Efficacy of neutral electrolyzed water vs. common topical antiseptics in the healing of full-thickness burn: Preclinical trial in a mouse model

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Abstract. Burn injuries impose challenges such as infection risk, pain management, fluid loss, electrolyte imbalance and psychological and emotional impact, on healthcare professionals, requiring effective treatments to enhance wound healing. The present study evaluated the efficacy superoxidized electrolyzed solution (SES), with low (SES-low) or high (SES-high) concentrations of active species, alone or in combination with a formulation in gel (G), in comparison with commonly prescribed treatments for burn injury, including nitrofurazone (NF) and silver sulfadiazine (S); normal saline was used as placebo (PI). A scald burn model was established in BALB/c mice. Measurements of the burned area and histological parameters such as inflammatory infiltration state, epithelial regeneration and collagen fibers were evaluated on days 3, 6, 9, 18 and 32 to assess healing score and status. All treatments achieved wound closure at day 32; histopathological parameters indicated that SES-low and SES-low + G performed better than the Pl and S groups (P<0.05). All treatments showed a lower count of inflammatory cells compared with S (P<0.05); for collagen deposition and orientation, SES-low + G showed a more uniform horizontal orientation compared with Pl, SES-high + G, NF and S groups (P<0.05). SES-Low was the most effective substance to induce favorable and organized healing, while S was the worst, inducing disorganized closure of the wound due to a pro-inflammatory effect.

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Introduction

Burn wounds are classified as first-, second- or third-degree according to the depth and severity of damage caused to the inner layers of the skin (1,2). Third-degree or full-thickness burns are particularly aggressive wounds that present considerable challenges for healthcare professionals since they extend below the epidermis and cause damage to the dermis and subcutaneous adipose tissue (3-5). This type of injury requires hospitalization and surgical intervention due to high risk of infection, shock and death; healing is slow and hypertrophic scarring is common (6). The worldwide incidence of burns is close to 9 million injuries each year with >2.2% being fatal (7,8). For non-fatal injury severe enough to hospitalization, long-term physical and psychological consequences include chronic pain, limited mobility, permanent scarring and post-traumatic stress disorder (9,10). The treatment of third-degree burns is individualized therapy depending on medical condition and affected organs. Considering only the wound healing aspect, the process is slower and more complex compared with other types of burn. However, it still follows several distinct stages: inflammation, proliferation and remodeling (6). Various treatments are employed to enhance skin

healing and improve patient outcomes, including skin grafts, polymeric membrane dressings, patches or gel (G) containing growth factors, delivery of adult mesenchymal stem cells to the wound and other skin substitutes (6,9,11-16). Additionally, because burn wounds are particularly susceptible to infection, topical antiseptics are crucial in wound care as they help prevent infections (16). Therefore, an antiseptic must be efficient and not hinder or negatively intervene in the healing process. Silver sulfadiazine (S; 1%) and nitrofurazone (NF; 0.2%) have been widely used due to their effective antimicrobial properties and general safety in improving healing (17-19). However, despite their effectiveness, it is important to consider factors such as availability, cost-effectiveness and the specific requirements of each treatment modality must be evaluated to make informed clinical decisions (20). In recent years, there has been growing interest in alternative antiseptics such as electrolyzed solutions (21-23). Superoxidized electrolyzed solution (SES) is noted not only for antimicrobial properties but also for its potential to efficiently promote wound healing, offering a promising addition to conventional treatment (22,23).

SES is produced from a saline solution of sodium chloride activated through controlled electrolysis process and pH control in a range of 6.5-7.5 (23). This process generates reactive species of chlorine and reactive oxygen species (ROS) (24). SES key reactive species include oxidant chlorine compounds, such as hypochlorous acid (HOCl), and ROS species, such as hydrogen peroxide (H_2O_2) (24-27). Several studies reported that SES exhibits properties similar to those of active compounds that are produced during the innate immune response; these include antimicrobial activity, an anti-inflammatory effects, decreased oxidative stress, promotion of tissue regeneration and angiogenesis and immune modulation, suggesting potential therapeutic benefits for wound healing and infection control (24-27).

SES has been shown to modulate inflammatory responses, promoting balanced healing and reducing excessive inflammation that can impede tissue regeneration (28-31). The regenerative properties of SES have also been explored in other areas, such as chronic wound and tissue engineering (32). SES also has showed the ability to stimulate angiogenesis, the formation of new blood vessels, which is key for supplying nutrients and oxygen to healing tissues (23,29,31,33). Moreover, SES has potential in modulating the production of growth factors and cytokines, facilitating tissue repair and regeneration (28-31,33).

Furthermore, some studies have highlighted the potential of SES in stimulating the proliferation and migration of various types of cells involved in wound healing, including keratinocytes and fibroblasts (26,28,32,34). These cells serve essential roles in re-epithelialization and production of extracellular matrix components necessary for tissue regeneration. By promoting cell activities, SES may contribute to accelerated wound closure and improved tissue regeneration (28-31,33,34).

The present study aimed to assess the efficacy of SES, at low (SES-low) and high (SES-high) concentrations of active species and compare it with commonly used antimicrobials for wound care in a preclinical murine burn wound model. Histological evaluation, focusing on wound closure, collagen fiber formation and orientation and the number of inflammatory cells, was performed to determine healing score and status, enabling a comparison of the efficacy of treatments and providing a comprehensive assessment of the wound healing process. By evaluating the advantages and disadvantages of treatments, the present results may contribute to the strategies to improve burn wound care.

Materials and methods

Treatments. SES was administered at two concentrations as a liquid solution and as gel formulations. The low-concentration SES (SES-low) contained 20 parts per million active species of chlorine and oxygen (0.002%), has a pH of 6.5-7.5 and oxidation-reduction potential of ~850 mV (Estericide® Solución Antiséptica; Esteripharma® S.A. de C.V.; cat. no. 0412C2016 SSA). Gel formulation is commercially available as Estericide® Gel Antiséptico (cat. no. 1594C2014 SSA). The high concentration SES (SES-high) contained <80 parts per million (>0.008%) chlorine (Microdacyn® Solución Antiséptica; Aerobal S.A. de C.V., México; cat. no. 1075C2003 SSA). The hydrogel formulation is commercially available as Microdacyn® Hydrogel Gel antiséptico and contains ≥40 parts per million of free chlorine (cat. no. 0176C2014 SSA). NF ointment was used at a concentration of 0.2% as the commercially available Furacin® (Siegfried Rhein® S.A. de C.V; cat. no. 31258 SSA) and S cream was used at a concentration of 1% as the commercially available Bioargirol-C (Bioresearch de México S.A. de C.V; cat. no. 489M2000 SSA). These products are widely available and frequently used in patients suffering burns covering <15 (adults) or 5% (children) of their total body surface area (19). Physiological saline solution (0.9% NaCl solution; PiSA Pharmaceuticals) was used as a placebo (Pl) in the control group.

Animals. Male BALB/c mice (n=252; Inotiv; age, 10-14 weeks; weight, 25-30 g) were used. The duration of the experimental procedure was 32 days. Mice were randomly assigned to seven groups, each comprising 36 animals as follows: Pl, SES-low, SES-low + G, SES-high, SES-high + G, NF and S. All animals were kept at 21±2˚C with 48% humidity in a 12/12-h light/dark cycle, with food and water provided *ad libitum*. The mice were kept in cages, with a maximum of 6 mice/cage.

The animal experiments were approved by the Research Ethics Committee of the Colima State Institute of Cancerology, Colima, Mexico (approval no. CIIECAN/06/19). Animals were handled in accordance with institutional guidelines (35) and the official Mexican standard for the care and use of laboratory animals (Official Mexican Standard NOM-062-ZOO-1999: Technical specifications for the production, care, and use of laboratory animals) (36-38), in addition to the eighth edition of the Guide for the Care and Use of Laboratory Animals prepared by the National Academy of Sciences of the USA (2011) (38). Mice were observed daily to assess for clinical signs of toxicity or distress, and behavioral changes were evaluated by functional observational battery parameters such ass salivation, lacrimation, signs of distress, changes in eating and drinking, activity levels and any signs of infection or discomfort at the wound site (39-41). Humane endpoints were weight loss >20% of body weight, severe illness, infection or necrosis at the wound site or any signs of severe distress, such as lack of grooming, abnormal posture or reduced activity (37,42). No

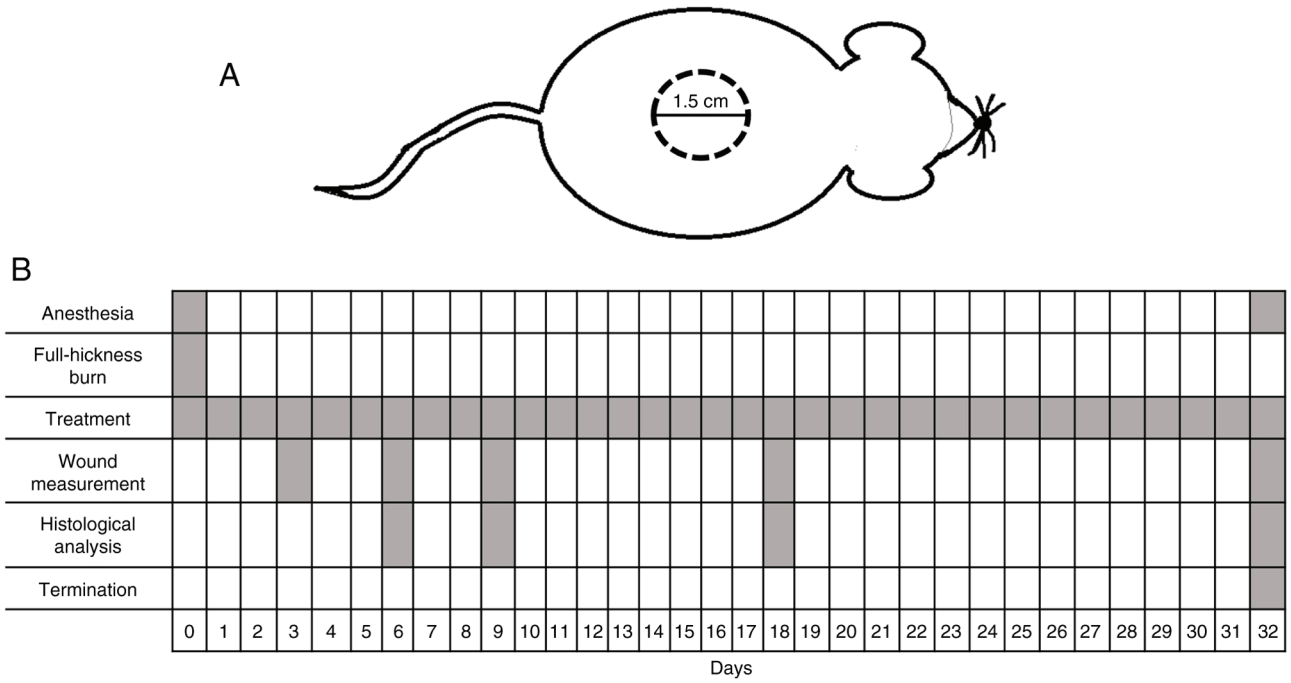

Figure 1. Experimental design. (A) Location and size of the full-thickness burn. (B) Experimental timeline. All treatments were applied once/day. Healing score/status was determined on day 32.

animals met the humane endpoints for euthanasia before the end of the experiment and none were found dead. Pain management included administering paracetamol (200 mg/kg) orally for the first 5 days (1,43-45) and ketamine (120 mg/kg) and xylazine (15 mg/kg) were used during burn induction and prior to euthanasia (45). Death was verified by cessation of heartbeat and respiration, as well as the absence of reflexes (46).

Full-thickness burn induction and treatment. The scald burn model was established as described by Abdullahi *et al* (47). At 1 day before the intervention, the dorsal area of the mice was shaved and depilated with cream (Nair Sensible, Reckitt Benckiser) for 30 sec and residues were removed with warm water. On the day of the intervention, mice were anesthetized using intraperitoneal ketamine (120 mg/kg) and xylazine (15 mg/kg; PiSA Pharmaceuticals®, Agropecuaria). Each mouse was placed in a supine position on a template of flame-resistant plastic mold, which included a window exposing the predetermined skin surface area. A test tube with 95˚C water was brought into direct contact with the exposed skin surface of the mouse for 7 sec, resulting in an oval burn with diameters of 1.5-2.0 cm (Fig. 1A).

A total of 12 mice/group was selected to investigate wound size change. Treatment was applied once/day for 32 consecutive days, starting on day 0 of the study (the day of the burn; Fig. 1B). Liquid was directly applied to the burn site, resulting in a total volume of ~1 ml product. For the gel, ~1 g was applied to the burn area using a sterile plastic applicator. On days 6, 9, 18 and 32, 6 mice/group were sacrificed by decapitation after being anesthetized as previously described (48).

Wound size and macroscopic evaluation. Periodic measurements of the wound area were taken on days 3, 6, 9, 18 and 32 as described by Zhang *et al* (49) with minor modification. The mice were immobilized and the contour of the wound was traced using a transparent graph sheet and marker. The resulting images were analyzed to determine the burn wound area. Wound area reduction was calculated using the following formula: Wound contraction (%)=100-[(wound size x100)/mean value of day 0 wound size]. Changes in morphology were documented by capturing images with a digital camera (Nikon AF-S VR Micro Nikkor; Nikon Corporation) at a constant focusing distance. The resulting images were analyzed using Fiji2.0 software (National Institutes of Health) (50). All images were captured under the same light and exposure.

Histopathological analysis. Samples of burn areas were surgically excised (1x1 cm) and rinsed with cold PBS following sacrifice and fixed in 10% neutral buffered formalin at room temperature for 24 h, washed, dehydrated with ethanol and embedded in paraffin. The obtained blocks were cut into 5-mm-thick tissue sections, mounted on glass slides, deparaffinized and rehydrated. Slides were stained with hematoxylin-eosin (H&E) at room temperature for 30 min for evaluation of inflammatory infiltration state and epithelial regeneration, and with Masson's trichrome at room temperature for 60 min to analyze collagen fibers (51-55). Each measurement was independently conducted by two qualified scientists in a blinded manner, ensuring unbiased data collection and analysis. Images were captured using a digital camera model Axiocam MRC-5 connected to a t bright-field optical light microscope model AxioPlan 2 M (Zeiss GmbH) with a motorized stage (total magnification, x100, 200 and 400). MosaiX and Autofocus modules were used to scan images of the entire sample surface and the lesions were measured using a calibration line. All images were captured under the same illumination and exposure times using the AxioVs

40 V.4.7.0.0 image software (Carl Zeiss Imaging Solutions GmbH). All histological data were obtained from 30 randomly selected fields of view from 6 mice (5 data/mouse). Counts of total inflammatory cells, polymorphonuclear neutrophils (PMNs) and mononuclear leukocytes (MNC) were manually determined using five randomly selected fields of view (magnification, x10 and 40). The inflammatory infiltration state was determined according to degree of inflammatory infiltrate, by assigning a semi-quantitative and discontinuous score: 1-plenty; 2-moderate and 4-few (51,52,54).

Samples stained with Masson's trichrome were analyzed using a Motic BA310E optical light microscope (Motic China Group Co., Ltd.; magnification, x10). A total of three microphotographs were captured for each tissue sample with a Moticam 1080 digital camera (Motic China Group Co., Ltd.) under the same lighting and exposure. The proportion, shape and type of collagen fibers were analyzed using Fiji 2.0 software. Collagen orientation was classified as follows: 1, vertical; 2 for mixed, and 4 for horizontal. The collagen patterns were categorized as: 1 for reticular, 2, mixed, and 4 for fascicular. The amount of early collagen was qualitatively evaluated as 1, profound; 2, moderate; 3, minimal and 4, absent. Mature collagen was classified as 1, profound; 2, moderate and 4, minimal (52-55).

Epithelial regeneration was evaluated by assessing the migration of cells to the wound edge, defined as the area where epithelial cells meet the edge of the wound, divided by the distance from the wound bed, the base of the wound where new tissue is forming, multiplied by 100% and scored as follows: 0-0; 1- >0 and<50; 2, ≥50 <100; 3-100% and irregular thickness, and 4, 100% and normal thickness (56). Additionally, quantitative (μm) and qualitative (yes/no) measurements of epidermal detachment visualized as separation of wound edges viewed at 2.5X magnification, as well as the thickness of the epidermal lesion (40X magnification), were performed (57). The number of blood vessels and follicles/field was included for evaluation, along with the presence or absence of scar tissue (58). Furthermore, a semi-quantitative assessment of granulation tissue (1, deep; 2, moderate; 3, scant and 4, absent) and a qualitative assessment of presence of the stratum corneum at 2.5X magnification was performed (51,52,59).

Calculation of healing score and status. Wound healing score and status were determined as described by Gupta and Kumar and Santos *et al* (51,52). The parameters assessed included granulation tissue amount, inflammatory infiltrate, collagen fiber orientation and pattern and early and mature collagen amount. The total healing score was calculated by adding the scores of individual criteria, with lower scores indicating poorer wound healing. Healing status was graded as follows: 8-11, poor; 12-15, acceptable and 16-19, good (51,52).

Statistical analysis. Data are presented as the mean and SEM (n=≥6. Normal distribution of data was determined using the Shapiro-Wilk test. Data were analyzed using one-way ANOVA for normally distributed data (parametric) or Kruskal-Wallis test for non-normal or ordinal data (non-parametric). Post hoc analysis was performed using Bonferroni's comparisons or Mann-Whitney U test (non-parametric) and Tukey's multiple comparison test (parametric). The statistical analysis was performed using IBM SPSS version 20 software (IBM Corp.) $P<0.05$ was considered to indicate a statistically significant difference.

Results

SES-low + G and nitrofurazone treatments induce the highest and early closure of the burn wound area. Fig. 2 shows representative pictures of the wound healing process and reduction of burn areas, providing a visual and quantitative assessment of the treatment outcomes.

On day 3, NF, SES-low + G, and S treatments exhibited the smallest wound areas, with closure of 59.7±5.9, 48.7±4.0 and 44.3±7.1%, respectively (Table SI). The NF group exhibited significantly greater wound closure compared with all other groups. SES-low + G showed better wound healing compared to SES-high, though not significantly different from the S group. Pl and SES-high groups had the poorest closure (Table SII).

On day 6, wound closure was highest in the SES-low + G (62.6±2.5%), NF (58.2±10.5%), and SES-high + G (59.2±9.2%) groups (Tables SI and SII). SES-low +G treatment demonstrated significantly better closure compared to the SES-low and SES-high groups.

At day 9, the SES-low + G (72.4±1.4%) and NF (67.8±12.0%) groups again exhibited the highest wound closure rates (Table SII). These were statistically different from the Pl and SES-high + G groups, showing superior wound healing outcomes.

From day 18 to 32, all treatments groups demonstrated similar wound healing progress, eventually reaching full wound closure (Fig. 2A). However, the SES-low + G and NF groups continued to exhibit the smallest final wound areas (Fig. 2B, Table SI), with the highest overall closure rates (Table SII). While SES-low + G outperformed the other treatments on days 6 and 9, the differences were not statistically significant (Table SII).

SES-low + G and SES-low treatment reduced inflammatory infiltrate in the burn wound area in the early and late states of healing. To assess the inflammatory response number of polymorphonuclear cells (PMNs) (Table SIII) and monocytes (Table SIV) at the days 6, 9, 18 and 32 of the wound healing process. The total inflammatory infiltrate was also measured, and multiple comparison tests were conducted to evaluate the significance of these counts across treatments (Table SV). As expected, abundant inflammatory infiltrate was observed in the early stages of wound healing as part of the typical course of the re-epithelization process (52,60-62) and reached maximum values on day 9 (Fig. 3A). On day 6, the S, NF and SES-high + G groups exhibited the highest cell/field values (92.90±7.19, 72.80±5.35 and 54.30±5.87, respectively). S showed the most abundant infiltrate, being significantly different from the rest of the groups except with NF. On the other hand, SES-low (29.33±3.20), SES-low + G (33.10±3.16) and SES-high (35.86±2.87) groups had similar effects to Pl (26.06±4.43).

On day 9, a general and significant increase in inflammatory infiltrate was observed (Table SV). However, SES-low (63.30±4.87) and SES-low + G (77.10±5.04 cells/field) groups exhibited significantly lower cell counts compared

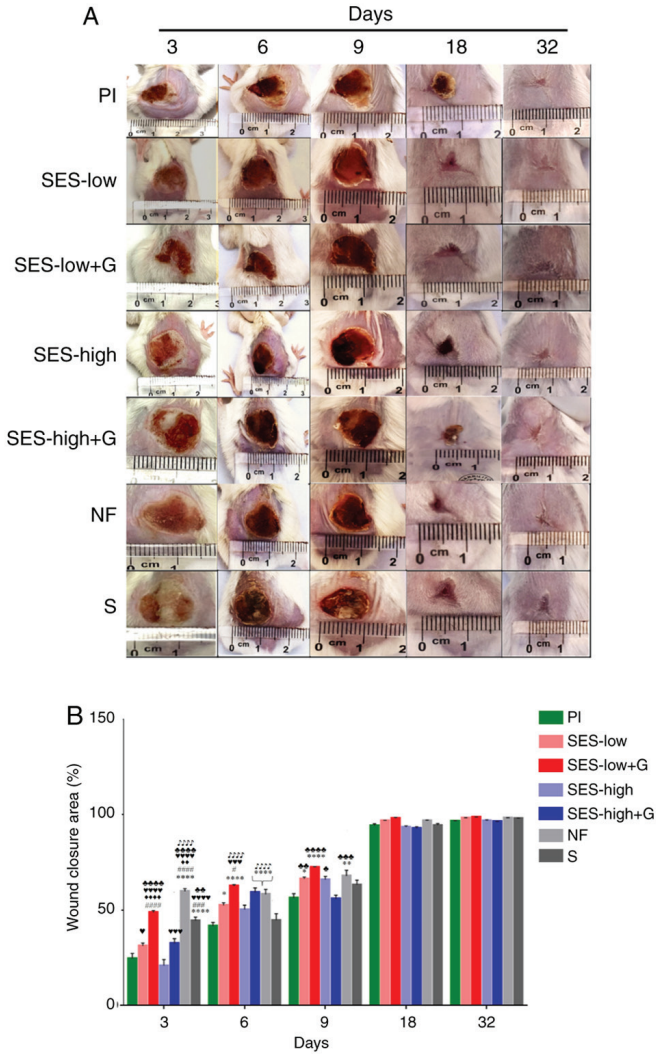

Figure 2. Macroscopic evaluation of burn wound Healing. (A) Morphology and macroscopic appearance of burn wound area. (B) Burn area reduction. *P<0.05, **P<0.01 vs. Pl, ****P<0.0001 vs. Pl, $^{\#}$P<0.05 vs. SES-low, $^{\#\#\#}$P<0.001 vs. SES-low, $^{\#\#\#\#}$P<0.0001 vs. SES-low, $^{\blacktriangledown\blacktriangledown}$P<0.01 vs. SES-low + G, $^{\blacktriangledown\blacktriangledown\blacktriangledown\blacktriangledown}$P<0.0001 vs. SES-low + G, $^{\blacktriangledown}$P<0.05 vs. SES-high, $^{\blacktriangledown\blacktriangledown\blacktriangledown}$P<0.001 vs. SES-high, $^{\blacktriangledown\blacktriangledown\blacktriangledown\blacktriangledown}$P<0.0001 vs. SES-high, $^{\blacklozenge}$P<0.05 vs. SES-high + G, $^{\blacklozenge\blacklozenge}$P<0.01 vs. SES-high + G, $^{\blacklozenge\blacklozenge\blacklozenge}$P<0.001 vs. SES-high + G, $^{\blacklozenge\blacklozenge\blacklozenge\blacklozenge}$P<0.0001 vs. SES-high + G, $^{\lozenge\lozenge\lozenge\lozenge}$P<0.0001 vs. S. SES, superoxidized electrolyzed solution; NF, nitrofurazone; Pl, placebo; S, silver sulfadiazine; and G, gel.

with all other groups. S (134.75±5.58) and NF (131.50±5.19 cells/field) groups had the highest levels of inflammatory infiltrate, followed by SES-high (120.93±5.77 cells/field), SES-high + G (119.93±5.00 cells/field) and Pl (110.90±6.47 cells/field) groups. No statistical differences were observed between SES-high, SES-high + G, NF, S and Pl groups. Fig. 3B shows histological images on day 9, demonstrating the differences in inflammatory infiltrate abundance, primarily macrophages (▲). SES-low and SES-low + G produced less infiltration in the tissue, indicating an anti-inflammatory.

On day 18, the inflammatory infiltrate all groups became similar, without no significant differences observed-The average cell count was 41.44±3.01 cells/field. By day 32, the counts of total inflammatory cells decreased in all groups. The S group exhibited the highest inflammatory infiltrate, with a mean value of 33.04±2.76 cells/field, which was significantly higher than the rest of the groups. SES-low and SES-low + G exhibited the lowest cell counts, with mean values of 16.53±1.20 and 18.00±1.01 cells/field, respectively. The cell counts in the SES-low group were significantly lower compared with those of the S and SES-high + G group (24.10±1.3). SES-low was better than SES-high at modulating the inflammatory process. Additionally, S and NF groups exhibited inflammatory infiltrate, suggesting an irritant effect. In the specifics counts of PMNs, the SES-low group had consistently lower cell counts throughout the study, maintaining modest values compared to the S and NF groups, particularly on day 32 where SES-low recorded 3.0±0.49 cells/field compared to S at 6.6±0.38 (Table SIII). Similarly, for monocytes, the SES-low group showed reduced counts at all time points, especially at day 32, with 13.5±1.54 cells/field compared to S, with 26.4±2.26 cells/field (Table SIV). This suggests that the treatment with SES-low and SES-low + G, may induce a regulated and balanced inflammatory response at the early and late stages of the healing process. S group induced a stronger pro-inflammatory reaction, particularly evident at later stages of wound healing.

SES-low + G, SES-low and Nitrofurazone treatments induced a more advanced progression in re-epithelization of burn wound area. On day 6, all groups exhibited epidermal detachment (Fig. 4). However, SES-low and SES-low + G groups showed a more defined lesion with indications of dermal recovery and greater differentiation of cutaneous layers, while Pl showed deeper burn damage. Subsequently, on day 9, a serohemorrhagic crust was present in all groups, indicating an ongoing repair process (+). By day 18, the repair and re-epithelization was indicated by epithelial edge junctions and the hair follicle presence (▲). The Pl, SES-high and NF groups did not show epithelial edge union, the process where wound edges come together as new skin forms, while SES-low, SES-low + G, S and SES-high + G-treated groups exhibited complete junction of epithelial edges. Additionally, traces of serohemorrhagic crust were observed in the Pl, SES-high + G and S groups. The presence of hair follicles in SES-low, SES-low + G and NF groups indicated a more advanced repair process (63-65). Furthermore, on day 32, complete healing and re-epithelization of the burn was observed in all groups, as evidenced by the union of edges, indicating full closure of the wound, and presence of a stratum corneum and hair follicles. The SES-low, SES-low + G, SES-high + G and NF groups showed a thicker stratum corneum (*), compared with Pl, SES-high, and S groups. Therefore, NF, SES-low + G and SES-low groups demonstrated a more advanced progression towards re-epithelization, characterized by a compact and well-defined serohemorrhagic crust.

SES-low + G, SES-low and Nitrofurazone generate better collagen matrix reorganization. The analysis of collagen parameters at days 6, 9, 18, and 32, was performed using Mason's staining (Fig. 5). The scores for orientation and amount of early or mature collagen at day 32 are presented in Fig. 6. Scores for days 6, 9 and 18 are shown in Figs. S1-S3, respectively while Tables SVI-SVIII provide the statistical analysis of these parameters. The collagen pattern showed no significant differences between groups on any of the days.

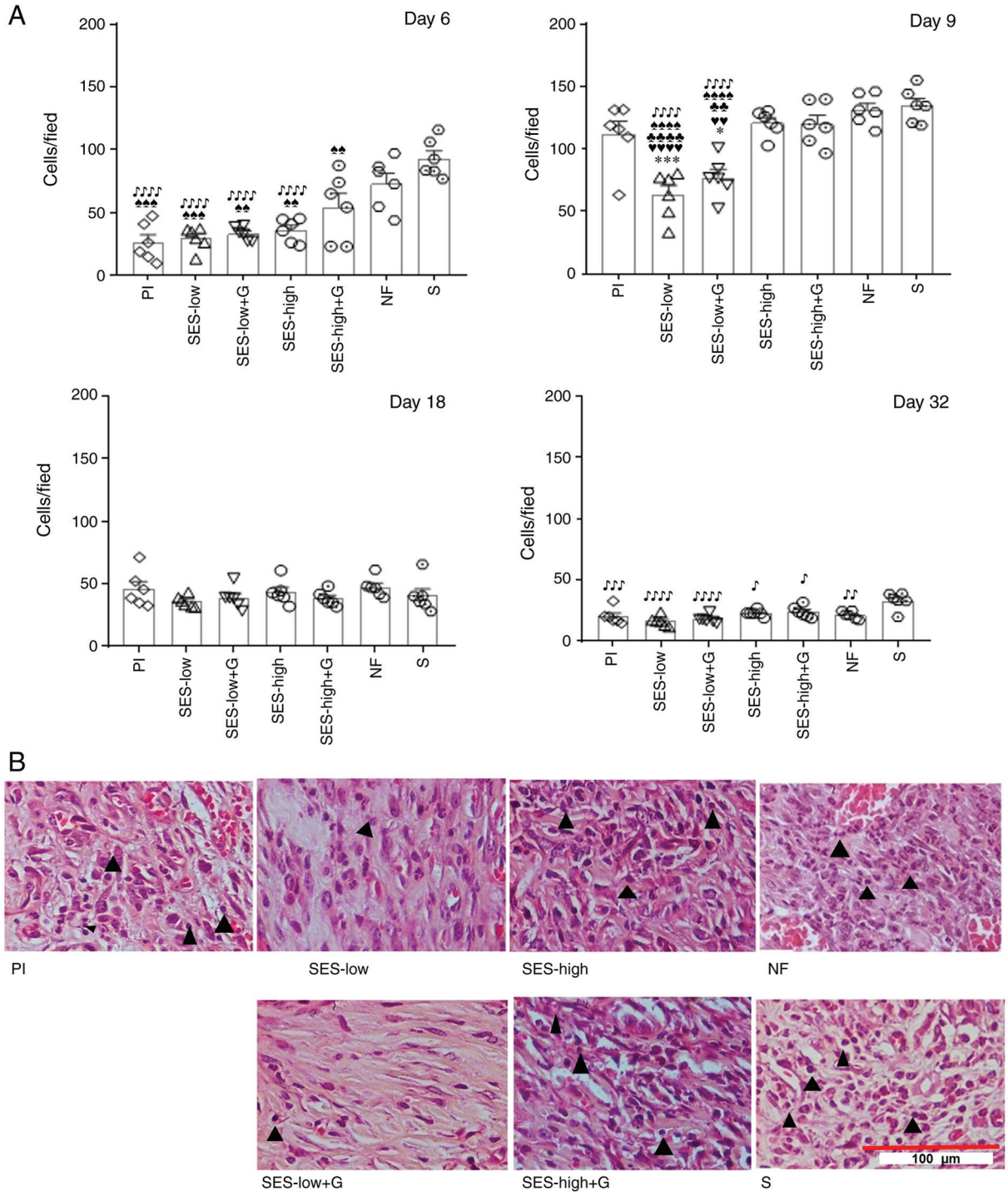

Figure 3. Inflammatory response and infiltrate abundance on day 9. (A) Amount of total inflammatory cells was significantly ifferent between the groups. *P<0.05, ***P<0.001 vs. Pl; ♥♥P<0.01, ♥♥♥♥P<0.0001 vs. SES-high; ♠♠P<0.01, ♠♠♠♠P<0.0001 vs. SES-high + G; ♣♣P<0.01, ♣♣♣P<0.001, ♣♣♣♣P<0.0001 vs. NF; ♪P<0.05, ♪♪P<0.01, ♪♪♪P<0.001, ♪♪♪♪P<0.0001 vs. S. (B) Inflammatory infiltrate abundance at day 9. Histological evaluation with hematoxylin/eosin staining, visualized with light microscopy (40X magnification). ▲ indicates macrophages. SES, superoxidized electrolyzed solution; NF, nitrofurazone; Pl, placebo; S, silver sulfadiazine; and G, gel.

Granulation tissue and inflammatory infiltrate were predominant at the early stages of wound healing with collagen deposition mainly early collagen (light blue) observed (Fig. 5). As wound healing progressed, increased collagen deposition was noted, with agglomeration of mature collagen, stained as deep blue, in SES-low, SES-low + G, SES-high, SES-high + G and NF groups, alongside a gradual decrease in inflammatory infiltrate and granulation tissue. By day 32 of follow-up, the collagen matrix reached its maximum in all groups, with statistical differences noted for minimal or absent deposition of young collagen in NF, SES-low and SES-low + G groups (Fig. 6) (66,67). Clear differences in the aggregation and organization of mature collagen were observed among treatments. In comparison with SES-low, S, NF and Pl showed thicker and more irregular deposition of mature collagen, with greater collagen matrix deposition. Vascularization and newly

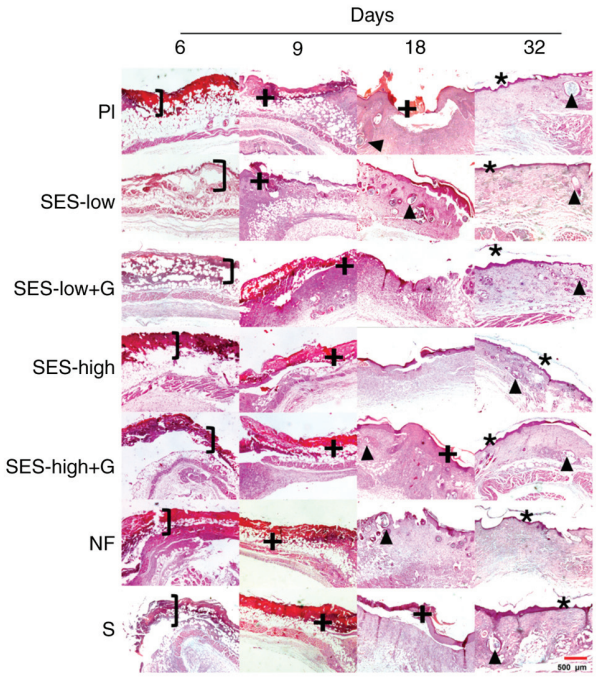

Figure 4. Repair of skin lesions. Histological evaluation with hematoxylin/eosin staining, visualized with light microscopy (2.5X magnification)] indicates epithelial detachment, ▲ indicates follicles; * indicates stratum corneum. The repair process includes formation of scar tissue (+), observed on days 9 and 18, showing re-epithelialization and healing of both the epidermis and the superficial and deep layers of the dermis. SES, superoxidized electrolyzed solution; NF, nitrofurazone; Pl, placebo; S, silver sulfadiazine; and G, gel.

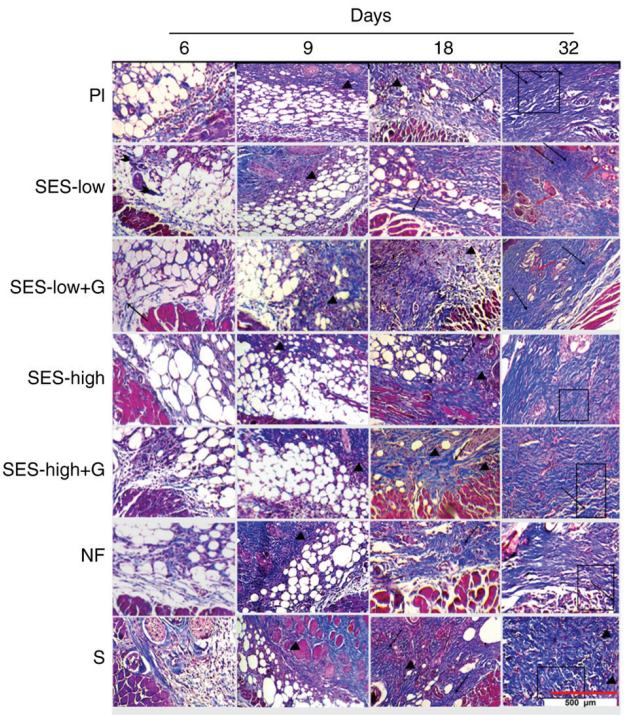

Figure 5. Collagen assessment using Mason's Trichrome staining Black arrows indicate mature collagen (black arrow). Boxes indicate disorganized or chaotic deposition of mature collagen. Red arrows indicate hair follicles and/or sebaceous glands. ▲ indicates inflammatory cellular infiltrate. SES, superoxidized electrolyzed solution; NF, nitrofurazone; Pl, placebo; S, silver sulfadiazine; and G, gel.

formed hair follicles were also noted in the SES-low group (Fig. 5) (66). The semi-quantitative analysis of collagen matrix deposition is shown in Figs. S1-S3.

At day 32, SES-low + G and SES-low exhibited the most uniform and horizontal orientation of collagen fibers, followed by SES-high (Fig. 5). This indicated better collagen matrix reorganization and improved final healing process (Fig. S4). Orientation analysis of collagen fibers at day 32 revealed significant differences between SES-low, SES-low + G and S, NF, Pl, and SES-high (Fig. 6). According to semiquantitative scores, NF was the third best treatment for inducing organized collagen matrix deposition, though no significant difference was observed compared to SES-high (Figs. S1-S3).

SES-low + G and SES-low treatments had the best Healing scores. At day 32, granulation tissue was absent and an uniform collagen pattern across the groups. This indicates that the healing process was complete and comparable in terms of collagen deposition, so all groups were rated with the highest score for this parameter. Additionally, scores for the amount of inflammatory infiltrate and type/abundance of deposited collagen were assigned. The highest scores were assigned to treatments that induced the lowest inflammatory infiltrate (Fig. 3; Tables SIII-SV). For quality of collagen deposition, the highest values were registered for treatments that produced an organized matrix, composed by horizontal deposition (Fig. 6; Tables SVI-SVIII). The higher the healing score, the more favorable outcome in terms of tissue repair. A high healing score suggested that the healing process progressed well and achieved the desired results. At day 32, SES-low + G and SES-low demonstrated the highest healing scores (20.85±0.36 and 20.03±0.19, respectively) compared with Pl with 17.50±0.22 and S with (17.11±0.20 (Fig. 7; Table SIX). SES-high, SES-high + G and NF had similar performance (~18 points) and without significant differences between them or the rest of the groups. It is interesting to notice that on day 6, treatments with the highest healing scores were NF, SES-low and SES-low + G; NF was significantly different compared with S. At day 9, SES-low + G, SES-low and Pl groups had the highest values; only SES-low + G was significantly different compared with S. These results partially coincide with the speed of wound closure observed in Fig. 2, which demonstrates that proper wound healing is not only matter of fast wound closure.

SES-low + G had the best Healing status. There was a significant difference in healing status at day 32 between Pl and SES-low and SES-low + G ($P<0.01$), as well as between S and SES-Low + Gel. At this day, SES-low + G yielded the best healing status (Fig. 8; Table SX). This effect can be attributable to the low inflammatory infiltrate observed in such groups, during wound healing evolution and particularly at day 9. On the contrary, treatments with Pl and S had the worst healing status, with no significant difference between them. No significant differences in healing status were observed between groups at days 6, 9, or 18 (Fig. 8; Table SX).

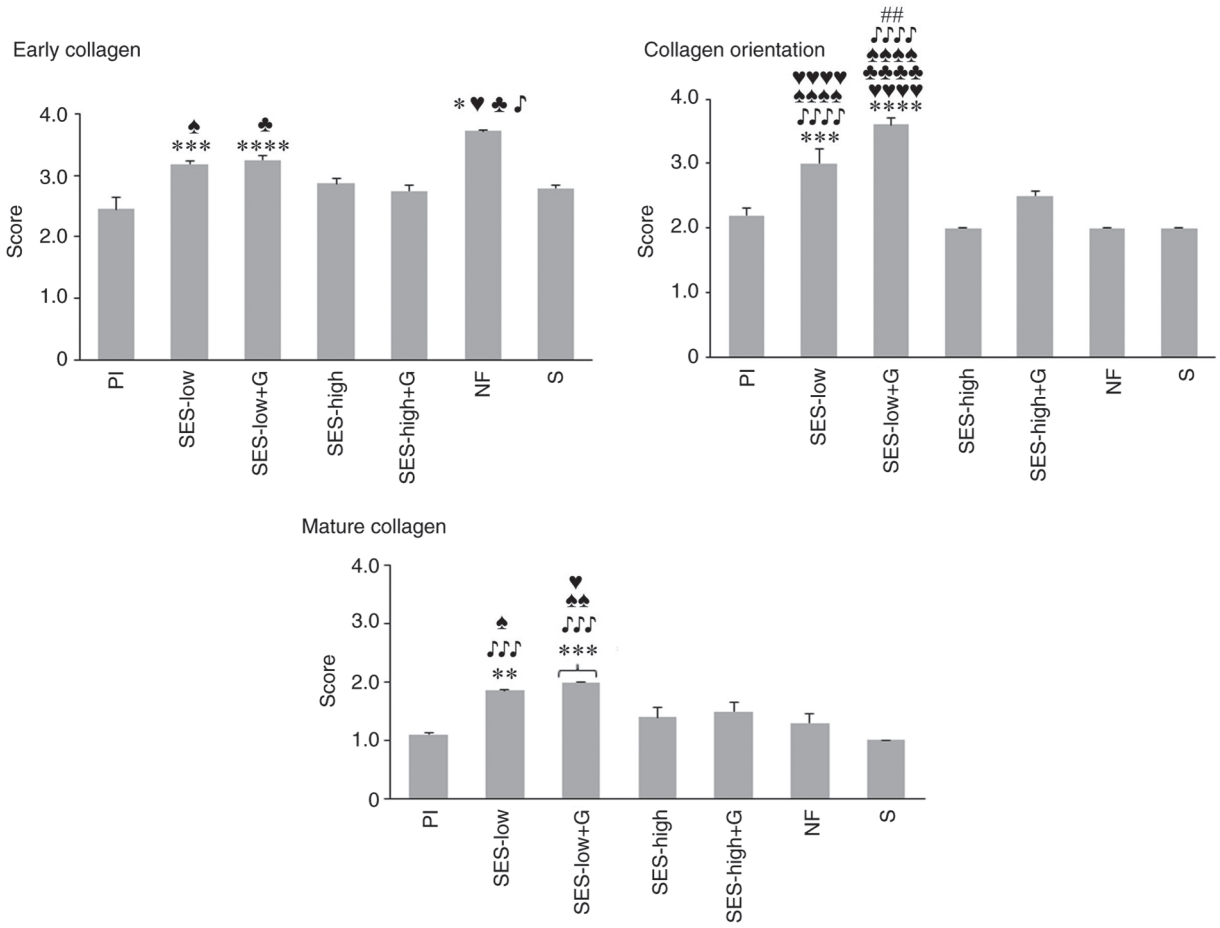

Figure 6. Semi-quantitative scores for early and mature collagen and collagen orientation at day 32. *P<0.05 vs. Pl, **P<0.01 vs. Pl, ***P<0.001 vs. Pl, ****P<0.0001 vs. Pl, ##P<0.01 vs. SES-low, ♥P<0.05 vs. SES-high, ♥♥♥♥P<0.0001 vs. SES-high, ♠P<0.05 vs. SES-high + G, ♠♠♠♠P<0.0001 vs. SES-high + G, ♪P<0.05 vs. NF, ♪♪P<0.01 vs. NF, ♪♪♪♪P<0.0001 vs. NF, ♫P<0.05 vs. S, ♫♫P<0.001 vs. S, ♫♫♫♫P<0.0001 vs. S. SES, superoxidized electrolyzed solution; NF, nitrofurazone; Pl, placebo; S, silver sulfadiazine; and G, gel.

Discussion

Successful treatment of full-thickness burns is a global challenge due to severity of the wounds and a health issue due to the impact in life quality of affected individuals (6,51,66). The healing of third-degree burns is a dynamic and complex process characterized by inflammatory, proliferative and remodeling phases, which result in regeneration and re-epithelization of affected tissues (11,60).

The inflammatory phase is characterized by chemotaxis of different cells to the injured site, release of histamine, pro-inflammatory factors, vasodilation, diapedesis and activation of white blood cells and fibroblasts (11,60). Pro-oxidant mechanisms, hemostasis and removal of dead tissue and foreign and microbial material also occur (11,60). In the proliferative phase, migration of keratinocytes, fibroblasts and endothelial cells occurs, resulting in new epithelization, with formation of fibronectin, collagen fibers, granulation tissue and neovascularization (53,60). A key driving force of this phase is growth factors produced by activated neutrophils and macrophages during the inflammatory phase (60,67). In the remodeling stage, the concentration of fibroblasts decreases, excess collagen is degraded and various enzymes and growth factors in the extracellular fluids that accumulate in and around a wound promote tissue repair (60,68,69). In severe burns, most affected tissue may be non-viable or necrotic, with poor or compromised vascularity; this inhibits the influx of white blood cells, such as neutrophils, into the injury site, complicating the healing process, while the risk of infection increases (68). Use of antiseptics and healing agents is key to prevent complications. Ideally, antiseptics must be effective to avoid infection and non-toxic to prevent wound healing inhibition.

The present study investigated the effect of different antiseptics on the quality of wound repair. SES-low and SES-low + G yielded the best wound healing parameters. NF showed a good performance but was significantly worse than SES-low + G in terms of orientation of the collagen and presence of mature collagen at day 32. All treatments produced wound closure, however SES-low significantly improved wound healing compared with Pl and S due to the anti-inflammatory effect. S exhibited significantly higher levels of inflammation infiltrate than the rest of the groups, while NF and SES-high produced less favorable healing process than SES-low.

As aforementioned, the active species of SES mimics the active species of chlorine and ROS produced by some white blood cells; this may explain the non-irritant effect of SES-low. Additionally, SES promotes wound healing by increasing

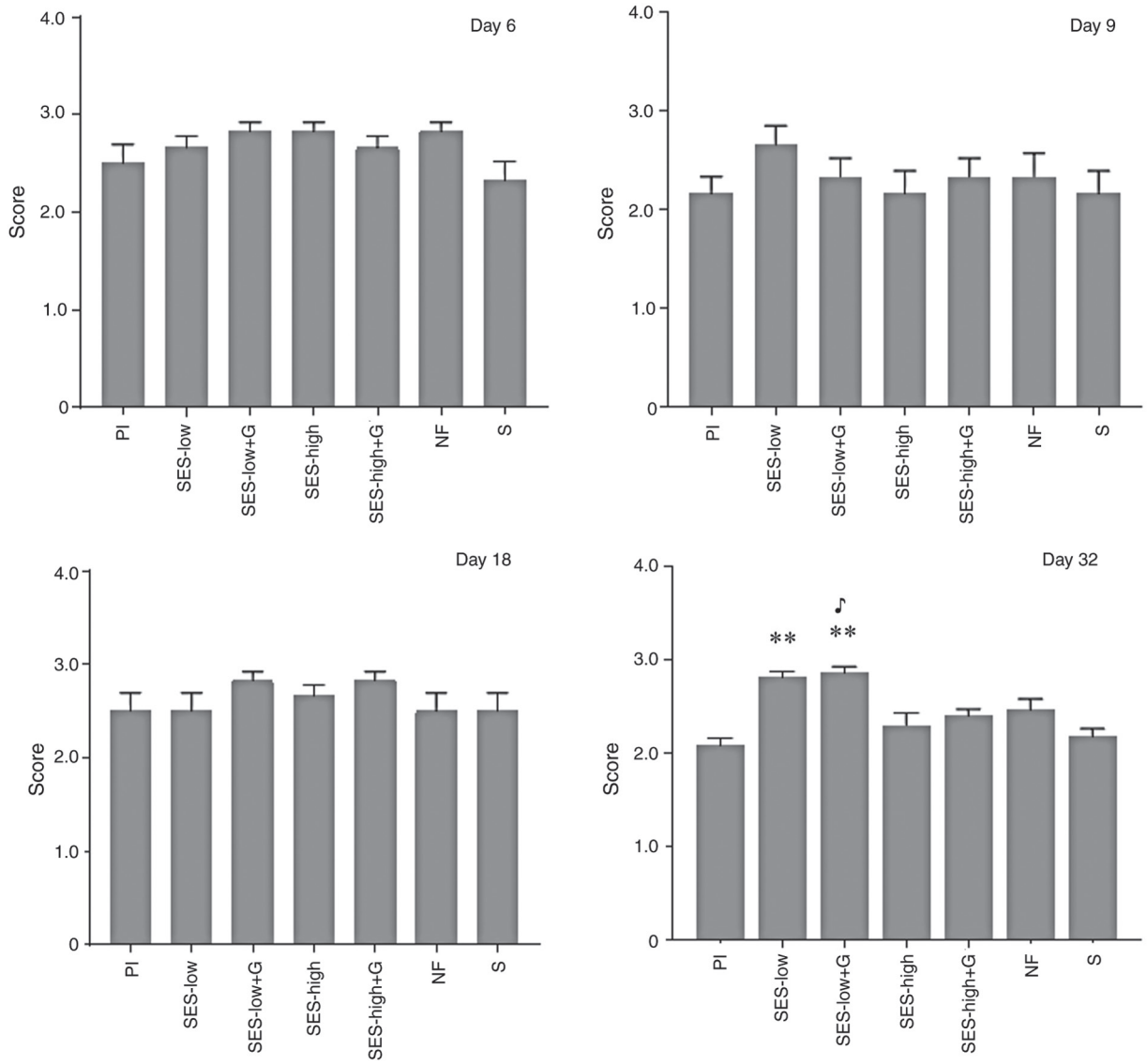

Figure 7. Healing score according to histopathological features **P<0.01 vs. Pl, ♪P<0.05 vs. S. SES, superoxidized electrolyzed solution; NF, nitrofurazone; Pl, placebo; S, silver sulfadiazine; and G, gel.

oxygenation of the lesion, regulating inflammatory activity and modulating several immune-redox processes (23,26,28,70,71).

ROS participate in the synthesis and deposition of collagen and other proteins such as elastin in a concentration-dependent manner (72-76). Exposure of fibroblasts to low concentrations of ROS increases elastin mRNA synthesis platelet activation is detected when dermal glycoprotein-VI is exposed to ROS (74-75). On the other hand, high concentrations of reactive oxygen species (ROS) are associated with oxidative damage, which can significantly impact wound healing (74,75,77). High ROS levels are known to cause oxidative damage to various cellular components, including lipids, proteins, and nucleic acids (74,75,77). This oxidative damage can inhibit collagen synthesis, leading to reduced fibroblast function and a weakened extracellular matrix (78). The fragmentation of collagen fibers due to high ROS levels further compromises the structural integrity of the tissue, making it more susceptible to complications and delayed healing (77). Excessive ROS can also lead to abnormal cross-linking of collagen fibers, resulting in stiffer and less elastic tissue, which negatively affects the remodeling process and can lead to the formation of fibrotic scars (77). Additionally, elevated ROS levels contribute to skin aging, wrinkles, and a loss of elasticity, which can impair the tissue's ability to heal effectively in epithelial tissue (73,74,76).

Active species of chlorine and oxygen in SES regulate the secretion/inhibition of specific cytokines, such as TGF-β and EGF, which attract and stimulate the proliferation of fibroblasts and keratinocytes, key cells in new tissue formation and re-epithelization (71). Also, SES serves as an immunomodulatory factor, inhibiting the secretion of TNF-α and IL-6 (26,28,70,71). SES can upregulate the production and utilization of intracellular calcium, as well as matrix metalloproteinases 1 and 9, which play an essential role in all stages of wound healing by modifying the wound matrix, allowing cell migration and promoting faster tissue healing and remodeling (71,78,79). However, the exact mechanisms by which these active species interact with cells, potentially causing damage or facilitating repair, have not been fully elucidated. Active species of chlorine and oxygen in SES can impact

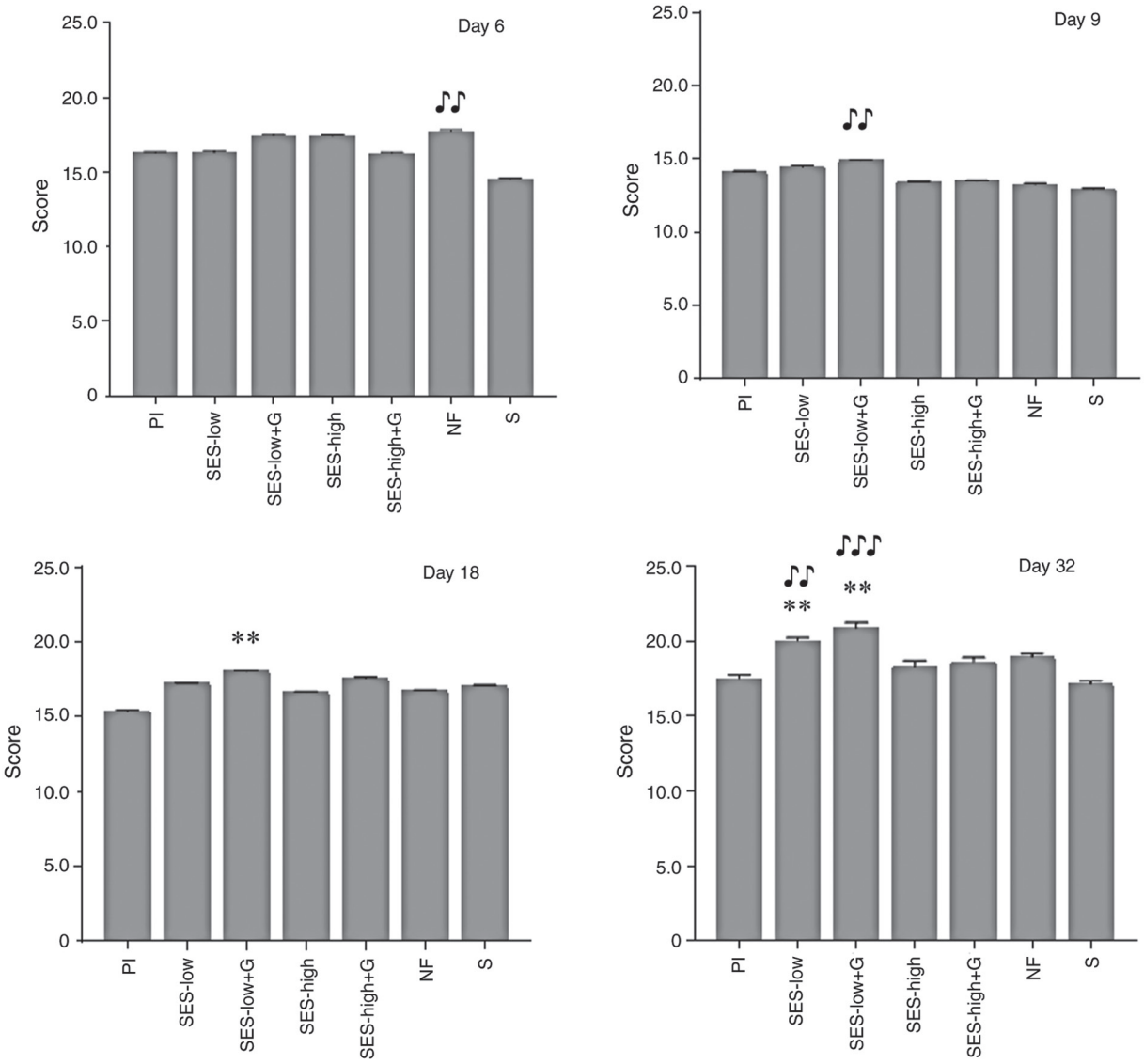

Figure 8. Semiquantitative analysis for healing status, according to histopathological features. **P<0.01 vs. Pl, ♪♪P<0.01 vs. S, ♪♪♪P<0.001 vs. S. SES, superoxidized electrolyzed solution; NF, nitrofurazone, Pl, placebo; S, silver sulfadiazine; and G, gel.

cellular integrity via oxidative stress and inflammation (25,70), however, both previous evidence and the present study demonstrate a beneficial effect of topical SES-low application on wound healing without notable adverse effects (25,30,80,81). Future research should focus on elucidating the underlying mechanisms, examining how these active species influence cytokine regulation, and understanding their impact on cellular function and integrity. This deeper understanding will provide a clearer picture of the dual role of chlorine and oxygen species in both potential cell damage and healing, contributing to more comprehensive understanding of how SES supports wound repair at the molecular and cellular level.

SES-low and SES-low + G exhibited better collagen scores and healing status than SES-high and SES-high + G. NF, SES-high and SES-high + G treatments did not significantly impede wound healing, but produced an irritant effect as evidenced by the increased inflammatory infiltrate and less organized collagen deposition observed in these groups resulting in a lower healing status. This was also observed for S treatment, due to the increased inflammatory cell infiltration and less efficient collagen matrix deposition. None of the treatments were worse than Pl. SES-low and SES-low + G treatments were significantly better than Pl and S groups. The controlled inflammatory cell infiltration response and increased wound healing quality in the SES-low groups were consistent with previous reports on wound healing and anti-inflammatory activity of SES (24,25,27). In a wound healing the transition from early to mature collagen is a positive indicator of proper healing because it promotes the generation of complex structures oriented for tensile strength restoration (66). However, excessive deposition and/or agglomeration is indicative of abnormal or hypertrophic wound healing (82,83). Then the organization of the collagen matrix is crucial for high-quality wound healing. horizontal orientation of collagen fibers facilitates recovery of the damaged area and is associated with a better organization and function of scar tissue (66).

The moisture of injury is also another factor for proper wound evolution. In comparison with dry environments, moisturized wounds show reduced necrosis and inflammatory infiltrate, as well as increased angiogenesis and

faster and better quality of healing (84-87). SES-low + G showed the best healing status, which may be explained by increased wound moisture and prevalence of the SES in the lesion; pattern of collagen formation was more structured (horizontal), with less accumulation of mature collagen and inflammatory infiltrate. SES-high resulted in similar performance to NF in terms of collagen formation/remodeling and fiber orientation but healing quality was lower compared with SES-low. Sulfadiazine, one of the most commonly antimicrobial topical dressings used by physicians for treatment of second and third-degree burns had the poorest performance in wound healing due to a prolonged inflammatory effect (88,89). Previous studies have reported that sulfadiazine slows wound healing (90,91). Additionally, silver particles may activate inflammatory responses when recognized as foreign material or as an antigen (92,93). Clinically, other adverse effects have been observed following the use of S, such as black scarring, restricted wound penetration, which refers to the reduced ability of the treatment to effectively reach deeper layers of the wound. Additionally, its use has been associated with hypersensitivity reactions, renal toxicity, and leukopenia, (89,91). Consequently, long-term use of is not recommended due to these potential risks and side effects (89).

The present pre-clinical model demonstrated that SES-low was the best antimicrobial substance for wound care since it did not compromise tissue repair and promoted high-quality wound healing, especially in combination with G. Nevertheless, the present study had limitations, such as use of the mouse model, which does not fully replicate complexities of wound healing in humans. For example, human skin is thicker and more complex than mouse skin with a wound healing processes that involves re-epithelialization and scar formation, while mice heal rapidly and primarily to wound contraction. Besides, human-wounds involve more prolonged and meticulous immune response that those in mice (47,55,94). However, it is an accepted model as a first approach to these skin repair processes and has been used to analyses the effect of SES on cutaneous wounds (29,71).

Additionally, more detailed studies such as immunohistochemistry and quantification of pro-/anti-inflammatory biomarkers are required to understand the wound healing mechanisms at molecular and cellular levels. Nonetheless, the present study demonstrated the potential of SES-low as an alternative, to promote high quality wound healing. Clinical trials or case reports are necessary to validate these findings.

While the present findings demonstrated the potential of SES-low as an effective treatment for enhancing wound healing in burn injury, translating these results into clinical practice requires consideration. Determining the optimal dosing and treatment frequency is key to achieve consistent therapeutic outcomes in human patients, as the frequency and concentration of SES application in the present study were tailored to the animal model and human skin may respond differently, necessitating dose adjustments. Although products based on SES are already available on the market as adjuvants for the treatment of acute and chronic wounds, it remains important to explore their precise formulation, dosing and application in clinical scenarios. For example, many of these products recommend cleaning the wound with water and soap, drying it and performing debridement with sterile gauze before applying SES directly to the wound three times/day or as directed by the physician (95,96), whereas the present laboratory study used a single daily dose. Therefore, it is necessary to establish new paradigms to confirm safety and effectiveness of SES in treating deep partial-thickness wounds and refine these guidelines for optimal use.

Moreover, it is key to consider and study potential interactions between SES and other topical products, commonly used in the integral treatment of these kind of injuries (6,9). As aforementioned before, third-degree burns require individualized therapy depending on medical condition and severity of the injurie, for example, moisturizers, polymeric membrane dressing with or without growing factors, and skin grafts are typically included in full-thickness burn management (11-16). These interactions may influence the efficacy of SES as antimicrobial, or the performance of the other adjuvants and dressing agents used to promote wound healing, or the tolerance of the patient to the integral treatment. For example, the present study demonstrated irritation and prolonged inflammation following treatment with SES-high and S but the specific impact of these side effects on the animal model was not elaborated. These adverse effects may lead to delayed wound closure or compromised tissue integrity, posing challenges in patient care. Strategies to mitigate these side effects, such as investigating the compatibility of antimicrobial substances with additional adjuvants and dressings, and adjusting their posology and frequency of administration should be explored to enhance patient outcomes.

More studies are needed to understand the dynamics of antimicrobials in these kind of integral burn treatments, especially the SES-based therapies, ensuring practicality and benefits in real-world scenarios while balancing therapeutic efficacy with safety in both preclinical and clinical settings. Patients with burns typically receive comprehensive treatment regimens, including hyperbaric oxygen therapy and pharmaceutical interventions, which were not considered in the present animal model. The present study demonstrates SES as a potential effective adjunctive therapy for wound healing, but its integration with established treatments such as hyperbaric oxygen therapy and pharmaceuticals requires further exploration. Future research should investigate the combined effects of SES with these standard therapies to determine how SES can be optimized alongside conventional burn care practices. This approach will provide a more comprehensive understanding of SES and its potential benefits when used in conjunction with existing treatments in clinical settings.

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Availability of data and materials

The data generated in the present study may be requested from the corresponding author.

Authors' contributions

IDE and BAPM conceived the study and revised the manuscript. NSAC, MLMF, CMR, DCC and MWG designed the methodology. AEHR, GAHF and MLMF analyzed and interpretation of data. MWG, ACL, and MDM, IPRS and GAHF analyzed the data. ARH, OBG, and CBCA performed the experiments. IDE, GAHF, BAPM and ACL wrote the manuscript. GAHF and ACL revised the manuscript. IDE and MLMF supervised the study. IDE and BAPM confirm the authenticity of all the raw data. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

The present study was approved by the Research Ethics Committee of the Colima State Institute of Cancerology (Colima, Mexico; approval no. CIIECAN/06/19).

Patient consent for publication

Not applicable.

Competing interests

BPM and ACL are employees at Esteripharma but did not participate in the decision to publish the results of the study, nor in its development or data collection. The rest of the authors declare that they have no competing interests.

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