Improved Wound Healing in Superficial Wound Infection Through α-SMA Upregulation and Increased Collagen Density by the Secretome of Hypoxic Mesenchymal Stem Cells

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Abstract

Background/Aim: Superficial wound infections cause delayed remodelling by suppressing the expression of alpha-smooth muscle actin (α-SMA), which inhibits collagen synthesis. Secretomes derived from hypoxic mesenchymal stem cells (SH-MSCs) contain a variety of cytokines and growth factors that contribute to accelerated wound healing and infection suppression. This study aimed to investigate the effects of SH-MSC gel on the expression of the α-SMA gene and the collagen density in the skin tissue of rats with superficial wound infections.

Methods: The research design utilised a post-test-only control group with a completely randomised format. The study sample consisted of 24 rats with superficial wound infections induced by *Staphylococcus aureus* bacteria at a concentration of 5 x 10^7 CFU/mL in 100 μL. The study included four groups: a healthy group, a negative control group, treatment group 1 (T1) receiving a 10 % dose of SH-MSCs gel and treatment group 2 (T2) receiving a 20 % dose of SH-MSCs gel. The expression of the α-SMA gene was analysed through qRT-PCR and collagen density was assessed using Masson's trichrome staining.

Results: The qRT-PCR analysis indicated that the α-SMA gene expression was significantly elevated in the T2 group, registering the highest level at 10.03 ± 3.78. This was followed by the T1 group, which had a mean α-SMA gene expression of 4.96 ± 2.77. The negative control treatment group exhibited the lowest level of α-SMA expression, measuring at 0.46 ± 0.09. Additionally, collagen density analysis showed an increase in the T2 group, which had the highest collagen density at 35.68 ± 5.28, followed closely by the T1 group at 34.78 ± 1.72. The negative control group exhibited the lowest collagen density, recorded at 17.39 ± 2.47.

Conclusion: The administration of SH-MSCs gel can enhance α-SMA gene expression and increase collagen density in a rat model of superficial wound infection.

Key words: Wounds and injuries, superficial; Wound infection; *Staphylococcus aureus*; Alpha-smooth muscle actin; Collagen; Mesenchymal stem cells; Secretome.

Introduction

Superficial infection wounds (SFW) are infections that occur on the skin's surface, caused by various external factors such as viruses, bacteria, fungi or parasites.[1] *Staphylococcus aureus* and *Streptococcus* are the most common bacteria that can enter a wound through cuts, scratches, or bites, leading to skin and soft tissue infections.[2] SFW is linked to an excessive inflammatory response, which is characterised by the activation of the nuclear factor kappa beta (NF-κB) transcription factor and the inhibition of wound closure processes due to collagen suppression.[3, 4] Current standard therapies for SFW, including antibiotics and corticosteroids, can lead to side effects and resistance, emphasising the need for safer and more effective treatment options.[5]

The incidence of SFW is notable, with 24.6 patients per 1000 annually; a significant proportion of cases is caused by *Staphylococcus aureus* bacteria.[6] There has been an increase in SFW cases caused by *Staphylococcus aureus*, with a significant percentage showing resistance. These statistics highlight the urgent need for alternative therapies for SFW treatment.[7] One potential therapeutic approach involves utilising secretome hypoxia mesenchymal stem cells (SH-MSCs). Preclinical studies indicate that SH-MSCs contain anti-inflammatory cytokines, including interleukin-10 (IL-10) and transforming growth factor (TGF-β),[8] which can trigger anti-inflammatory effects and induce collagen production.[9] SH-MSCs are mesenchymal stem cells cultured in a hypoxic medium that has undergone filtration using tangential flow filtration (TFF)[8] The MSC secretome with high levels of IL-10 and TGF-β can inhibit excessive inflammatory responses caused by infections in SFW.[10] Previous studies have shown that IL-10 can reduce inflammation by inhibiting the activation of NF-κB.[11] In addition, TGF-β plays a role in inducing α-SMA, causing fibroblasts to transform into myofibroblasts,[12, 13] which stimulate collagen production, crucial for the healing process and wound closure.[14-16]

Multiple studies indicate that MSC secretomes can reduce the production of pro-inflammatory cytokines and accelerate wound healing in animal models.[17] There is currently limited research on the use of SH-MSC cream in relation to α-SMA gene expression and collagen levels in a rat model of superficial infection wounds. Therefore, further investigation is necessary to assess the impact of SH-MSC administration on α-SMA gene expression and collagen levels in Wistar rats with superficial infection wounds.

Methods

Material and study design

This post-test-only control group study design was conducted at the Stem Cell and Cancer Research in Semarang, Indonesia, from July to September 2023. The study received approval from the Ethics Committee of Sultan Agung Islamic University (decision No 225/VI/2023/Komisi Bioetik).

MSCs culture and isolation

Rat mesenchymal stem cells (MSCs) were isolated from a 19-day pregnant female rat. Donor rats were first anaesthetised, after which their abdomens were carefully dissected. Under sterile conditions, the umbilical cord (UC) was collected and rinsed with phosphate-buffered saline (PBS). The UC artery and vein were removed and the cord was then cut into segments measuring 2–5 mm using a sterile scalpel. These sections were evenly placed in T25 flasks containing Dulbecco's modified Eagle's medium (DMEM) (*Sigma-Aldrich*, St. Louis, MO), supplemented with 10 % PBS and 100 IU/mL of penicillin/streptomycin (*GIBCO*, Invitrogen). The flasks were incubated at 37 °C with 5 % CO_2. The culture medium was refreshed every three days and the cells were passaged once they reached 80 % confluence. Umbilical cord MSCs at passages 4–6 was used for subsequent experiments.[18, 19]

Osteogenic and adipogenic differentiation assay of MSCs

The MSCs were cultured in a 24-well plate at a density of 1.5 x 10^4 cells per well, using a standard medium comprised of DMEM (*Sigma-Aldrich*, Louis St, MO) supplemented with 10 % FBS (*Gibco™ Invitrogen*, NY, USA) and 1 % penicillin (100 U/mL) and streptomycin (100 µg/mL) (*Gibco™ Invitrogen*, NY, USA). The cultures were maintained at 37 °C in a humidified atmosphere with 5 % CO_2 until they reached 80 % confluence, at which point the osteogenic and adipogenic differentiation protocols were initiated. For osteogenic differentiation, the standard medium was removed and replaced with an osteogenic dif-

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ferentiation medium. This medium included Human MesenCult™ osteogenic differentiation basal medium (*Stem Cell Technologies*, Singapore), supplemented with 20 % Human MesenCult™ osteogenic differentiation 5x supplement (*Stem Cell Technologies*, Singapore) and 1 % L-Glutamine (*Gibco™ Invitrogen*, NY, USA). The differentiation medium was refreshed every three days. After bone matrix formation, osteogenic differentiation was confirmed by staining with 1 mL of 2 % alizarin red solution. For adipogenic differentiation, the growth medium was switched to Human MesenCult™ adipogenic differentiation basal medium (*Stem Cell Technologies*, Singapore). The medium was changed every other day and on day 35, the cultures were stained with Oil Red O and observed under a microscope.

Characterisation of MSCs surface marker

MSCs were analysed for specific surface marker expression using flow cytometry. The cultured cells were initially incubated in the dark with primary antibodies, including mouse anti-human CD29, mouse anti-human CD90 and mouse anti-human Lin negative (CD45/CD31). This was followed by the addition of a secondary conjugated antibody. The MSCs were stained with the specific antibody for 30 minutes at 4 °C. After staining, the samples were examined using a BD Accuri C6 Plus flow cytometer (*BD Biosciences*, San Jose, CA, USA) and the data were analysed with BD Accuri C6 Plus software (*BD Biosciences*, San Jose, CA, USA).[18, 20]

Hypoxic secretome MSCs (SH-MSCs) isolation

Once the mesenchymal stem cells (MSCs) reached 70 % confluence in a 75 cm² flask containing complete medium, they were washed twice with 5 mL of phosphate-buffered saline (PBS) and then incubated in DMEM (Dulbecco's modified eagle medium, *Gibco*, USA). The cells were placed in a hypoxic chamber (anaerobic environment; *ThermoForma*, Waltham, MA, USA) with 15 mL of DMEM for 12 h. This airtight, humidified hypoxic chamber was maintained at 37 °C and continuously supplied with 5 % carbon dioxide (CO_2), 10 % hydrogen (H_2) and 85 % nitrogen (N_2), resulting in an oxygen level of approximately 0.5 %. After the incubation period, the hypoxic secretome was collected and centrifuged twice at 1,500 rpm for 3 minutes at 4 °C to eliminate debris and dead cells. The supernatant was then filtered using a tangential flow filtration (TFF) system (*Formu-latrix*, USA) equipped with sterile hollow fibre polyethersulfone membranes that have a molecular weight cut-off of 30-50 kDa to remove larger biomolecules.[21-23]

Animal studies

The sample size for this research was determined using the resource equation formula: E = N - T (where T is the number of treatments and N is the number of repetitions), resulting in E being between 10 and 20. A total of twenty-four male Wistar rats, aged 6 to 8 weeks, were purchased from local breeders in Semarang, Indonesia. The rats were raised in a controlled environment with a regular 12 h light-dark cycle and were randomly assigned to four groups: sham, negative control, SFW with SH-MSCs at 10 % in hydrogel (T1) and SFW with SH-MSCs at 20 % in hydrogel (T2). For the experimental procedure, 2x2 cm excisions were made on the backs of the rats, reaching a depth of 1 mm. A bacterial infection was induced using *Staphylococcus aureus* at a concentration of 5×10^7 CFU/mL, with a total volume of 100 μL applied using a micropipette and spread evenly with a spatula. The infected wounds received daily topical treatment with gels containing SH-MSCs at concentrations of 10 % and 20 %, applied in 100 mg doses for 14 days, starting one day after the wound creation. The healthy rats received no treatment, while the control group was treated with a base gel. The rats were euthanised on the 15th day after the treatment began.

Collection of tissue samples

After 14 days of SH-MSCs administration (day 15), animal skin samples were collected for histological and gene expression studies.[24]

α-SMA gene expression by qRT-PCR

Total RNA from rat skin tissue was extracted with TRIzol (*Invitrogen,* Shanghai, China) according to the manufacturer's protocol. Briefly, first-stranded cDNA was synthesised with 1 g of total RNA using Super-Script II (*Invitrogen*, Massachusetts, USA). SYBR No ROX green I dye (*SMOBIO Technology Inc*, Hsinchu, Taiwan) was used for reverse transcription in a real-time PCR instrument (PCR max Eco 48) and mRNA levels of the α-SMA genes were measured using the respective primers. The thermocycler conditions were as follows: initial step at 95 °C for 10 minutes, followed by 50 cycles at 95 °C for 15 seconds and 60 °C for 1 minute. The gene expression was recorded as the Cycles threshold (Ct). Data were obtained using

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Eco Software v5.0 (*Illumina Inc*, San Diego, CA, USA). All reactions were performed in triplicate and data analysis used the $2^{-\Delta\Delta}$ Ct method (Livak method).[25, 26]

Collagen density analysis

Skin tissue samples from all groups were collected for histological examination. The collected tissue samples underwent processing using the paraffin method followed by Masson's Trichrome staining to visualise collagen fibres. Stained tissue sections were observed under a light microscope at various magnifications (eg, 100x, 400x).

Statistical analysis

Data were presented as the mean ± SD. The statistical significance of differences between the groups was examined on SPSS 26.0 (*IBM Corp*, Armonk, NY, USA) using ANOVA with post-hoc Fisher's LSD analysis. $P < 0.05$ was considered significant.

Results

Mesenchymal stem cells (MSCs) were evaluated for their ability to adhere under standard culture conditions. After the fourth passage, the MSCs exhibited typical adherent behaviour, forming monolayers of spindle-shaped fibroblast-like cells (see Figure 1A). To assess their *in-vitro* differentiation potential, the MSCs were treated with osteogenic and adipogenic differentiation media. The presence of calcium deposits, indicated by red colouring in the osteogenic differentiation assay (Figure 1B), confirmed that the MSCs differentiated into osteogenic cells. Adipogenic differentiation was evidenced by the accumulation of neutral lipid vacuoles, which stained positively with Oil Red O (Figure 1C). Further characterisation of umbilical cord-derived MSCs (UC-MSCs) was performed using flow cytometric analysis, revealing that the MSCs were positive for CD90 (97.60 %) and CD29 (97.70 %), while being negative for CD45 (1.50 %) and CD31 (3.20 %) (Figure 1D). To enhance the production of cytokines and growth factors, the MSCs were cultured under hypoxic conditions with 5 % O_2 for 12 h.
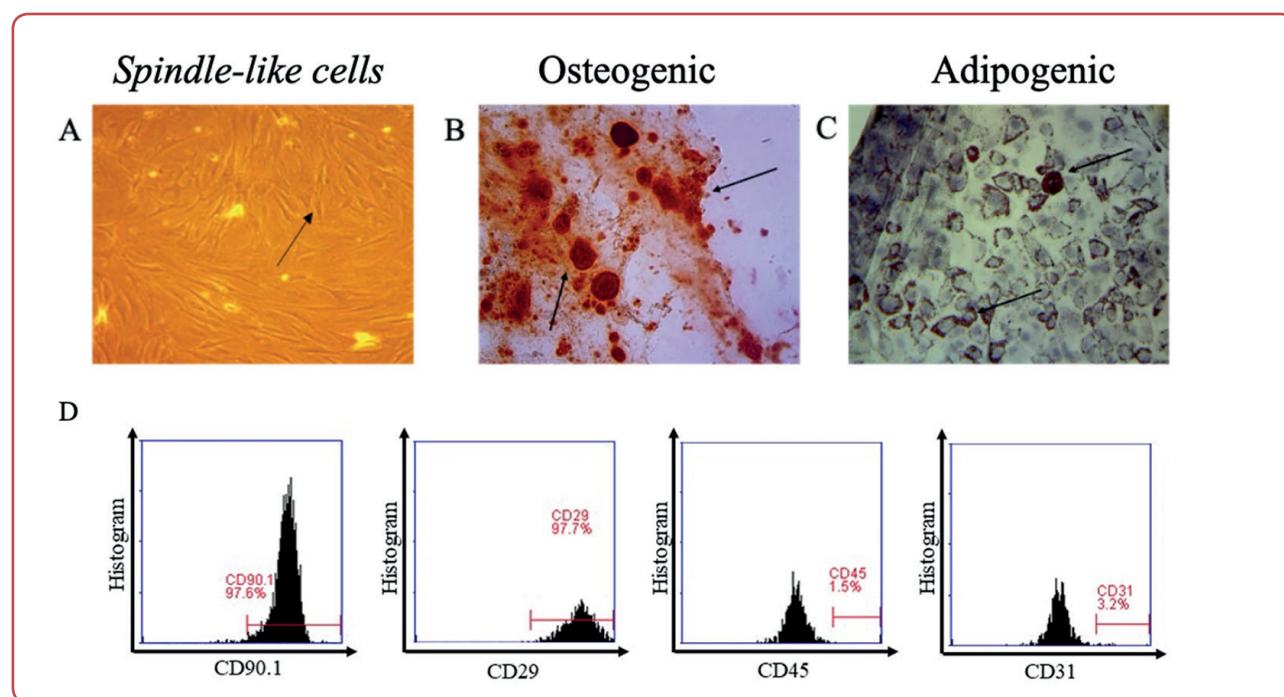
Figure 1: *Characterisation and validation of mesenchymal stem cells (MSCs). (A) Morphological MSCs. The cells appeared as homogeneous spindle shaped. (B) Calcium deposition under osteogenic differentiation assay following Alizarin Red staining. (C) The accumulation of neutral lipid vacuoles stained with oil red O (D) graphs displayed the phenotype of MSCs CD90, CD29, CD45 and CD31.*

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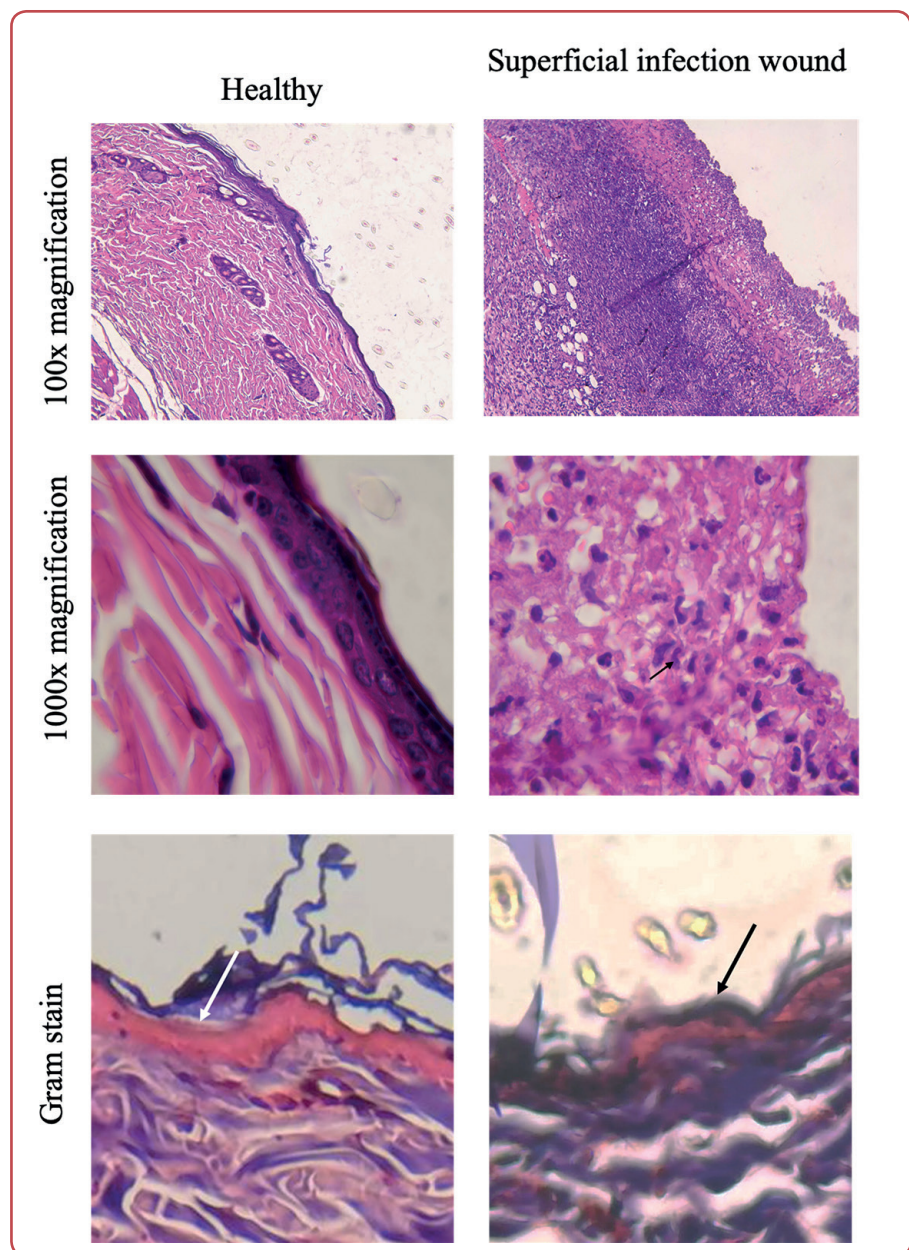
Figure 2: *The superficial infection wound was validated using haematoxylin and eosin (HE) and Gram staining. The black arrows indicate Gram-positive bacterial infection.*

The animal model was created by making an incision in the epidermis and introducing *Staphylococcus aureus* bacteria to induce infection. Validation of the superficial infection wounds was observed on the sixth day after induction. In the haematoxylin and eosin (HE) staining, signs of inflammation were noted in the superficial infection wound group, evidenced by the presence of numerous neutrophils (indicated by black arrows at 100x magnification) (Figure 2). Additionally, Gram staining revealed blue-coloured colonies in the epidermal area (also indicated by a black arrow), in contrast to the healthy group, where no blue coloration was observed in the epidermal region. These findings confirm that the animal model developed a superficial infection wound.

This study revealed significant findings showing that SH-MCs gel significantly increased the expression of α-SMA in a superficial infection wound model in rats, in a dose-dependent manner (Figure 3). The average α-SMA gene expression in Group T2 was the highest at 10.03 ± 3.78, followed by Group T1 with a mean expression of 4.96 ± 2.77. The negative control treatment group exhibited the lowest expression, measuring 0.46 ± 0.09. These results
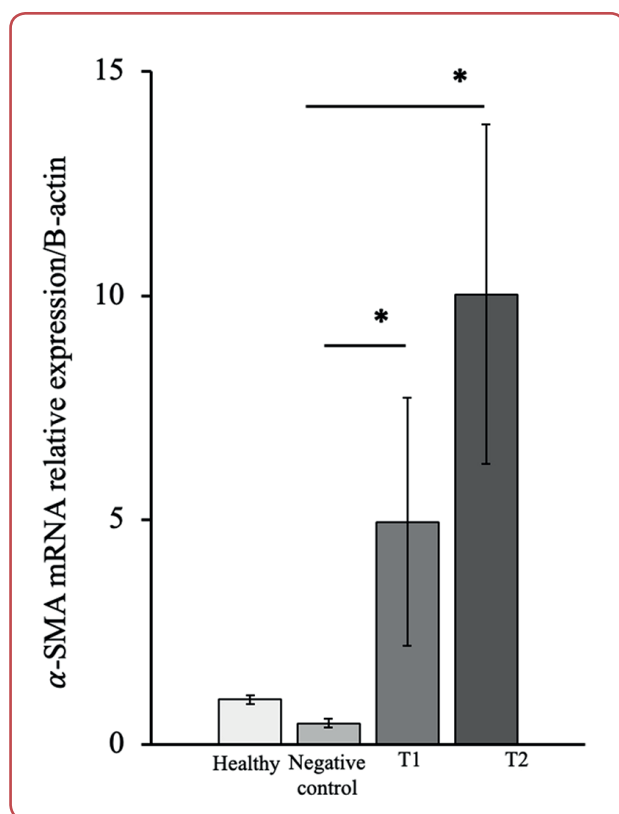
Figure 3: The effect of hypoxic secretome mesenchymal stem cells (SH-MSCs) on the alpha-smooth muscle actin (α-SMA) SOD gene expression on superficial infection wounds (SFW) rat models. RNA was extracted from the rat's skin tissue and analysed for mRNA expression by qRT-PCR (n = 6 ± SE). Data are presented as fold gene expression changes relative to the SFW unexposed group. *p < 0.05; ns: statistically non-significant different at p > 0.05.
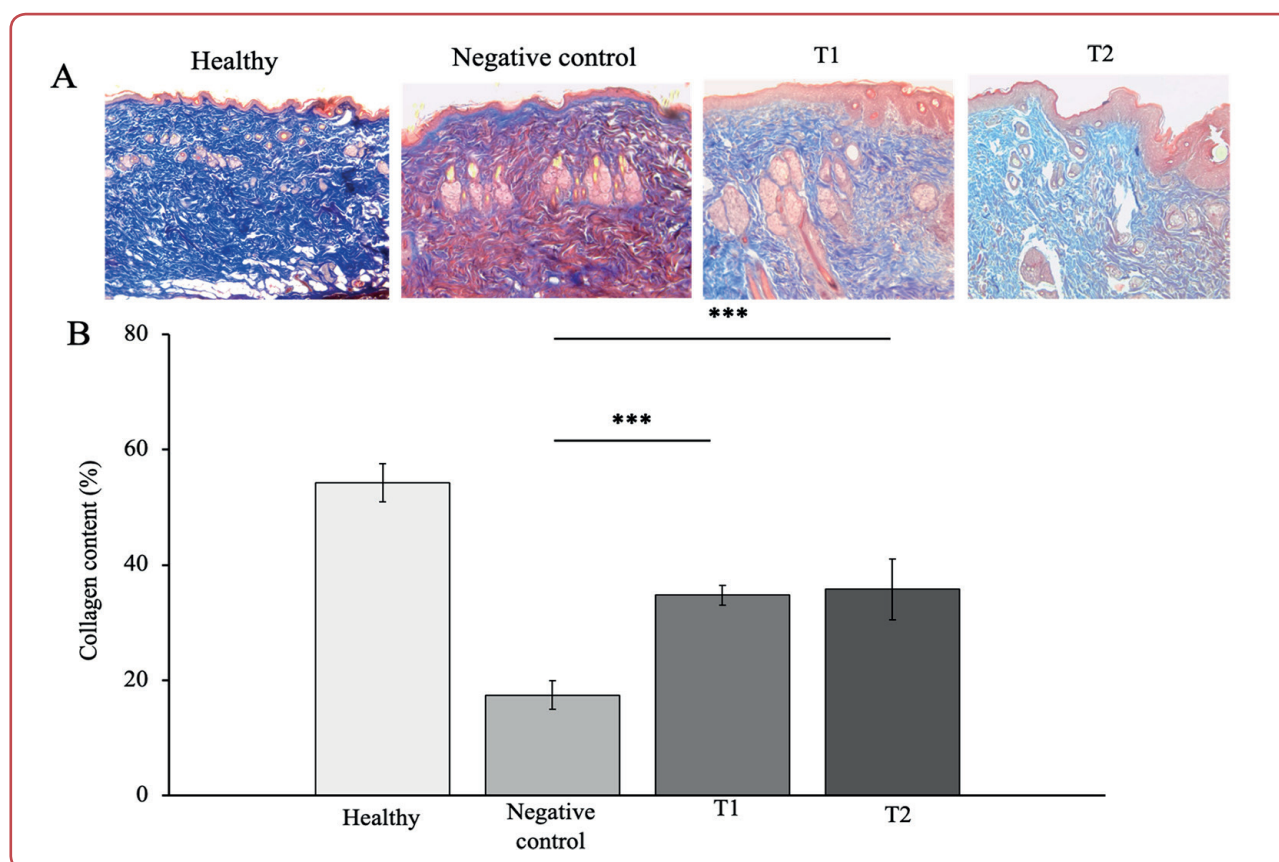
Figure 4: (A) Collagen density staining shows the qualitative results using Masson Trichrome across various experimental groups. This staining highlights collagen fibres in different colours, allowing us to visualise collagen distribution and tissue density. (B) The quantitative measurements of collagen density provide a detailed understanding of the changes in collagen in response to the experimental treatments.

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demonstrate that the application of SH-MCs gel leads to a dose-dependent increase in α-SMA gene expression in the superficial infection wound model.

The study demonstrated that SH-MCs gel significantly increased collagen density in a superficial infection wound model in rats and this effect was dose-dependent (Figure 4). The highest mean collagen density was observed in Group T2, measuring 35.68 ± 5.28, followed closely by Group T1 with a mean collagen density of 34.78 ± 1.72. In contrast, the negative control group showed the lowest collagen density, at 17.39 ± 2.47. These findings emphasise the dose-dependent increase in collagen density resulting from the application of SH-MCs gel in the superficial infection wound model.

Discussion

The study findings indicate that the expression of α-SMA significantly increased in the T1 (SH-MSCs 10 %) and T2 (SH-MSCs 20 %) groups compared to the superficial infection wound control group. This suggests that the upregulation of the α-SMA gene can enhance wound healing by increasing the number of dermal fibroblasts and blood vessels, as well as promoting collagen formation, which is a crucial component of the extracellular matrix. Additionally, a previous study revealed that TGF-β derived from SH-MSCs activates the PI3K pathway through the TGF-β pathway, which involves TGF-β receptors I and II.[27] The activation of PI3K triggers the activation of Akt, which in turn affects the activation of mTOR.[28] Active mTOR promotes the expression and activation of SMAD2/3. The activation of SMAD2/3 leads to the release of α-SMA, resulting in the transformation of fibroblasts into myofibroblasts and enhancing wound healing.[29, 30]

The observed increase in α-SMA expression corresponds with the higher collagen density, resulting in faster wound closure. The notable rise in collagen density in the T1 and T2 groups, compared to the negative control, indicates that SH-MSCs may accelerate the inflammatory phase. This is likely due to the presence of IL-10, which acts as an anti-inflammatory agent by inhibiting the NF-κB pathway through the IL-10 STAT3

signalling pathways.[31-33] Furthermore, the study findings are consistent with previous research demonstrating the regenerative and immuno-modulatory effects of MSC secretomes, which can enhance wound healing and promote angiogenesis through paracrine interactions.[34, 35] The secretion of various cytokines and chemokines, such as TGF-β, VEGF and IGF-1, by SH-MSCs enhances fibroblast and keratinocyte migration and proliferation, leading to collagen formation and tissue repair.[33, 36] It is important to recognise the limitations of the study. Notably, there was no direct assessment of IL-10 levels after treatment with SH-MSC gel, no analysis of TGF-β gene expression and no examination of bacterial presence in the wounds at the conclusion of the observation period. Addressing these limitations in future research would enhance our understanding of the mechanisms behind the therapeutic effects of SH-MSCs in healing superficial infection wounds.

Conclusion

This study adds to the expanding evidence that suggests SH-MSCs may be a promising method for tissue repair and wound healing. The findings highlight the need for additional research to clarify the complex molecular mechanisms behind the therapeutic effects of SH-MSCs. This research could lead to innovative treatments for superficial infected wounds.

Ethics

Acknowledgement

Conflicts of interest

The authors declare that there is no conflict of interest.

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Data access

The data that support the findings of this study are available from the corresponding author upon reasonable individual request.

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