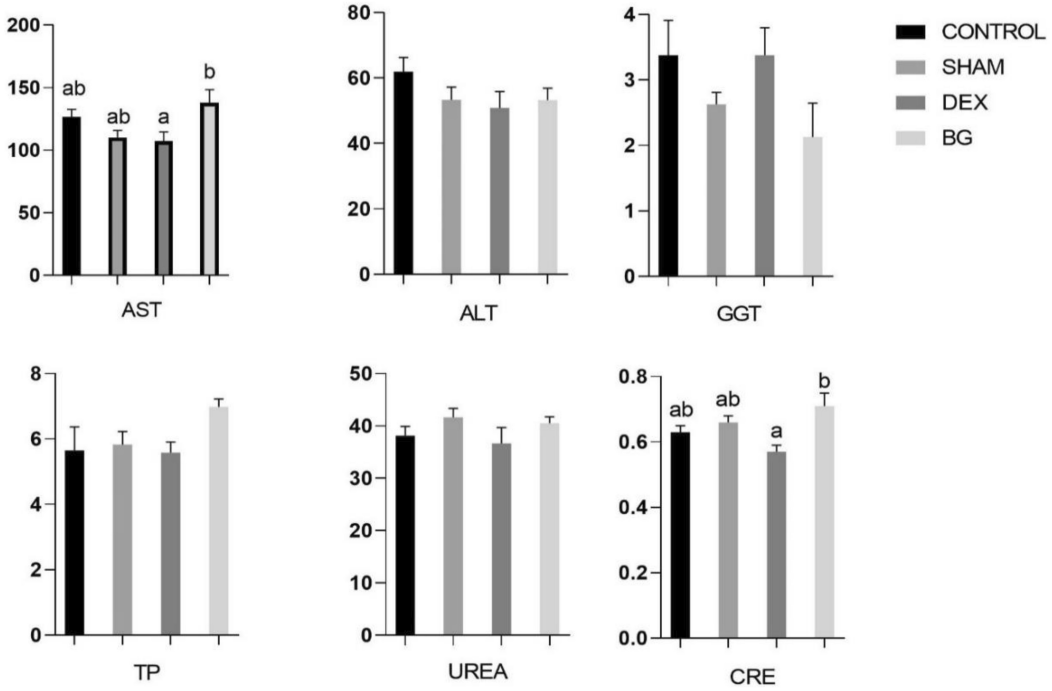

FIGURE 4 | Statistical comparison of some biochemical parameters between groups (Mean ± SD). $p < 0.05$. a,b: Different letters in the same line indicate the difference between groups.

(Bepanthene Plus Bayer, Germany) containing the active ingredient Dexpanthenol, which is used commercially as a wound cream and has a positive effect on wound healing (Figure 2).

Conditions such as oxidative stress and inflammation in the organism are under the control of complex molecular mechanisms [28–30]. While TNFα gene expression levels were expected to be high in all experimental groups, it was found to be similar to the CONTROL group in the BG group. As it is known, TNFα is an important inflammation mediator that plays a role in the response to inflammation in maintaining homeostasis in tissues and organs [28, 31]. The fact that this cytokine was similar to the CONTROL group in tissue mRNA level in the BG group suggests that the BG used in the study acts on TNFα in suppressing inflammation. In the study, TNFα gene expression levels as well as TNFα protein

levels detected in the tissue showed similarities between the groups, but they were found at similar levels to the CONTROL group in both DEX and BG groups. This indicates that TNFα is suppressed at both mRNA and protein levels due to the use of bioactive glass.

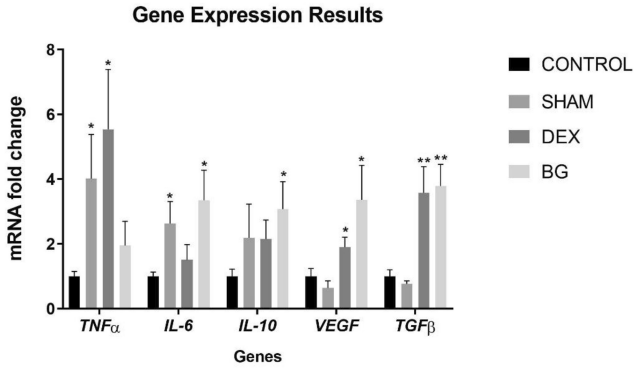

FIGURE 5 | Gene expression levels in groups (*$p < 0.05$, **$p < 0.01$).

Following inflammation in tissues and organs in the organism, neutrophil infiltration increases and proinflammatory cytokines such as TNFα and IL-6 are activated [28, 32, 33]. The levels of IL-6, another important cytokine that plays a role in the response to inflammation, were detected in the DEX group at similar levels to the CONTROL group, both at the mRNA and protein levels. Although BG suppresses TNFα level, its effectiveness on IL-6 was not as much as DEX, however, it was determined that it provided protein amounts at similar levels to the CONTROL group.

IL-10 is an important cytokine in providing anti-inflammatory activity [34, 35]. In line with the findings obtained in the study, it was determined that the significant increase in IL-10 gene expression levels among the experimental groups was only in the BG

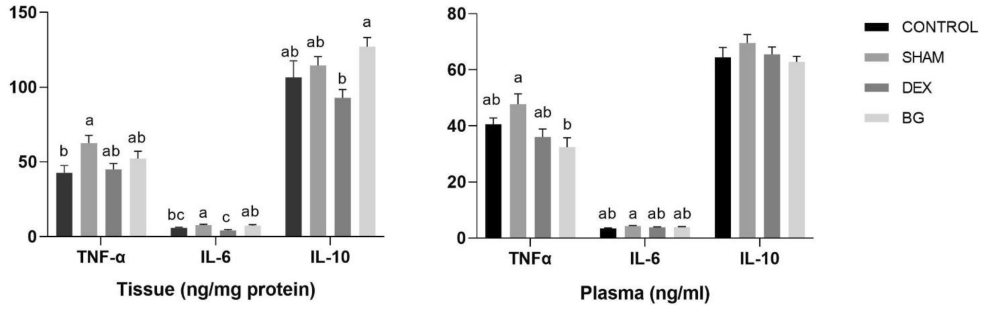

FIGURE 6 | Levels of TNFα, IL-6, and IL-10 proteins in tissue and plasma samples (Mean ± SE). $p < 0.05$. a,b: Different letters in the same line indicate the difference between groups.

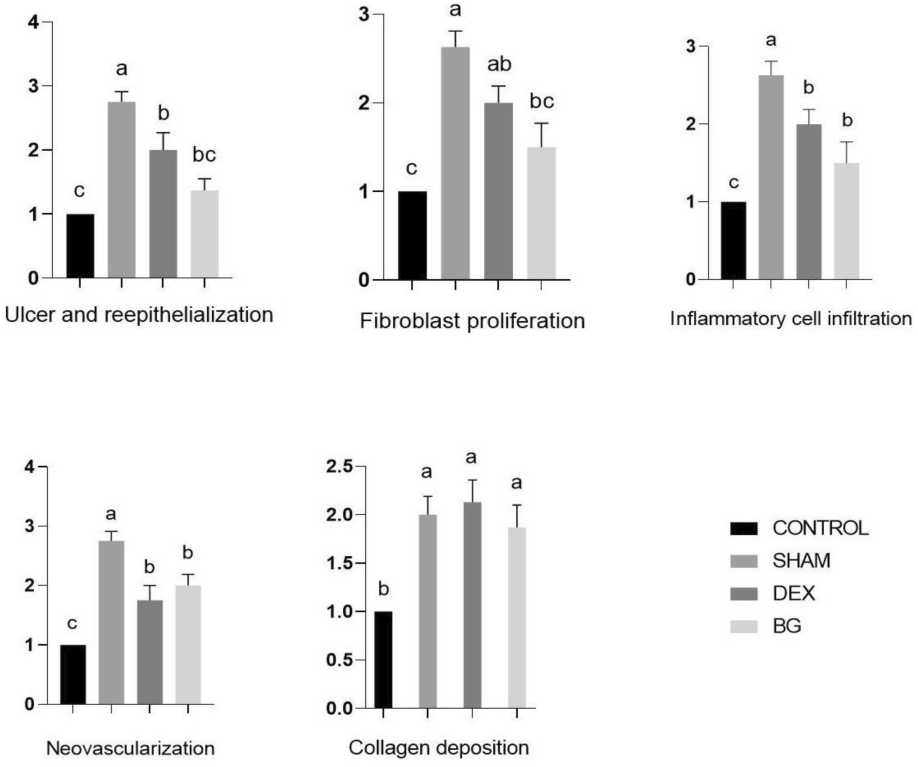

FIGURE 7 | Statistical values of histopathological results between groups (Mean ± SE). a,b,c: Different letters in the same line indicate the difference between groups.

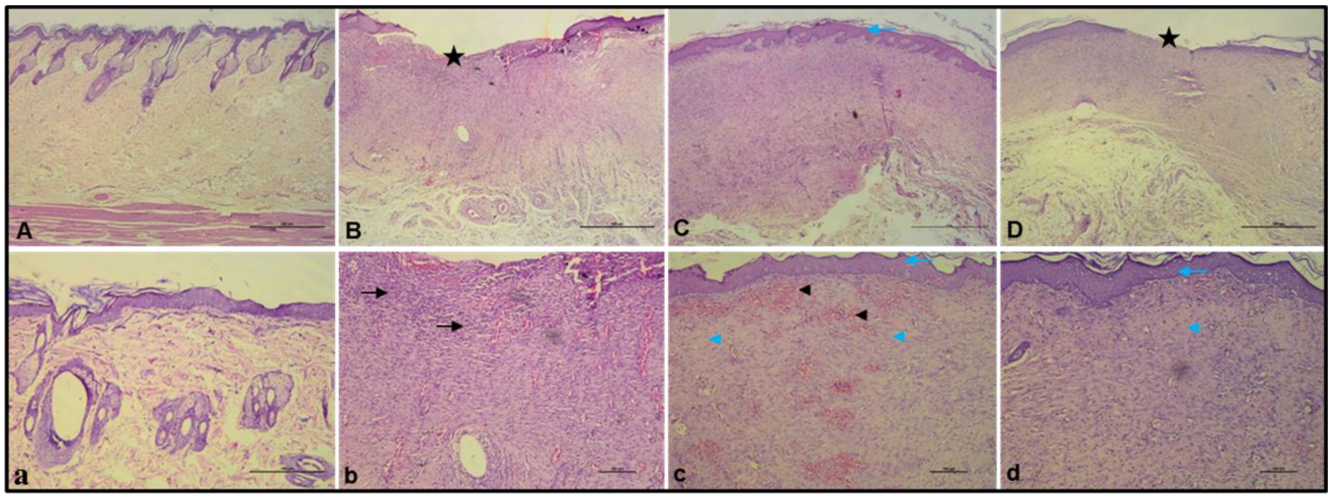

FIGURE 8 | Intergroup histopathological examination, H-E, ×10, 40. A, a; CONTROL, B, b; SHAM, C, c; BG, D, d; DEX, Ulcer (star), re-epithelialization (blue arrows), inflammatory cell infiltration (black arrows), neovascularization (black arrowheads), collagen deposition (blue arrowheads).

group. In addition, although the protein levels of IL-10, which has a major role in the anti-inflammatory response, increased in tissue in all experimental groups, the highest increase was found in the BG group, similar to gene expression levels. In a study conducted on the effect of BG on human macrophages and monocytes, they reported that BG increased IL-10 levels, although not significantly, and decreased IL-6 and TNFα levels significantly [36]. Xie et al. [37] reported that the *TNFα* level of BG was high on the first day in their diabetic wound model, but decreased on the fifth day and was the same as the CONTROL group. The number of studies examining BG applications in the wound healing model is limited. However, in parallel with the literature presented in our study, it is possible to say that the BG in question suppresses inflammation through the TNFα pathway and has an anti-inflammatory effect due to the increase in IL-10. Unlike other studies, the levels of TNFα, IL-6, and IL-10 in plasma of BG applied locally to the wound area were investigated systemically. The findings confirm the tissue levels of these cytokines, and only IL-10 plasma protein levels were found to be similar between the groups. This showed that IL-10 was more locally effective.

Vascular endothelial growth factor is the most functional angiogenic factor in wound healing [38–40]. It contributes significantly to tissue nutrition and healing by increasing vascularization in the injured area [41]. As expected, VEGF gene expression levels were upregulated in the DEX group, which was considered as the positive CONTROL group in the current study. In addition, VEGF gene expression levels were significantly increased in the BG group, which was hypothesized to have positive effects on wound healing, compared to the CONTROL. Mao, Lin, and Chen [13] reported in a diabetic wound model study that when BG is used together with Greek Baio oil, it increases VEGF activity, increasing vascularization and has a positive effect on healing. In another study, it was reported that BG kept VEGF levels at similar levels to the CONTROL group in a diabetic wound model [37]. Considering that different results were given in the literature, it was determined that the BG used in this study supported wound healing in the tissue by showing an effect on VEGF. Transforming growth factor-beta (TGF-β), another important gene whose activity was investigated in the study, is reported to have functions such as circulating fibroblast proliferation and infiltration in the wound healing process, supporting wound healing and stimulating collagen production [42, 43]. In the study, it was determined that TGF-β gene expression levels were significantly upregulated in both DEX group and the BG group compared to the CONTROL. Considering its role in wound healing, the increase in the mRNA level of this factor in the BG group was evaluated as positive. In line with the results obtained in the study, it is possible to say that BG supports wound healing at the molecular level.

Wound healing involves different biological processes such as epithelialization formation, contraction, and fibrosis. The healing process largely depends on the biosynthesis, accumulation, and subsequent maturation of new collagens [44, 45]. In our study, ulcer and re-epithelialization and fibroblast proliferation scores were found to be reduced in the BG and DEX groups compared to the SHAM group (Figure 7). In this context, the statistical similarity to DEX in the BG-treated group indicates that it has a significant effect on the wound healing process. In addition, during the wound healing process, on the 21st day, the number of fibroblasts in the wound area decreases, the amount of collagen reaches balance and mature granulation tissue forms [46]. In this study, the fibroblast proliferation statistical score was lower in the BG group compared to the DEX group, revealing that it accelerated the healing process at the end of the 21st day.

Inflammatory cells that infiltrate the area in the early stages of wound healing undertake many pathophysiological tasks such as phagocytosis, migration of endothelial cells, and induction of proliferation, affecting the healing process [44, 47, 48]. Long-term inflammation in the later stages of the wound healing process can lead to some negative effects that affect wound healing [49]. Recently, many different agents have been used on wound healing and the anti-inflammatory properties of these agents have been highlighted [19, 48]. In this study, statistically significant increases in inflammatory cell infiltration were detected

in the treatment groups compared to the CONTROL group, in accordance with the literature. It was observed that the scores in the BG and DEX groups decreased significantly compared to the SHAM group (Figure 7d). It was also found that the scores of the BG group were one step ahead compared to the DEX group. In light of our qPCR and ELISA findings, it can be concluded that the anti-inflammatory feature of bioactive glass plays an important role in its effect on wound healing.

5 | Conclusion

According to the findings of our study, it is thought that topical application of BG has anti-inflammatory effects and at the same time accelerates healing by increasing vascularity and makes positive contributions to tissue healing. In addition, the general similarity of BG results with DEX results, which has been proven to be effective on wounds, shows that BG may have a positive effect on wound healing. In conclusion, although the data of this study presented the positive effects of BG on healing of skin wounds with different analysis methods, more detailed studies are needed to fully reveal the healing mechanism.

Author Contributions

Kırgız Ö. conducted the experimental trial and wrote the original text. Altuğ M.E. developed the concept and designed the experiment and edited the final draft. Özkan H. analyzed the data and graphic design. Han M.C. analyzed the data. Akçakavak G. analyzed the data. Özarslan A.C. conducted the preparation of bioactive glass and bioactive glass-ointment. Yücel S. conducted the preparation of bioactive glass and bioactive glass-ointment.

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Ethics Statement

The experimental procedures employed in the investigation were approved by the Local Ethics Board of Animal Experiments of Hatay Mustafa Kemal University (Decision no: 2020/03-1). The research was conducted in accordance with the internationally accepted principles for laboratory animal use and care as found in the European Community guidelines (EEC Directive of 1986; 86/609/EEC).

Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

The authors have nothing to report.

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