Article

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The Role of Mechanically Isolated Stromal Vascular Fraction on Epithelial Proliferation during Burn Healing

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Background: Burn healing consists of four stages, homeostasis, inflammation, proliferation, and remodeling. Burn treatment aims to avoid infection, enhance tissue recovery, and prevent scarring. Recently, regenerative therapy shifted from using fat that contains mesenchymal stem cells to using various types of cells isolated from fat tissue called Stromal Vascular Fraction (SVF). It can be isolated either enzymatically, using collagenase, or mechanically, using emulsification of fat and filtration of cells. Previous research showed that both enzymatical and mechanical isolation of SVF can enhance neovascularization, re-epithelization and reduce inflammation. However, the mechanism behind its therapeutic effect needs to be explored. This study aims to investigate the role of mechanical isolation of SVF on the re-epithelization stage of deep-partial thickness burn in Wistar rats.
Methods: Eighteen Wistar rats were used in this experiment. Three rats were used for fat isolation. After burn induction, fifteen rats were grouped randomly as follows: the control group (5 rats) received intradermal injection of 1 mL saline, Silver Sulfadiazine (SSD) Cream group (5 rats) was treated with the cream, and the SVF group (5 rats) received intradermal injection of (1×10^6 cells/mL). All rats were euthanized at day 32 post-treatment. Morphological and histological examination for re-epithelization, measuring epithelial thickness and Ki-67 immunostaining was compared between all groups.
Results: Wound contraction and re-epithelization were completed in all experimental groups. However, the epithelial thickness of the epidermis was higher in the SVF group than in the SSD group ($p = 0.034$). Ki-67 staining was higher in the SVF group than in the SSD group ($p = 0.025$).
Conclusion: Mechanically-isolated SVF showed a positive effect on re-epithelization by increasing cell proliferation via the activation of Ki-67 thus increasing the epidermis thickness in a regulated way.

Keywords: Stromal Vascular Fraction; burn; re-epithelization

Introduction

Burn injuries can be fatal and affect millions of people globally. Wound closure and tissue regeneration are the most important factors that affect the healing process. Recently, research has shifted from using stem cells to various populations of cells extracted from fat tissue, called the Stromal Vascular Fraction (SVF). Despite the efficacy of stem cells in treating many diseases, SVF provides faster isolation techniques with less invasive procedures, avoids culture expansion, and lowers costs.

Burn healing consists of four overlapping phases, hemostasis, inflammation, proliferation, and remodeling. Hemostasis aims to stop bleeding through vasoconstriction. In the inflammatory phase, edema and inflammatory cell infiltration characterize this stage to initiate immune response post-injury. It starts within hours after injury and lasts for up to a week. The proliferation phase aids wound closure through re-epithelization, and neovascularization, to replenish tissues with oxygen and nutrients. This phase is stimulated by different growth factors [1]. Re-epithelization occurs after stimulation with different mediators, such as keratinocyte growth factors and epidermal growth factors, and the migration of cells from the surrounding tissues to rebuild the epithelial layer. Excess re-epithelization and collagen deposition could result in hypertrophic scar formation. This phase takes from one to two weeks post-burn. Lastly, the remodeling phase lasts for a long time. Depending on the burn size, it could take months [2].

Burn treatment aims to restore the skin structure and function while preventing infection, scarring, and other complications. Treating deep partial-thickness burns depends on avoiding infection, reducing hypertrophic scar formation, and tissue regeneration. Silver Sulfadiazine (SSD) Cream has been used for decades as a standard burn treatment for its antimicrobial properties [3]. However, this compound causes toxicity to the wound bed, forming a pseudo-eschar which can enhance bacterial colonization and impair wound healing. Additionally, SSD treatment requires a daily change of the dressing, which could affect the healing process and increase the patient's pain [4]. It was reported that using silver-based dressing was asso-

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ciated with delayed re-epithelization, hypersensitivity, and discoloration of scars [5,6]. For severe burn injuries, excision and grafting are performed to close the wound. However, each treatment modality has its drawbacks. Therefore, new interventions are needed to prevent these complications.

Recently, regenerative therapy has been used to develop several strategies for skin regeneration, skin functionality retention, and preventing adverse outcomes. Stem cell therapy is one of the treatment strategies for burn injuries. In recent decades, SVF has emerged as a new therapeutic option for many diseases, including burn injury [7]. SVF is a mixed population of cells, including mesenchymal stem cells, pre-adipocytes, endothelial cells, pericytes, and macrophages, that are isolated from the adipose tissue [8]. In SVF, cells have the potential to secrete different mediators such as anti-inflammatory factors, chemokines, growth factors, and secretomes, which play a role in the healing process of burn injury [1]. Mesenchymal stem cells can differentiate into various cell lineages. SVF was found to promote burn healing. In animal models, SVF treatment reduced inflammation and increased neovascularization, fibroblast activity, collagen deposition, and re-epithelization [7,9,10]. Treatment with both SVF and platelet-rich plasma accelerated wound closure, re-epithelialization, neovascularization, hair growth, and decreased inflammation compared to either therapy alone [11,12]. Therefore, SVF has a therapeutic potential for treating burn injuries.

SVF isolation can be either enzymatic or mechanical. The enzymatic method involves proteolytic enzymes such as collagenase, trypsin, or dispase. After liposuction and washing of fat, collagenase hydrolyzes the collagen fibers within the extracellular matrix to release the cellular component. Subsequently, cells are collected by centrifugation. This methodology has been used in clinical settings and most previous burn injury research. However, this technique is time-consuming, requires an enzyme purification step, and can cause cell contamination. This method also digests collagen which damages the microenvironment. The microenvironment role benefits the healing process, including cell communication, proliferation, and differentiation. Mechanical isolation has recently been developed to avoid these issues and restore the microenvironment to enhance healing outcomes. Mechanical isolation depends on fat emulsification by shifting the adipose tissue sample between Luer-Lock syringes multiple times to destroy adipose cells while retaining stromal and other cell types [13]. Emulsification is followed by centrifugation and filtration to obtain a cell suspension. Many devices and tools have been developed for mechanical isolation to speed up the isolation process, minimize cell manipulation, and make the process suitable for use in clinical settings. This study used a Microlyzer SVF kit (BSLrest Inc., Turkey) for mechanical isolation. This tool is based on micro-fragmentation using a sharpened-edge microblade with a honeycomb design. High cell viability (approximately 90%), cell count, and the presence of mesenchymal cell markers have been reported using this tool [14]. Therefore, this tool is a costless option for producing efficient viable cells.

Previous studies, including our work, showed the positive impact of mechanical isolation of SVF on the healing stages of deep-partial thickness burn [15,16]. However, the mechanism behind its effect remains to be explored. This study extends our previous work with mechanically isolated SVF in burn injury. This study aimed to evaluate the proliferation ability of SVF by investigating the epithelial thickness histologically, and the proliferating marker (Ki-67) immunobiologically of deep-partial thickness burn at 32 days post-treatment with mechanically-isolated SVF in Wistar rats.

Methods

Experimental Design

This study was conducted under the ethical approval of the Animal Care and Use Committee office (ACUC), King Fahad Medical Research Center, King Abdulaziz University (No. ACUC-20-11-35). Eighteen male Wistar rats, weighing between 150–200 grams, were used in this experiment. Rats were housed in a 14:10 light/dark cycle at 21 °C. All rats had free access to water and food. Three rats were used for fat isolation. Fifteen Wistar rats were divided randomly as follows: the control group (5 rats) was injected intradermally with (1 mL) saline, the SSD group (5 rats) was treated with SSD (1% Flamazine®) cream only, and the SVF group (5 rats) was injected intradermally with SVF (1×10^6 cells/mL) only. SSD cream was used as a positive control since this cream is used as standard care for deep-partial thickness burn injury. This study considered the day of the induction of deep-partial thickness burn (day 0). The next day, rats received the treatment via intradermal injection in the burned location. The SVF group was injected intradermally with SVF cells at (1×10^6 cells/mL) for each wound since this cell count was reported to impact tissue regeneration [13,17,18]. All rats were euthanized at day 32 by cervical dislocation (60 mg/kg Ketamine and 10 mg/kg Xylazine® were used to anesthetize rats by intraperitoneal injection).

Deep-partial Thickness Burn Induction

To ensure the induction of deep-partial thickness, histological analysis was conducted to compare scalding of 100 °C for 6 and 10 seconds. In a pilot study, eight rats were divided into two groups (four rats/group). The first group received scalding with a 22-gram iron rod (1.8 cm in diameter) and heated up to 100 °C for 10 seconds while the second group received scalding for 6 seconds. The scalding was done in the dorsal area for each rat. This method was validated previously [19]. The same researcher conducted them to ensure the consistency of burns. Post-burn, 2 mL

per 100 g body weight of saline was injected into all rats intraperitoneally. Every rat was kept in a cage for five days to avoid contamination. A water containing Paracetamol (2 mg/mL) was given for three days to prevent pain. After 24 hours, skin samples were collected for histological analysis to compare the damage between these two-time frames and to determine which time frame induces deep-partial thickness burn for this study.

Mechanical Isolation of SVF

The fat tissue was collected from the inguinal area of three rats as previously described [7]. The collected fat was washed and minced with a sterile scalpel for 30 minutes. Fat emulsification was achieved using a microlyzer (BSLrest Inc., Turkey) designed for mechanical isolation of SVF as previously described [20]. SVF was separated from the emulsified fat layer by centrifugation at 400× for 5 minutes [21]. SVF pellet was collected, washed twice with saline, and centrifuged twice to remove any residuals. Cells were filtered using the sterile nylon cloth (100 µm) and then collected in a tube after centrifugation at (400 ×g for 5 min). Cell count and viability were detected using the trypan blue test.

Morphological Analysis

As described previously, the morphometric evaluation was evaluated by assessing wound contraction between all experimental groups [9]. Images were analyzed using ImageJ program (ImageJ; National Institute of Health, Bethesda, MD, USA). Results were compared between all experimental groups at day 1, 7, 21, and 32 post-burn induction.

Re-epithelization Evaluation

Hematoxylin and eosin (H&E) staining was used for histological analysis as previously described [22]. Re-epithelization was assessed by calculating the difference between the distance of the wound gap and the length of the newly formed epithelial tongue. Measurement was conducted at 5× magnification as previously described [23].

Epithelial Thickness Evaluation Histologically

Measuring the length between the basal and outer layers of the epidermis in each tissue specimen was done from 20 different fields (at ×20 magnification) for each sample using a grid (0.1 mm^2) [10]. Results were presented by calculating the mean of all measurements for every sample. This measurement was done by using Philips IntelliSite Pathology Solution software (Veenpluis 6, Philips Medical Systems, Veenpluis, Netherlands).

Ki-67 Immunohistochemical Staining

For immunostaining, slides were heated up to 75 °C for 1 hour. They were deparaffinized and then rehydrated through graded ethanol alcohol solutions. After washing slides, they were rehydrated with phosphate-buffered saline (PBS). Antigen retrieval was conducted, using 10 mM sodium citrate buffer (pH 6.0), by heating slides at 95 °C for 10 minutes. After 30 minutes, rinsing slides with PBS was conducted. A visualization agent (#ab64264, Abcam, Cambridge, UK) was used. The hydrogen peroxide block (ab64259, Abcam, Cambridge, UK) was used by incubating the slides for 10 minutes. Protein (ab64259, Abcam, Cambridge, UK) was blocked by incubating slides for an hour at room temperature, then rinsing with PBS. Anti-Ki-67 (a Rabbit monoclonal; Cat# of 790-4286, CiteAb, Ventana, England) was used at 1:100 dilution for 20 minutes. The negative control was incubated with PBS instead. After incubation, slides were rinsed with PBS four times. Biotinylated goat anti-polyvalent (ab64255, Abcam, Cambridge, UK) was applied for ten minutes and washed 4 times with PBS. Streptavidin peroxidase (ab64259, Abcam, Cambridge, UK) was applied for ten minutes onto slides, then they were washed four times with PBS. Diaminobenzidine (ab236466, Abcam, Cambridge, UK) was applied for 5 minutes, and then rinsed with distilled water. Staining with hematoxylin for 30 seconds and then washing with distilled water were conducted for all sections. Finally, dehydration of slides was established by using graded ethanol alcohol, and xylene. Positive control was tonsil samples.

Statistical Analysis

Data was analyzed using SPSS (Win 10.0) software (IBM-SPSS Statistics, Chicago, IL, USA). Data was presented as mean ± standard deviation (SD). One-way ANOVA (Kruskal Wallis test) was used to compare groups. Results were considered significant if the *p*-value was lower than 0.05.

Results

Deep-partial Thickness Burn Induction

Pathohistological examination by H&E staining in our laboratory for scalding for 6 or 10 seconds confirmed that scalding at 100 °C for 6 seconds induces deep-partial thickness burn in Wistar rats. In the 10-second samples, the epidermal layer was damaged and lost its adherence to the dermal layer. Hair follicles were destroyed with blocked vessels to the skin thickness. The damage extended to the subcutaneous fat, and the muscle layer showed an irregular shape compared to the normal skin (Fig. 1A). Therefore, the burn damage was considered a full-thickness burn injury. For 6 seconds scalding, histological analysis showed complete damage to the epidermis layer with shrinkage in the nuclei of the basal layer. The collagen fibres were damaged at the upper side of the dermis layers. Results showed the presence of damaged hair follicles and blocked vessels. Vessels' dilation was observed at the lower dermis. The subcutaneous fat layer was damaged as well (Fig. 1B). Nor-

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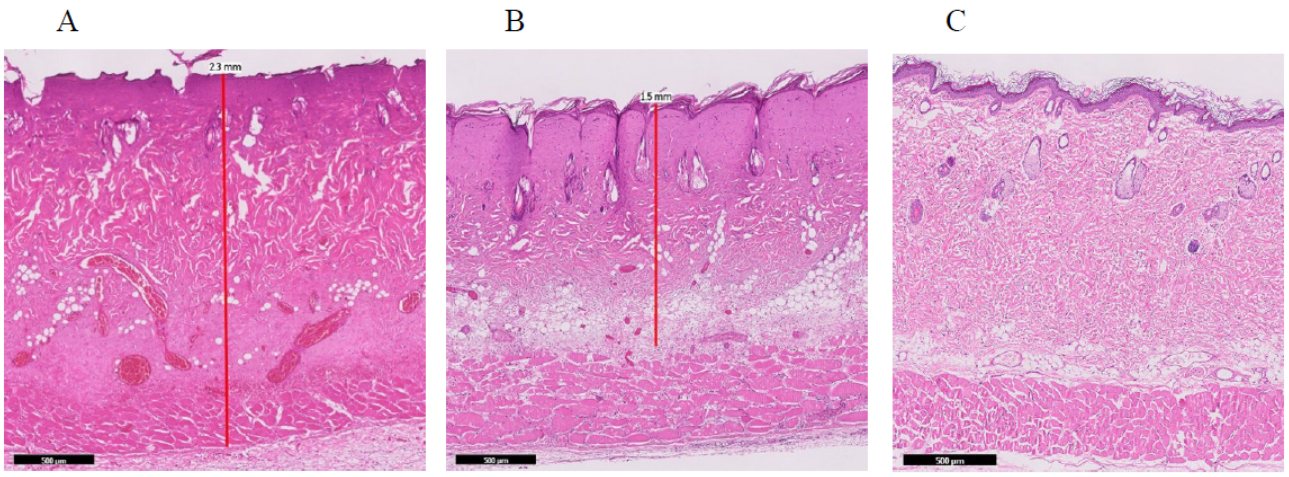

Fig. 1. Histological evaluation showing burn degree 24 hours after burn induction in Wistar rat skin. (A) Scalding for 10 seconds showing complete damage up to the dermis and hyperdermis layers. (B) Scalding for 6 seconds showing complete damage up to the dermic layer. (C) Normal skin. Red lines represent the measurement of the damaged area. Scale bar: 500 µm.

mal skin sample was presented in (Fig. 1C). Statistical analysis indicated that the average depth of the stasis zone of 6 seconds burn duration was significantly lower than the 10 seconds duration (1.66 ± 0.274 vs. 2.1525 ± 0.075, p = 0.0067). The average depth of the coagulation zone of 6 seconds burn duration was significantly lower than the 10 seconds duration (0.53 ± 0.0523 vs. 0.7375 ± 0.0850, p = 0.003). Therefore, this study considered 6 seconds of scalding for inducing deep-partial thickness burn. All figures were taken from the coagulation and stasis zones to detect damage to skin layers after scalding, thus inflammatory infiltration was not detected in these figures. However, the previously published article measured inflammation during the inflammatory phase of burn healing [16].

Morphological Evaluation

Morphological assessment of wound contraction showed no significant differences between all groups (p = 0.5). After burn induction, all burns appeared pale with no redness, then dried eventually. At 32 days post-treatment, burn wounds in all groups were closed without any formation of scar hyperplasia (Fig. 2A). The rate of wound contraction for all groups at days 1, 21, and 32 are presented in (Fig. 2B).

Re-epithelization

Evaluation of re-epithelization showed no significant differences between groups 32 days post-treatment. All groups showed a complete epidermis layer by the end of the experiment (Table 1).

Epithelial Thickness

At 32 days post-treatment, the epidermis layer was formed for all samples. However, the epidermis thickness was different between groups. The epidermis thickness was higher in the SVF group than in the SSD group (p = 0.034) (Fig. 3). However, there were no significant differences between the control and SSD groups or the SVF (p = 0.287 and p = 0.436, respectively).

Ki-67 Protein Expression

Ki-67 protein expression was higher in the SVF group than in the SSD group (p = 0.025). However, it was not statistically significant between other groups (Fig. 4).

Discussion

Routine treatment of deep partial-thickness burns depends on preventing infection, enhancing tissue recovery, and avoiding scarring. Stem cell therapy is a promising therapeutic option. SVF contains adipose-derived stem cells (ADSCs) that have the potential to differentiate and regulate different mediators. The SVF cellular population was previously detected from mechanical and enzymatical isolation [24]. Therefore, it was not examined in this study. Previous research has shown treatment efficacy with enzymatically isolated SVF on burn healing. Treatment with SVF was found to enhance neovascularization and re-epithelialization and decrease inflammation [7,9,10,25,26]. The SVF used in most previous studies was isolated enzymatically, using collagenase. Recently, similar results were reported for mechanically-isolated SVF [16]. In this study, SVF was isolated mechanically without enzyme exposure. This research evaluated the efficacy of mechanically isolated SVF on re-epithelization histologically and via Ki-67 (proliferation marker) protein expression at 32 days post-treatment.

Hemostasis, inflammation, proliferation, and remodeling are the stages of burn healing. The goal of the proliferation stage is wound closure to restore the skin's func-

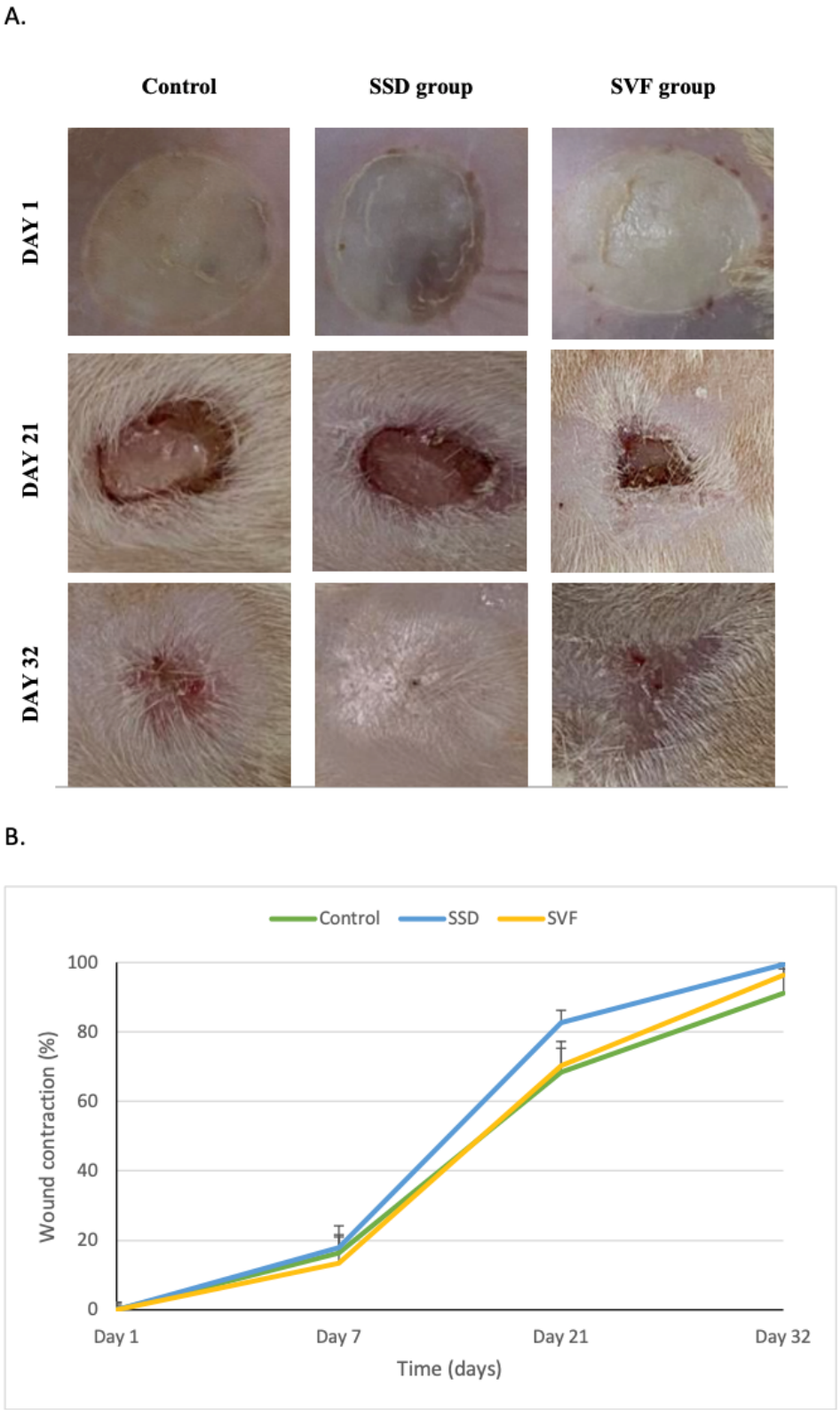

Fig. 2. Morphological evaluation of Wound contraction for days 1, 21, and 32 post-treatment. (A) Images of wound contraction comparison between all experimental groups on days 1, 21 and 32. Control (saline injection), SSD (cream treatment), and SVF (SVF injection). (B) Rate of wound contraction for all experimental groups. SSD Cream, Silver Sulfadiazine Cream; SVF, Stromal Vascular Fraction.

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Table 1. Histological evaluation of re-epithelization (mm) (grading 0–3) in deep partial-thickness burn wounds in Wistar rats on day 32.

Time	Control	SSD	SVF	*p*-value
Day 32	3.00 ± 0.00 (n = 5)	3.00 ± 0.00 (n = 5)	3.00 ± 0.00 (n = 5)	$p = 1$

Data are expressed as mean ± standard deviation (SD). SSD, Silver Sulfadiazine; SVF, Stromal Vascular Fraction.

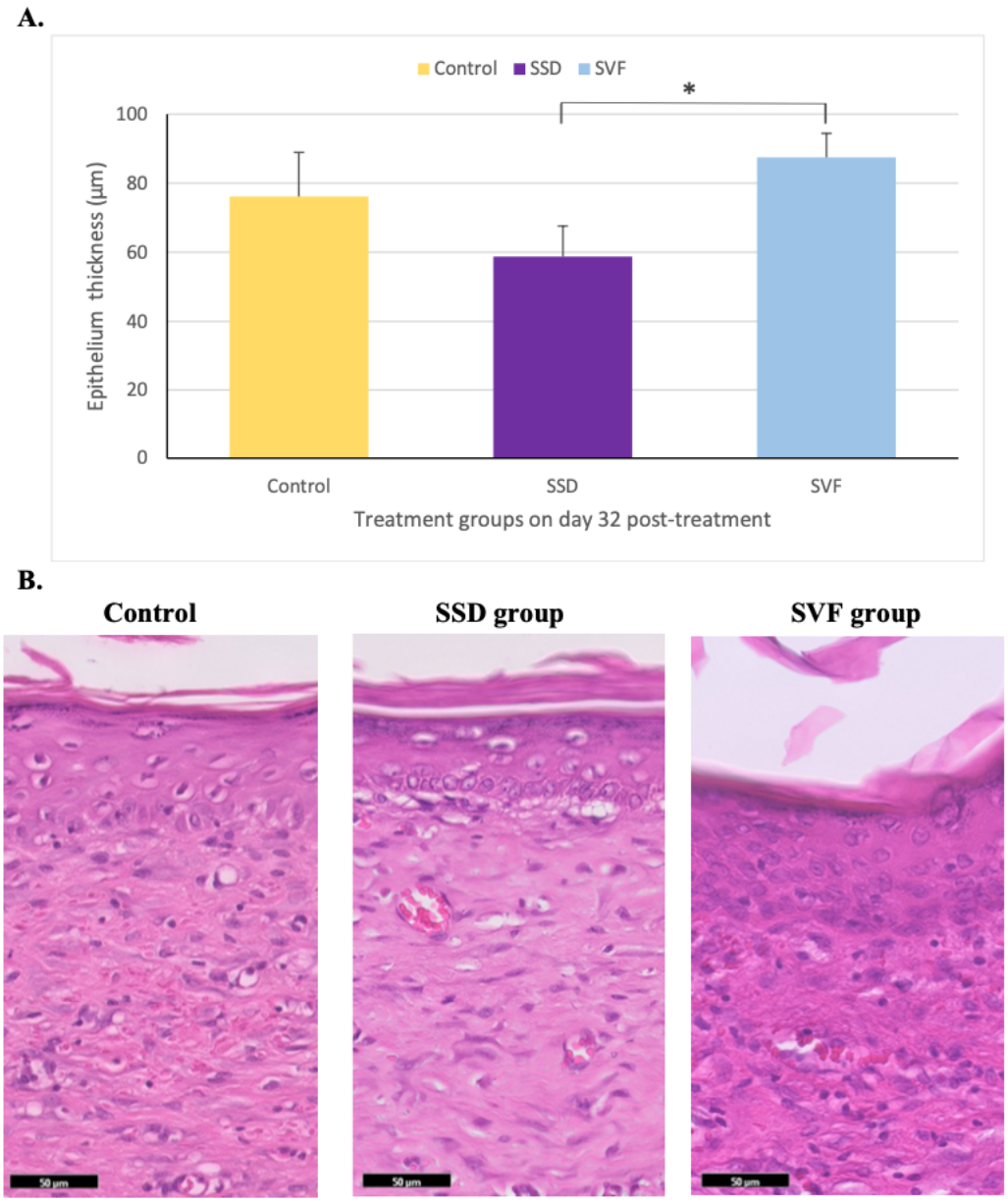

Fig. 3. Epithelial thickness measurement in all experimental groups on day 32. (A) Comaprison between all groups. Data re presented as mean ± SD. * indicates $p < 0.05$ between SSD and SVF groups. (B) Histological photographs of all experimental groups. Scale bar: 50 µm.

tionality. During this stage, re-epithelialization depends on keratinocyte and fibroblast proliferation. Fibroblasts secrete collagen to form an extracellular matrix while keratinocytes migrate to form a new epithelium layer. The epithelial layer is rebuilt from viable keratinocytes that migrate from the surrounding uninjured tissue to the edges of the burn wound to cover the damaged area. That depends on the migration of cells to the edges of the surrounding

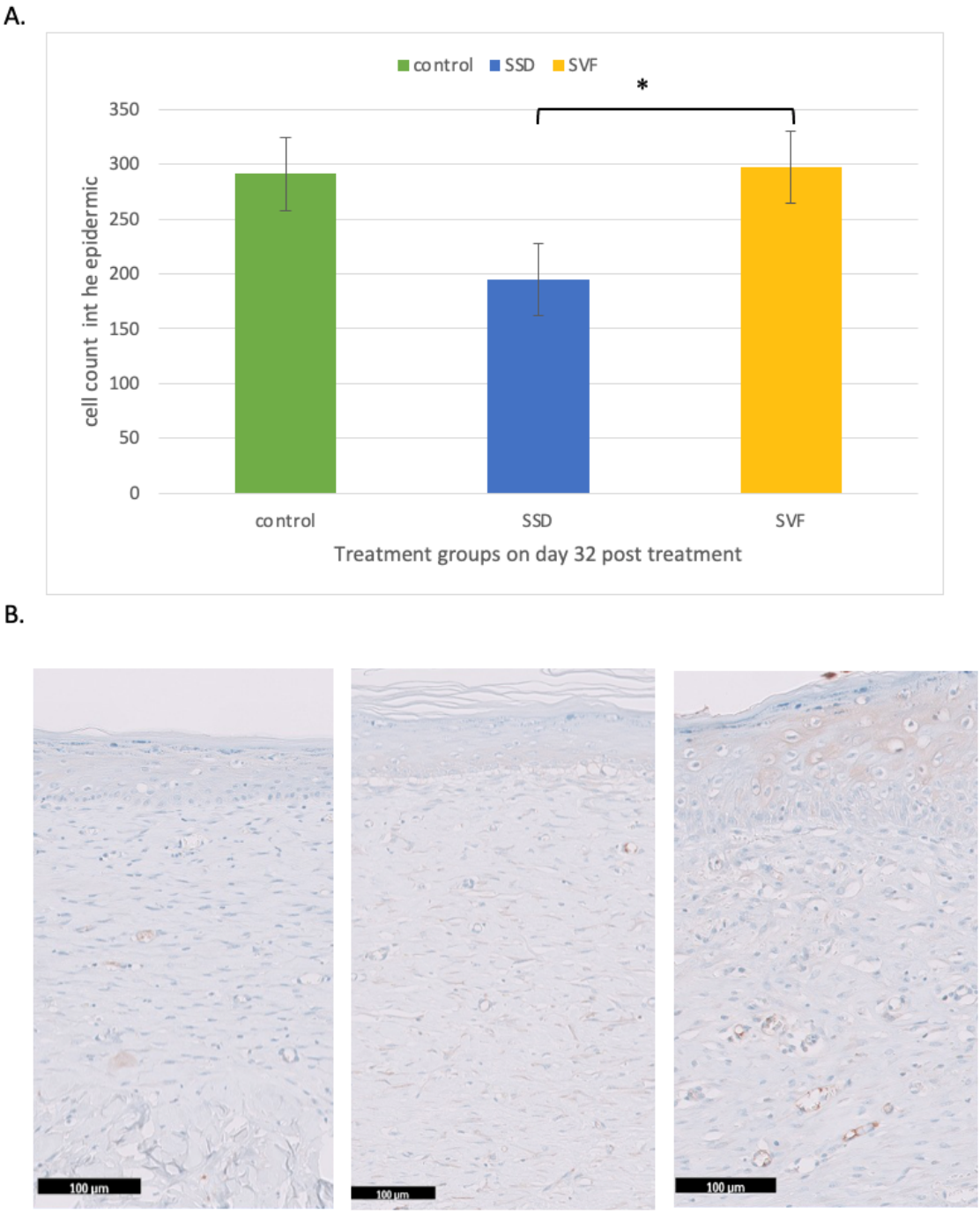

Fig. 4. Ki-67 protein expression in all experimental groups. (A) Ki-67 comparison between all experimental groups. Data are expressed as mean ± SD. * indicates $p < 0.05$ for comparison between SSD and SVF groups. (B) Histological images of IHC staining for all groups. Scale bar: 100 µm.

viable cells. Re-epithelization is essential for wound closure. In this study, there were no significant differences in wound contraction between all groups at day 1, 21 and 32 post-treatment. The epithelialization was complete in all groups at 32 days post-treatment. Results were not significant between experimental groups. Similar result has been reported for complete epithelization at approximately 21–25 days post-injury [26]. Adding platelet-rich plasma to enzymatically-isolated SVF enhanced re-epithelialization completion by day 10 compared with treatment with SVF alone [27]. Therefore, mechanically-isolated SVF does not harm re-epithelialization or burn healing.

Since there were no significant differences in wound contraction and re-epithelization between all groups, the

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thickness of the epithelium layer was compared between all treatment groups on day 32. This study showed increased epithelial thickness in the skin of rats treated with mechanically-isolated SVF compared to the SSD group. Similar results were obtained using enzymatically-isolated SVF. Studies using a deep partial-thickness burn rat model showed that SVF treatment was associated with thicker epithelium than the control group [10,11]. A recent study in patients with third-degree burns treated with SVF showed higher epithelial thickness (19%) [28]. Both isolation methods of SVF enhanced cell proliferation in the epidermis layer compared to other treatment groups. Fortunately, the hypertrophic scar was not reported in all previous studies. That indicates that SVF enhances proliferation in a regulated way. The mechanism behind this effect remains to be explored.

The proliferation of cells in skin layers can be used as an indicator of burn healing. Ki-67 protein is a cell's proliferative marker. To confirm these results, Ki-67 protein expression was used as a marker of cell proliferation in this study. It is expressed in cell cycle stages and used as an indicator of replication in active cells. Our results showed a significant increase in the expression of Ki-67 in the SVF group than in the SSD group. Similarly, results obtained using enzymatically-isolated SVF, either with collagenase types I and II or with collagenase type I alone, showed higher levels of Ki-67 in the treatment groups with full-thickness burns than control [26]. Similarly, treatment with SVF increased Ki-67 in radiation-induced gastrointestinal syndrome in mice [28]. This effect could be due to the presence, or a result of the activity, of ADSCs in the SVF. ADSCs differentiate into keratinocytes and other cell types to rebuild the epidermis and dermis layers. In an animal model, treatment with ADSCs, in diabetic rats, enhanced neovascularization and re-epithelialization in wounds treated with grafted cells [29]. The indirect effect could be through the secretion of several growth factors, such as transforming growth factor β1 (TGF-β1), which can repair damaged tissue [30]. The signaling pathway of TGF-β1 is important for the epithelization of the wound area. That increases Smad2 phosphorylation, which is essential for wound closure [31]. In addition, the mechanism could involve the upregulation of other growth factors, such as fibroblast growth factor-7 (FGF-7), keratinocyte growth factor-1 (KGF-1), or platelet-derived growth factor-BB (PDGF-BB), which are all involved in keratinocyte proliferation, growth, and differentiation, thus enhancing re-epithelization [32]. Therefore, SVF can enhance epithelial proliferation through cellular differentiation or paracrine signaling.

Besides the role of cell migration, re-epithelization is affected by many factors, such as pro-inflammatory cytokines and growth factors, through intrinsic interaction between cells in adjacent tissue. For instance, TNF-α stimulates keratinocyte proliferation and intracellular adhesion molecule-1 expression. IL-6 inhibition may delay keratinocyte proliferation [33]. Therefore, different mediators can affect the proliferation phase and should be investigated.

Cell proliferation can also be associated with scar hyperplasia formation that results from excessive proliferation and collagen secretion. Results from this study did not show scar hyperplasia formation in the SVF group. This indicates that SVF controls re-epithelization in a regulated way.

It is important to understand the mechanisms underlying the efficacy of SVF in burn treatment. Previous studies explored the role of different cells in the healing stages using enzymatically-isolated SVF. Treatment with SVF increases a variety of markers, such as Ki-67, keratinocyte differentiation marker cytokeratin 17 (CK17), and stem cell marker (CD44), that play a role in epithelial proliferation [34]. However, the role of mechanical isolation of SVF requires further investigation. Other markers, such as CCK8, and analysis need to be investigated to emphasize these results.

The results of this study suggest that mechanically isolated SVF has a similar effect to enzymatic isolation on re-epithelization and burn healing. Mechanical isolation stimulates epithelial cell proliferation by stimulating the proliferating marker Ki-67. In the future, other markers and mediators should be investigated. Our study provides an alternative option for SVF treatment using mechanical isolation, which is faster and more applicable than enzymatic isolation in clinical settings. However, further research is required to examine the mechanisms underlying the efficacy of mechanically isolated SVF in burn treatment.

Conclusion

This study found the role of SVF, when isolated mechanically, in activating the proliferating marker (Ki-67) in the epithelial layer during the healing stages of burned skin. Mechanical isolation of SVF has the same efficacy as the enzymatic one.

Availability of Data and Materials

The author has all the data, materials, and ethical approval included in this study and takes responsibility for the integrity of these data.

Author Contributions

FK contributed to the design of the work; analysis, interpretation of data for the work, and drafting and revising the manuscript. FK approves the final manuscript. The author has participated sufficiently in the work and agreed to be accountable for all aspects of the work.

Ethics Approval and Consent to Participate

This study was conducted under the ethical approval of the Animal Care and Use Committee office (ACUC), King Fahad Medical Research Center, King Abdulaziz University (No. ACUC-20-11-35).

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Conflict of Interest

The author declares no conflict of interest.

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