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Regenerative Capability of Secretome-Derived Bone Marrow Stem Cells for Treating Traumatic Oral Ulcers in Albino Rats (Immunohistomorphometric Study)

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Aim: Ulcers can weaken the oral mucosa's innate defenses. The purpose of the study was to examine the therapeutic advantages of using secretome derived from bone marrow stem cells to heal traumatic ulcers in albino rats.

Materials and Methods: Thirty male albino rats with extensively damaged ulcers were randomly assigned to three groups: control group, group that received oracure gel treatment, and group that received secretome treatment. Tissues from the buccal mucosa were taken on days three, seven, and twelfth. Assessment was done via clinical evaluation, histological examination, Masson's trichrome stain, and vascular endothelial growth factor (VEGF)-specific immunohistochemistry assays. Statistical analysis was then performed on the outcomes.

Results: The group treated with secretome had the greatest percentage of wound contraction and the fastest rate of healing. Histological examination of the secretome-treated group showed improved re-epithelialization and better healing capacity. In addition, this group showed an increase in collagen content, the formation of new blood vessels, and the capacity to promote their maturation.

Conclusions: Secretome-derived therapy may be a safe and effective way to encourage mucosal repair. It might function as a novel, cell-free treatment strategy. As so, it offers regenerative medicine as a possible substitute for conventional cell therapy.

Key words: Oral ulcers, Regeneration, Bone marrow stem cells, secretome, albino rats.

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Introduction

The soft tissue lining the mouth cavity is called the oral mucosa. It has various functions and a layered structure like the skin.[1]

The oral cavity is constantly in danger due to a variety of stimuli encountered in our daily lives. The oral mucosa's primary function is to shield the underlying tissues from outside effects.[2]

Moreover, substances found in tobacco, alcohol, and betel nuts may induce cancer of the oral mucosa. Any compromise in the oral cavity's barrier function might lead to significant dysfunction.[3]

An ulcer is defined as a break in the skin's or mucosa's surface epithelial integrity. It may cause traumatic removal or molecular death on the surface of the epithelium.[4]

Oral ulcer incidence ranges from 5% to 20%. The most typical complaints are redness, burning feeling, soreness, and discomfort. They can appear anywhere in the oral cavity, but they may be uncomfortable if they do so in the moveable section.[5]

Prolonged and severe ulceration can make it difficult to swallow, eat, speak, or maintain good oral hygiene since the oral mucosa has a dense network of nerves.[6]

The use of painkillers, mouthwashes containing steroids and antibiotics, and other prescription drugs are recommended to lessen discomfort and hasten the healing of lesions. But using these medications often and for long periods might have a lot of unfavorable side effects.[7]

Corticosteroids and antibiotics are frequently used to manage extensive chemically-induced lesions, though they may result in oral candidiasis.[8]

The effectiveness of standard ulcer therapies is not always guaranteed; hence modern procedures should be created. In this situation, regenerative medicine has been used extensively.[9]

Stem cells are widely used in regenerative medical applications. As innovative methods to generate stem cells from diverse sources improve, there could be new possibilities for cell-based therapies.[10]

A greater variety of stem cell-based treatments have been studied in preclinical and clinical settings to assess how they affect wound healing. Numerous studies have been conducted in this area, particularly using mesenchymal stem cells (MSCs).[11]

Numerous studies on compounds produced by stem cells have shown that they can promote tissue regeneration even when the stem cells are not present.[12]

Soluble compounds produced by stem cells make up the secretome. These compounds consist of a complicated mixture of soluble products, of which a portion is soluble in proteins. This fraction contains several different substances, such as serum proteins, cytokines, growth factors, and angiogenic agents.[13]

Secretome is a protein-based biotechnology product that has the potential to be a safer substitute for products made using living cells. Moreover, long-term storage of the secretome does not pose a risk to its biological properties.[14]

VEGF is a highly effective proangiogenic growth factor in wound healing. The amount of VEGF that is present in a wound is a critical component that has a substantial impact on the healing process.[15]

To date, there has been little research done on the role of secretome in mucosal healing. Thus, this study aimed to shed light on the healing benefits of secretome therapy in severely traumatized oral ulcers in albino rats.

Material and Methods

Ethical Statement

All animal procedures were conducted following ARRIVE guidelines. The Institutional Animal Care and Use

Committee of Minia University, Egypt submitted its approval for the current study on **March 28, 2023. Committee approval number: 95.**

I. Material

1. Experimental Animals

Thirty adult male albino rats (weighing 200–250 g and 8–12 weeks old) were kept at the Medical Pharmacology Laboratory of Assiut University in Egypt. For the duration of the trial, the animals were housed in wire mesh cages with sawdust flooring and were allowed unlimited access to a balanced diet and tap water.

2. The Used Agents

a) Oracure® Gel: it contains two active ingredients: cetylpyridinium chloride and lidocaine HCL. It is produced by Amoun Pharmaceutical Industries Company.
b) The Bone marrow mesenchymal stem cells (BM-MSCs): They were obtained from albino rat femur bones in the Assiut University, Egypt's Molecular Cell Biology Research Lab's Zoology Department.

II. Methods

1. Isolation and Culture of Bone Marrow-Derived Mesenchymal Stem

In this investigation, two males, five-week-old albino rats were put to death via cervical dislocation and total body immersion in 70% ethanol. The femurs were then washed and dissected, and they were then placed on ice in 10 milliliters of full Dulbecco's Modified Eagle Medium (DMEM/F12).

Three or four passes through an 18-gauge needle connected to a 30-milliliter syringe were made with the recovered marrow plugs. Next, a 70 µm strainer was used to filter the cell suspension.

Trypan blue was removed, and then the cells were counted using a hemocytometer to determine the vitality of the cells. About 4×10^7 bone marrow cells were recovered as the cell yield.[16]

2. Harvesting of Secretome from the Bone Marrow Stem Cells

After removing the media at the third passage, when cell confluence was between 60% and 80%, the cells were repeatedly washed with PBS. After that, they were given DMEM/F12 Medium without serum and left overnight.

The stem cell secretome was then gathered, promptly aliquoted into 1 ml vials, and stored at 80°C in an ultra-freezer.[17]

3. Experimental Procedure for Inducing Oral Ulcers

All of the animals were anesthetized with intraperitoneal injections of xylazine hydrochloride 2% and ketamine hydrochloride 10%, at doses of 0.03 and 0.06 ml/100 g, respectively. After they were rendered unconscious, Allis forceps were used to hold their mouths open while 0.12% chlorhexidine digluconate was used to sanitize the buccal mucosa.

A symmetrical circular ulcer measuring 5 mm in diameter and 2 mm in depth was induced in the mucosa of the right cheek using Mitex Rotary Trephine Burs, Stainless Steel Germany (Ø 5.0). To maintain uniformity, the same operator carried out every surgical procedure.

4. Study Design

Thirty albino rats were randomly divided into three groups for this study:

I. Control group (n=10): saline was used to treat rats.

II. Oracure gel-treated group (n=10): twice a day, topically applied gel.

III. Secretome-treated group (n=10): the bone marrow stem cells-derived

secretome was injected locally with 0.1 ml.

Each group was divided into three subgroups (A, B, C) according to the date of performance of the investigating tests (3, 7, 12 days respectively).

5. Clinical Evaluation

Using a William's periodontal probe, the diameter of the wounded area (mm) was measured to determine the ulcer's size. The measurements were taken in each group on days 3, 7, and 12 following ulcer induction.

To determine the degree of wound contraction, the ulcer size data were used.[18]

$$\text{Wound Contraction} = \frac{\text{Inital Wound Size} - \text{Special Day Wound Size}}{\text{Initial Wound Size}} \times 100$$

6. Histopathological Assessment via Hematoxylin and Eosin Staining Technique

After being collected, the buccal mucosa biopsy samples were stored in 10% neutral buffered formalin. The samples were dried using increasing amounts of alcohol, and any leftover alcohol was removed by immersing them in xylene.

The samples were then embedded in blocks of paraffin wax. Hematoxylin and eosin staining was performed on sections that were mounted onto glass slides after they were cut to a thickness of around 4-5 microns.

7. Masson's Trichrome Staining Procedure

The tissue samples were deparaffinized, rehydrated using varying alcohol strengths, and then cleaned in distilled water. To improve the staining quality, formalin-fixed tissues were refixed in Bouin's solution and heated to 56°C for an hour.

The staining process was initiated using Weigert's iron hematoxylin and subsequently washed with warm tap water. The sections were washed with distilled water after the Biebrich scarlet-acid fuchsin solution was applied.

After completing the earlier procedures, an aniline blue solution was applied, followed by a quick rinse and two to five minutes of differentiation in a 1% acetic acid solution. Quick dehydration was accomplished, and then xylene was cleared. Ultimately, a resinous substance was used to install the parts.

Histological examination showed that the colors blue symbolized the different types of collagen fibers: dark blue for native collagen, light blue for newly formed collagen, and black for nuclei.

8. VEGF-A Based Immunohistochemical Analysis for Angiogenesis Detection

Tissue slices were arranged on poly-lysine-coated slides. These sections were painstakingly deparaffinized, rehydrated, and then stained with avidin-biotin peroxidase complex for immunohistochemical analysis.

The primary rabbit anti-human VEGF-A IgG (5 mg/mL), von Willebrand factor (1:1600), HHF35 (1:100), HAM56 (1:100), UCHL 1 (1:100), and MX-PanB (1:100) antibodies were then incubated for an extra night at 4°C in a moisture chamber after the steps of blocking endogenous peroxidase activity and microwaving antigen retrieval.

9. Statistical Analysis

The experimental data was statistically analyzed using the Statistical Package for the Social Sciences (SPSS). To compare various variables, One-way Analysis of Variance (ANOVA) and Post Hoc Tukey's Honestly Significant Difference (HSD) test were utilized. The data were expressed using the mean ± standard deviation in the ANOVA test. The results were significant if the ***P***-value was less than 0.05.

Results

1. Clinical Evaluation

In all groups, mouth ulcers appeared crateriform, edematous, and hyperemic over the first three days. By the seventh day, all groups' pseudomembranes had more definite shapes, and ulcer boundaries had become more regular. The ulcer area contraction was greater in the secretome-treated group than in the other groups.

Conversely, the control group showed ulcers with jagged margins and a pale ring encircling the residual wound site. The oral ulcers in the group treated with secretome were cured after twelfth day. Ulcers were still clinically evident in the control group and the groups treated with oracure gel (Fig.1).

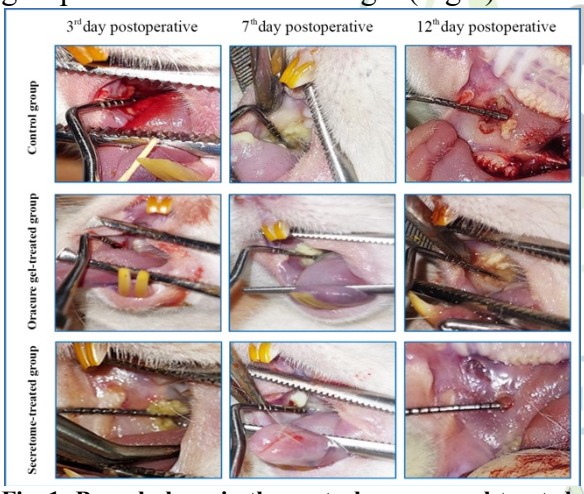

Fig. 1: Buccal ulcers in the control, oracure gel-treated, and secretome-treated groups.

2. Histopathological Assessment via Hematoxylin and Eosin Staining Technique

a. Group I

On the third day, subgroup IA: The ulcerated areas showed degenerative areas, blood vessels (BVs), disordered basal cell layers, ill-defined basement membranes, and epithelium loss due to apoptotic activity (Fig. 2A).

On the seventh day, subgroup IB: Proliferation and migration of epithelial cells were noted near the ulcer margins, collagen fibers, and a high infiltration of inflammatory cells (Fig. 2B).

On the twelfth day, subgroup IC: Thin, atrophic stratified squamous epithelium layers with flat rete ridges covered the healed areas, fibroblasts, minor inflammatory cell infiltration, and a few degenerative regions were observed in lamina propria (LP) (Fig. 2C).

b. Group II

On the third day, subgroup IIA: Newly formed BVs, fibroblasts, collagen fibers, and infiltration of inflammatory cells encircled the ulcer gaps inside keratinized epithelial margins (Fig. 2D).

On the seventh day, subgroup IIB: Several apparent degenerative patches were present, and the epithelial margins were approaching each other (Fig. 2E).

On the twelfth day, subgroup IIC: The freshly formed non-keratinized epithelial linings included short rete ridges, poorly developed rete pegs, weakly defined basement membranes with few inflammatory cells, and collagen fibers (Fig. 2F).

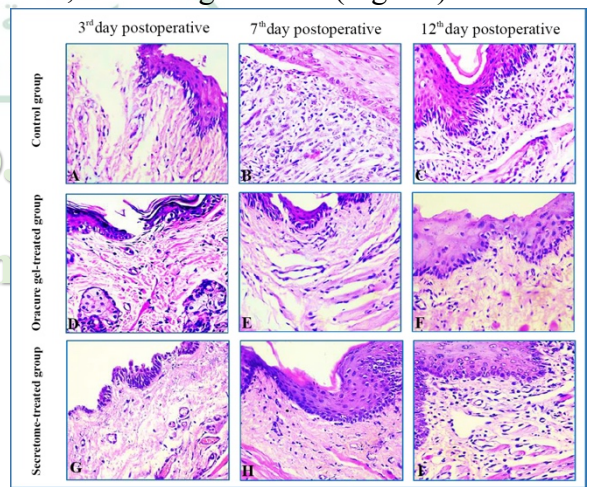

Fig. 2: Histopathological images showed the healing of ulcers in the control group (A-C), the oracure gel-treated group (D-F), and the secretome-treated group (G-I) on days 3, 7, and 12 (H&E stain, mag. X400).

c. Group III

On the third day, subgroup IIIA: The healed areas included a high concentration of inflammatory cells, BVs engorged with

RBCs, fibroblasts, and collagen fibers in addition to newly produced epithelial lining from basal and parabasal layers (Fig. 2G).

On the seventh day, subgroup IIIB: The regenerated areas had newly continuous epithelial linings, thin layers of keratin, few inflammatory cells, and a significant number of fibroblasts (Fig. 2H).

On the twelfth day, subgroup IIIC: At the regenerated areas, a continuous keratinized stratified squamous epithelium was seen. In the underlying LP, several newly formed BV, collagen fibers, fibroblasts, and inflammatory cell infiltration was seen (Fig. 2I).

3. Masson's Trichrome Staining Procedure

a. Group I

On the third day, subgroup IA: The central regions of the ulcers showed thin, fragmented collagen fibers (Fig. 3A).

On the seventh day, subgroup IB: The collagen fibers were thin, immature, and included spaces between them (Fig. 3B).

On the twelfth day, subgroup IC: The healed areas showed thick, randomly organized immature collagen fibers (Fig. 3C).

b. Group II

On the third day, subgroup IIA: The ulcers' centers included thin, discrete, and jumbled collagen fibers (Fig. 3D).

On the seventh day, subgroup IIB: Immature collagen bundles were found beneath the ulcer borders (Fig. 3E).

On the twelfth day, subgroup IIC: Bundles of immature collagen fibers oriented irregularly radially were present beneath the healed sites, together with a few inflammatory cells (Fig. 3F).

c. Group III

On the third day, subgroup IIIA: Dense bundles of immature collagen fibers oriented radially were present beneath the regenerated sites, together with a few inflammatory cells (Fig. 3G).

On the seventh day, subgroup IIIB: Collagen fibers were evenly spaced and closely packed (Fig. 3H).

On the twelfth day, subgroup IIIC: Increased vascularity, a high concentration of fibroblasts, and well-organized collagen fibers were among the characteristics of the regenerated epithelium that suggested a return to its original architecture (Fig. 3I).

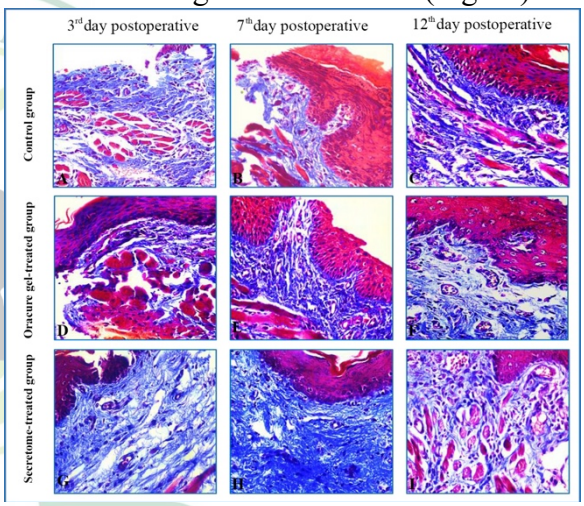

Fig. 3: Histochemical analysis revealed newly formed collagen fibers in the control group (A-C), the oracure gel-treated group (D-F), and the secretome-treated group (G-I) on days 3, 7, and 12 respectively (MT stain, mag. X400).

4. VEGF-A Based Immunohistochemical Analysis for Angiogenesis Detection

a. Group I

On the third day, subgroup IA: Fibroblasts, inflammatory cells, and endothelial cells all showed a mild immunological positive response (Fig. 4A).

On the seventh day, subgroup IB: The basal layer surrounding the ulcers showed considerable immunological positivity, and some immuno-positive nuclei were seen in the fibroblasts, inflammatory cells, and endothelial cells of the blisters (Fig. 4B).

On the twelfth day, subgroup IC: The healed regions showed a clear immunological

expression of VEGF, with some positive reactions in the LP and positive stained nuclei in the basal and prickle cell layers (Fig. 4C).

b. Group II

On the third day, subgroup IIA: Immunoexpression of VEGF revealed moderately positive stained nuclei in the basal and prickle cell layers of the ulcer margins (Fig. 4D).

On the seventh day, subgroup IIB: VEGF immunohistochemistry showed moderately positive-stained nuclei in the healed areas (Fig. 4E).

On the twelfth day, subgroup IIC: VEGF expression was elevated in the fibroblasts, endothelial cells, infiltrating inflammatory cells, and epithelium (Fig. 4F).

c. Group III

On the third day, subgroup IIIA: In the regenerated areas, VEGF was visibly seen in many nuclei within the ulcer margins (Fig. 4G).

On the seventh day, subgroup IIIB: epithelial cells showed signs of VEGF immune positivity (Fig. 4H).

On the twelfth day, subgroup IIIC: The regenerated mucosa had moderately positive nuclei, according to VEGF immune-staining (Fig. 4I).

Statistical Analysis

The one-way ANOVA showed a significant difference in wound contraction among the control group, oracure gel-treated group, and secretome-treated group. Additionally, the Post Hoc Tukey's HSD test compared the percentage of wound contraction between each pair of these groups (table 1).

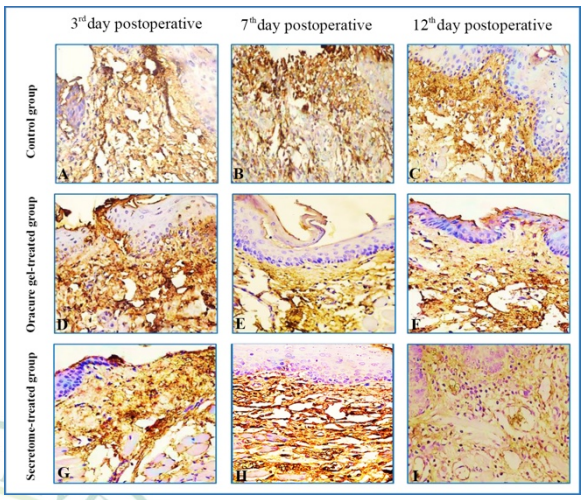

Fig. 4: VEGF immunohistochemistry images of buccal mucosa healing on days 3, 7, and 12 in the control group (A-C), oracure gel-treated group (D-F), and secretome-treated group (G-I) (VEGF, mag. X400).

Table 1: The percentages of wound contraction observed in the various groups were evaluated using the One-way ANOVA followed by the Post Hoc Tukey's HSD test.

	Control group	Oracure gel-treated group	Secretome-treated group	F	*P*-value
Day 3	0	13.33±5.77	36.67±5.77	46.50	<0.0001*
		P1≤0.031*	*P1*≤0.0001*		
			P2≤0.002*		
Day 7	16.67± 5.77	33.33 ± 5.77	63.33±5.77	50.33	<0.0001*
		P1≤0.028*	*P1*≤0.0001*		
			P2≤0.002*		
Day 12	33.33±5.77	70±10	96.67±5.77	54.60	<0.0001*
		P1≤0.002*	*P1*≤0.0001*		
			P2≤0.011*		

Data reported as mean±SD, *P*: Probability, *significance <0.05. *P*1: significance vs control group. *P*2: significance vs oracure gel-treated group.

Discussion

Traumatic oral ulcers constitute one of the most prevalent inflammatory conditions. These lesions have a high risk of infection and can be extremely painful. The cheek mucosa is the most particularly vulnerable to trauma.[19]

One of the biggest challenges in treating oral ulcers is the search for more potent medications with fewer adverse effects that can restore oral function, lessen discomfort, reduce inflammation, and minimize the duration of the ulcer.[20]

Numerous studies have been conducted to expedite the healing process of

oral ulcers; nevertheless, no permanent cure for ulceration has been established.[21]

Stem cell therapy has influenced medicine over time, giving intriguing potential for the treatment of several ailments.[22]

Through stem cell therapy, the tissue can be returned to its pre-injured state. Angiogenesis is promoted and wound healing has been demonstrated by biologic therapies such mesenchymal stem cell transplantation.[23]

Several issues arise from the widespread usage of BM-MSCs. Among these obstacles are concerns connected to immunological rejection, the potential for malignant tumor formation, spontaneous modifications in cellular features and behavior, as well as the risk of transmitting infectious diseases.[24]

To solve the obstacles associated with cell-based therapy, researchers are turning increasingly to cell-free approaches.[25]

The purpose of the current study was to evaluate the ability of locally delivered BM-MSCs-derived secretome to repair traumatic oral ulcers in albino rats' cheek mucosa.

The therapeutic impact of oracure gel and bone marrow-dervied secretome therapy was assessed using clinical evaluation, routine Hematoxylin and Eosin (H&E) stains, Masson's trichrome (MT) special stain, and immunohistochemistry for angiogenesis.

When compared to the control group, the clinical evaluation of the oracure gel-treated group showed increased wound contraction at 3, 7, and 12 days after ulceration. Additionally, the histological and immunohistochemical evaluation revealed relative improvement in the rats treated with oracure. However, the rats treated with secretome revealed significant improvement in healing the clinical evaluation and histological and immunohistochemical features compared to both the control group and oracure group.

Our findings coincide with the previous work reported that acellular derivatives of MSCs were shown to be the most effective technique for wound repair, according to ***de Mayo, Conget et al.***[26], who noted that the secretome of the BM-MSCs group obtained much higher percentages of wound closure.

According to *Ichi, Triwahyuni, et al.*[27], these results can be explained by the ability of secretome to stimulate wound healing. These cells produce inflammatory mediators in vitro, such as prostaglandin E2, interleukin-1, and interleukin-6, which can reduce neutrophil activity and accelerate wound closure.

Park, Kim, et al.[28] showed that increased levels of hepatocyte growth factor, basic fibroblast growth factor, and epidermal growth factor are linked to secretome-mediated wound healing. Fibroblast migration and proliferation are stimulated by EGF. In addition, it has been discovered that HGF stimulates the growth of endothelium and epithelial cells and inhibits apoptosis.

In reference to the MT stain, the results originally revealed the greatest quantity of dense and thick immature collagen fibers in secretome treated rats. By seventh day, these collagen fibers had transformed into precisely structured collagen bundles, and by twelfth day, the architecture had been restored with mature collagen fibers.

El-Tookhy, Shamaa, et al.[29] indicated that exosomes generated from MSCs significantly impacted the healing of wounds. Significant improvements in collagen deposition and fibroblast migration were seen after 14 days after treatment, as indicated by MT staining, which suggests ongoing tissue remodeling.

At days three and seven after the treatment, the VEGF immunoreaction

showed strong positive reactions; by twelfth day, the healing process had been fully achieved.

In our investigation, the secretome-treated group showed a significant decrease in the percentage of area fraction on the twelfth day as opposed to the third and seventh days.

This finding supports earlier studies' hypothesis that angiogenesis would be suppressed during the last phases of healing. The reduction of tissue hypoxia and inflammation results in a decrease in the quantity of growth factors present in the wound.[30]

The findings of *Johnson and Wilgus*[15], which showed that VEGF protein levels in injured tissue begin to rise one day after injury relative to control intact skin, may also explain this conclusion. VEGF levels are much higher in injured skin three and five days later than in control skin. Protein levels return to normal after seven to fourteen days.

Ultimately, statistical examination demonstrated that, in comparison to the control and oracure gel-treated groups, the secretome-treated group exhibited a highly statistically significant difference. ***P***-value <0.0001.

Finally, through the coordination of several processes, the secretome of MSCs plays a pivotal role in wound healing. It involves promoting inflammation resolution, modulating fibrosis, and reducing scarring, while also boosting angiogenesis and granulation tissue formation. Additionally, it exhibits antimicrobial properties, neutralizes reactive oxygen species, and enhances dermal fibroblast function. MSCs' conditioned medium stimulates nearby stem cells for tissue repair.[31]

Furthermore, soluble substances produced by BM-MSCs are recognized by the β2-adrenergic receptor, which promotes keratinocyte migration and the epithelial-mesenchymal transition necessary for re-epithelialization.[32]

Conclusions

The effectiveness of bone marrow stem cells-derived secretome in curing oral ulcers is demonstrated by our research. It also improves angiogenesis and encourages the production of new collagen fibers, which enhance the quality of the healed tissue. In light of this, this study provides new insights into more safe, cell-free-based therapies instead of cell-based therapeutics.

Conflict of Interest

The authors have no financial conflicts of interest.

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