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Effect of Local Transplantation of Mesenchymal Stem Cell/Macrophage Culture Supernatants on Oxidative Stress Markers in Wound Healing

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ABSTRACT

Skin wound healing is a complex biological process that involves a series of coordinated steps that ultimately restore the skin's integrity and function. Stem cell and macrophage secretions show promise in promoting this natural repair process. This study aims to explore the impact of locally transplanted mesenchymal stem cell/macrophage culture supernatants on oxidative stress markers during wound healing. Full-thickness wounds were created on rats. One group received local injections of a 1:1 mixture of MSC and macrophage culture supernatants, while the control group did not. After 21 days, researchers measured markers of oxidative stress and antioxidant enzyme activity in the wound tissue. The group receiving the culture supernatant mixture exhibited significantly lower levels of malondialdehyde (MDA) and total oxidant status (TOS). Additionally, they showed higher activity of glutathione peroxidase (GPx) and higher total antioxidant capacity (TAC). Local transplantation of the culture supernatant mixture improved wound healing by reducing oxidative stress and increasing antioxidant activity. These findings suggest this approach may be a promising cell-free therapy for wound healing.

Introduction

Wounds disrupt tissue integrity, often causing discomfort and increasing infection risk.[1] The wound healing process involves intricate cellular and chemical interactions, forming new blood vessels, collagen, and scar formation.[2] This process requires precise coordination of various cell types, releasing cytokines, growth factors, and extracellular matrix components.[3-5]

Normal tissue repair hinges on a carefully regulated oxidative environment. Extreme fluctuations in ROS levels can compromise the wound healing process.[6] Various research findings have indicated that decreased ROS levels, induced by magnetic fields or pro-oxidant enzyme reduction, facilitated wound healing in a diabetic mouse model.[7,8] Additionally, extensive research indicates that non-healing wounds, whether caused by diabetes or chronic conditions exhibit elevated levels of ROS.[9-11]

The regenerative abilities and therapeutic potential of mesenchymal stem/stromal cells (MSCs) extend beyond the cells. Cell-free products derived from their secretions offer a promising avenue for clinical trials. These products, found in the culture medium MSCs, demonstrate therapeutic potential similar to the cells. MSCs and these derivative products exert beneficial paracrine effects, meaning they act on nearby cells. These effects include regulating inflammation, influencing fibroblast activation and collagen production, promoting the formation of new blood vessels, and re-epithelialization.[12] When inflammation occurs, macrophages are drawn to the area of injury. There, they exhibit remarkable flexibility

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by adopting either a classical or alternative activation state. Macrophage activation states are influenced by a complex interplay of factors they produce themselves, including cytokines, oxidants, lipids, and growth factors.[13-15] Macrophages are adaptable, adjusting their function based on the evolving wound environment. They play a multifaceted role, actively contributing to several overlapping stages of wound healing. Notably, they are effective at neutrophil removal from the wound site.[16] As the wound remodels, macrophages undergo clearance through apoptosis or migration to nearby lymph nodes. Throughout this process, they contribute to both the remodeling of the extracellular matrix and the resolution of fibrosis. Potentially, macrophages act as regulators of the initial blood vessel growth in wounds by delivering a balanced mix of proangiogenic and antiangiogenic signals. This helps control the angiogenic response during tissue granulation and scar formation.[13] This study aims to investigate the effects of local transplantation of mesenchymal stem cell/macrophage cultured supernatants on oxidative stress markers in wound healing.

Materials and Methods

The procedures of this work were approved by the University Ethical Committee and filed under IR-UU-AEC-3/47 code. We followed instructions of National Academy of Sciences Publication with number of 85-23 that was revised in 1985.

Animals

Sixteen healthy adult male Wistar rats, aged 8-10 weeks and weighing around 200 grams, were used in this study. Animals were housed individually in cages within a controlled environment with 12-hour light/dark cycle. The room temperature was maintained at a constant 23 ± 2°C. The rats had unlimited access to food and water. Surgery was performed using general anesthesia. Anesthesia was induced by injecting a combination of xylazine hydrochloride 2% (5 mg/kg, Alfasan International, Woerden, Holland) and ketamine hydrochloride 10% (80 mg/kg, Alfasan International, Woerden, Holland) intraperitoneally. Following excisional wound creation, the rats were randomly divided into two groups of eight animals each as follows: 1) Control group. 2) Treatment group: The wound bed received a local application of one milliliter solution containing a 1:1 mixture of mesenchymal stem cell and macrophage culture supernatants (MAC-MSC/SN). On day 21 of the study, the rats were euthanized with an intraperitoneal injection of ketamine-xylazine at a dose five times higher than the anesthesia induction dose.[17] Following euthanasia, tissue samples were collected. Throughout the 21-day study period, the rats exhibited no signs of illness or distress, including weight loss, lethargy, or wound infection.

Macrophage Supernatants Collection

To isolate and culture rat macrophages, a previously described method was employed with some modifications (Figure 1A).[18,19] Briefly, rats were euthanized by cervical dislocation and disinfected with 75% alcohol. Peritoneal macrophages were collected by lavage with PBS, isolated by centrifugation, and washed with RPMI-1640 (Sigma Chemical Co., Munich, Germany) containing 10% FBS (Gibco, Germany). Cell viability was assessed by trypan blue exclusion (Figure 1B). Adherent macrophages were cultured in RPMI-1640 with 10% bovine serum, penicillin, and streptomycin for 4 days. Subsequently, cells were cultured in FBS-free RPMI-1640 containing antibiotics for 24 hours. The supernatant was collected, centrifuged, filtered, and stored at -80 °C.

Mesenchymal Stem Cell Supernatant Collection

Eight rats were used for mesenchymal stem cell isolation. The procedure followed a previously described protocol with some adjustments for bone marrow extraction (Figure 2).[20] Briefly, rats were euthanized by cervical dislocation and tibia and femur bones were dissected, cleaned for removing muscles and connective tissue, and sterilized in 10% ethanol. Bone marrow was flushed with DMEM (Sigma Chemical Co., Munich, Germany), the resulting solution filtered, and cultured in DMEM supplemented with 10% FBS (Gibco, Germany). Cells were subcultured every 4 days until the third passage. The culture supernatant from the third passage was collected, centrifuged, filtered, and stored at -80 °C.

Wound Creation

Following anesthesia, the hair on the rat's back was shaved and the surgical site was prepared for aseptic surgery. A full-thickness, circular wound measuring 20 millimeters in diameter was created on the thoracic region between the scapulas, using a size 10 scalpel blade. This excisional wound involved the complete removal of epidermis and dermis (Figure 3A).

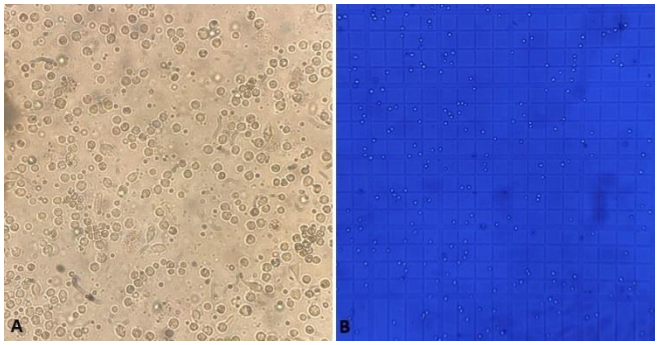

Figure 1. A: Morphological observation of a representative primary macrophage under an inverted microscope. B: Trypan blue dye exclusion assay determined the viability of peritoneal cells.

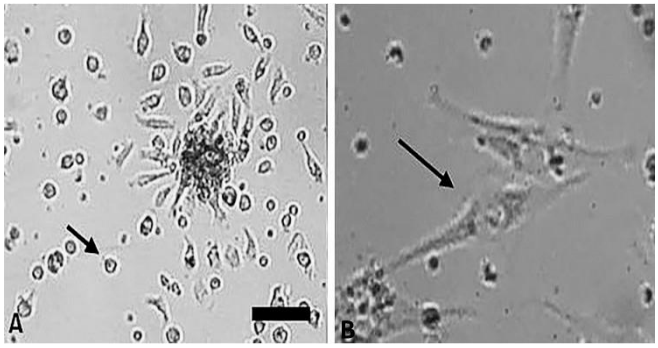

Figure 2. Morphological characteristics of cultured BMSCs. Small rounded (A), and fibroblast-like morphology (B) in the confluent culture (arrows), Scale bar: 100 µm.

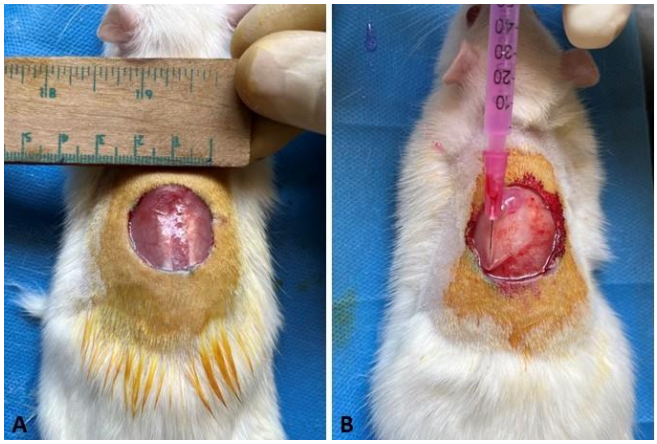

Figure 3. A: Excisional wound was created on the thoracic region between the scapulas. B: Injection of supernatant in the wound bed.

Treatment

The treatment group received 1 ml of a 1:1 (v/v) mixture of mesenchymal stem cell and macrophage culture supernatants. During local treatment, 1 ml of a cell culture supernatant mixture was injected subcutaneously at four locations around the wound bed, maintaining a 3 mm distance from the edge (Figure 3B). The treatment group received a single dose injection right after the wounds were created.

Oxidative Stress Levels and Antioxidant Enzyme Evaluations

On day 21, tissue samples were collected for biochemical analysis. The samples were immediately frozen at -20 °C for preservation. The tissues were manually homogenized in a mortar. Next, one gram of each homogenized sample was combined with 4.5 ml of appropriate buffer and homogenized on ice for 15 minutes using an Ultra-Turrax homogenizer (IKA, Werke, Germany). The homogenates were centrifuged in a refrigerated centrifuge at 4 °C. Finally, the resulting supernatants were used to measure enzyme activity. All assays were performed at room temperature. Antioxidant capacity (TAC), oxidative stress level (TOS), and malondialdehyde (MDA) content of the supernatants was assessed using a spectrophotometer and commercially available assay kits (Navand Salamat, Urmia, Iran). All procedures followed the manufacturer's instructions. To assess glutathione peroxidase (GPx) activity, the supernatant collected on day 21 was used according to a previously established protocol.[21] In this procedure, the sample was first treated with KH_2PO_4, EDTA, GSH, B-NADPH, NaN_3, and GR, followed by incubation. After adding H_2O_2, the change in light absorption at 340 nm was measured every 15 seconds for a total of 5 minutes.

Statistical Analyses

The Kruskal-Wallis test was used to analyze differences between groups. If the *p*-value from this test was statistically significant (indicating a difference between groups), multiple comparison tests were utilized to get the differences. The analyses were conducted using SPSS 11.5 (SPSS Inc. USA), and we considered a *p*-value less than 0.05 to be statistically significant.

Results

Oxidative Stress Levels and Antioxidant Enzyme Evaluation of Healed Wound Area

This study investigated the effects of locally transplanted mesenchymal stem cell/macrophage cultured supernatants on oxidative stress markers during wound healing. The levels of antioxidant enzymes (specifically GPx), a byproduct of lipid peroxidation (MDA), TAC, and TOS were measured (Table 1). Biochemical analysis revealed a significant increase in TAC and GPx activity in the treatment group compared to the control group. Conversely, MDA and TOS levels were significantly lower in the treatment group compared to the control group ($p < 0.05$).

Discussion

In the inflammation stage, tissue injury triggers the production of numerous radicals. These radicals are often linked to oxidative stress, leading to lipid peroxidation and impairing wound healing.[22] In 2015, Li *et al.* demonstrated that mesenchymal stem cell-conditioned medium could eliminate reactive oxygen species and reactivate the Erk pathway. This suggested that MSC-CM

Table 1. Comparison of the activities of TAC, TOS, MDA, and GPx in the tissue samples taken from experimental groups on day 21. Data are expressed as Mean ± SD.

Indices	Sham	Treatment
TAC	0.49±0.06	0.88±0.14
TOS	100.75±16.90	61.62±8.85
MDA	0.91±0.29	0.51±0.25
GPx	0.12±0.1	0.25±0.05

TAC: Total antioxidant capacity, TOS: Total oxidant status, MDA: Malondialdehyde, GPx: Glutathione peroxidase dismutase. * $p < 0.05$ vs. MAC-MSC/SN and MAC.

held great potential for serving as a therapeutic modality for developing safe and effective cell-free regenerative strategies to improve poor wound healing conditions.[23] In 2021, Saleem *et al*. investigated the antioxidant effects of MSC-CM on cultured human BMSCs using an in vitro oxidative stress model. Their findings demonstrated that MSC-CM contained a group of proteins with antioxidant properties and exerted a protective effect against oxidative stress-induced damage on hBMSCs.[24] Liang et al. demonstrated that the conditioned medium from induced pluripotent stem cell-derived mesenchymal stem cells prevented apoptosis in human umbilical vein endothelial cells and reduced both cellular and mitochondrial ROS levels induced by H2O2.[25] In 2022, Zhang *et al*. reported that administering Exosomes Derived from Adipose Mesenchymal Stem Cells subcutaneously to diabetic mice for three consecutive days mitigated oxidative stress within the wounds. To assess oxidative stress markers, they employed MDA, TAC, and superoxide dismutase (SOD) kits to analyze wound tissue from each experimental group. Their findings indicated a reduction in MDA levels and an increase in SOD and TAC levels within the exosome-treated group. In 2022, Mousavi *et al*. investigated the coactivity of mast cells and stem cells on antioxidant potentials during the inflammation, proliferation, and tissue remodeling phases of wound healing in albino Wistar rats. Their research measured levels of MDA and SOD. The findings revealed increased SOD levels in groups that received cells. This was likely due to the presence and antioxidant properties of bone marrow-derived mesenchymal stem cells, which presumably stimulated SOD production. Additionally, MDA, a marker of tissue stress, was found to be lower in animals that had received cells.[26] In agreement with previous findings indicating the antioxidant effect of MSC culture supernatant, our study found that local transplantation of mesenchymal stem cell/macrophage co-cultured supernatants significantly improved oxidative stress markers and antioxidant enzyme activity. This was evidenced by a decrease in MDA and TOS and an increase in GPx and TAC levels compared to the control group.

Conflict of Interest

There is no conflict of interest to declare.

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