Original Article

The Regenerative Potential of Adipose-Derived Stem Cells Versus their Conditioned Media on Tongue Defects of Adult Male Rats

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ABSTRACT

Background and objectives: Tongue is a strategic organ in the oral cavity that is commonly affected by different diseases and malignancies, that may require surgical resection leaving resistant defects. Adipose derived stem cells (ADSC) are an available, accessible and abundant mesenchymal stem cells source that have a wide range of differentiation potentials and secrete a large variety of growth factors and cytokines. Cell conditioned media (CM) hold all the biologically active components secreted by the cultured cells providing a safe, cell free therapy. This study was conducted to assess the regenerative capacity of adipose derived stem cells and their conditioned media on mechanically induced tongue defects.

Materials and Methods: Male, Sprague Dawley rats were subjected to mechanical circular defects on the dorsum of the tongue and then allocated to three groups (n=15): group I (control), group II (ADSC treated), and group III (ADSC-CM treated). On the fourth, seventh and fourteenth days after defect preparation, five rats from each group were euthanized and specimens were subjected to clinical, histological, histomorphometrical, and immunohistochemical examination followed by statistical analysis.

Results: In both ADSC and ADSC-CM treated groups, the tongue defects showed reduced defect depth, better histological regeneration including the epithelial layers, the connective tissue and the tongue musculature, as well as enhanced cellular proliferation, and less inflammation as compared to the control untreated group. ADSC treated group showed earlier healing than the ADSC-CM treated group, but the results didn't express statistical significance.

Conclusions: ADSC and their CM can be good candidates for treating tongue defects.

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Key Words: Cell proliferation, inflammation, mesenchymal stem cells, oral ulcers, regeneration.

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INTRODUCTION

Tongue is a complex muscular organ that functions in speech, mastication, deglutition, and swallowing. However, it can be affected by a variety of diseases and neoplasms such as erythroplakia, granular cell tumor, squamous cell carcinoma, and kaposi sarcoma that may require surgical resection of the affected part of the tongue[1]. Tongue defects are mostly caused by oncological resection. However, severe trauma can also cause extensive defects[2].

Tongue can also be affected by oral ulcers which are described as loss of connective tissue and overlying epithelium which could be attributed to a variety of reasons. They may either be acute or chronic depending on the ulcers' characters and advancement[3]. Unfortunately the ulcerated oral tissue are in constant contact with oral microbial flora making them susceptible to infection and delayed healing, which requires early management[4].

Several studies have been conducted with the aim of improving recovery of oral pathologies or wounds and relieving pain such as antibiotics, analgesics[5], anti-inflammatories, immunomodulators[6], herbal medicines[7], and targeted lesion treatments, such as chemical cauterization, debridement, surgical removal, low-density ultrasound, and photo-biomodulation treatment[8]. However, these treatment modalities may not achieve the desired outcomes or can even result in undesired adverse effects[6,9].

The recent advancement in tissue engineering and stem cells based strategies raised tremendous hopes for regenerating surgically resected tissues[10]. Adipose-derived stem cells (ADSCs) are an encouraging type of stem cells isolated from adipose tissue. They are capable of secreting a variety of growth factors and signaling molecules in a paracrine manner. ADSCs can be easily isolated in abundant quantities. They are capable of self-renewal and multilineage differentiation. Moreover, they are constantly available, less immunogenic, possess immunosuppressive functions and have relatively lower cost. ADSCs were highly successful in resolving chronic ulcers compared to other therapeutic modalities[11].

The serum-free conditioned media (CM) from ADSC offer a cell-free therapy that is more convenient and safer to apply. ADSCs secrete many growth factors such as basic fibroblast growth factor (bFGF), transforming

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growth factor (TGF)-β, keratinocyte growth factor (KGF), hepatocyte growth factor (HGF), and vascular endothelial growth factor (VEGF) into the conditioned media, which might mediate the regenerative effect of ADSCs[12]. This study was performed to compare between the effectiveness of ADSCs and its CM on healing of mechanically induced tongue defects.

MATERIALS AND METHODS

Ethical statement

The animal experimental procedures were conducted following the regulations recommended by Mansoura University animal care and use committee (MU-ACUC) (ID: DENT.R.22.09.1) according to the biosecurity and biosafety procedures described by Alderman *et al*.[13]

Animals

Forty-eight male, Sprague Dawley, pathogen free rats, with the weight of 250-300 gm were chosen. Animals were kept in medical experimental research center (MERC) (Mansoura University, Mansoura, Egypt) under standardized housing requirements, in coops of five rats each, in a room with a light-dark cycle of 12h. Relative moisture of 65-70% and temperature of 22 ºC were kept constant. Animals had access to commercially available diet and water as desired and were housed for at least 2 weeks before the beginning of the study.

Study design

This was a randomized controlled experimental study. Sample size calculation was based on mean PCNA antibody expression among studied groups with ADSC and control group retrieved from a previous study[14]. Calculation of sample size was performed using G*power program (version 3.1.9.4) depending on effect size of 2.66, 2-tailed test, 0.05 α error and 90.0% power. It was calculated to be at least five in each group.

Three animals were sacrificed at the beginning of the experiment as a negative control to evaluate the normal histology of rat's tongue. The rest of the animals were subjected to mechanical tongue defect induction and then randomly allocated following simple random sampling strategy into three groups (n=15) according to the treatment that was injected circumferentially around the wound margins as a single injection directly after defect preparation, as follows:

Group I (Positive control): Tongue defects received 0.1 ml phosphate buffered saline (PBS).

Group II (ADSC): Tongue defects received ADSCs (25×104) suspended in 0.1ml PBS.

Group III (ADSC-CM): Tongue defects received 0.1 ml ADSC-CM.

On the fourth, seventh and fourteenth days after defect preparation, five rats from each group (n=5) were euthanized by intraperitoneal injection of sodium pentobarbital (≥0.86 mg/kg) and tongue specimens were excised for clinical, histological and immunohistochemical examination.

ADSCs Isolation

For the isolation of ADSCs, six Sprague Dawley rats of 200-250 g weight (5-6 months of age), were used following our previous protocol[15]. Briefly adipose tissue was obtained from rats' supra-renal fats, washed extensively, minced into pieces, and then digested in 0.1% type I collagenase (cat. #SCR103, EMD Millipore Corp, Billerica, USA), for 1h at 37ºC. Action of collagenase enzyme was neutralized by adding DMEM culture media (cat. #L0066-500, BioWest, Nuaillé, France) with 10% fetal bovine serum (FBS) (cat. #S1810-500, BioWest, Nuaillé, France). Cells were then kept in 5% CO_2 and 37 ºC in an incubator and media changed every 2-3 days thereafter. Cell cultures were evaluated each day by an inverted microscope (Olympus, CKX41SF, Tokyo, Japan) till reaching 80% confluence.

ADSCs characterization

For ADSCs characterization, a BD Accuri C6 flow cytometer (BD Biosciences, California, USA) and program software was used for flowcytometric immunophenotype determination. Third passage cells were treated with trypsin, followed by washing with PBS and incubation with the following primary antibodies; anti-CD90 PE (BD Biosciences, cat. #551401, California, USA), anti-CD73 purified (BD Biosciences, cat. #551123, California, USA), anti-CD45 FITC MAB (BD Biosciences, cat. #561867, California, USA), and anti-CD34 purified (R&D systems, cat. #AF6518-SP, Minneapolis, USA). For purified antibodies, Fluorescein isothiocyanate fluorophores (FITC, cat. #F143, Thermo Fisher Scientific, Massachusetts, USA) were added to each antibody, followed by their incubation in the dark for 30 minutes at 4 °C. PBS was then used to rinse the labeled ADSCs, which were then centrifuged for 5 minutes at 200×g and suspended in PBS.

Conditioned media preparation

Conditioned media were prepared as described by Yang *et al*.[16]. ADSCs (4 x 10^6) from the third passage were collected. After reaching 80-90% confluence, cells were rinsed three times with PBS and then media were replaced by serum-free media and left for 2 days, followed by media collection, centrifugation, filtration through a 0.2 µm filter and storage at -80°C.

Surgical procedures

Prior to any procedures, animals were weighed to adjust drug doses. Rats were anesthetized by injecting ketamine hydrochloride (35-45 mg/kg) and xylazine hydrochloride (5-7 mg/kg) intramuscularly. Rats' tongues were wiped with Betadine and defects measuring 5 mm diameter and 2 mm depth[17] were produced in the midline of the middle thirds of the tongues' dorsum utilizing a tissue punch rotary drill (cat. #4159, IQ implants USA, Maryland,

USA) (Figures 1A, B,C). The bases of the defects were incised with surgical scissors. Immediately after defect preparation, different treatments were injected around the wound margins. As postoperative care measures, Oxytetracycline hydrochloride 20% and Analgin 0.5 mg were administered to the rats for three days.

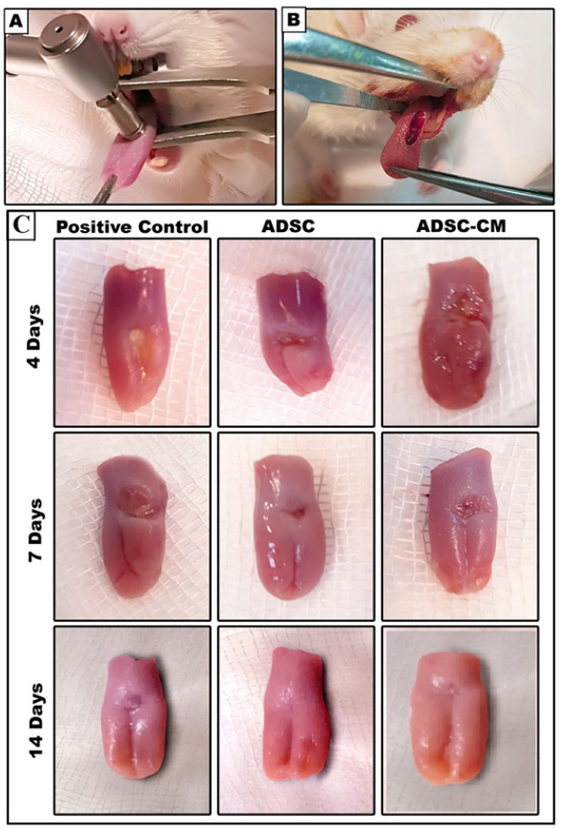

Fig. 1: A: Surgical procedures for tongue defect preparation. **B:** Circular tongue defect. **C:** Clinical picture of the tongue defects of different groups at different time intervals.

Histological assessment

After fixation with neutral-buffered formalin, tongue specimens were processed into paraffin blocks, sliced into 4µm sections and prepared according to our previous protocol[18]. Serial sections were prepared using microtome, deparaffinized, rehydrated, and stained with hematoxylin and eosin (H&E). For immunohistochemical staining, endogenous peroxidase blockage was performed using H_2O_2 and antigens were retrieved by boiling in citrate buffer. Slides were then incubated with the primary antibodies for proliferating cell nuclear antibody (PCNA monoclonal antibody, cat. #307901, Biolegend, California, USA) and Interleukin-6 (IL-6 polyclonal antibody, cat. #A0286, ABclonal, Massachusetts, USA), then incubated with the secondary biotinylated antibody and streptavidin biotin complex. Diaminobenzidine chromogen (DAB kit, cat. # ab64238, Abcam, Cambridge, UK) was applied then counterstaining with Harris hematoxylin was performed.

Digital image analysis

Digital image analysis was carried out on H&E-stained slide photographs that were obtained by ToupCam® digital camera (XCAM1080PHA). The camera was coupled with 0.5 photo adaptor on a light microscope (Olympus®, CX22, Japan), using 4x objective lens. The vertical defect depth was measured for each slide.

For immunohistochemically stained histological sections, two blinded examiners photographed five different sites (1x1 mm^2) in each slide using a 20x objective lens. For PCNA immunostaining, the percentage of positively stained nuclei was calculated by dividing the number of the positive nuclei by the total number of nuclei. While for IL-6, the percentage was calculated by dividing the area of positive brown staining over the total area. Brown staining was considered regardless of stain intensity.

Quantification of defect depth and staining was carried out by the image processing software Fiji ImageJ (version 2; NIH, Maryland, USA) by modifying the method established by Patera *et al*.[19]. The histological dyes digital separation was performed on the microphotographs and three independent digital images were obtained (H&E, DAB, and a complementary image). Then, stain-specific values for the area percentage and positive cell number in DAB and H&E images were determined. Data was presented as the mean area percentage or positive cells percentage ± standard deviation.

Statistical analysis

Data was analyzed using IBM SPSS. (Version 22.0, 2013. Armonk, NY: IBM Corp). Data normality was tested by Shapiro-Wilk test. Data was tabulated as mean ± standard deviation. Result significance was calibrated at *P-value* of 0.05. The combined effect of the two independent factors which were group and time on the dependent continuous outcomes which were defect depth, PCNA, and IL-6 antibody immunostaining was tested using two-way ANOVA test succeeded by Post Hoc Tukey test for pairwise comparison.

RESULTS

Flow-cytometric phenotypic characterization

The third passage ADSCs were subjected to cell surface phenotypic marker analysis where the mesenchymal markers CD90 (93.3%) and CD73 (92.7%) were found to be highly positive, while the hematopoietic markers CD45 (3.2%) and CD34 (4.2%) were negative (Figure 2).

Clinical examination

Two animals died during the first 4 days after injury and were replaced. This may be caused by difficulties in feeding after tongue injury. On examination of the tongue specimens at 4 days, the tongue defects in all experimental groups showed marked ulceration and deformity. Some of the control defects showed a white colored mucosal covering. On day 7, the defects were more healed, but

still surface continuity was not restored in all groups. Defects of the control group appeared wider and deeper than the treated groups and defects of group II (ADSC) showed better healing. On day 14, the rats' tongues showed continuous surface with slight depression especially in the control group. Group II (ADSC) and group III (ADSC-CM) showed better regeneration with group II showing almost normal textured mucosa (Figure 1C).

Haematoxylin and Eosin (H & E) histological staining results

Hematoxylin and Eosin staining of tongue specimens of the negative control group, C-K. Hematoxylin and Eosin staining of tongue specimens of the positive control, ADSC-treated and ADSC-CM treated groups at 4,7 and 14 days (4x). E: epithelium, CT: connective tissue, M: muscles, K: keratin, Fg: fungiform papilla, Fl: filiform papilla, Arrowhead: taste buds, D: defect, Arrow: granulation tissue, Asterisk: inflammatory infiltrate, V: blood vessel, L: lateral wound margins (Figures 3 A,B).

Hematoxylin and Eosin staining of tongue specimens of the positive control, ADSC-treated and ADSC-CM treated groups at 4,7 and 14 days (10x). J,K. Hematoxylin and Eosin staining of the mucosal overgrowths. E: epithelium, CT: connective tissue, M: muscles, K: keratin, Fl: filiform papilla, D: defect, Arrow: granulation tissue, Asterisk: inflammatory infiltrate, V: blood vessel, L: lateral wound margins, Thick arrow: exophytic outgrowth (Figures 4 A-I).

Histological evaluation of the negative control slides revealed normal tongue histology with normal regular thickness of keratinized tongue epithelium and well-arranged lingual papillae. Filiform papillae showed regular, conical, tapered shape while fungiform papillae were observed between the filiform papillae carrying normal taste buds. The underlying connective tissue (CT) presented regular CT papillae interdigitating with the overlying epithelium and well-organized tongue musculature with different orientation (Figures 3A,B).

After 4 days the control group (Figures 3C, 4A) showed tongue defects extending into the underlying CT and including tongue musculature with numerous inflammatory cells at wound margins and base. The epithelium directly adjacent to the wound lacked lingual papillae. While after 7 days (Figures 3D, 4B)., defects were still deep but less than the depth at 4 days. Newly formed blood vessels and numerous inflammatory cells at wound margins and depth were observed with no signs of regenerating muscles. After 14 days (Figures 3E, 4C)., wounds showed depth reduction and a thin layer of regenerating covering epithelium was formed. However, lingual papillae and tongue muscles were not restored at the wound site.

In group II (ADSC treated group) after 4 days (Figures 3F, 4D), some defects were almost filled with granulation tissue with new vascularization and inflammatory cell infiltrate. After 7 days (Figures 3G, 4E), the wound was filled with better organized granulation tissue with more newly regenerated blood vessels and more organized collagen fibers. Regenerating migrating epithelium was observed at the lateral wound margins with definite keratin layer. Some bundles of regenerating muscles were seen. After 14 days (Figures 3H, 4F), the wound showed complete healing and normal histological picture with full epithelial thickness and even keratin layer with normal lingual papillae. The connective tissue had well-arranged collagen bundles and connective tissue papillae were evenly interdigitating with the epithelium. Tongue musculature showed marked regeneration with newly formed muscle bundles.

In group III (ADSC-CM treated group) after 4 days (Figures 3I, 4G), tongue wounds showed some new granulation tissue formation with numerous inflammatory cells at wound margins and base and no signs of regenerating muscles. After 7 days (Figures 3J, 4H), the defects showed marked decrease in depth relative to the same group after 4 days, but still deeper than defects in group II at the same time interval. Numerous inflammatory cells and newly formed vessels were observed in the granulation tissue. A delicate layer of overlying regenerating epithelium was seen at the lateral wound margins, but no definite regenerating muscle bundles were noticed. After 14 days (Figures 3K, 4I), the wound showed reduced depth, but the surface was still depressed than the surrounding normal epithelium. The wound surface was covered by a well-organized, regenerated epithelium with regular keratin layer, but the lingual papillae were not completely restored. The underlying CT showed more arranged collagen bundles and minimal muscle regeneration. However, in some histological slides of different groups after 14 days, polyp-like overgrowths of tongue mucosa were observed at the defect site as connective tissue cores covered by full epithelial layer and keratin (Figures 4J, K).

Defect depth results

The bar graph (Figure 5A) shows the results of the two-way ANOVA statistical analysis. Post Hoc Tukey test for comparison of factors affecting defect depth revealed that the treated groups showed significantly shallower defects at all time intervals than the control group. At 4 days, the ADSC and the ADSC-CM groups didn't differ significantly, while at 7 days the ADSC treated group showed earlier healing and reduced depth as compared to the ADSC-CM treated group. However, after 14 days, both groups revealed comparable regeneration with less depth in the ADSC group, but no significance was detected between them (Table 1). Two-way ANOVA revealed that the effect of time alone and the effect of group alone were statistically significant with a statistically significant interaction between group and time factors ($P<0.05$).

Immunohistochemical staining results

Proliferating cell nuclear antibody (PCNA)

As shown in (Figure 6), the anti PCNA immunostaining

appeared as brown nuclear deposits. PCNA was expressed within epithelial basal and parabasal cell layers and in the lamina propria. It was also expressed in the granulation tissue and around blood vessels. The bar graph in (Figure 5B) shows the statistical analysis of PCNA immunostaining positive cell percentage, where the ADSC treated group showed the highest proliferation rates at all time periods followed by the ADSC-CM then the control group. The treated groups expressed significantly higher proliferation indices than the control group at all time intervals. ADSC-treated group showed more cellular proliferation than the ADSC-CM group at 4 and 7 days with significant difference however, after 14 days the difference didn't reach statistical significance. As per the effect of time, the control and the ADSC-CM treated groups revealed significant increase in proliferation rates between days 4 and 14 while the ADSC group showed early high proliferation index at 4 days (Table 2). The two-way ANOVA revealed significant effect of group, time, and group-by-time interaction (P<0.05).

Interleukin 6 (IL-6) antibody

(Figure 7) shows the anti-IL6 immunostaining results where positive reaction was observed as brown cytoplasmic or nuclear deposits. The inflammatory reaction as measured by IL-6 was significantly decreased in the experimental groups compared to control group at each time period with the lowest values expressed in the ADSC followed by the ADSC-CM treated groups. However, the statistical analysis revealed no significant difference between the treated groups at any time interval (Table 3). Through the period of the study, as shown in the bar graph (Figure 5C), the inflammatory reaction decreased in all groups with significant decline between days 4 and 14. The two-way ANOVA revealed significant effect of both group and time factors on IL-6 expression (P<0.05), but the interaction between group and time didn't reveal significant effect.

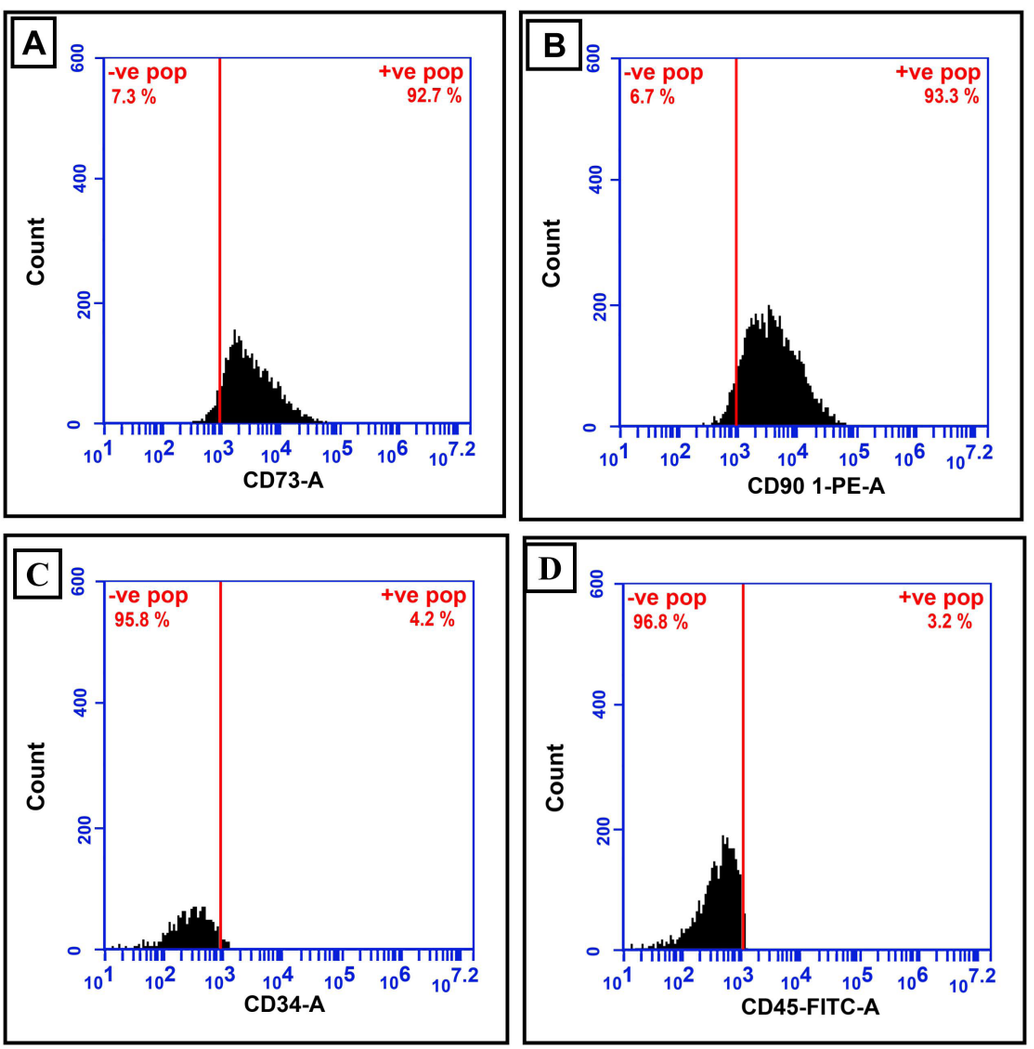

Fig. 2: Flowcytometry histograms for ADSC phenotypic characterization: A. CD73, B. CD90.1, C. CD34, D. CD45

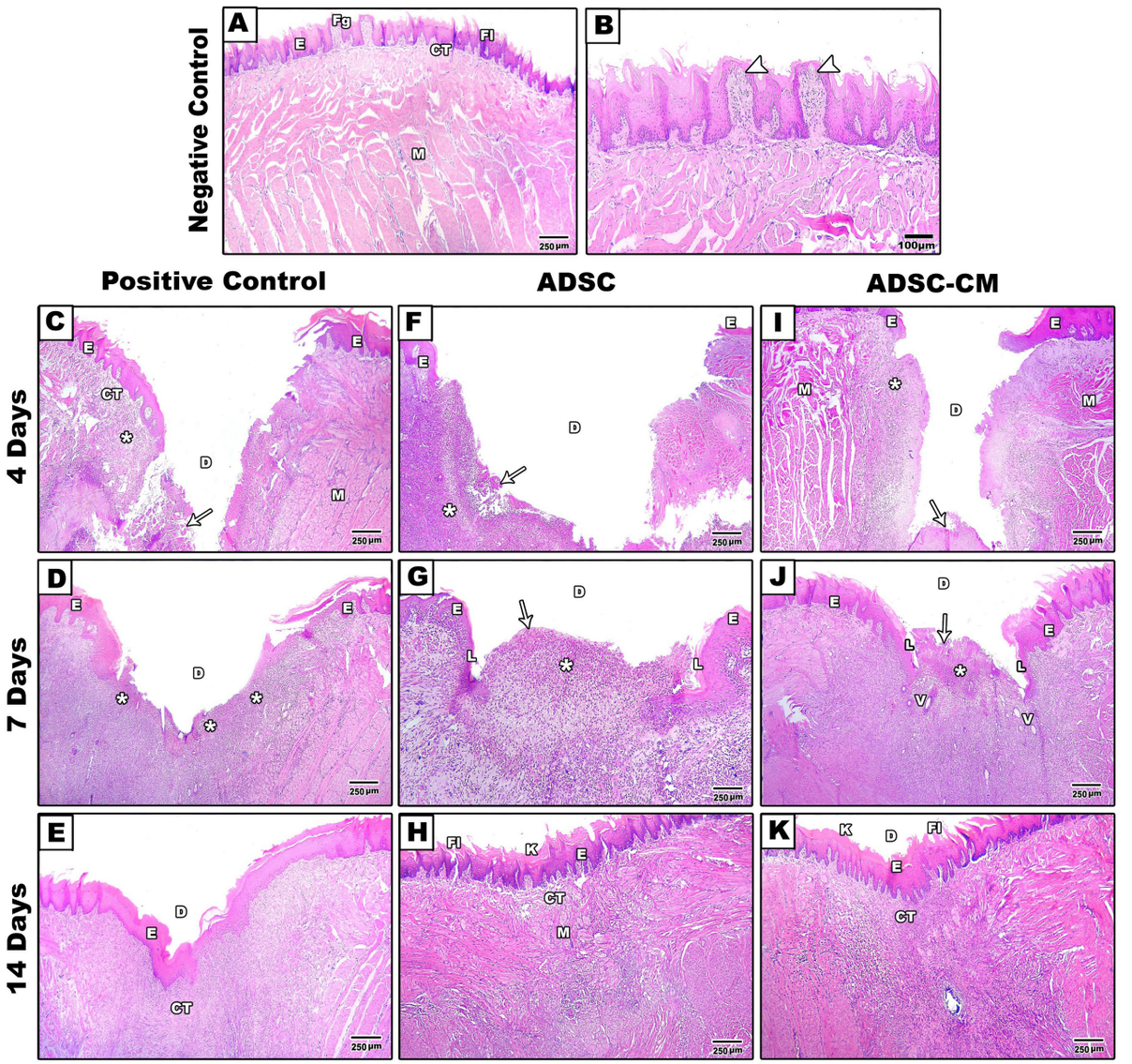

Fig. 3: A,B: Hematoxylin and Eosin staining of tongue specimens of the negative control group, **C-K:** Hematoxylin and Eosin staining of tongue specimens of the positive control, ADSC-treated and ADSC-CM treated groups at 4,7 and 14 days (4x). E: epithelium, CT: connective tissue, M: muscles, K: keratin, Fg: fungiform papilla, Fl: filiform papilla, Arrowhead: taste buds, D: defect, Arrow: granulation tissue, Asterisk: inflammatory infiltrate, V: blood vessel, L: lateral wound margins.

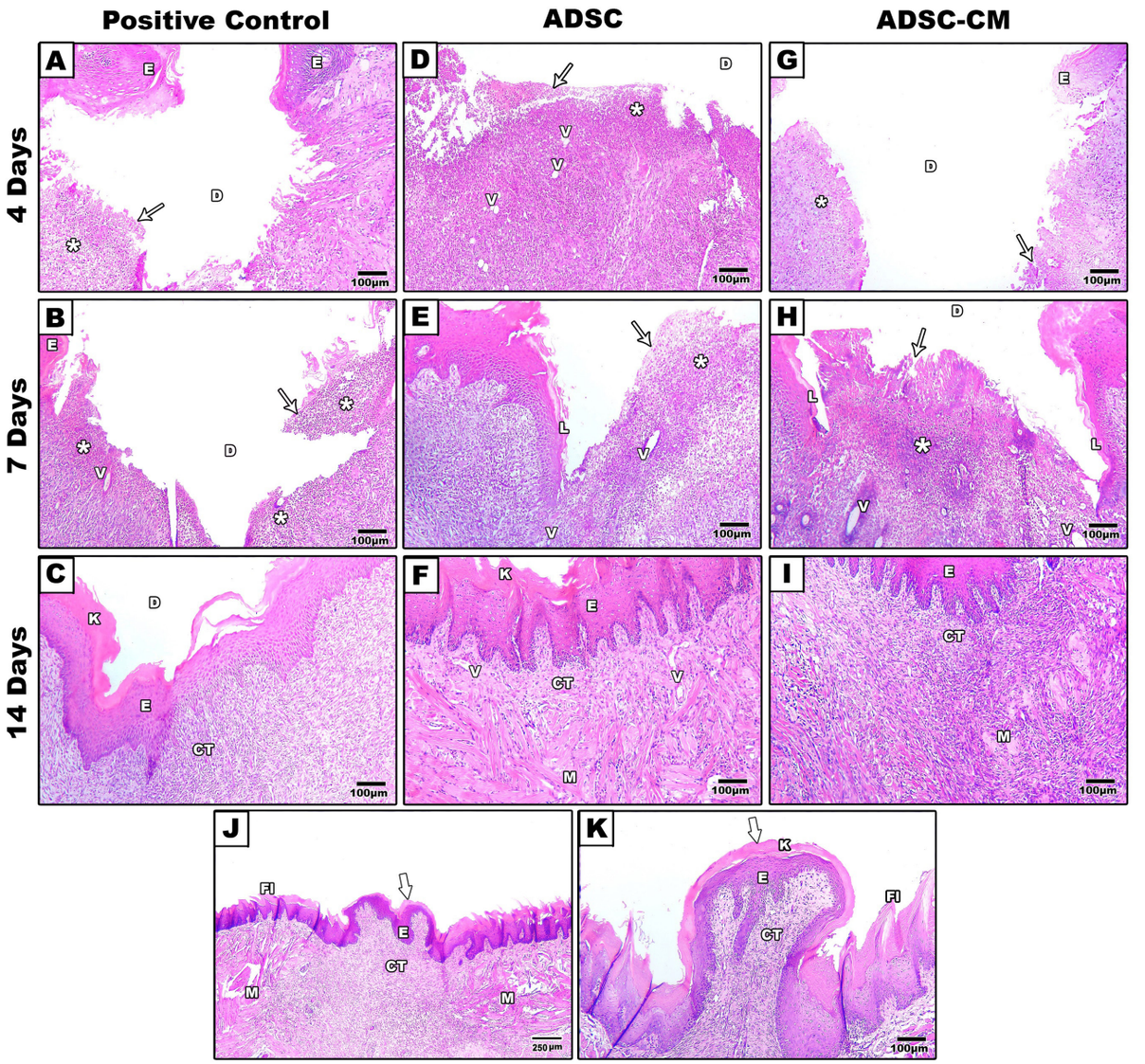

Fig. 4: A-I: Hematoxylin and Eosin staining of tongue specimens of the positive control, ADSC-treated and ADSC-CM treated groups at 4,7 and 14 days (10x). **J,K:** Hematoxylin and Eosin staining of the mucosal overgrowths. E: epithelium, CT: connective tissue, M: muscles, K: keratin, Fl: filiform papilla, D: defect, Arrow: granulation tissue, Asterisk: inflammatory infiltrate, V: blood vessel, L: lateral wound margins, Thick arrow: exophytic outgrowth.

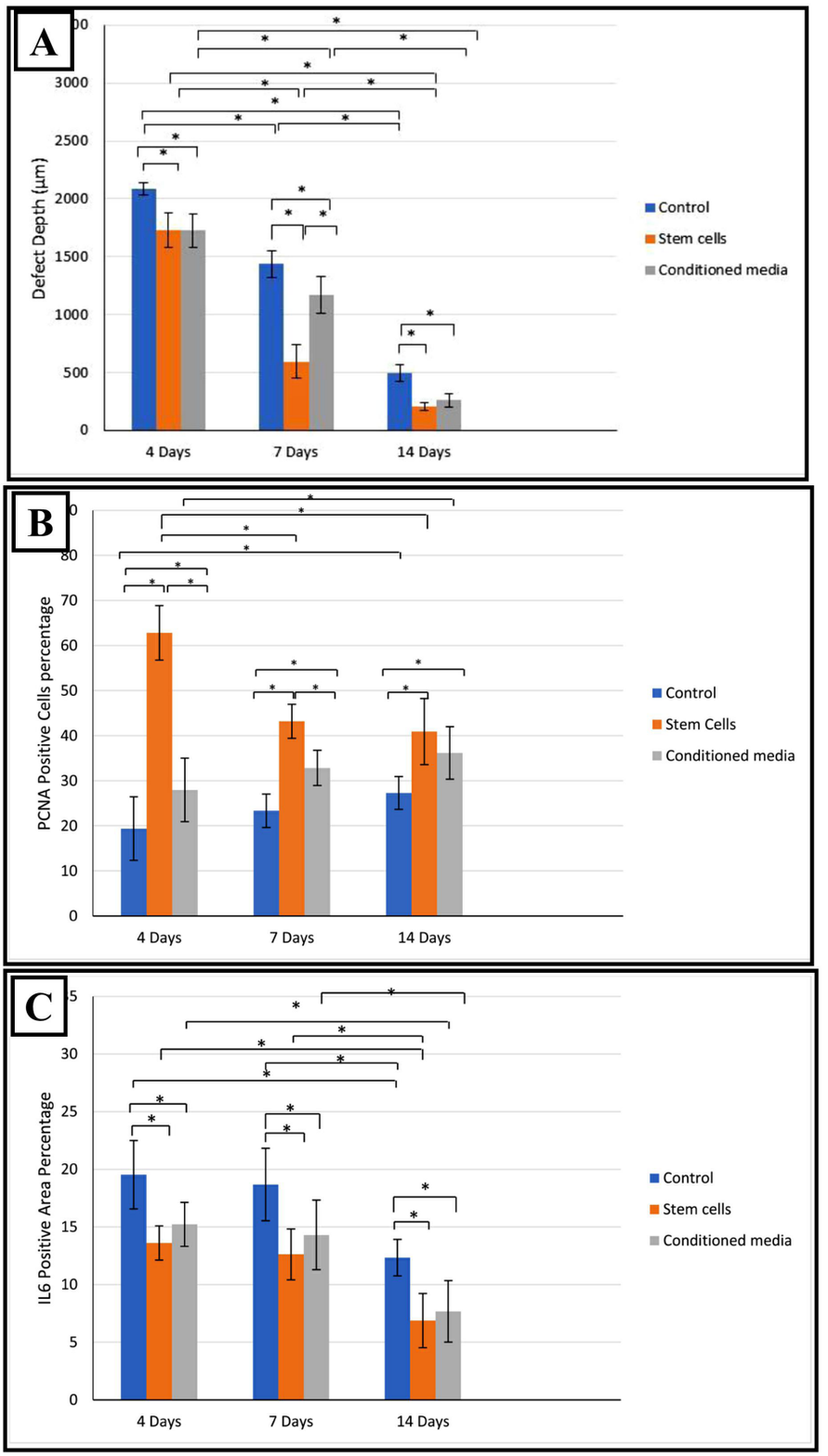

Fig. 5: Bar graphs showing the statistical analysis for: A. Defect depth, B. anti-PCNA immunostaining and C. anti-IL-6 immunostaining. * Denotes statistical significance

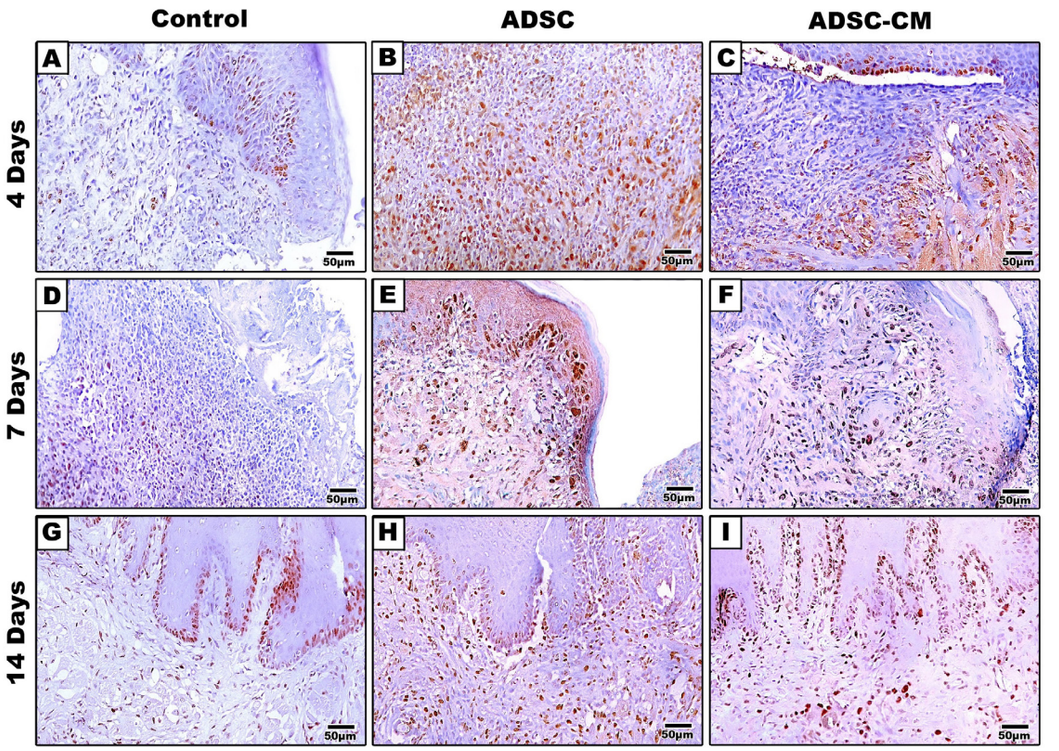

Fig. 6: Immunostaining for PCNA antibody

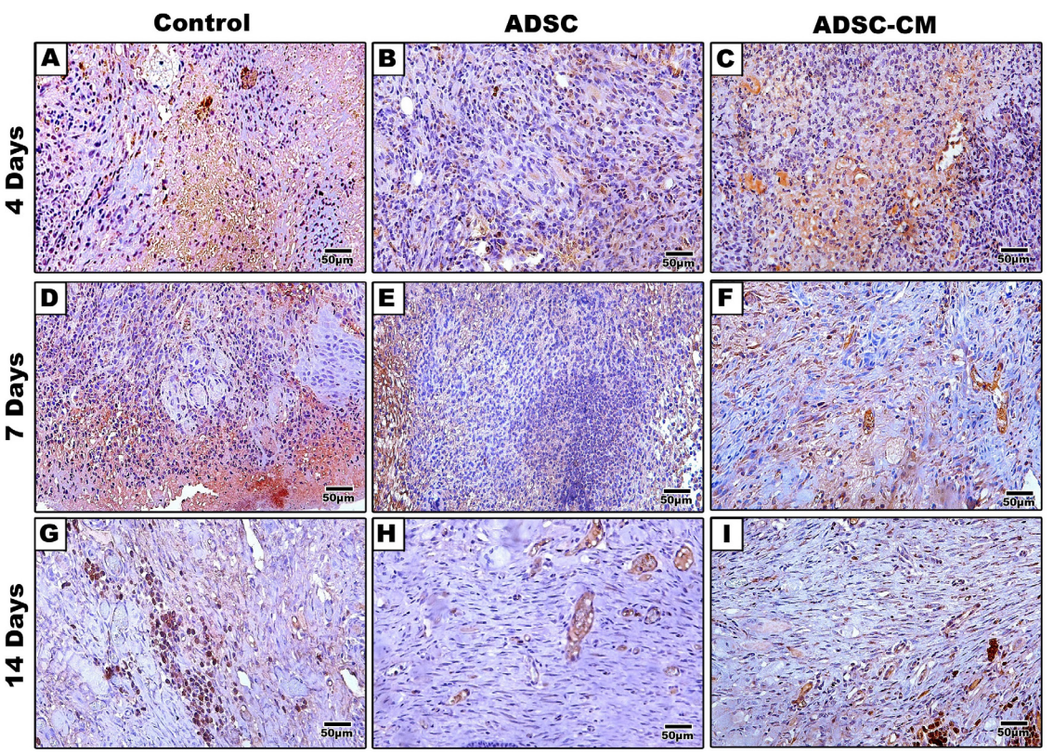

Fig. 7: Immunostaining for IL-6 antibody

Table 1: Post Hoc Tukey test for pairwise comparison of factors affecting defect depth.

Time \ Group	Control	Stem cells	Conditioned Media
4	2085.0±52.69	1731.16±150.27*	1726.5±147.21*
7	1436.83±117.93^{A}	593.33±146.06^{A*}	1166±159.71^{A*+}
14	491.83±72.51^{AB}	207.50±35.21^{AB*}	260.17±55.61^{AB*}

^{A} Significance vs 4 days group, and ^{B} significance vs 7 days group within the same intervention. * Significance vs control group, and $^{+}$ significance vs stem cells group, within the same time point.

Table 2: Post Hoc Tukey test for pairwise comparison of factors affecting PCNA positive cells percentage

Time \ Group	Control	Stem cells	Conditioned Media
4	19.37±7.05	62.80±6.04*	27.97±7.04^{*+}
7	23.32±3.71	43.18±3.76^{A*}	32.82±3.89^{*+}
14	27.27±3.65^{A}	40.90±7.34^{A*}	36.14±5.83^{A*}

^{A} Significance vs 4 days group, and ^{B} significance vs 7 days group within the same intervention. * Significance vs control group, and $^{+}$ significance vs stem cells group, within the same time point.

Table 3: Post Hoc Tukey test for pairwise comparison of factors affecting IL-6 positive area percentage

Time \ Group	Control	Stem cells	Conditioned Media
4	19.52±2.97	13.61±1.49*	15.22±1.91*
7	18.68±3.15	12.62±2.21*	14.31±3.02*
14	12.34±1.58^{AB}	6.88±2.35^{AB*}	7.67±2.67^{AB*}

^{A} Significance vs 4 days group, and ^{B} significance vs 7 days group within the same intervention. * Significance vs control group, and $^{+}$ significance vs stem cells group, within the same time point.

DISCUSSION

Wound healing in the oral cavity represents a challenge due to the bacterial-laden oral environment, and the continuous physical insult within the oral cavity making chronic oral wounds common. Different regenerative approaches have been employed to enhance the re-epithelization and extracellular matrix remodeling of oral tissues during the wound healing process[20]. This study was conducted to compare the regenerative effect of ADSCs versus their conditioned media on the tongue oral wound model in rats.

A circular wound model of 5 mm diameter and 2 mm depth was done using a tissue punch rotary drill to ensure that all defects were of identical sizes and that complete, spontaneous healing will not occur till the end of the experiment which was evidenced by the clinical and histological results.

Wound healing in different tissue types follows four main overlapping stages which are hemostasis, inflammation, proliferation, and maturation[20]. Dense inflammatory infiltrate was observed at the margins and bases of the wounds at days 4 and 7 after surgery. This conforms to the fact that the inflammatory phase begins as early as 24 or 48 hours and can last up to a week after injury where inflammatory cells are recruited as a result of the chemokine secretion at the injury site[21].

In all groups in our study, granulation tissue started to form in the wound site at 4 days post-surgery with variable degrees in different groups and became more arranged and vascularized at 7 days. Wound reepithelization was observed after 7 days of surgery beginning at the lateral margins of the defects. This agrees with des Jardins-Park *et al*.[21], who clarified that during the proliferative stage of healing, that takes about 3 weeks in response to the released signaling molecules, the extracellular matrix is replaced by a vascular stroma that leads to granulation tissue formation and reepithelization starts at the wound edges.

The maturation and remodeling stage was observed in this study by day 14, where granulation tissue was replaced with organized oral mucosa to variable extents in different groups. Different studies as well, reported that the maturation stage begins after day 8 of injury and may extend up to a year[22,23].

In the current study, the control defects showed some inflammation. The depth of the wound decreased gradually and by day 14, a thin epithelial covering was restored but without complete lingual papillae or tongue musculature regeneration. In coincidence with our results, Xu *et al*.[24] did not observe gross epithelization of the wounds of mechanical rat tongue defects by day 5 in all groups. However, they detected some re-epithelization in the control defects after 14 days, but tongue musculature was not restored. Similarly in another study by Faruk *et al*.[25], the control defects of chemically induced tongue ulcers showed inflammatory infiltrate and dilated blood vessels but didn't show complete healing or epithelization after 14 days.

Tongue wounds of group II (ADSC- treated) in this study showed better healing through the experimental period with well-organized granulation tissue filling the defects at the beginning and almost normal keratinized epithelium with regular papillae and regenerating underlying muscle bundles after 14 days. According to Aly *et al*.[26], ADSC can be applied for oral ulcer healing as an efficient, available, and accessible alternative to BMSC. In a study by Lee *et al*.[27], ADSC sheets aided in regeneration of buccal and gingival chemically induced oral wounds showing normal mucosa before day 5 and complete epithelial healing by day 7 with significant decrease in ulceration area. This fast-healing rate may be attributed to the different wound induction model where chemically induced ulcers may be more superficial than mechanically induced ones. Also, the mucosal covering of tongue's dorsal surface is a unique structure different than any other oral mucosa with more complex structure including an extensible masticatory mucosa covered with different lingual papillae with taste buds and overlying a dense muscular system[28] that may require more time for healing.

In another study, gingival derived mesenchymal stem cells (GMSC) loaded on small intestinal submucosa extracellular matrix (SIS-ECM) constructs enhanced the healing of mechanical rat tongue dorsum defects where by day 14 post-surgery, defects in the experimental groups showed better clinical healing and less wound contraction than the untreated group. However, the GMSC/SIS-ECM group demonstrated better reepithelization and muscle regeneration histologically than the SIS-ECM constructs alone indicating the regenerative potential of MSC[24]. According to Ayavoo *et al*.[29], stem cells can enhance the wound healing process via different mechanisms including cell recruitment to the site of injury, secretion of different signaling molecules as well as direct trans-differentiation into other cell types.

In group III (ADSC-CM) in the current study, tongue wounds showed reduced depth with full reepithelization, but lingual papillae and tongue musculature where not fully regenerated as in group II (ADSC). In a study by Gao *et al*.[30], ADSC-CM ameliorated the recovery of rat full-thickness skin wounds and after 11 days of injury, the wounds were almost healed with normal epidermal thickness. ADSC-derived conditioned media was found to express different growth factors that can assist wound healing and cell regeneration such as TGF-b1, TGF-b2, VEGF-2, FGF, Vascular Cell Adhesion Molecule (VCAM)-1, and Epidermal Growth Factor (EGF)[31].

Histological examination of some specimens showed the presence of small exophytic masses at the defect sites. According to Allon *et al*.[32], such lesions may be caused by the irritation resulting from local trauma as that caused by mechanical disruption of the tongue tissues in the current study.

In the present study cell proliferation was quantified using PCNA immunostaining where PCNA is considered one of the most reliable immunohistochemical markers for cell proliferation[33]. The statistical analysis showed higher proliferation index for the ADSC treated group indicating earlier and more rapid regenerative effects however, after 14 days of injury, no significance was detected between the ADSC and the ADSC-CM treated groups. Similarly, in another study gingival derived stem cells were injected into chemotherapy induced tongue mucositis mouse model and PCNA immunostaining showed higher proliferation for the stem cell treated groups relative to the control group[34]. In the same context, in a study by Rashed *et al*.[14], circular, 5 mm, mechanically induced cheek ulcers in rats were injected circumferentially with bone marrow stem cells (BMSC) and PCNA immunostaining results showed high proliferation rates for the experimental group when contrasted to the control group.

In this study, the inflammatory response of the injured tissues was assessed by IL-6 antibody immunostaining as IL-6 is one of the cytokines that are involved in immune responses during inflammation and is commonly used as an inflammatory marker[35,36]. Our results showed decreased inflammatory reaction in the ADSC and ADSC-CM treated groups as compared to the control group. Similarly in a research conducted by Gao *et al*.[30], ADSC conditioned media were used to treat rat skin wounds and the histological inflammatory infiltrate was found to decrease in the treated groups at days 3, 9 and 11 after injury. In another study a herbal extract was used to treat tongue ulcers and interleukin-2 (IL-2) and tumor necrosis factor (TNF) were used to evaluate the inflammatory reaction where the treated groups showed reduced inflammatory response than the control group[37]. In the same context, Camacho-Alonso *et al*.[38] detected gradual decrease of inflammation through the study period after induction of mechanical dorsal tongue ulcers as well as less inflammation related to the treated groups when compared to the control one.

As per our study, ADSC as well as ADSC-CM enhanced the healing of the induced tongue defects when compared to the control defects. However, ADSC group showed better histological regeneration of the tongue tissues. Similarly in a study of acute renal injury rat model conducted by Gabr et el.[39], BMSC showed histological regeneration of the kidneys while BMSC-derived CM didn't show significant results when injected intravenously. On the contrary, in another study on the healing effect of BMSC and BMSC-CM on induced liver fibrosis in rats, Abdel Aal *et al*.[40] detected better effect of CM.

This relatively higher regenerative effect of ADSC than their derived CM may be attributed to the preparation methodology of CM, where the collected ADSC culture media were filtrated through a 0.2 µm filter, while stem cells secrete a variety of heterogenous extracellular vesicles populations (EVs) that play role in intercellular communication and tissue repair and vary in size from 30-10000 nm in diameter[41] which is a larger size range than the applied filters. Also, during CM preparation, the collected media were subjected to centrifugation and according to

Baskoro *et al*.[42], different speeds of centrifugation affects the protein profile emergence and expression. This may have affected the protein content of the ADSC media.

CONCLUSION

Adipose derived stem cells and their conditioned media can improve tongue defects regeneration through enhancing cellular proliferation and suppressing inflammation and according to the present model, ADSC showed earlier and more rapid regenerative effects than the conditioned media.

CONFLICT OF INTERESTS

There are no conflicts interest.

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الملخص العربى

الإمكانات التجديدية للخلايا الجذعية المشتقة من الدهون مقابل وسائطها المغذية على عيوب اللسان لدى ذكور الفئران البالغة

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مقدمة: اللسان هو عضو استراتيجي في تجويف الفم يتأثر عادة بأمراض وأورام خبيثة مختلفة ، والتي قد تتطلب استئصالا جراحيا يترك عيوبا مقاومة للعلاج. الخلايا الجذعية المشتقة من الانسجة الدهنية (ADSC) هي مصدر متاح، وفير، وسهل الوصول إليه للحصول على الخلايا الجذعية الوسيطة التي لديها قدرة عالية للتمايز وتفرز مجموعة كبيرة ومتنوعة من عوامل النمو والسيتوكينات. تحتوي الوسائط المغذية المستخلصة من الخلايا (CM) على جميع المكونات النشطة بيولوجيا التي تفرزها الخلايا المستزرعة مما يوفر علاجا آمنا وخاليا من الخلايا.

أهداف البحث: أجريت هذه الدراسة لتقييم القدرة التجديدية للخلايا الجذعية المشتقة من الانسجة الدهنية ووسائطها المغذية على عيوب اللسان المستحثة ميكانيكيا.

المواد والطرق: تعرضت ذكور فئران سبراغ داولي لعيوب دائرية ميكانيكية على ظهر اللسان ثم تم تقسيمها لثلاث مجموعات (n = ١٥): المجموعة الأولى (الضابطة) ، المجموعة الثانية (معالجة ADSC) ، والمجموعة الثالثة (ADSC-CM المعالجة). في الأيام الرابع والسابع والرابع عشر بعد التحضير ، تم القتل الرحيم لخمسة فئران من كل مجموعة وخضعت العينات للفحص السريري والنسيجي والنسيجي الكيميائي المناعي متبوعا بالتحليل الإحصائي.

النتائج: في كل من المجموعات المعالجة ب ADSC و ADSC-CM ، أظهرت عيوب اللسان تقلص في عمق العيوب ، وتجديدا نسيجيا أفضل بما في ذلك الطبقات الظهارية والنسيج الضام وعضلات اللسان ، بالإضافة إلى تعزيز الانتشار الخلوي ، والتهاب أقل مقارنة بالمجموعة الضابطة غير المعالجة. أظهرت المجموعة المعالجة ب ADSC شفاء مبكرا عن المجموعة المعالجة ب ADSC-CM ، لكن النتائج لم تعبر عن دلالة إحصائية.

الاستنتاجات: يمكن أن تكون الخلايا الجذعية المشتقة من الانسجة الدهنية (ADSC) و وسائطها المغذية (ADSC-CM) مرشحين جيدين لعلاج عيوب اللسان.