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Research

Sequential Applications of Adipose Tissue–derived Stem Cells Enhance Healing in Complex Wounds: Experimental Model

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Background: Complex wounds represent a challenge to global health, incurring significant healthcare costs and compromising patients' quality of life. Recently, research has investigated the potential of adipose tissue–derived stem cells (ASC) for therapeutic stem cells' immunomodulatory properties. This study aimed to evaluate the effect of sequential applications of ASC in an experimental model of a complex wound.

Methods: Thirty male Wistar rats were subjected to a complex wound model of enterocutaneous fistula. After 4 weeks, the rats were divided into 3 groups: control, 2 applications of culture medium, and 2 applications of 1×10^6 allogeneic ASC. The animals were euthanized and analyzed 4 weeks after the first application regarding the macroscopic reduction of the wound, histopathologic changes, and gene expression related to wound healing (*Cd68*, *Il1rap*, *Il10*, *Mmp3*, *Mmp9*, and *Tnf*).

Results: Animals treated with ASC showed a reduction in wound diameter of 68% compared with the control group ($P = 0.002$) and a reduction of 65% compared with the culture medium group ($P = 0.011$). The ASC group also showed a more than 100% increase in blood vessel count compared with the control group ($P = 0.02$). Gene expression analysis showed a decrease in the *Mmp9* levels in the ASC group compared with the control group.

Conclusions: This study demonstrated that the treatment with ASC improves the healing of enterocutaneous fistula wounds. *(Plast Reconstr Surg Glob Open 2025;13:e6718; doi: 10.1097/GOX.0000000000006718; Published online 25 April 2025.)*

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INTRODUCTION

Complex wounds are a specific type of chronic wound that represent a significant challenge to global health today. They are characterized by healing through secondary intention, tissue loss, prolonged treatment duration, association with infection, and the presence of comorbidities.[1–3]

According to data from 2018 in the United States, they affect 8 million Medicare beneficiaries, compromising patients' quality of life and generating annual costs of US $97 billion.[4] In Europe, the estimated prevalence of complex wounds in the German population is 2%, whereas the annual impact on the United Kingdom's public healthcare system is £5.0 billion, generating physical, mental, and social repercussions.[5,6]

Unlike acute wounds, in which tissue repair mechanisms act in an organized manner to resolve the wound, the environment of a complex wound is characterized by several metabolic and immunologic changes.[7,8] Some health conditions are associated with the appearance of these injuries, such as age, diabetes, obesity, infection,

Disclosure statements are at the end of this article, following the correspondence information.

vascular insufficiency, and mechanical impairment.[9–11] The progressive aging of the population and the association of these chronic-degenerative diseases with older individuals makes the scenario more challenging for the health system.[12,13]

Treating a patient with a complex wound is challenging and requires a multidisciplinary effort. In addition to treating the underlying conditions, it also involves local wound care with the aim of reversing the metabolic and immunologic changes. Recent studies on the application of adipose tissue–derived stem cells (ASCs) have demonstrated their modulatory effects on the inflammatory and immunologic responses, showing potential benefits in diseases marked by immunologic alterations, such as Crohn's disease, axillary hidradenitis, and systemic lupus erythematosus.[14–16]

The main actions of ASC can be grouped into 6 different groups: immunomodulation, angiogenesis, stimulation of growth and differentiation of local stem cells, chemotaxis, inhibition of apoptosis, and inhibition of fibrosis.[17–19] This regulatory action of the immune and inflammatory response is capable of contributing to the treatment of complex wounds, by contributing to the reversal of the metabolic and immunologic changes presented.[14,17,20]

Considering the importance of treating complex wounds and the potential therapeutic benefits of ASC, this study aimed to evaluate the effect of sequential ASC application in an experimental model of the complex wound, both at the macroscopic levels of wound reduction, as well as histopathologic and gene expression changes.

Takeaways

Question: Can adipose tissue–derived stem cell (ASC) application improve the healing of complex wounds?

Findings: In this experimental model, rats were subjected to a complex wound model of enterocutaneous fistula. After 4 weeks, rats were divided into 3 groups: control, 2 applications of culture medium, and 2 applications of allogeneic ASCs. Results showed that animals treated with ASCs presented a reduction in wound diameter, an increase in blood vessel count, and a decrease in the *Mmp9* gene expression levels compared with the control group.

Meaning: The treatment with ASCs can improve the healing of complex wounds, as shown in this experimental model of enterocutaneous fistula.

METHODS

We conducted a study on 30 male Wistar rats 8–12 weeks of age between January and June 2021. The rats weighed between 300 and 350 g and were kept in species-specific cages at a temperature of 24°C, with 12/12-hour day-night cycles and an ad libitum diet. Our project followed the recommendations for laboratory animal handling established by the National Council for the Control of Animal Experimentation and the ARRIVE guidelines.[21] The animal research ethics committee of our institution approved the study (1278/2019).

Harvesting and Production of ASCs

Three Wistar rats were anesthetized with intraperitoneal ketamine hydrochloride (Pfizer) at a dose of 100 mg/kg and xylazine hydrochloride (Syntec, Brazil) at a dose of 10 mg/kg. Adipose tissue was harvested from the abdominal region, mechanically fragmented, and enzymatically digested at 37°C using collagenase IA 0.1% dissolved in Dulbecco's modified Eagle's medium (DMEM, Sigma Aldrich, Germany). The material was filtered and centrifuged at 2000 rpm for 5 minutes, and the stromal vascular fraction (SVF) was isolated in a culture medium (CM) comprised of 80% DMEM, 20% fetal bovine serum (FBS, Sigma Aldrich, Germany), and antibiotics (penicillin, Sigma Aldrich, Germany; streptomycin, Biofarm, Brazil; amphotericin B, Cristalia, Brazil). The cells were then incubated at 37°C. Upon reaching 80% confluence, the cells were released using trypsin (Sigma Aldrich, Germany) and ethylenediaminetetraacetic acid (Sigma Aldrich, Germany). These cells were amplified until the fourth passage, at which they were used.

The presence of ASCs was confirmed, and the immunophenotyping was determined through flow cytometry assays.[22] The confirmation of viable ASCs is in accordance with the criteria established by the International Society for Cellular Therapy.[23]

Induction of Complex Wound: Enterocutaneous Fistula

Twenty-seven animals were submitted to the surgical procedure. After anesthetization with ketamine hydrochloride (Pfizer) at a dose of 100 mg/kg and xylazine hydrochloride (Syntec, Brazil) at a dose of 10 mg/kg, a complex wound was created. The procedure consisted of executing a previously established model of enterocutaneous fistula (ECF).[24] A midline laparotomy was performed to identify and exteriorize the cecum through a counter incision. The intestinal loop was then opened and attached to the abdominal wall and skin.

After a 4-week recovery period, the animals were randomly assigned to 1 of 3 groups, with each group consisting of 9 animals:

- Control: no treatment was administered.
- CM: animals received a perilesional subdermal injection of 0.5 mL of 80% DMEM and 20% FBS CM. The same procedure was repeated after 1 week.
- ASC: animals received a perilesional subdermal injection of 1×10^6 ASCs in 0.5 mL of CM solution. The same procedure was repeated after 1 week, according to the previous results.[22]

Four weeks after the first intervention, the effects of the treatment were evaluated. The experimental design is depicted in Figure 1.

Macroscopic Analysis

The animals were examined weekly to check their general condition. Four weeks after the intervention, the diameter of the wound was measured with the animals in dorsal decubitus with a millimeter ruler at the largest

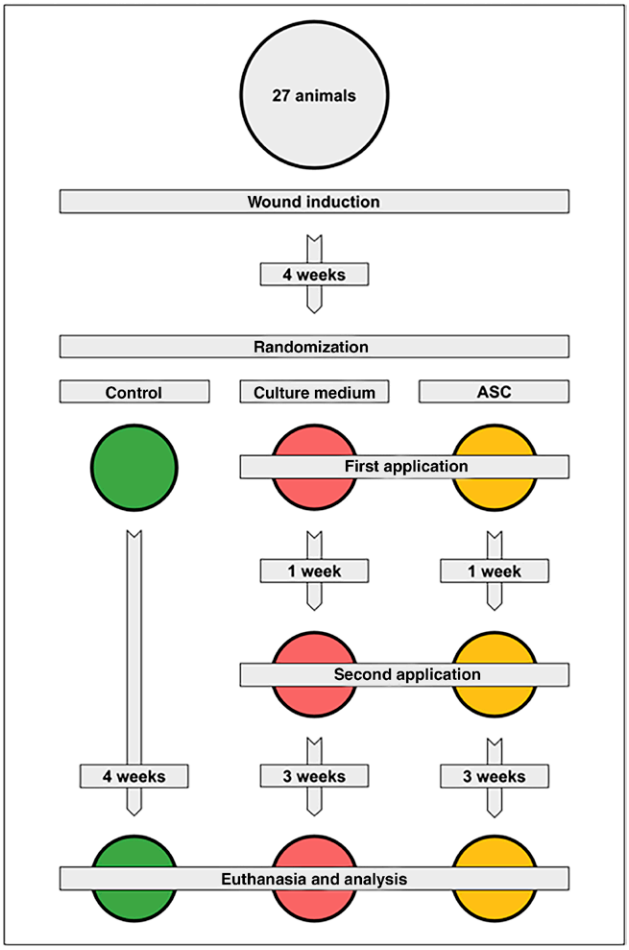

Fig. 1. Schematic diagram of experimental design. The animals were submitted to the procedure for making a complex wound (ECF) and, after 4 weeks, randomized between the control, CM, and ASC groups, when the animals were submitted to the first intervention. After 1 week, they received a new application of CM or stem cell. Finally, 3 weeks later, the animals were euthanized, and the results were analyzed.

diameter, following specific recommendations for this type of wound.[25]

Histopathologic Analysis

Wound samples were collected and analyzed using optical microscopy (Nikon Eclipse E600, Tokyo, Japan). The following variables were evaluated: inflammatory cell count (macrophages and polymorphonuclear cells) and blood vessel count (arterioles), in addition to the analysis of fibrosis and inflammation.

The quantification of inflammatory cells and blood vessels for vascular density was analyzed by directly counting these structures at 40× magnification in 10 random fields per slide.

The analysis of fibrosis and inflammation was also performed at 40× magnification in 10 fields per slide. The scenarios observed on the slides were subjectively classified according to a score from 0 to 5, where 0 represents the best possible scenario, with little or no fibrosis and abnormal infiltration of inflammatory cells. Score 5 presents the worst scenario, with high levels of fibrosis and abnormal infiltration of inflammatory cells.

Gene Expression Analysis

The gene expression analysis was performed on a selection of genes associated with wound healing and inflammation, including *Cd68, Il10, Il1rap, Mmp3, Mmp9,* and *Tnf.*[26–29]

Tissues stabilized in RNAlater (30 mg) were used for total RNA extraction using the RNeasy Mini Kit (Qiagen, Germantown, MD). The tissues were disrupted using the TissueLyser LT apparatus (Qiagen, Germantown, MD). To avoid DNA contamination during the extraction, TRIzol Reagent (Thermo Fisher Scientific, Waltham, MA) was used in place of the Buffer RLT reagent from the RNeasy Mini Kit, according to the manufacturer's instructions. One milliliter of TRIzol Reagent (Thermo Fisher Scientific, Waltham, MA) and stainless-steel beads were added to the microcentrifuge tubes. Tissue disruption was carried out for 5 minutes at 50 Hz. After removing the beads, 0.2 mL of chloroform was added. After homogenization in the vortex for 15 seconds, samples were incubated for 3 minutes at room temperature followed by centrifugation at 12.000*g* for 15 minutes at 4°C. The upper aqueous phase was transferred to a 1.5 mL tube, and a 1:1 volume of 70% ethanol was added, then homogenized by vigorous pipetting. A volume of 0.7 mL of prepared sample, including any type of precipitate, was transferred to an RNeasy kit column. From this stage on, extraction was carried out following the kit manufacturer's recommendations. RNA purity (evaluated through the ratios 260:280 and 260:230) and integrity were determined by spectrophotometry (NanoDrop 1000 Spectrophotometer, Thermo Fisher Scientific, Wilmington, NC) and gel electrophoresis, respectively. RNA samples were stored at –80°C until use.

The quantitative reverse transcription polymerase chain reaction was performed using 2000 ng of total RNA, which was reverse transcribed to cDNA using the High-Capacity RNA-to-cDNA Kit (Thermo Fisher Scientific, Waltham, MA) as recommended by the manufacturer. The expression levels of *Cd68* (Rn01495634_g1), *Il10* (Rn01483988_g1), *Il1rap* (Rn01404183_m1), *Mmp3* (Rn01495634_g1), *Mmp9* (Rn00579162_m1), and *Tnf* (Rn01525859_g1) genes were quantified using TaqMan methodology (Thermo Fisher Scientific, Waltham, MA) in the StepOnePlus real-time polymerase chain reaction system (Thermo Fisher Scientific, Waltham, MA), using the default cycling conditions recommended by the manufacturer. Each sample was run in duplicate, and *Gapdh* (Rn01775763_g1) was used as the endogenous control. Fold change (FC) was calculated using the comparative CT method ($2^{-\Delta\Delta CT}$).[30] The control group was used as the reference.

Statistical Analysis

Statistical analyses were conducted using STATA (v. 14, StataCorp, College Station, TX), with a significance level of 5% and a study power of 80%. The Shapiro–Wilk test was initially applied to assess data normality and guide the

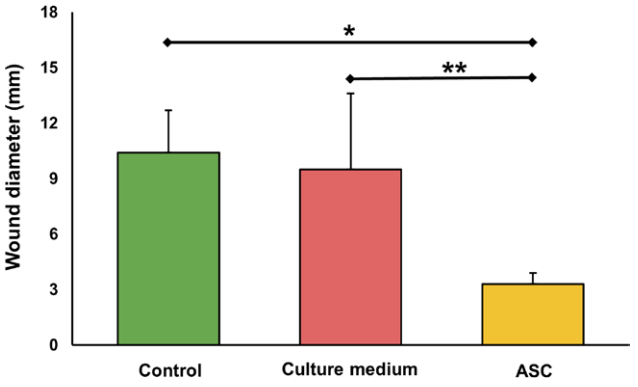

Fig. 2. Comparison of ECF wound diameter (mm) among the control, CM, and ASC groups. Data are expressed as mean ± SD. *P = 0.002, **P = 0.011. The Kruskal–Wallis test was followed by the Dunn posttest.

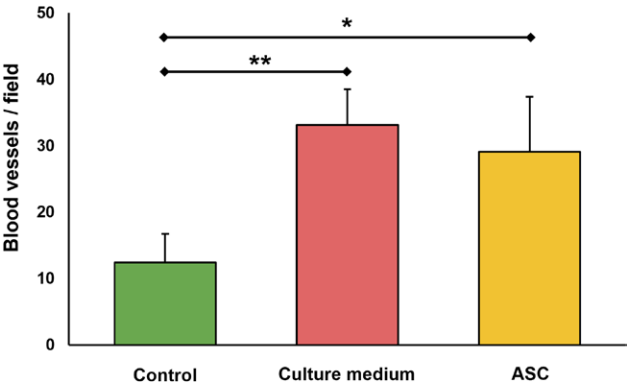

Fig. 3. Comparison of blood vessel count among the control, CM, and ASC groups. Data are expressed as mean ± SD. *P = 0.02, **P < 0.001. The Kruskal–Wallis test was followed by the Dunn posttest.

choice between parametric and nonparametric statistical tests. Continuous variables with normal distribution were expressed as mean and SD. The analysis of variance test was used for parametric variables, whereas the Kruskal–Wallis test was used for nonparametric variables to compare groups. If significance was observed, a post hoc Bonferroni test was performed. Regarding gene expression analysis, we used the parametric test analysis of variance with Tukey post hoc for comparison among groups, with a significance level of 5% using SPSS software version 25.0 (IBM, Armonk, NY).

RESULTS

Out of the 27 animals that underwent the complex wound surgical procedure, 26 survived until the end of the evaluation period. Specifically, 8 rats in the control group and 9 rats each in both the CM and ASC groups were included in the study. One animal died before the randomization stage and was excluded from the study.

Flow Cytometry

The analysis showed that CD31 and CD45 markers showed a negative result, whereas the CD29, CD73, CD90, and CD105 markers showed a positive result, confirming the isolation of viable ASCs.

Macroscopic Assessment

Wound measurements (Fig. 2) indicated that the diameter in the ASC group (3.3 ± 0.6 mm) decreased by 68.3% compared with the control group (10.4 ± 2. 3 mm, P = 0.002) and by 65.3% compared with the CM group (9.5 ± 4.1 mm, P = 0.011). No significant differences were observed between the control and CM groups.

Histopathologic Analysis

The number of blood vessels in both the ASC and CM groups increased by more than 100% compared with the control group (P < 0.001 and P = 0.02, respectively), as illustrated in Figure 3 and Table 1.

Regarding inflammation, there were no significant differences in the number of inflammatory cells (Table 1) or the inflammation score (Table 2) among the groups.

The evaluation of the fibrosis score of the wound tissue is shown in Table 2. There were no significant differences among the studied groups.

Figure 4 presents the histopathologic sections from the 3 groups, showing blood vessels, inflammatory cells, and tissue fibrosis.

Gene Expression Analysis

For gene expression analyses, the expression levels of each gene of interest were compared among the CM (n = 4), ASC (n = 9), and control (n = 6) groups, using

Table 1. Number of Blood Vessels and Inflammatory Cells in Control, CM, and ASC Groups

	Control	CM	ASC
Blood vessels	12.4 ± 4.3	33.1 ± 5.4*	29.1 ± 8.3**
Inflammatory cells	453.4 ± 170.2	618.4 ± 253.1	508.4 ± 294.7

Data are expressed as mean ± SD.
*Versus control, P < 0.001.
**Versus control, P = 0.02. The Kruskal–Wallis test was followed by the Dunn posttest.

Table 2. Comparison of Inflammation and Fibrosis Scores Among the Control, CM, and ASC Groups

	Control	CM	ASC
Inflammation score	3.75 ± 0.5	3.7 ± 0.7	3.6 ± 0.7
Fibrosis score	4.25 ± 0.5	3.9 ± 0.9	3.6 ± 0.7

Data are expressed as mean ± SD. The Kruskal–Wallis test was followed by the Dunn posttest.

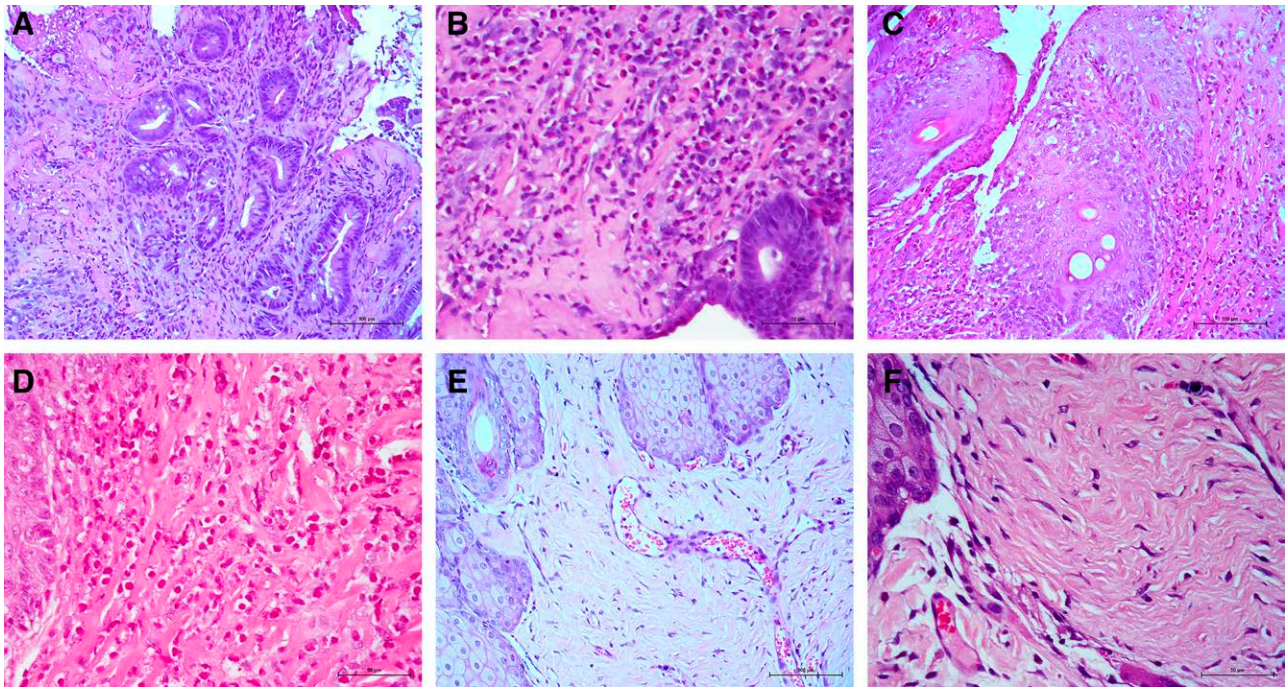

Fig. 4. Representative histopathologic sections of ECF wound samples stained with hematoxylin/eosin of control group (A and B), CM group (C and D), and ASC group (E and F). Magnification: 200× (A, C, and E) and 400× (B, D, and F).

Table 3. Comparison of the Gene Expression Between Control (N = 4), CM (N = 9), and ASC (N = 6) Groups, Using the Control Group as a Reference

	Control	CM	ASC	*F*	*P*
Cd68	1.19 ± 0.78	0.23 ± 0.09	0.58 ± 0.65	5.9	0.012*
Il10	1.19 ± 0.92	1.59 ± 1.79	4.04 ± 4.03	1.8	0.199
Il1rap	1.04 ± 0.33	0.75 ± 0.36	1.27 ± 0.66	2.7	0.099
Mmp3	1.43 ± 1.31	2.59 ± 3.32	6.09 ± 7.59	1.0	0.379
Mmp9	1.66 ± 1.50	0.15 ± 0.23	0.19 ± 0.18	6.5	0.009*
Tnf	1.02 ± 0.23	1.68 ± 1.01	2.11 ± 0.96	1.9	0.184

Data are expressed as FC mean ± SD. One-way analysis of variance test.
*$P < 0.05$.

the control group as the reference. We found significant differences in *Cd68* ($P = 0.012$) and *Mmp9* ($P = 0.009$) gene expression levels among groups (Table 3). Tukey post hoc showed that the *Cd68* gene expression level was 0.23-fold in the CM group in relation to the control group ($P = 0.009$). For the *Mmp9* gene, we demonstrated an FC of 0.15 in the CM group when compared with the control group ($P = 0.007$) and an FC of 0.19 in the ASC group in relation to the control group ($P = 0.043$), as shown in Figure 5.

DISCUSSION

Our understanding of the mechanisms underlying chronic wound healing is constantly improving, thanks in large part to the development and refinement of wound models and diagnostic tools. The complex wound model used in this study is consistent with what has been found in clinical practice, particularly those involving wounds that remain unresolved for more than 30 days. Additionally, all ECFs, regardless of their cause, are marked by elevated levels of inflammatory markers.[31–33] This sustained inflammatory activity in ECFs impedes tissue healing, highlighting the need for alternatives that target and modulate this inflammatory mechanism.

The findings of this study were consistent with previous research in the literature on the application of stem cells derived from adipose tissue.[34–41] It is important to highlight that, despite all demonstrating positive and well-tolerated results, the methods used were heterogeneous. The studies differ in the origin of stem cells, the number of cells administered, the time frame of the analysis, the method of application, and whether the cells were autologous or allogeneic. These variations in protocols highlight the need for further research to standardize treatment approaches. Regarding the translational aspects of applying this protocol in human subjects, immunologic incompatibility should not be a concern, as the objective is to use autologous ASC in the recipients. In contrast, allogeneic cells may trigger high inflammatory and immune responses. Although elevated inflammation was not observed in this study, it is essential to address this

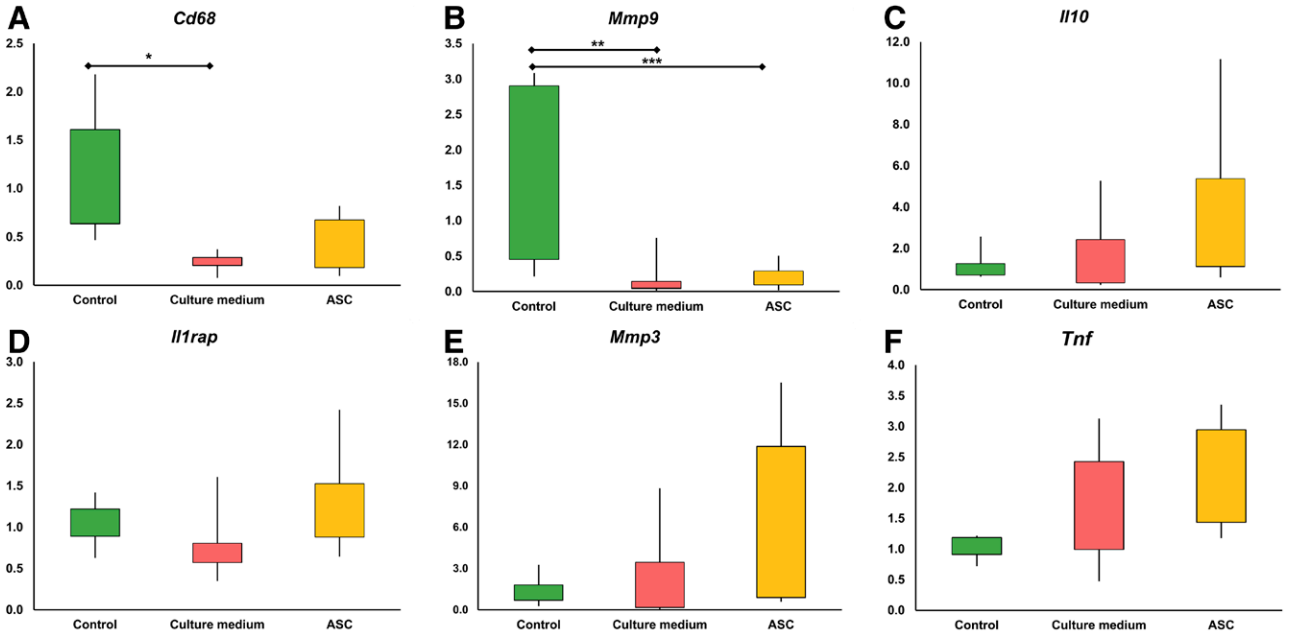

Fig. 5. Comparison of the expression levels of *Cd68* (A), *Mmp9* (B), *Il10* (C), *Il1rap* (D), *Mmp3* (E), and *Tnf* genes (F) among control (n = 4), CM (n = 9), and ASC (n = 6) groups. FC was calculated by the $2^{-\Delta\Delta Ct}$ method, with the control group used as reference. **P* = 0.009, ***P* = 0.007, ****P* = 0.043. A one-way analysis of variance test with the Tukey posttest was performed.

potential issue when translating the protocol to human subjects to mitigate the risk of host-versus-graft disease.

Histopathologic evaluations in the present study revealed an increase in vascular density following ASC infiltration and no notable change in the quantities of inflammatory cells, despite a significant reduction in wound diameter. Wound healing is known to be a continuous process, often divided into stages in a didactic manner, according to the main findings of the moment.[26] The initial inflammatory phase is characterized by the intense migration of neutrophils, macrophages, and lymphocytes, which produce various cytokines and growth factors.[26,27] Subsequently, the proliferative phase is characterized by the production of extracellular matrix by fibroblasts, which move through the scar tissue through the degradation of the previous matrix via production of matrix metalloproteinases (MMPs). This phase also sees intense angiogenesis and epithelialization. Finally, during the remodeling phase, the number of inflammatory cells decreases, and tissue tensile strength progressively increases. In chronic wounds, where these processes are not well coordinated, elevated levels of TNF-∂, IL-1, IL-6, and MMPs are commonly observed.[26] Thus, the timing of tissue sample collection in this study suggests a transition toward the resolution of the inflammatory phase, as evidenced by stable inflammatory cell counts, increased vascular density, and reduced wound diameter.

As the wound progresses toward resolution, the activation state of macrophages and their function undergo significant changes. One key marker of this phenotypic change is the glycoprotein *Cd68*.[42,43] Although the number of inflammatory cells did not differ significantly among the studied groups, gene expression analysis indicated a trend in the reduction of *Cd68* expression in tissue samples subjected to ASC application, which might show a change in the healing process toward wound resolution.

MMPs play a crucial role in the degradation of extracellular matrix proteins such as collagen, proteoglycans, and elastin, contributing to tissue remodeling.[27] Although they are involved in several diseases, including cancer, arthritis, chronic obstructive pulmonary disease, and sepsis, MMPs also have important functions in physiological processes such as wound healing, angiogenesis, and inflammatory regulation. In this study, the reduction in *Mmp9* expression in the ASC group in relation to the control group indicates the capacity of these cells to modulate the immune response and is compatible with the intense reduction in the size of the wound, moving toward resolution. Different types of MMPs show preferences for specific substrates, and a reduction in the activity of 1 MMP can often trigger a compensatory increase in the activity of others, maintaining the overall balance of their effects.[28]

IL-10 is a cytokine that plays a crucial role in regulating the immune response and has anti-inflammatory properties. It inhibits the production of pro-inflammatory cytokines by Th1 cells, macrophages, monocytes, neutrophils, and other antigen-presenting cells.[44] Our study indicated an upward trend in IL-10 in the group treated with ASCs, which, although not statistically significant, might suggest one of the possible mechanisms related to the immunomodulatory effect of ASCs.[29]

TNF stimulates vasodilation, edema formation, and adhesion of leukocytes to the epithelium; regulates blood clotting; contributes to oxidative stress at sites of inflammation; and, indirectly, induces fever.[45] Additionally, TNF induces angiogenesis and regulates tissue remodeling

through its stimulatory action on MMPs. The evaluation of *Tnf* gene expression in this study showed no change among groups, despite the increase in blood vessels and the reduction of the wound in the ASC group.

Finally, IL-1 plays an important role in healing, being involved in the innate inflammatory response and in the activation of immune system cells during this process.[46] IL-1, found constitutively in healthy cells, is released under conditions of cell death, stimulating the production of chemokines and the infiltration of cells such as neutrophils and monocytes. Our study found no difference among groups in *Il1rap* expression, indicating that wound resolution advanced despite remaining similar, suggesting another mechanism of action.

In the same way that this study indicated an increase in the number of blood vessels in the group subjected to infiltration of CM only, in addition to a decrease in the expression of *Mmp9*, previous studies have demonstrated some effect on tissue repair mechanisms. Guo et al[47] demonstrated the highest concentration of vascular endothelial growth factor in an ex vivo experimental skin model in samples subjected to DMEM applications when compared with those treated with ASC or CM–ASC application. Jiang et al,[48] using a murine model of scleroderma, found greater thickness of the dermis and collagen fraction in the skin of animals treated with phosphate-buffered saline, when compared with fat, fat enriched with SVF, and only SVF.

Some limitations of the study should be acknowledged. The evaluation of the ASC effects could be expanded to a broader range of gene expression targets, including collagen and growth factors, to capture the complexity of the immunologic response involving various cellular and molecular agents. The changes observed in some markers in the CM group are likely attributed to the composition of DMEM, combined with fetal bovine serum, which may have stimulated the healing process through the presence of several proteins and growth factors. Finally, caution is required when translating experimental findings to humans, considering that there are biological differences that can impact the outcomes.

CONCLUSIONS

In this study, stem cells derived from adipose tissue were evaluated in the treatment of complex wounds in a murine experimental model. The results demonstrated that, after the application of these cells, there was a reduction in the diameter of the wounds, an increase in vascular density, and alterations in *Mmp9* expression levels, revealing clinical, histopathologic, and biomolecular concordances in this sample. These findings indicate that the application of stem cells derived from adipose tissue can favor wound resolution through the formation of new blood vessels and modulation of the inflammatory response, making it a potential therapeutic strategy in complex wounds.

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DISCLOSURE

The authors have no financial interest to declare in relation to the content of this article.

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