Original
ISSN 2413-0516

The Effect of Adipose Derived Stromal Vascular Fraction on The Early Inflammatory Phase of Burn Wound Healing in Male Rat Model

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(Submitted: 23 February 2023 – Revised version received: 14 March 2023 – Accepted: 02 April 2023 – Published online: 26 June 2023)

Abstract

Objectives: Severe burn treatment remains challenging. Research has investigated the efficacy of stem cells and stromal vascular fraction (SVF) in many types of injuries. SVF has advantages for its heterogenous population of cells and avoidance of culturing and ethical issues. SVF can be isolated either enzymatically or mechanically. Enzymatically isolated SVF reduced inflammation and enhanced neovascularization and re-epithelization in the treatment of burn injury. This study highlighted the efficacy of applying mechanically isolated SVF on the early inflammatory phase of deep partial-thickness burn by investigating toll-like receptor 4 (TLR4) signaling pathway.

Methods: 30 male Wistar rats with deep partial-thickness burns were assigned into: The control group, silver sulfadiazine (SSD) cream group, and mechanically isolated SVF group. Morphological, histopathological evaluation of inflammation, and immunohistochemical analysis for (TLR4, TNF-α, IL-1β, and IL-6) were evaluated for the first eight days post-treatment.

Results: Morphologically, the SVF group significantly reduced edema and increased wound bed dryness on day one compared to the control group ($P = 0.0001$) and to the SSD group ($P = 0.001$). Histopathologically, the SVF group significantly reduced inflammation four days post-treatment compared with the control group ($P = 0.045$). The SVF group significantly reduced TLR4 protein expression on days four and eight post-treatment compared with the control group ($P = 0.001$, $P = 0.042$, respectively). The TNF-α protein expression in the SVF group was significantly lower on days four and eight post-treatment compared with the control group ($P = 0.046$, $P = 0.046$, respectively) and with the SSD group ($P = 0.008$, $P = 0.001$, respectively). The IL-1β expression was significantly reduced in the SVF group compared to the control group on day four post-treatment ($P = 0.017$). There were no significant differences in IL-6 expressions between all groups on both days.

Conclusion: The mechanical isolation of SVF has an early anti-inflammatory impact on deep partial-thickness burn injury. This effect could be through inhibiting TLR4, TNF-α, and IL-1β pathways. This could partially explain the mechanism behind SVF efficacy in the healing process of burn injury.

Keywords: Stromal vascular fraction, burn injury, inflammation, toll-like receptor 4

Introduction

Burns are among the most devastating injuries worldwide.[1] Nonfatal burns injuries cause morbidity, prolonged hospitalisation, disfigurement, and disability, which affects the lives of patients.[2] Skin wounds are defined as structural and functional damage or disruption of normal skin.[3] Thermal skin burns are defined as the degradation of skin tissue due to heat exposure. Heat is the most common cause of skin burns; however, it can also be caused by radioactivity, electricity, chemicals, or friction.[4] Damage can range from simple damage to the epithelial layer to damage to deeper layers or other structures, such as muscles and blood vessels.[3] Burns can be categorised according to their depth into superficial dermal burns, superficial partial-thickness burns, deep partial thickness burns, and full thickness burns.[5] Management aims to provide rapid and comprehensive treatment to restore the skin's structure and functionality.

The healing process involves four overlapping phases: haemostasis, inflammation, proliferation, and remodeling.[5] In acute burn injuries, several types of cell types, such as platelets, fibroblasts, neutrophils, macrophages, and lymphocytes, are present at the wound site. They are regulated by the complex interaction between cytokines and neuroendocrine mechanisms.[6] During the early inflammatory phase (1-2 days), neutrophils, granulocytes, and macrophages at the wound site release an influx of inflammatory mediators and growth factors.[7] Neutrophils migrate at the wound site to phagocyte bacteria and other foreign particles. Moreover, epithelial cells around the wound site begins to proliferate and deposit components of the basal membrane. During the late inflammatory phase (2-3 days), several mediators attract macrophages to the wound site, including cytokines, leukotrienes, platelet-derived growth factors, and transforming growth factors-ß. Macrophages play both phagocytic and proliferative roles in wound healing. They also secrete further growth factors, such as transforming growth factor- α, basic fibroblast growth factor, and heparin-binding epidermal growth factor, which further stimulate the inflammatory response. Prolonged inflammation can impair the healing process and lead to chronic burn injury.

Toll-like receptors (TLRs) are recognition receptors that activate the innate immune system and inflammatory response, damage tissue sensing, infection control, and enhance tissue repair. Ten TLR members have been identified in humans; six are located on the cell surface (TLR1,2,4,5,6, and 10), whereas (TLR3,7,8, and 9) are intracellular (8). TLR4 is a cell surface receptor of the circulating and skin cells, including leukocytes, keratinocytes, endothelial cells, myofibroblasts, and primary fibroblasts.[8] TLR4 is a well-recognised receptor for bacterial lipoproteins and lipopolysaccharide (LPS).[9] After a burn injury, damage-associated molecular patterns (DAMPS) and pathogen-associated molecular patterns (PAMPs) emanate from a burn injuries and activate TLRs. Activation of TLR4 pathway results in an inflammatory response, which stimulates pro-inflammatory cytokine secretion, such as IL-1β, IL-6, IL-8, and TNF-α.[8]

Burn injury treatment depends on the degree of burn, the affected area, the age of patient, and health status. Preventing

infection, reducing fibrosis, and enhancing skin's thickening is crucial for deep-partial thickness burn.[5] However, interventiona are needed to improve functional recovery and morphological construction. Recently, research has shifted to using stem cell and stromal vascular fraction (SVF) as treatment options. The SVF is considered a promising therapy because of its advantages over stem cells; it has a heterogeneous population of cells, and its isolation methods avoid culturing and ethical issues. The SVF can be isolated either enzymatically or mechanically. Studies have used enzymatically isolated SVF to treat burn injuries. The results showed enhanced re-epithelisation, and neovascularization, and reduced inflammation and scar formation.[1,10-21] This study highlights the therapeutic effect of mechanically isolated SVF on the early inflammatory phase of deep partial-thickness burns morphologically, histologically, and immunohistochemically, for TLR4 and pro-inflammatory cytokines.

Methodology

Experimental Animal

This study was approved by the Research Ethics Committee, Faculty of Pharmacy, King Abdulaziz University (Ref: PH-1443–21). This study included thirty male Wistar rats, weighing 265 ± 31 g, and six rats for fat isolation. All rats were kept at a controlled temperature (22 ± 1°C) with a 12-hour light/dark cycle. They were fed with standard laboratory chow and given water ad libitum. Each animal was kept individually in a separate cage for acclimatization for 3 days prior to the experiment.

Deep Partial-Thickness Burn Induction

In this study, Ketamine/Xylazine was used to anesthetize rats by intraperitoneal injection (60 mg/kg Ketamine and 10 mg/kg Xylazine®).[22] A deep partial-thickness burn was created to the rats' dorsal area after it was shaved and disinfected as previously described.[23-25] A heated iron stamp (1.8 cm diameter and weighted 22 g) up to 99–100°C was applied directly to the skin for 6 seconds. Intraperitoneal injection of ringer lactate (2 ml/100 g body weight) was used to resuscitate the animals immediately to prevent spinal shock.[26,27] Post-burn induction, every rat was kept individually in a separate cage bedded with cotton under-pad and was changed daily for 5 days to minimize wound infection. Early post-burn analgesia was provided by adding 2 mg/ml paracetamol in rats drinking water for three days.[28] The burn degree was confirmed histopathologically using H&E staining.

Study Design

Thirty rats were assigned into three groups, and six rats were used as donors for adipose tissue collection. Study groups of, 10 rats each, were assigned as follows: the control group was treated with normal saline, the SSD group was treated with silver sulfadiazine cream (Flamazine®) as standard burn treatment, and the SVF group was treated with mechanically isolated SVF. The control group received an intradermal injection of normal saline solution, the SVF group had an intradermal injection of (1×10^6 cells/ml), whereas the SSD group received a thin layer of the cream. The duration of the experiment was for 8 days. The day of burn induction was considered as day (0) of the experiment, and day (1) was the treatment day. Five rats from each group were euthanized on days 4 and 8. The cervical dislocation was used to euthanize all rats at the end of the study period.

SVF Mechanical Isolation and Preparation

The inguinal area was shaved and disinfected in six rats. The fat pad was washed with normal saline solution and finely minced using two sterilized scalpels. The minced fat was transferred into two 10 mL syringes connected to a Luer-Lok connector with a 2 µm internal diameter and shifted mechanically 30 times to achieve emulsification of fat. The emulsified fat was washed with saline and centrifuged at 400x for 5 mins to separate the SVF. The fat layer and supernatant were discarded. SVF pellet was washed with saline and centrifuged twice. Then, cells were filtered over the sterile nylon cloth (100 µm and 40 µm), and the effluent was collected in a sterile tube to remove the connective tissues' remnants and centrifuged at 400x g for 5 mins.[29,30]

Total cell count was conducted using Trypan blue dye exclusion test. After counting cells, SVF solution was prepared with normal saline resulting in a cell volume equivalent to (1×10^6 cells/ml). Total cells were injected intradermally at the four edges of the burn to cover the wound bed. Total cell concentration was chosen according to previous data showing that the concentration of 1×10^6 cells per wound or more has an impact on the tissue regeneration.[29,31,32]

Morphological Analysis

The morphological changes were examined based on three criteria: redness color, edema, and wound bed dryness, as described previously.[1,33] Skin-burned wound photographs were taken to observe the morphological changes on 1- and 7 days post-treatment. The wound changes were examined and recorded daily by the same researcher.

Histopathological Evaluation of Inflammation

The burn area and surrounding skin were collected after euthanasia. Each sample was embedded in 10% formaldehyde for a standard H&E staining.[14,33] Prior to sectioning, paraffin blocks were placed in potassium hydroxide (KOH) for 10 minutes to soften the burned tissue and prevent it from fragmentation.[34] Sections were cut (4 µm) and placed on slides for staining.

The inflammation was evaluated in the entire wound on days 4 and 8 of the experiment duration by using a light microscope corroborated by a grid and ruler measurement via the Philips IntelliSite Pathology Solution (Philips Digital Pathology Solution, Netherlands) software.

Inflammation was assessed by the inflammatory cell density. It was calculated by counting the polymorphonuclear and mononuclear numbers and graded for every tissue specimen at x40 of magnification from 20 different areas using a grid (0.1 mm^2). Every measured field was graded as follows: Score 0: normal (cell count <20/field), score 1: moderate—many inflammatory cells (cell count 20-300/field), score 2: severe - exaggerated inflammatory cellularity (cell count > 300/field). The mean of the total count was calculated for every tissue specimen. The examination was performed by the same researcher blindly.

Immunohistochemistry Staining

Skin samples were collected on days 4 and 8 post-treatment for IHC analysis. For IHC, slides were (4 µm thickness) deparaffinized in xylene, rehydrated through 100, 95, and 70% graded ethanol alcohol solutions, washed, then rehydrated with phosphate-buffered saline (PBS pH 7.4). Subsequently, antigen retrieval was performed by boiling slides at 95°C for 10 minutes with 10 mM sodium citrate buffer (pH 6.0). A visualization agent was used from the detection system, Abcam (#ab64264, Abcam mouse and rabbit specific HRP/Dab (ABC) detection IHC Kit, Abcam, Cambridge, UK). Slides were incubated with primary antibodies TLR4, TNF-α, IL-6 (#ab22048, #ab220210, and #ab9324, Abcam, Cambridge, UK) and IL-1β (#pa5-105048, Thermo Fisher Scientific, USA). All primary antibodies were diluted to 1:100. Slides were placed in humidity chambers and incubated with TLR4, TNF-α, and IL-1β at 4°C overnight, and IL-6 at 4°C for 45 minutes. All sections were scanned by using Philips IntelliSite Pathology Solution (Philips Digital Pathology Solution, Netherlands) software. Optical density was used across 4 different fields by light microscopy at x10 magnification. Staining intensity was scored as follows: 0, no staining; 1, weak staining; 2, moderate staining; 3, strong staining; and 4, intense staining as previously described.[35,36] The mean was calculated for every tissue specimen.

Statistical Analysis

Statistical analysis was performed using SPSS Win 10.0 software and presented as mean ± standard deviation. Results were compared between groups using one-way ANOVA Mann-Whitney U and Kruskal-Wallis H tests. *P*-value of < 0.05 was considered statistically significant.

Results

Deep-Partial Burn Induction

Histopathological analysis results showed that scalding with 100°C for 6 seconds caused epidermal damage, with the presence of shrunken and elongated nuclei at the basal layer. The collagen fiber had a basophilic color and disorganized fibers at the upper dermis layers. Moreover, minor damage to the hair follicles with a blocked vessel was observed. At the deep lower dermis, dilation of the blood vessels was observed with damage to the subcutaneous fat layers. The skeletal muscles layer was unaffected. Therefore, the burn damage was considered a deep partial-thickness burn injury (Figure 1).

SVF Mechanical Isolation

About 8×10^6 cells/ ml were isolated from six rats. Trypan blue results showed around 96 % of cells' viability.

Morphological Analysis

The macroscopic changes were measured on days 1, 4, and 8 for all experimental groups. Redness of the wound bed was observed in all groups ($P = 0.60$, $P = 0.129$, and $P = 0.522$, respectively) (Table 1).

On day one, the burn wounds area exhibited swelling and edema. The SVF group showed a significant reduction in edema formation compared to the control group ($P = 0.000$) and the SSD ($P = 0.001$). There were no significant differences between the control and SSD groups ($P = 0.268$). However, all groups had no significant differences on days 4 and 8 ($P = 0.541$ and $P = 1$, respectively) (Table 1).

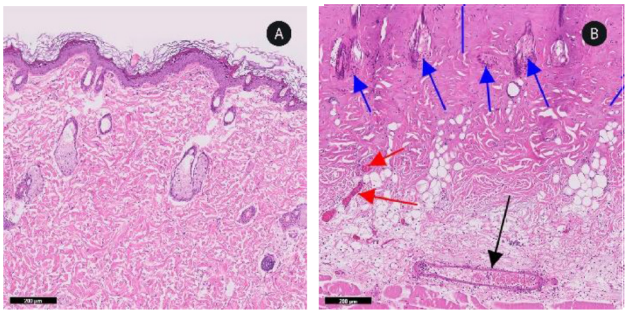

Fig. 1 **Histopathological image of a burn skin stained with H&E 24 hours after burn induction. (A) Normal skin, (B) Deep partial-thickness burn. Scale bar: 200 µm. Hair follicles (blue arrow), Blocked vessels (red arrow), and Dilated vessels (black arrow).**

The wound bed dryness was significantly higher in the SVF group compared to the control group ($P = 0.000$) and SSD group ($P = 0.001$) on day 1. There were no significant differences between the control and SSD groups ($P = 0.268$) on day 1. All groups had no significant differences on days 4 and 8 ($P = 0.765$ and $P = 1$, respectively) (Table 1).

Skin-burned wound photographs showed a minor and non-significant prominent ring (redness) representing the zone of hyperemia surrounding the wounds in all groups. Additionally, photographs showed the morphological changes in edema and wound bed dryness post-treatment with SVF compared with other groups (Figure 2).

Histopathological Evaluation of Inflammation

On day one, the inflammation intensity was significantly lower in the SVF group compared to the control group ($P = 0.045$), but it was not significant compared to the SSD group ($P = 0.465$). On day 8, all did not show any significant differences ($P = 0.558$) (Figure 3).

Table 1. **Wound bed analysis on days 1,4, and 8 post-treatments of deep partial-thickness burn wound. Control: Normal saline ($n = 10$), SSD: Silver sulfadiazine ($n = 10$), and SVF: Stromal vascular fraction ($n = 10$).**

Criteria	Time	Control	SSD	SVF	*P*-value
Color (redness)	Day 1	0 ± 0.00	0.07 ± 0.26	0.07 ± 0.26	$P = 0.60$
	Day 4	1.00 ± 0.00	1 ± 0.00	0.87 ± 0.35	$P = 0.129$
	Day 8	0.80 ± 0.42	0.30 ± 0.67	0.90 ± 0.57	$P = 0.522$
Edema	Day 1	1 ± 0.85*	0.67 ± 0.72**	0 ± 0.00*,**	$P = 0.001^{(+)}$
	Day 4	0.13 ± 0.35	0.40 ± 1.06	0.07 ± 0.26	$P = 0.541$
	Day 8	0 ± 0.00	0 ± 0.00	0 ± 0.00	$P = 1$
Wound bed dryness	Day 1	3 ± 0.85*	3.33 ± 0.72**	4 ± 0.00*,**	$P = 0.001^{(+)}$
	Day 4	3.87 ± 0.35	3.93 ± 0.26	3.93 ± 0.26	$P = 0.765$
	Day 8	4 ± 0.00	4 ± 0.00	4 ± 0.00	$P = 1$

Data are expressed as mean ± standard deviation.
(+)Indicates $P < 0.05$ by comparison of all groups (Kruskal-Wallis test).
*Indicates $P < 0.05$ for comparison between control and SVF groups.
**Indicates $P < 0.05$ for comparison between SSD and SVF groups.

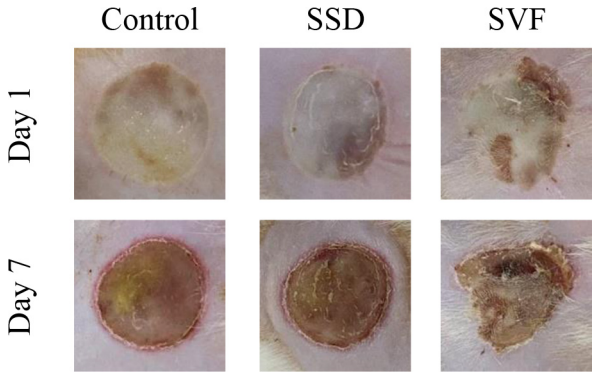

Fig. 2 **Photographs showing wound of rat skin burned wounds at days 1 and 7 post-treatment. Control: Normal saline ($n = 10$), SSD: Silver sulfadiazine ($n = 10$), and SVF: Stromal vascular fraction ($n = 10$).**

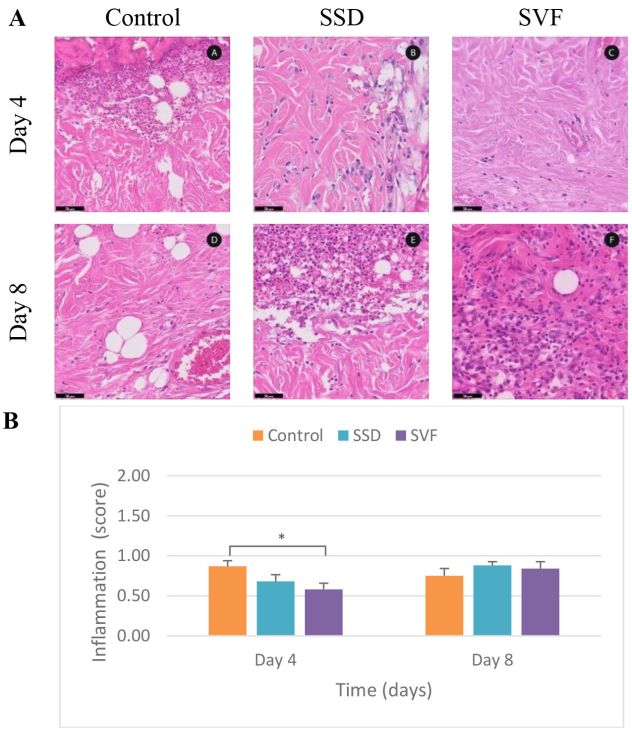

Fig. 3 **(A) Histopathological image showed the inflammatory cell density of deep partial-thickness burn skin wounds on days 4 and 8 post-treatment at x40 (H&E, scale bar: 50 µm). Grade 0: B, C, and D; Grade 1: A, E, and F. (B) Comparison of inflammation scores between the study groups in deep partial-thickness burn wounds on days 4 and 8. Control: Normal saline ($n = 10$), SSD: Silver sulfadiazine ($n = 10$), and SVF: Stromal vascular fraction ($n = 10$). Data are expressed as mean ± standard error. *Indicates $P < 0.05$ for comparison between control and SVF groups.**

Immunostaining for TLR4 and Pro-Inflammatory Cytokines

The slides were scored according to the optical density of all antibodies. TLR4, IL-1β, and IL-6 expressions were observed in the epidermis layer, while TNF-α expression was detected in the dermis layer.

On day 4, TLR4 protein expression was significantly lower in the SVF and SSD groups compared to the control group ($P = 0.001$ and $P = 0.000$, respectively). However, no significant differences were recorded between the SVF and SSD groups ($P = 0.229$). On day 8, the SVF group showed a significant reduction in TLR4 expression compared to the control group ($P = 0.046$). However, no significant differences were recorded in the SSD group compared to the control group and SVF group ($P = 0.127$ and $P = 0.532$, respectively) (Figure 4).

Treating burned skin with mechanically isolated SVF significantly reduced TNF-α expression 4 days post-treatment compared to the control and SSD groups ($P = 0.046$ and $P = 0.008$, respectively). Moreover, the SSD group showed a significant increase in TNF-α compared to the control group ($P = 0.026$). On day 8, the SVF group showed a significant reduction of TNF-α expression compared to the control group ($P = 0.046$) and compared to the SSD group ($P = 0.001$). However, no significant differences were recorded between the control and SSD groups ($P = 0.089$) (Figure 5).

The expression of IL-1β in the SVF group showed a significant reduction on day 4 compared to the control group ($P = 0.017$). However, the SSD group showed no significant differences compared to the control and SVF groups ($P = 0.166$ and $P = 0.225$, respectively). On day 8, there were no significant differences in IL-1β expression between all groups ($P = 0.168$) (Figure 6).

The IL-6 expression results showed no significant differences between all groups on both days ($P = 0.474$ and $P = 0.845$, respectively) (Figure 7).

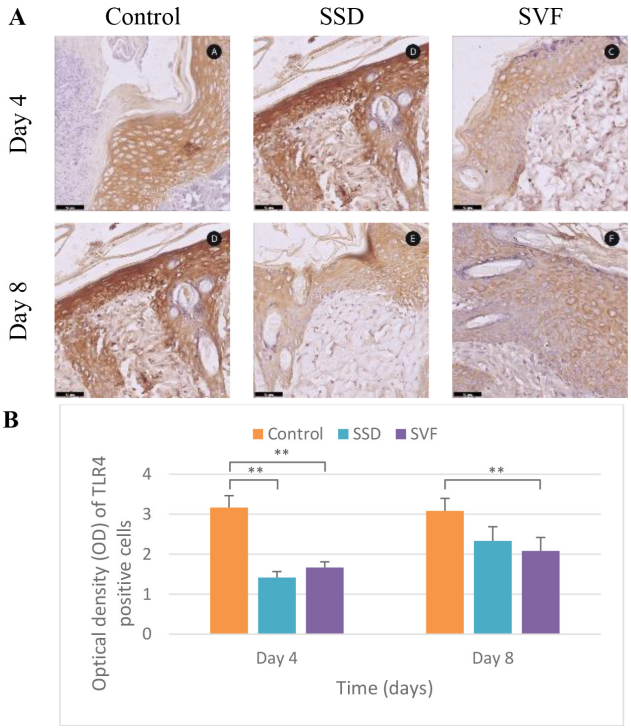

Fig. 4 **(A) Expression of TLR4 in deep partial-thickness burn skin wounds on days 4 and 8 post-treatment detected with immunohistochemistry staining at x20 (scale bar: 50 µm). (B) Comparison of immunohistochemical scores of TLR4 expression between the study groups in deep partial-thickness burn wounds on days 4 and 8. Control: Normal saline ($n = 3$/day), SSD: Silver sulfadiazine ($n = 3$/day), and SVF: Stromal vascular fraction ($n = 3$/day). Data are expressed as mean ± standard error. *Indicates $P < 0.05$ for comparison between control and SSD groups. **Indicates $P < 0.05$ for comparison between control and SVF groups.**

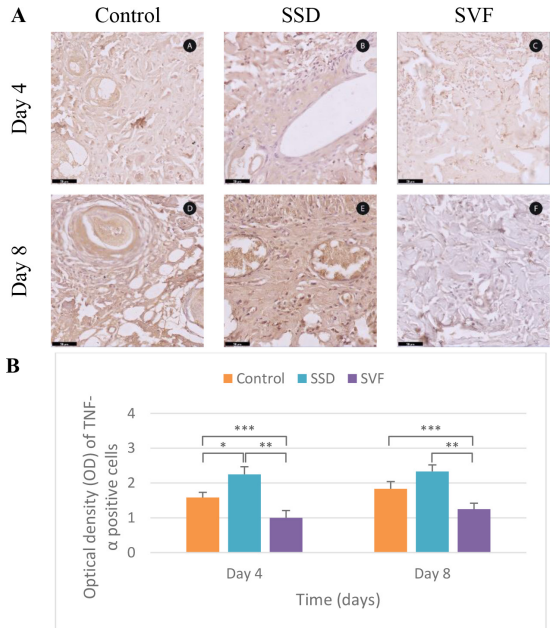

Fig. 5 **(A) Expression of TNF-α in deep partial-thickness burn skin wounds on days 4 and 8 post-treatment detected with immunohistochemistry staining at x20 (scale bar: 50 µm). (B) Comparison of immunohistochemical scores of TNF-α expression between the study groups in deep partial-thickness burn wounds on days 4 and 8. Control: Normal saline (*n* = 3/day), SSD: Silver sulfadiazine (*n* = 3/day), and SVF: Stromal vascular fraction (*n* = 3/day). Data are expressed as mean ± standard error. *Indicates *P* < 0.05 for comparison between control and SSD groups. **Indicates *P* < 0.05 for comparison between SSD and SVF groups. ***Indicates *P* < 0.05 for comparison between control and SVF groups.**

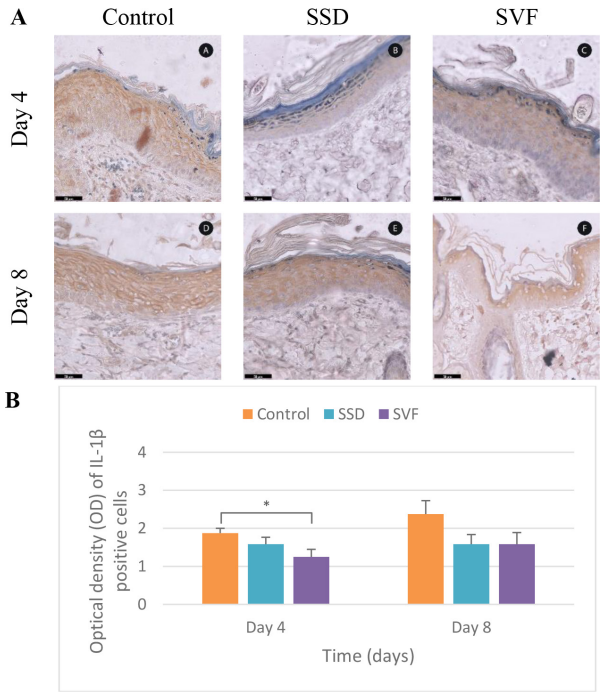

Fig. 6 **(A) Expression of IL-1β in deep partial-thickness burn skin wounds on days 4 and 8 post-treatment detected with immunohistochemistry staining at x20 (scale bar: 50 µm). (B) Comparison of immunohistochemical scores of IL-1β expression between the study groups in deep partial-thickness burn wounds on days 4 and 8. Control: Normal saline (*n* = 3/day), SSD: Silver sulfadiazine (*n* = 3/day), and SVF: Stromal vascular fraction (*n* = 3/day). Data are expressed as mean ± standard error. *Indicates *P* < 0.05 for comparison between control and SVF groups.**

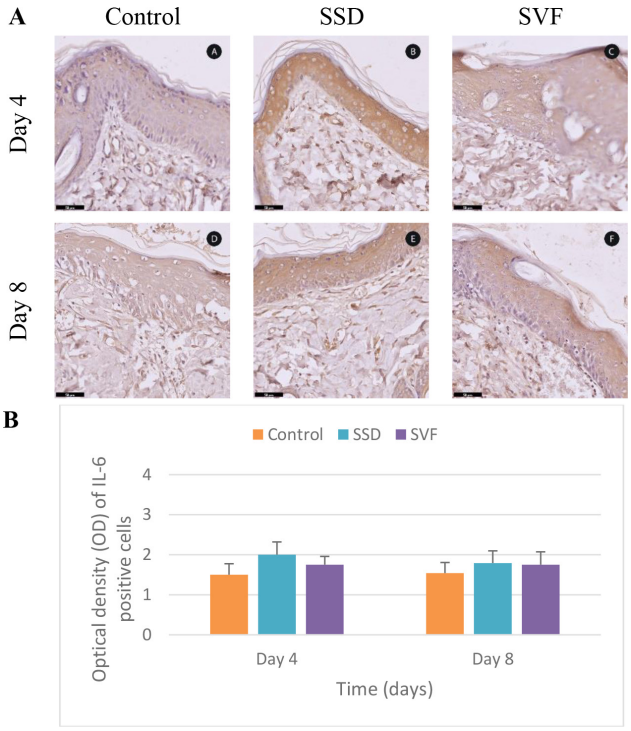

Fig. 7 **(A) Expression of IL-6 in deep partial-thickness burn skin wounds on days 4 and 8 post-treatment was detected with immunohistochemistry staining at x20 (scale bar: 50 µm). (B) Comparison of immunohistochemical scores of IL-6 expression between the study groups in deep partial-thickness burns wounds on days 4 and 8. Control: Normal saline (*n* = 3/day), SSD: Silver sulfadiazine (*n* = 3/day), and SVF: Stromal vascular fraction (*n* = 3/day). Data are expressed as mean ± standard error.**

Discussion

The healing process of burn injuries involves four overlapping phases, haemostasis, inflammation, proliferation, and remodeling. During the inflammatory phase, polymorphonuclear leukocytes (PMNs) and mononuclear cells (MNs) infiltrate and participate in removing dead tissue and infections.[5,37] They are regulated by complex interactions between different mediators, including TLRs and inflammatory cytokines. After burn injury, TLR4 is activated in response to DAMPs and PAMPs. TLR4 activation produces pro-inflammatory cytokines, such as TNF-α, IL-1β, IL-8, and IL-6.[8] Targeting the TLR4 signaling pathway could represent a treatment option to reduce inflammation and improve the healing of burn injuries. Therefore, the focus of this study was to investigate TLR4 and pro-inflammatory cytokines during the inflammatory phase of deep-partial burn healing in a rat model.

Treatment of severe burn injuries remains a challenge for therapists. The aim of treating deep-partial thickness burns is to avoid infection and fibrosis and to restore the structure and functionality of the skin. Regenerative medicine has shifted from using stem cells to SVF owing to its reparative capacity, differentiation potential, and ease of harvesting from adipose tissue. Two isolation techniques of SVF have been established: enzymatic and mechanical. Previous studies have investigated the efficacy of enzymatical isolation of SVF in the treatment of burn injury.[1, 10–21] Enzymatically isolated SVF enhanced the healing of burn injuries by increasing re-epithelization, neovascularization, collagen deposition, and reducing

inflammation. However, mechanical isolation of the SVF may offer a viable clinical option because of its ease of isolation, preparation, safe application, and preservation of the microenvironment. Using mechanical isolation methods, the enzyme purification step is avoided; thus the microenvironment that aids in cell communication and differentiation is preserved for better outcomes. Therefore, this study investigated the efficacy of mechanically isolated SVF during the inflammatory phase of deep-partial thickness burn injuries.

This study evaluated the quality of SVF harvested from inguinal fat via the mechanical isolation technique, with a high proportion of cell count and viability (96%). These results were similar to those of studies that obtained around (93–97.55%) of viable cells from mechanically isolated SVF. The cell count in this study was higher than that in similar studies that mechanically isolated SVF at approximately 0.019–0,889 × 10^6 cells/ml of cell count.[29,38-42] The differences in the results depended on the total volume of the extracted fat. These results indicate that mechanically isolated SVF potentially generates a high cell count and viability.

The morphological evaluation of inflammation showed a minor and non-significant prominent ring (redness) representing the zone of hypaeremia surrounding the wounds for up to eight days post-treatment in all groups. These results are consistent with those of similar studies that showed no significant differences in wound redness in all study groups up to seven days post-treatment of deep partial- and full-thickness burn wounds that are treated with enzymatic SVF.[1,14] However, the results of this study showed that the mechanically isolated SVF significantly reduced oedema at the wound site on day one post-treatment compared to the other groups. These results agreed with those of a previous study conducted in a deep partial-thickness burn model, which demonstrated a reduction in oedema formation seven days post-treatment at the wound site in the group treated with SVF enzymatically isolated.[1] Therefore, mechanically isolated SVF has an early impact on oedema formation. A previous study revealed that treatment with SVF improves vasodilation and reduced cellular infiltration in a focal model of arterial inflammatory insult.[43] The reduction in oedema formation could be due to the adipose-derived mesenchymal stem cells (ADSCs) in the SVF.[44] The ADSCs can reduce vascular permeability, leading to a reduced fluid retention, and decreased oedema formation. Therefore, these results suggest that the early oedema reduction effect of mechanically isolated SVF could be due to the anti-hyperpermeability role of ADSCs in SVF. Further studies are required to clarify the effects of SVF on microvascular permeability and osmotic activity in burn models.

Based on the histopathological evaluation of inflammation, the SVF treatment group showed a significant early reduction in inflammatory cell infiltration on day four compared with the other groups. Other studies have shown that treating deep partial-thickness burns with enzymatically isolated SVF inhibits inflammation by reducing inflammatory cells infiltration seven and ten days post-treatment.[1,10,15] This shows that the mechanically isolated SVF has an early anti-inflammatory response when compared to the enzymatic method for treating deep-partial thickness burn injuries. However, others reported an increase in cellular infiltration three days post-treatment in deep partial- and full-thickness burn with enzymatically isolated SVF.[14,19] The differences in these results suggested that SVF could modulate inflammation in a bi-directional manner. This anti-inflammatory effect can be mediated by reducing vascular permeability, which decreases cellular infiltration.[43,44] This may be due to the paracrine effects of ADSCs, which reduce the synthesis of various cytokines and chemokines.[14,45]

To further investigate the anti-inflammatory effects of mechanically isolated SVF, TLR4 and proinflammatory cytokines expression was examined during the inflammatory phase of deep partial-thickness burns. Treatment with mechanically isolated SVF showed an inhibitory effect on TLR4 protein expression on days four and eight post-treatment compared with the control. Previous studies have shown that ADSCs have an inhibitory effect on TLR4 expression in the treatment of sepsis induced by LPS.[46] Therefore, the reduction in TLR4 levels could be due the anti-inflammatory role of ADSC in the SVF. During burn injury, DAMPS are generated by necrotic tissue to activate the immune response via TLRs, producing pro-inflammatory cytokines. TLRs represent the link between burn injury, infection, and inflammation. In the burn wound, myeloid-derived suppressor cells (MDSCs) infiltrate to regulate the immune response by suppressing T-cells and regulating cytokines synthesis.[47] They are also present in the adipose tissue and play a role in tissue hemostasis. Taken together, the cellular composition of SVF is involved in TLR4 inhibition as an immune response to burn injury.

In this study, the early anti-inflammatory effect in the mechanical SVF group was associated with substantial inhibition of TNF-α protein expression at four and eight days after treatment compared to the other groups. These results are consistent with several studies that exhibited a suppression in TNF-α protein expression after treatment with enzymatically isolated SVF.[48,49] A reduction in TLR4 expression could result in suppression of TNF-α expression. In addition, the reduction of TNF-α level suggests that the anti-inflammatory effect could be due to the paracrine signalling of SVF that directly activates macrophage migration to the wound site.[50] Previous studies have suggested that the anti-inflammatory role of SVF in reducing TNF-α levels is due to its ability to significantly increase M2 macrophages up to 14 days post-treatment.[48] This suggests that SVF controls the inflammatory response by suppressing TNF-α expressions and regulating cytokine-cytokine receptor interaction during wound healing.

The SVF group showed a significant early reduction in IL-1β protein expressions on day four compared with the control group. These results were consistent with a study applying SVF to treat full-thickness burns that previously showed a significant reduction of IL-1β expression two days post-treatment.[49] This could be a result of the suppression of the TLR4 pathway. These results suggested that mechanical SVF has an anti-inflammatory ability to minimise IL-1β levels, which could promote deep partial-thickness wound healing.

The results from this study did not show any significant differences between all groups in IL-6 protein expressions post-treatment between the groups. In skin wound injuries, ADSCs and SVF exhibited high expression levels of IL-6 after treatment.[49,51,52] SVF studies indicated that high expression of IL-6 suppresses the inflammatory response by directly reducing TNF-α and IL-1β expressions and changing its behavior.[53] This effect of the SVF paracrine activity on TNF-α and IL-6 expressions directly activates macrophage migration to the wound site.[50] In addition, activated macrophages indirectly participate in inhibiting inflammation events by

secreting anti-inflammatory cytokines, such as IL-10, and growth factors, such as the vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF).[48] These changes indicated that SVF shifts the IL-6 pro-inflammatory reaction and induces an anti-inflammatory effect, which could accelerate wound healing.[50,54,55]

The anti-inflammatory response is essential for preserving the tissue surrounding burns from damage and for controlling the hyperinflammatory state in burn injuries.[56] These findings indicate that the mechanical isolation technique of the SVF has a positive impact on reducing the early inflammatory response in deep partial-thickness burn injuries. This effect could occur directly through the cellular components of the SVF or indirectly via a paracrine effect. The early inflammatory role of mechanically isolated SVF could be modulated partially by the suppression of TLR4, TNF-α, and IL-1β. Finally, these results suggest that applying mechanically isolated SVF to treat deep partial-thickness burns is a development as a treatment option for burns.

Conclusions

Mechanically isolated SVF exerts an anti-inflammatory effect and paracrine interactions; suppression of the initial inflammation response by inhibiting TLR4, TNF-α, and IL-1β in deep partial-thickness burned rats. Further investigation for other markers is required.

Acknowledgment

This work was funded by the University of Jeddah, Jeddah, Saudi Arabia, under grant No. (UJ-22-DR-28). The authors, therefore, acknowledge with thanks the University of Jeddah for its technical and financial support.

Conflict of Interest

The authors state that there was no conflict of interest with regard to this manuscript.

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