

Healing Capacity of Gingival-Derived Stem Cells' Conditioned Media and LASER Photobiostimulation on Albino Rat's Induced Tongue Defect

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Research Article

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Abstract

Objective

The tongue is often affected by trauma, ulceration, and neoplasms requiring surgical removal, leading to difficult-to-heal defects. Novel cell-free treatments have shown significant regenerative potential. Gingiva-derived mesenchymal stem cells conditioned medium (GMSCs-CM) contains trophic factors with anti-inflammatory, immunomodulatory and angiogenic properties. Low-level LASER treatment (LLLT) is recognized for its anti-inflammatory, and healing-enhancing effects. This study aimed to explore the individual and combined healing effects of GMSCs-CM and low-level LASER therapy on tongue defects in rats.

Design:

Eighty male Sprague Dawley rats were randomly allocated to five groups: control negative, control positive, GMSCs-CM treated, LASER treated, and combined GMSCs-CM + LASER treated. Standardized circular tongue defects were created and animals were euthanized at 1 and 2 weeks post-surgery. Specimens were subjected to histological, histomorphometric analysis for epithelial thickness and defect depth in addition to ELISA detection of TNF- α and IL-1 β expression. One-way ANOVA and Tukey's test were used for statistical analysis.

Results

All treated groups exhibited enhanced re-epithelialization, angiogenesis, connective tissue remodeling, and a reduction in defect depth compared to untreated controls. GMSCs-CM enhanced vascularization and reduced inflammatory infiltrates; however, LASER therapy enhanced muscle regeneration and epithelial thickness. Defect depth was significantly reduced in the combination group, nearly normal histological architecture, and the lowest levels of TNF- α and IL-1 β ($p = 0.001$).

Conclusions

The combined use of GMSCs-CM and LLLT markedly improves tongue wound healing relative to individual therapies, demonstrating its promise as an effective, minimally invasive cell-free regenerative strategy for oral soft tissue regeneration.

Introduction

Tongue is a sophisticated muscular organ used for communication, swallowing, and mastication. It is characterized by specialized dense skeletal muscle fibers, considerable vascularization, and constant mobility. These attributes make the effective structural and functional regeneration more difficult following injury. Delayed re-epithelialization, fibrosis, and restricted muscle remodeling might hinder functional recovery [1]. A number of illnesses and cancers, such as erythroplakia, squamous cell carcinoma, granular cell tumor, and Kaposi sarcoma, might harm the tongue and require the damaged part to be surgically removed [2]. Oral ulcers, defined as loss of connective tissue and the overlying epithelium, can affect the tongue for several reasons [3]. Early care is necessary because ulcerated oral tissue is prone to infection and delayed healing due to its continuous contact with oral microbial flora [4]. Several potential causes of oral ulcers affect the tongue. They can be classified as acute or chronic based on their course and characteristics [5]. The social, physical, and mental well-being of patients is adversely affected by these lesions. Therefore, treating these

oral conditions is essential to improve patients' quality of life [6]. Antibiotics, analgesics [7], anti-inflammatory drugs [8], angiogenic factors [9], herbal remedies [10], and specialized regional treatments, such as: chemical cauterization, surgical excision, and LASER therapy, can all be utilized to treat oral and maxillofacial disorders. These therapies, however, might not provide much of an improvement or might even have a number of unanticipated negative effects [11]. There are great expectations for the regeneration of surgically removed tissues due to the novel developments in tissue engineering and stem cell-based techniques [12].

Almost all adult tissues contain mesenchymal stem cells (MSCs), a subset of multipotent stromal cells with strong immunomodulatory and anti-inflammatory properties, the potency to differentiate into multiple cell lineages, and the capacity to evade allogeneic immune responses [13, 14]. Many researches indicated that both endogenous and exogenous MSCs have the ability to sense their microenvironment and migrate to damaged areas, where they collaborate with other immune and structural cell types to coordinate the complex process of site-specific tissue healing [13, 15]. Because of these special characteristics, MSCs are a special source of potential cells for stem cell-based therapy across a variety of therapeutic settings, including tissue engineering and organ and tissue regeneration [13, 15, 16]. With their potent self-renewal, multilineage differentiation, and immunomodulatory abilities, gingival-derived mesenchymal stem cells are anticipated to be applied in tissue regeneration. They have emerged as a promising tool in regenerative medicine due to their significant paracrine activity. They release a variety of growth factors and cytokines that can modulate inflammation, promote angiogenesis, and enhance epithelial and connective tissue regeneration. Gingival-derived MSCs offers several advantages when compared to other stem cell sources, including ease of isolation, less invasive harvesting, rapid proliferation potential, immunomodulatory characteristics, and diminished ethical issues [17]. It has been determined that Conditioned Medium (CM) derived from several mesenchymal stem cell sources benefits the receiver by having anti-inflammatory, anti-scarring, and immunomodulatory features. The study of tissue regeneration has made extensive utilize of Mesenchymal Stem Cells Conditioned Medium (MSC-CM) in recent years [18]. MSC-CM has been shown to improve regeneration in numerous animal wound models and to exhibit cell growth-supporting properties, and immunomodulating, angiogenesis-promoting in several studies [19].

In normal dental practice, Light Amplification by Stimulated Emission of Radiation (LASER) therapy has emerged as one of the most effective forms of treatment. latterly, a focal lens was utilized to focus the diode beam in the Low-Level LASER Therapy (LLLT) setting. It has been evaluated for its biostimulatory characteristics, including enhanced cellular metabolism, increased ATP synthesis, improved microcirculation, and apparent anti-inflammatory effect that reduces edema and postoperative discomfort, encourages tissue healing, and permits good tissue penetration while respecting the tissues and preventing excessive overheating through the photobiostimulation effect, these effects contribute to creating a favorable microenvironment for tissue repair [20]. Photobiostimulation is induced by low-level LASER therapy, also known as cold or soft LASER therapy. The word "LASER" describes the use of coherent, monochromatic light to treat wounds and promote the healing of various lesions. The methods via which the energy from therapeutic LASER and light-emitting diodes (LEDs) functions at the cellular and organismic levels remain unknown. The therapeutic window for LLLT is around 600–1400 nm, which is in close proximity to the hemoglobin and water absorption spectrums [21]. Therefore, the combination of GMSCs-CM and LLLT may provide a combined therapeutic approach that enhances wound healing and accelerates the regeneration of tongue tissue.

The purpose of this study was to assess the impact of low-level laser therapy and medium conditioned with gingiva-derived mesenchymal stem cells on the healing of tongue wounds. The null hypothesis stated that there would be no discernible variations in the inflammatory response and histological healing between the experimental groups.

Materials and methods

Ethical statement:

In accordance with the biosecurity and biosafety protocols outlined by Alderman et al., the animal experimental procedures were completed in compliance with the recommendations made by the Mansoura University Animal Care and Use Committee. (MU-ACUC) (ID: DENT.R.25.04.23) [22]. Eighty male Sprague Dawley rats that were free of pathogens and weighed between 250 and 300 grams were obtained from the Animal House, Faculty of Medicine, Mansoura University, Egypt. The animals were housed in coops of five rats each in a room with a 12-hour light-dark cycle at the Medical Experimental Research Center (MERC) (Mansoura University, Mansoura, Egypt), in accordance with standard housing protocols. A steady temperature of 22 °C and a relative moisture content of 65–70% were maintained. The animals were housed for at least two weeks prior to the start of the trial and were given access to commercially available food and drink as they needed.

Study design:

This study was a randomized controlled experiment. The sample size was determined a priori using G*Power software (version 3.1, Heinrich-Heine University Düsseldorf, Germany). An effect size ($f = 0.40$) was assumed based on effect sizes published in previous studies on rat tongue wound healing models [23]. The minimum required sample size for a one-way ANOVA, with an α error probability of 0.05 and a study power of 80%, was estimated to be 16 rats per group. Sixteen rats were added to each experimental group. All histological, histomorphometric evaluations, ELISA, immunohistochemical, image analysis and statistical analyses were conducted by two blinded investigators to ensure an unbiased assessment. All animals were subjected to mechanical tongue defect induction except 16 rats of control negative group and then randomly allocated following a simple random sampling strategy into four groups as follows: **Group I (Positive control)**: Tongue defects was injected by 0.1 ml phosphate-buffered saline (PBS). **Group II (GMSCs-CM)**: Conditioned medium (0.1 ml) was injected at three equidistant locations around the incision borders, penetrating roughly 2–3 mm into the muscle tissue. **Group III (LASER treated)**: Tongue defects received irradiation every 48 h for 7 days. **Group IV (combination)** (CM+ LASER treated): Tongue defects received 0.1 ml GMSC-CM circumferentially injected around the wound margins as a single injection directly after defect preparation and irradiation every 48 h for 7 days. On the seventh and fourteenth days following defect preparation, eight rats from each group were euthanized by intraperitoneal injection of ketamine (80 mg/kg) and xylazine (10 mg/kg) to guaranty profound deep anesthesia prior to sample collection and surgical steps. Euthanasia was then carried out by cervical dislocation while the animals were completely unconscious. Tongue specimens were removed for histological and immunohistochemical analysis and for ELISA to measure TNF α and IL-1 expression. Additional 4 rats were used for extracting gingival stem cells.

Surgical procedures:

Animals were weighed before any operations to modify the dosages of drugs. Ketamine hydrochloride (35–45 mg/kg) and xylazine hydrochloride (5–7 mg/kg) were injected intramuscularly into rats to induce anesthesia. After the rats' tongues were cleaned with betadine, a biopsy punch tool was used to create defects in the midline of the middle thirds of the tongues' dorsum that were 2 mm deep and 5 mm in diameter [24] (**FIG.1**). The surgical scissors were used to cut the bases of the defects. Treatments were injected around the edges of the wounds as soon as the defect was prepared. The rats were given 20% oxytetracycline hydrochloride and 0.5 mg of analgin for three days as postoperative care.

GMSCs Isolation:

Zhang's method for extracting gingival stem cells was used. After four pathogen-free Sprague Dawley rats weighing 80–150 grams were euthanized by inhaling an excessive amount of halothane, tissue samples were collected from their clinically healthy gingiva [25]. The samples were randomly incubated and treated with dispase (2 mg/mL, Sigma-Aldrich (St. Louis, MO, USA) for an entire night at 4 °C in order to separate the epithelium from the lower spinous layers. After cutting the remaining connective tissues into segments measuring 1–3 mm², they were digested for 2 hours at 37

°C in sterile phosphate-buffered saline (PBS) (Sigma-Aldrich, Steinheim, Germany) containing 4 mg/ml Worthington Biochemical collagenase IV. Following that, the samples were cultured in (DMEM-F 12), a medium that contained 1% streptomycin, penicillin, and amphotericin along with 10% (FBS) (Sigma Aldrich, Steinheim, Germany). The explants were humidified with 5% CO₂ and incubated at 37°C. Cells were trypsinized and subcultured once confluence reached 80% between days 14 and 21 [26].

Characterization of GMSCs:

A BD Accuri C6 flow cytometer (BD Biosciences, California, USA) and software were utilized for flowcytometric immunophenotype identification in order to characterize GMSCs. Trypsin treatment was applied to 3rd passage cells, which were then washed with PBS and incubated with the primary antibodies: BD Biosciences provided purified versions of anti-CD90 (cat. #554897, California, USA), anti-CD44 (cat. #553134, California, USA), anti-CD45 FITC MAB (cat. #553772, California, USA), and anti-CD34 (cat. #560238, California, USA). Each purified antibody was mixed with fluorescein isothiocyanate fluorophores (FITC, cat. #F143, Thermo Fisher Scientific, Massachusetts, USA) and incubated for 30 minutes at 4 °C in the dark. The tagged cells were then rinsed with PBS, centrifuged at 200×g for 5 minutes, and suspended in PBS [27].

GMSCs Conditioned media preparation:

GMSCs were cultured in complete medium containing 10% FBS until they reached 80% confluency. The cells were then cultured for an additional 48 hours at 37 °C with 5% CO₂ after the medium was changed to serum-free α-MEM. To extract CM, the GMSC supernatants were collected, centrifuged for 5 minutes, and then filtered through 0.22-µm filters. The obtained CM were then concentrated 100 times using ultrafiltration centrifuge pipes (Ultra-15 10 kD centrifugal filter, EMD Millipore, Billerica, MA, USA) at 5000g and 4 °C for 40 minutes. The conditioned media were standardized by culturing gingival mesenchymal stem cells at the same seeding density under similar conditions. Upon reaching the required confluency, cells were cultured in serum-free media for a fixed duration. Furthermore, the CM volume was normalized compared to the cell count to ensure standardization of all samples. The grades of platelet-derived growth factor (PDGF) and interleukin 10 (IL-10) in the CM were then measured using the ELISA technique. Concentrated CM was kept at -80 °C after being subpackaged [18,19].

ELISA assay of GMSCs-CM:

IL-10 and PDGF concentrations were measured by ELISA using pooled GMSC-CM, according to the producer's guidelines. A quantitative enzyme immunoassay technique was utilized with specific antibodies targeting PDGF or IL-10 proteins. The immobilized antibody bound to any target protein that was present after standards and samples were pipetted into the wells. A biotin-conjugated antibody specific for either PDGF (LS Bio, catalog No. Ls-F27550) or IL-10 (Rat Interleukin ELISA kit, MyBiosource, Cat. No. MBS269138) was added to the wells after any unbound materials had been eliminated. Following washing, the wells were supplemented with avidin-conjugated horseradish peroxidase. After washing the wells to remove any unbound avidin-enzyme reagent, a substrate solution was inserted, and the amount of protein bound in the first stage was reflected in the color that formed. A stop solution was inserted to halt the color development, and a microplate reader that could measure absorbance at 450 nm with the correction wavelength set at 560–630 nm was used to measure the color's intensity. PDGF and IL-10 quantitative concentration results were plotted, and the results were compared to a standard curve. After the addition of a substrate solution, the signal was revealed and was proportional to the target's concentration. PDGF had a detection range of 100–2500 pg/mL, while IL-10 had a range of 15.6 pg/mL. Rat PDGF-AB usually had a minimum detectable dose of less than 1 pg/mL, while IL10 usually had a minimum detectable level of up to 5 pg/mL. The sensitivity of this examination, or the lower detection limit, was realized as the lowest protein concentration that could be differentiated from zero [28].

Low-Level Laser Therapy protocol:

Low-Level Laser Therapy was done using a 976 nm diode laser device (Solase-976 Dental Diode Laser, Lazon Medical Laser Co., Ltd., China) that works with an InGaAs semiconductor active medium. Irradiation was administered in constant mode at a power output of 0.2 W for 40 seconds per session, providing a total energy delivery of 8 J. Using a 30 mm diameter tip, this equaled to an energy density of around 1.13 J/cm² and a power density of 0.03 W/cm². Laser treatment was applied every 48 hours for 7 days.

Determining the tissue levels of TNF- α & IL-1 β using the ELISA test:

An ELISA was used to quantify TNF- α (pg/mL) and IL-1 β (pg/mL), markers of inflammation and immune system activation. To get rid of any leftover blood, tongue tissues were cleansed by ice-cold phosphate-buffered saline, as directed by the manufacturer. Butylated hydroxytoluene and a proteolysis inhibitor were inserted to the samples to prevent proteolysis and oxidation. After homogenization, the samples were stored in 20 milliliters of 1x phosphate-buffered saline at -20 degrees Celsius overnight. To break the cell membranes and strip any particulate components, the homogenate was centrifuged for 5 minutes at 5,000 x g after 2 freeze-thaw cycles. The supernatant was stored at -20 degrees Celsius after separation. Utilizing a Ray to Stat Fax 3200 microplate reader, and Rat IL-1 β ELISA Kit, Catalogue Code: CSB-E08055r, Sensitivity: less than 15.6 pg/mL, Detection range: 62.5 pg/mL-4000 pg/mL and TNF- α (Catalogue Code: CSB-E11987r, Rat TNF- α , Detection range: 6.25 pg/mL-400 pg/mL, Sensitivity: less than 1.56 pg/mL). ELISA test kits were supplied by CUSABIO[®]. The obtained samples were stocked at -80 °C and then allowed to come to room temperature before being utilized. As instructed by each kit's manufacturer, tissue samples with volumes ranging from 40 to 100 μ l per well were added to the microplate's experimental wells. The experimental and control wells were then filled with antibody combination. The microplates were blocked with an adhesive and allowed to stand at room temperature for two hours before being shaken on a microplate shaker. Three gentle washes of 350 μ l of wash buffer per well were applied on the experimental wells. The plate was agitated for an hour at room temperature in the dark after 200 μ l/well of the antibody conjugate was added. After adding 200 μ l of the substrate solution to each well, the shaker plate was incubated for 30 minutes. Each well was turned yellow when 50 microliters of stop solution were added for 30 minutes, and the absorbance at 450 nm was measured [29].

Histological evaluation:

Following neutral-buffered formalin fixation (Sigma-Aldrich (St. Louis, MO, USA)), tongue specimens were prepared in accordance with our earlier methodology, processed into paraffin blocks, and cut into 4 μ m sections. Hematoxylin and eosin (H&E) (Sigma-Aldrich (St. Louis, MO, USA)) staining was applied after serial sections were deparaffinized, rehydrated, and produced using a microtome [30]. Endogenous peroxidase blocking was carried out with H₂O₂ for immunohistochemical staining, and antigens were recovered by boiling in citrate buffer. After that, the slides were treated with the primary interleukin-6 antibody (IL-6 polyclonal antibody, cat. #A0286, ABclonal, Massachusetts, USA), followed by the streptavidin-biotin complex and a secondary biotinylated antibody. After applying the diaminobenzidine (DAB) chromogen (DAB kit, cat. #ab64238, Abcam, Cambridge, UK), Harris hematoxylin counterstaining was performed [31].

Histomorphometric Evaluation

A quantitative morphometric assessment was conducted to objectively measure epithelium thickness and defect depth. Digital photomicrographs were obtained at standardized magnification and calibrated with the integrated scale bars.

Epithelial thickness was measured from high-magnification images calibrated with a 100 μ m scale bar. In contrast, defect depth was evaluated from low-magnification images calibrated with a 250 μ m scale bar to ensure complete visualization of the defect margins.

For each specimen, six equidistant linear measurements were acquired at predefined anatomical landmarks using image analysis software (ImageJ, National Institutes of Health, USA). The mean value per specimen was calculated and presented as mean $\pm$ standard deviation (SD) for each experimental group.

Digital Image Analysis:

IL-6-stained slide photos captured by the TouPCam® digital camera (XCAM1080PHA) were subjected to digital image analysis. The camera was connected to A light microscope (Olympus®, CX22, Japan) with a 0.5 photo adapter and a 4x objective lens. The area of positive brown staining was divided by the total area to determine the percentage. Regardless of stain strength, brown staining was taken into consideration. The data was displayed as the proportion of positive cells or the mean area percentage $\pm$ standard deviation.

Statistical analysis and data interpretation:

Data analysis was carried out by SPSS software, version 26 (SPSS Inc., PASW Statistics for Windows version 26. Chicago: SPSS Inc.). Qualitative data were characterized by numbers and percentages. Quantitative data were characterized by mean $\pm$ standard deviation for normally distributed data, after testing normality using the Shapiro-Wilk test. Significance of the obtained results was judged at the 0.05 level. A one-way ANOVA was performed to compare more than 2 independent groups, with the Post Hoc Tukey test used to detect pairwise comparisons.

Results

Morphology of the cultured GMSCs:

By using adherent separation, GMSCs were successfully separated. As shown in **FIG. 2**, the inverted phase-contrast microscope revealed a number of rounded, non-adherent cells on the first day of isolation. A few fibroblast-like cells attached to the flask during the first culture on the sixth day after isolation. The adherent cells achieved 80% confluence in 21 days.

Phenotypic characterization using flow cytometry:

Cell surface phenotypic marker examination of the third passage GMSCs revealed that the hematopoietic markers CD45 (8.1%) and CD34 (6.2%) were negative. In contrast, the mesenchymal markers CD90 (91.3%) and CD44 (96.3%) were significantly positive, as shown in **FIG. 3**. These findings demonstrate that the culture did not contain any hematopoietic cells and that the cells were unequivocally of ectomesenchymal origin.

ELISA assay results of GMSCs-CM:

In the pooled GMSCs-CM, PDGF and IL-10 were identified. High levels of IL-10 and PDGF were detected, with respective values of 263.32 and 251.13 pg/mL and 155.25 and 141.39 pg/mL.

Tongue tissue levels of TNF- α and IL-1 β :

After one and two weeks, the control positive samples had noticeably higher levels of TNF- α and IL-1 β than the control negative group. The samples injected with GMSCs-CM had lower levels of TNF- α and IL-1 β than the control-positive group, but higher levels than the LASER group. Among all treated groups, the combined group has the lowest levels of TNF- α and IL-1 β (**FIG. 4**). An ANOVA test showed a significant difference among all groups ($p = 0.001$; **Table 1**).

Table (1): Comparison of IL1 β and TNF- α between different studied groups at 1 & 2 weeks.

	Control -Ve	Control +Ve	Conditioned Media	LASER	combination-	Test of significance
IL1 β (pg/mL) At 1 week (n=4)	65.69 $\pm$ 4.47 ^A	756.08 $\pm$ 7.64 ^B	500.49 $\pm$ 6.11 ^C	344.25 $\pm$ 8.02 ^D	217.51 $\pm$ 9.66 ^E	F=8347 P=0.001*
IL1β (pg/mL) at 2 weeks (n=4)	65.69 $\pm$ 4.47 ^A	667.44 $\pm$ 14.35 ^B	459.84 $\pm$ 8.94 ^C	281.45 $\pm$ 11.56 ^D	148.47 $\pm$ 6.32 ^E	F=3847.58 P=.001*
TNF-α (pg/mL) At 1 week (n=4)	81.69 $\pm$ 0.92 ^A	954.23 $\pm$ 6.23 ^B	534.58 $\pm$ 8.82 ^C	391.62 $\pm$ 11.87 ^D	258.56 $\pm$ 9.58 ^E	F=9356.12 P=0.001*
TNF-α (pg/mL) at 2 weeks (n=4)	81.69 $\pm$ 0.92 ^A	857.04 $\pm$ 13.76 ^B	437.45 $\pm$ 5.34 ^C	313.52 $\pm$ 4.68 ^D	189.56 $\pm$ 7.01 ^E	F=9324.76 P=0.001*

F: One-Way ANOVA test, * different superscript letters within the same row indicate statistically significant differences between groups (p < 0.05)

Hematoxylin and Eosin histological staining results

The negative control slides' histological analysis showed normal tongue histology, with lingual papillae distributed in a regular pattern and normal thickness of keratinized tongue epithelium. Fungiform papillae were seen in between filiform papillae, which had a regular, conical, tapered shape. Regular connective tissue papillae interdigitating with the overlaying epithelium and well-organized tongue musculature with varying orientations were seen in the underlying connective tissue, as shown in **FIG. 5**.

After 7 days, **FIGs. 6 and 7** show that the control positive group displayed tongue abnormalities that extended into the underlying CT, as well as tongue musculature and a large number of inflammatory cells at the base and edges of the incision. There were no lingual papillae in the epithelium next to the wound. A small layer of regenerating covering epithelium emerged after 14 days. Although the defects remained deeply present, there were no indications of muscle regeneration; only newly formed blood vessels and a large number of inflammatory cells were observed at the depth and edges of the lesion.

After 7 days, defects in the GMS-CM-treated group were nearly filled with granulation tissue, new vascularization, and an infiltration of inflammatory cells. Defects were less severe after 14 days than they were after 1 week. More recently regenerated blood vessels and better-organized collagen fibers were present in the wound's granulation tissue. There was a thin layer of renewing epithelium on top. After seven days, tongue wounds in group III (the group that received laser treatment) displayed some new granulation tissue formation, fewer inflammatory cells at the base and borders of the wound, and no indications of muscle regeneration. Additionally, regenerated epithelium was observed on the surface. Full-thickness keratin-layered regenerating epithelium was shown after 14 days, and the depth of the defects had significantly decreased in comparison to the same group after 7 days. Bundles of regenerating muscles were seen (**FIGs. 6 and 7**).

After seven days, the wound in group IV (combined group) was shallower, but the surface remained lower than the normal epithelium around it. The lingual papillae were not fully recovered, but the wound surface was covered by a well-organized, regenerated epithelium with a regular keratin coating. Muscle regeneration and better-organized collagen bundles were visible on the underlying CT scan. After 14 days, the wound had fully healed and shown a normal histological picture, including an even keratin layer, normal lingual papillae, and full epithelial thickness. Collagen bundles were organized neatly in the connective tissue, and the connective tissue papillae were uniformly interdigitating with the epithelium. Newly developed muscle bundles demonstrated a notable regeneration of the tongue's musculature. At the defect site, polyp-like overgrowths of tongue mucosa were observed, with connective tissue cores covered by keratin and the entire epithelial layer, as shown in **FIGs. 6 and 7**.

At two weeks, the control positive group still shows incomplete epithelialization and persistent inflammation. In the Condition Media group, there is a delicate layer of regenerating epithelium on the surface. The LASER group shows nearly complete re-epithelialization, with fewer inflammatory cells and denser collagen. The Combination group displays fully re-epithelialized tissue with a well-developed keratin layer, mature connective tissue, an organized collagen matrix, and minimal inflammation.

At 2 weeks, the control positive and Condition Media groups exhibit further re-epithelialization with decreased inflammation and increased collagen. The LASER group shows well-formed stratified epithelium and reduced granulation tissue. Remarkably, the Combination group shows complete re-epithelialization, dense connective tissue (CT), mature vasculature (V), and normal epithelial layers.

Quantitative Histomorphometric Findings.

Defect Depth:

Quantitative analysis demonstrated statistically significant differences in defect depth across groups at both time intervals ($p < 0.001$), as shown in **Table 2**. At one week, all treated groups exhibited significant decrease in defect depth relative to the positive control, with the combination group showing the most marked decrease. At two weeks, defect depth was significantly diminished in the treatment groups, especially in the combination and LASER groups.

Epithelial Thickness:

Epithelial thickness assessments revealed statistically significant differences among groups at both one and two weeks ($p < 0.001$). The treatment groups demonstrated markedly increased epithelial thickness relative to the positive control, with the combination group showing the most pronounced regenerative response. By the second week, epithelial thickness in the combination and LASER groups approached normal histological architecture.

Table (2): Comparison of defect depth and epithelial thickness between different studied groups at 1 & 2 weeks.

Parameter		Control +ve	Conditioned Media	LASER	Combination	F-value	P-value
Defect Depth (Week)	(1	959.92 ± 132.21 ^a	470.67 ± 78.95 ^c	547.37 ± 94.00 ^b	410.00 ± 111.24 ^c	32.96	P < 0.001
Defect Depth (Weeks)	(2	958.19 ± 138.46 ^a	327.02 ± 24.61 ^b	192.32 ± 48.42 ^c	189.41 ± 44.44 ^c	133.89	P < 0.001
Epithelial Thickness (1 Week)		7.91 ± 2.15 ^d	129.09 ± 24.08 ^c	231.68 ± 25.89 ^b	288.28 ± 81.41 ^a	46.29	P < 0.001
Epithelial Thickness (2 Weeks)		98.54 ± 15.45 ^d	134.18 ± 26.90 ^c	328.88 ± 28.45 ^a	302.22 ± 28.71 ^b	125.32	P < 0.001

Data expressed as mean ± SD (n=4). Different superscript letters within the same row indicate statistically significant difference (one-way ANOVA followed by Tukey post hoc test, p < 0.05).

Interleukin-6 antibody immunostaining results:

The results of the anti-IL-6 immunostaining showed brown nuclear or cytoplasmic deposits, indicating a positive reaction. At every time point, the experimental groups' inflammatory responses, as assessed by IL-6, were significantly lower than those of the control group, with the combination group exhibiting the lowest values, followed by the LASER-treated groups (**FIG. 8**). However, according to the statistical analysis, there was a significant difference between the treated groups at all time points (**Table 3**). The bar graph (**FIG. 9**) shows that the inflammatory response declined over the course of the study across all groups, with a notable drop between days 7 and 14. Both group and time factors affected IL-6 expression, as indicated by the two-way ANOVA (P=0.001).

Table (3): Comparison of IL 6 % area between different studied groups at 1 and 2 weeks

	Control -Ve	Control +Ve -1week	Conditioned Media -1W	LASER-1W	combination-1W	Test of significance e
%Area 1 week	0.570±0.07 ^A	20.71±0.33 ^B	7.46±0.28 ^C	2.60±0.19 ^D	1.49±0.24 ^E	F=7268.32 P=0.001*
%Area 2 weeks	0.570±0.07 ^A	14.55±0.22 ^B	4.11±0.14 ^C	1.76±0.14 ^D	0.817±0.08 ^E	F=10274 P=0.001*

F: One-way ANOVA test, * different superscript letters within the same row indicate statistically significant differences between groups (p < 0.05).

Discussion

The current work showed that both LASER and gingival-derived mesenchymal stem cell-conditioned medium greatly accelerated the healing of surgically created tongue defects in rats, with the combined treatment group showing the strongest regenerative effects. Because of the tongue's continuous exposure to mechanical stress, microbial colonization, and continuous salivary flow, all of which impede the early clot formation and the initial inflammatory response, healing is thought to be particularly challenging [32]. Previous studies have investigated many regeneration approaches for the healing of oral mucosa. Squillaro et al. noted that mesenchymal stem cell transplantation enhances

tissue regeneration; however, its clinical application constrained by challenges associated with donor heterogeneity, ex vivo expansion and immunogenicity [33]. Barrientos et al. also stated that the injection of growth factors accelerates wound healing, but the effect is often limited [34]. Pawitan highlighted the regenerative properties of stem cell-conditioned medium as a cell-free alternative [35].

The present study demonstrated enhanced tongue regeneration using conditioned media. The addition of LASER therapy apparently enhanced this effect, suggesting a synergistic interaction. This combined method may thus provide a safer and more effective alternative to conventional regenerative approaches. GMSCs-CM's paracrine secretome, which is abundant in bioactive substances such as growth factors, cytokines, chemokines, and extracellular vesicles, is responsible for its capacity for regeneration [36]. PDGF and IL-10 were chosen as typical markers because of their well-documented roles in angiogenesis, fibroblast proliferation, and the regulation of inflammatory responses during wound healing. PDGF serves as a powerful mitogenic and chemotactic agent for mesenchymal cells, whereas IL-10 is essential for its anti-inflammatory function in mitigating excessive tissue damage [37]. High quantities of interleukin-10 and platelet-derived growth factor were found in this investigation. These two substances are recognized for accelerating wound healing by enhancing fibroblast migration, promoting angiogenesis, and suppressing excessive inflammatory responses. According to reports, PDGF functions as a chemical adsorbent for fibroblasts and mesenchymal cells and is a powerful mitogen. It is essential for numerous activities, including cellular proliferation, differentiation, wound healing, embryonic development, inflammation, migration, immunity, and survival [38]. Previous research suggests that PDGF may facilitate vascular regeneration and wound healing [39]. Similarly, by suppressing pro-inflammatory mediators such as IL-1 β and TNF- α , the anti-inflammatory cytokine IL-10 helps prevent tissue damage and promote tissue regeneration. This is in line with recent research indicates that MSC-derived secretomes can facilitate the polarization of macrophages from a pro-inflammatory M1 phenotype to a pro-regenerative M2 phenotype, thereby altering the inflammatory environment towards a regenerative state [36]. It is recognized that the secretome of GMSCs is a multifaceted biological mixture that likely includes additional trophic factors such as VEGF, TGF- β , FGF, and extracellular vesicles [37], which may have facilitated the enhanced angiogenesis and muscle regeneration observed, especially in the combination group. Still, the enhanced keratinization, epithelial thickness, and angiogenesis seen in the treated specimens indicate that LASER facilitated the regeneration of epithelial and connective tissues. The fundamental mechanisms of LASER therapy are thought to involve the activation of mitochondrial cytochrome c oxidase, which enhances ATP synthesis, increases reactive oxygen species (ROS) signaling, and stimulates downstream transcription factors related to migration, proliferation, and distinctiveness [40]. LLLT has been shown to induce local vasodilation predominantly via nitric oxide (NO) release, thereby enhancing microcirculation and improving tissue perfusion. In dense muscle tissues like the tongue, increased blood flow may improve the distribution and diffusion of injected conditioned media, thereby increasing the local bioavailability of its bioactive components [41]. Thus, LLLT could create a more favorable environment that enhances the absorption and activity of paracrine mediators, supporting the observed enhanced healing response.

Optimal results were achieved with the combination of GMSC-CM and LASER. This combination exhibited nearly complete regeneration of the tongue's musculature, connective tissue, and epithelium. The improvement of local tissue perfusion, vascular permeability, and cellular responsiveness induced by LASER may explain this combined effect. These improvements can intensify the effects of the bioactive components found in GMSCs-CM. The polyp-like mucosal overgrowth observed in the combination group at two weeks may signify a hyperplastic epithelial response rather than a pathogenic, dysplastic transformation. The significant increase in epithelial thickness and surface projections, without any evident indications of cellular atypia, supports this. This reaction may be due to the combined treatment, which increase proliferative activity and accelerate epithelial regeneration and tissue remodeling.

The pro-inflammatory cytokines IL-1 β , TNF- α , and IL-6 were significantly reduced, with the combined group showing the lowest levels, further confirming the immunomodulatory impact of the treatments. Both GMSCs-CM and LASER have

the potential to significantly reduce persistent inflammation, which is a major contributor to delayed wound healing. According to earlier research, MSC-derived conditioned media can inhibit NF- κ B activation and reduce oxidative stress, thereby lowering inflammation and cell death while promoting tissue repair and regeneration. This therapeutic effect is achieved through paracrine signaling, in which conditioned media delivers a variety of molecules, including growth factors, cytokines, and extracellular vesicles such as exosomes, which protect against oxidative damage, reduce inflammation, and support cellular homeostasis [42]. This strategy has great potential for use in oral and maxillofacial surgery, especially in treating chronic oral ulcers or partial glossectomy, where rapid tissue healing is necessary to restore speech, mastication, and swallowing. Because of its lower immunogenicity, decreased risk of tumorigenesis, and ease of storage and clinical application, GMSCs-CM is a better cell-free therapy than cell transplantation [43]. In conclusion, although GMSCs-CM and LASER individually facilitated the healing of tongue defects, their combined application yielded superior outcomes by markedly minimizing inflammation and accelerating muscle bundle development, connective tissue organization, angiogenesis, and epithelium regeneration.

Limitations

The complexity of human oral wounds is not entirely replicated by the rat model used in this study, which had a brief follow-up. Additional research is required to assess the long-term safety and to harmonize the dose of GMSCs-conditioned media and the repeated use of low-level laser therapy. Despite the improvement in histological architecture, functional assessment such as tongue mobility and muscular contraction was not assessed. A future study must include long-term follow-up and functional assessments to verify the clinical applicability of this combined therapy.

Abbreviations

Full name	abbreviation
Light Amplification by Stimulated Emission of Radiation	LASER
Conditioned media	CM
Mesenchymal stem cells	MSCs
Gingival-Derived Mesenchymal Stem Cells	GMSCs
Low-Level Laser Therapy	LLLT
Phosphate-Buffered Saline	PBS
Fetal Bovine Serum	FBS
Platelet-Derived Growth Factor	PDGF
Interleukin 10	IL-10

Declarations

Ethical approval

The current study was approved by Mansoura University Animal Care and Use Committee (MU-ACUC).

Ethics and consent to participate: not applicable.

Clinical trial number: not applicable

Availability of data and materials: All data generated during this study are included within this article.

Authors contributions

Elham H. Ahmed, Mohammed Abdelrahim Abdeen, and Esraa Abdelraouf Elsayed were responsible for the study's idea and design, data collection, analysis, and interpretation.

Elham H. Ahmed, Mohammed Abdelrahim Abdeen, and Esraa Abdelraouf Elsayed wrote, reviewed, and edited this.

Writing the article and critically editing it for significant intellectual content: Mohammed Abdelrahim Abdeen, Elham H. Ahmed, and Esraa Abdelraouf Elsayed

The final text was reviewed and approved by all authors.

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Figures

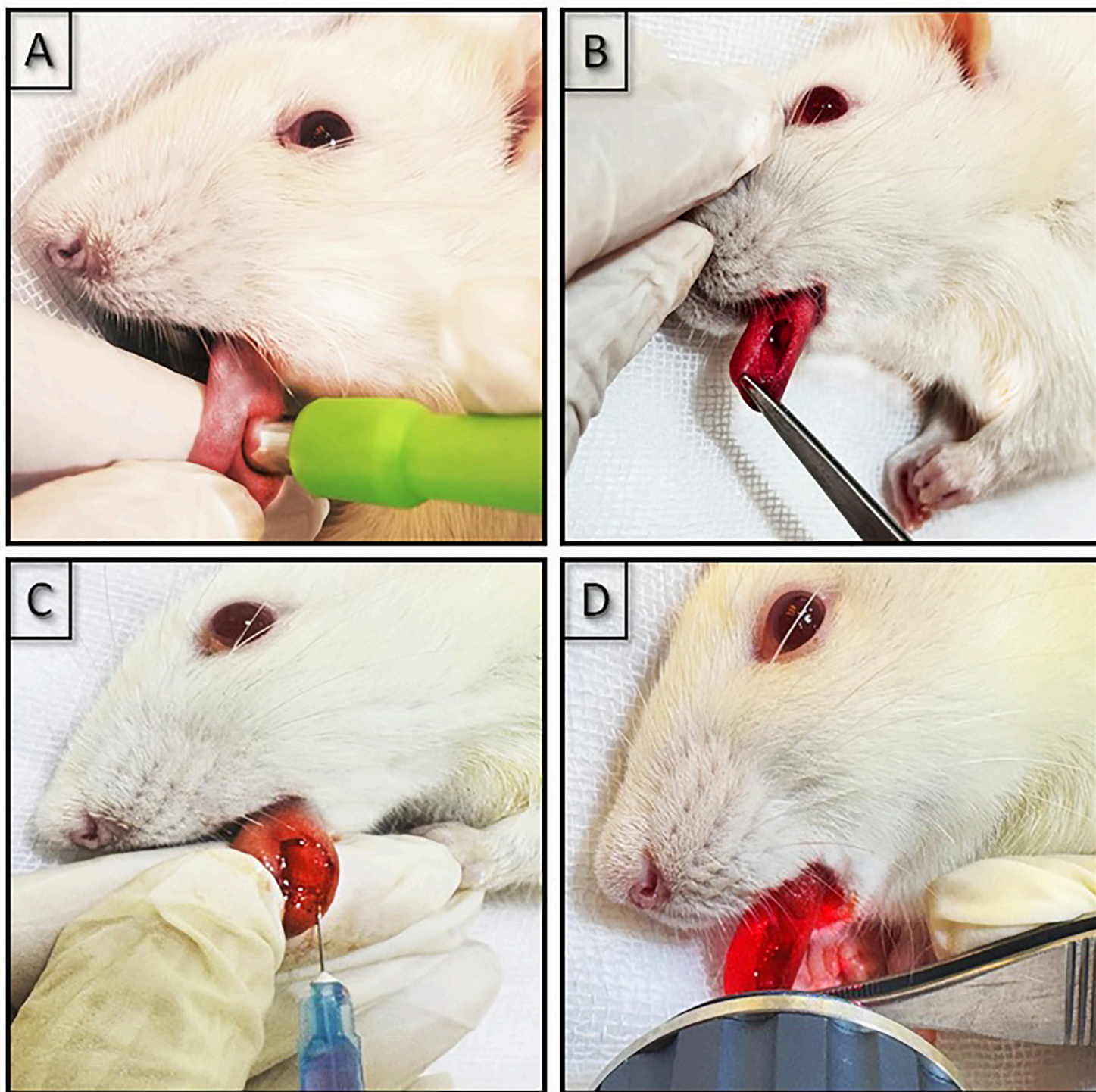


Figure 1

(A): A standardized tongue ulcer is made using a biopsy punch tool. **(B):** The created ulcer is seen on the tongue's lateral surface. **(C):** Injection of conditioned media directly around the ulcerated area using a syringe. **(D):** LASER therapy is applied to the defect area.

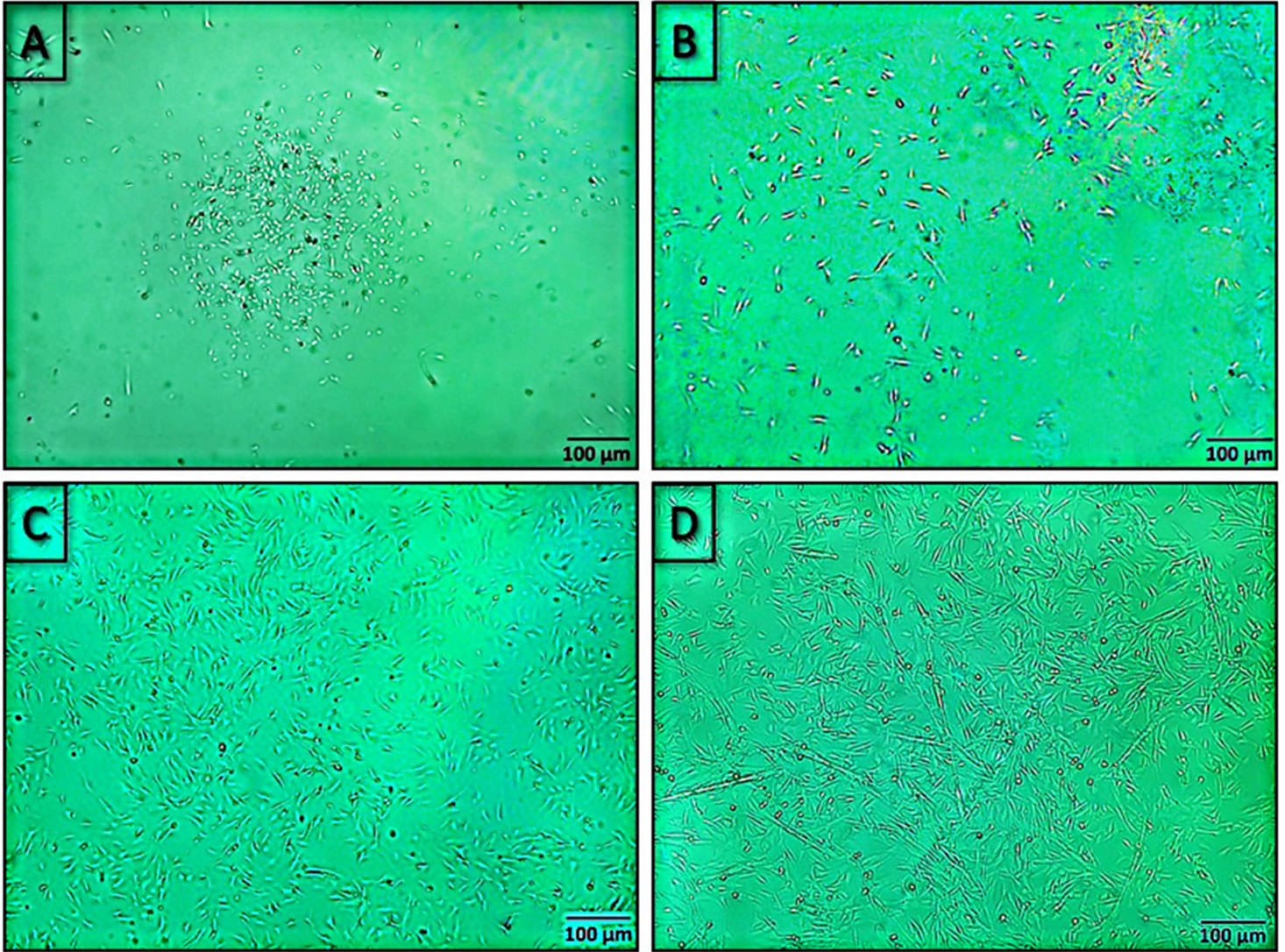


Figure 2

Gingival-derived mesenchymal stem cells photomicrographs taken during primary culture and later passages using an inverted microscope. (A) A primary culture displaying clusters of tiny, spherical cells. (B) Early adherent cells with a spindle-like appearance. (C) Passage 2 cells that exhibit fibroblast-like characteristics and enhanced proliferation. (D) The uniform spindle-shaped morphology and parallel organization of passage 3 cells. (Scale bar = 100 µm)

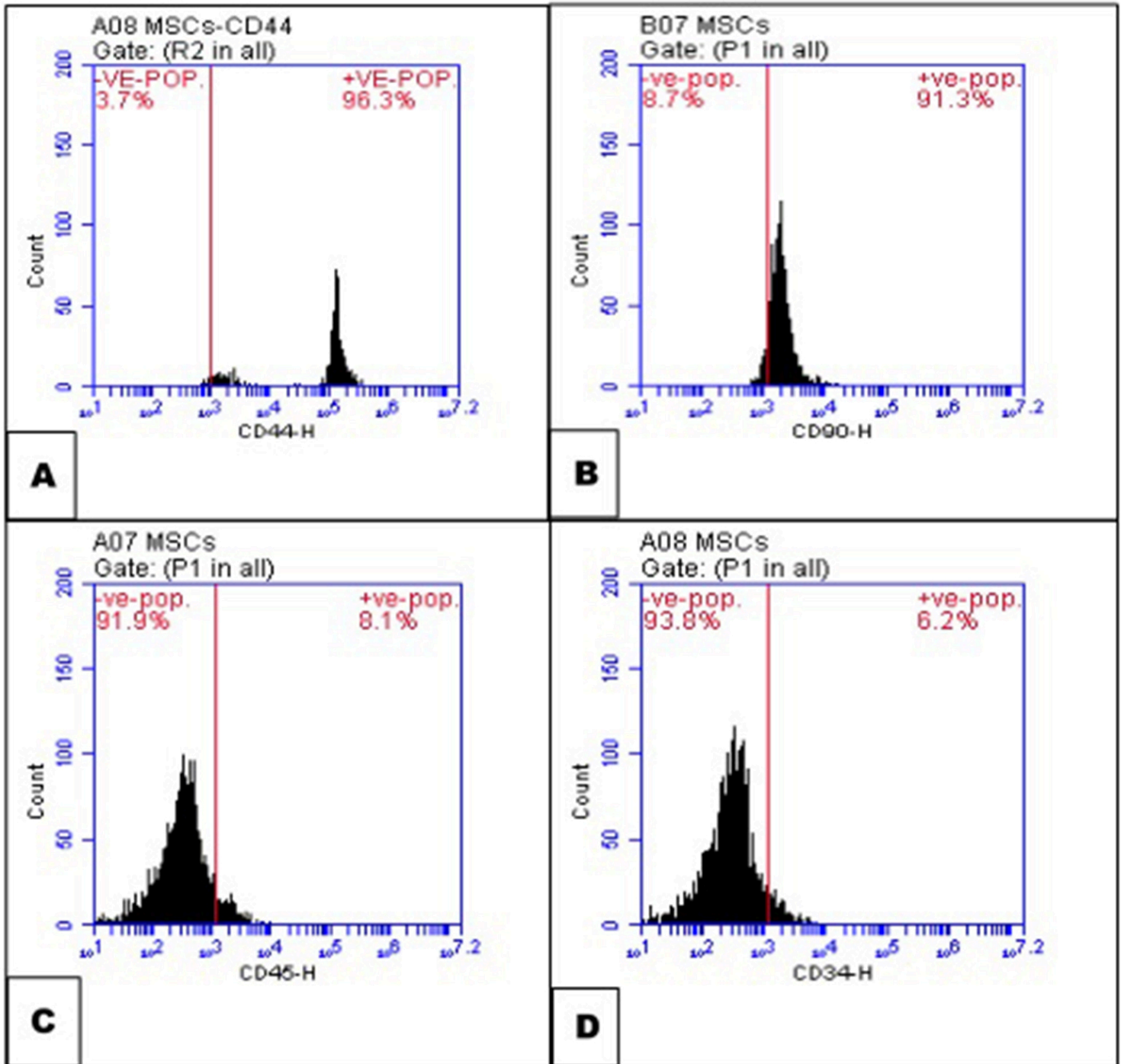


Figure 3

Flow cytometry chart with single-parameter histograms for CD44 (**A**), CD90 (**B**), CD45 (**C**), and CD34 (**D**). Flow cytometry analysis shows that GMSCs react positively to CD44 (96.3%) and CD90 (91.3%), but negatively to CD45 (8.1%) and CD34 (6.2%).

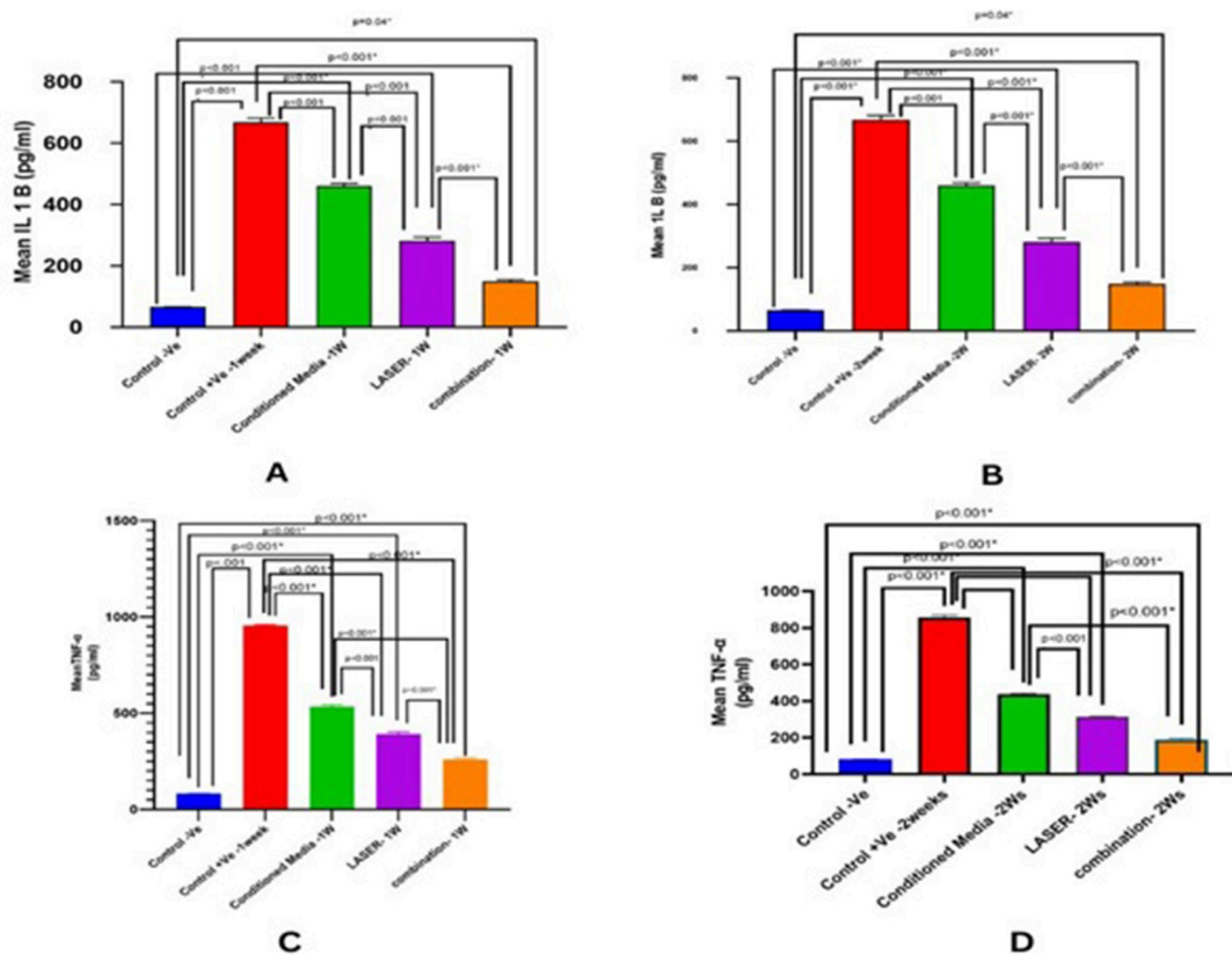


Figure 4

Bar chart represents the tissue levels of: (A) IL1β after 1 week, (B) IL1β after 2 weeks, (C) TNFα after 1 week & (D) TNFα after 2 weeks between different studied groups.

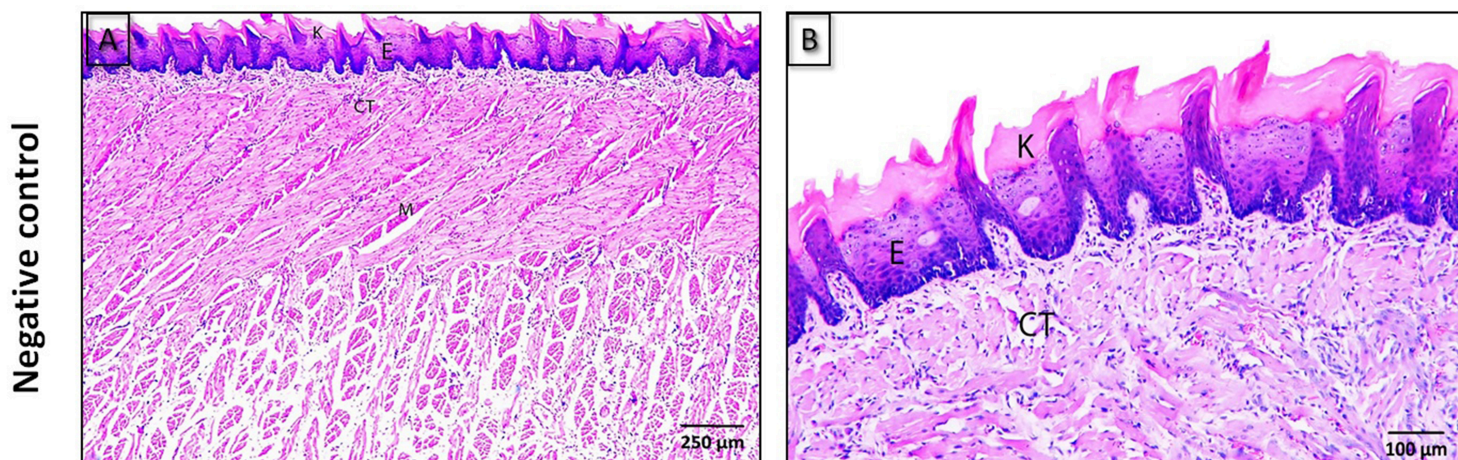


Figure 5

Histological examination of the negative control group's tongue mucosa.

(A) A low magnification image demonstrates the tongue mucosa's typical architecture, with a well-organized stratified keratinized squamous epithelium covering the muscle and connective tissue layer underneath. There are no indications of disruption or abnormal proliferation, and the epithelial layer seems to be continuous and intact.

(B) Higher magnification shows distinct layers of keratin, epithelium, filiform, and fungiform papilla, and underlying connective tissue. There were no indications of ulceration, inflammation, or epithelial hyperplasia. (H&E staining, scale bar = 250 μ m) (K): keratin, (CT): connective tissue, (M): muscle, (E): epithelium.

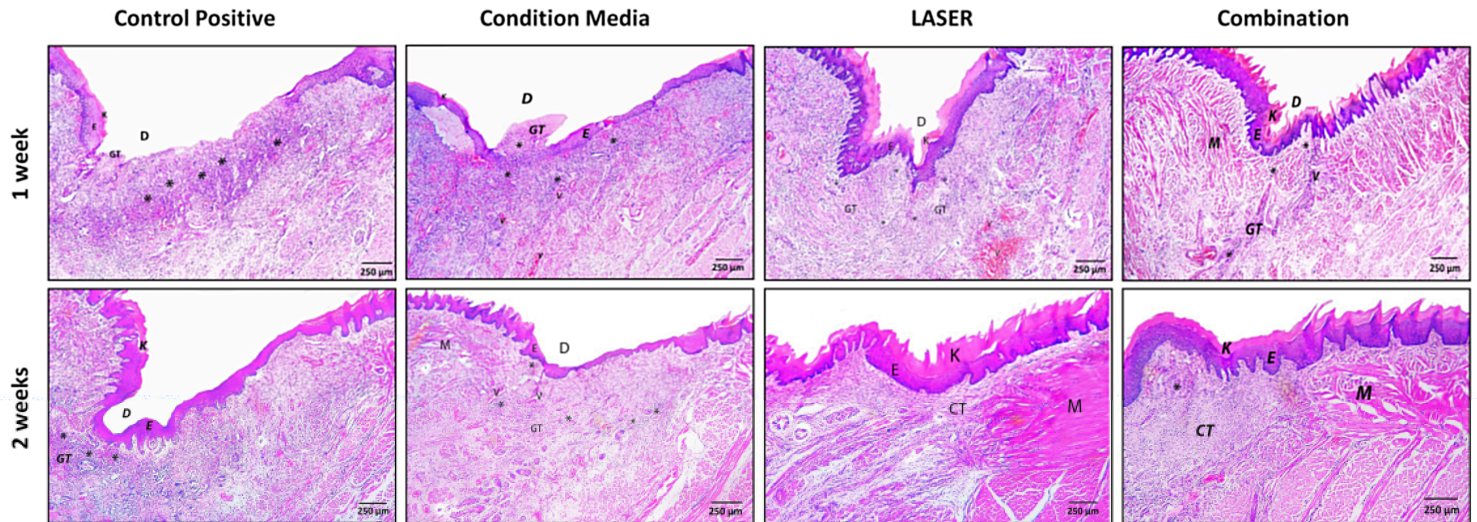


Figure 6

Histological evaluation of tongue healing at 1 and 2 weeks. (H&E staining, scale bar = 250 μ m). After one week, the control positive group exhibits a significant defect (D), with the epithelium next to the wound lacking lingual papillae (E), granulation tissue (GT), and a large presence of inflammatory infiltrates (*). The Condition Media treatment shows early development of the epithelial lining, moderate vascularization (V), and granulation tissue formation, along with a decrease in inflammatory cells. The LASER group demonstrates enhanced epithelial thickness, keratinization (K), and more structured granulation tissue, accompanied by moderate vascular proliferation. The Combination treatment results in notable epithelial stratification, formation of a keratin layer, bundles of regenerating muscles (M), neovascularization, and reduced inflammation.

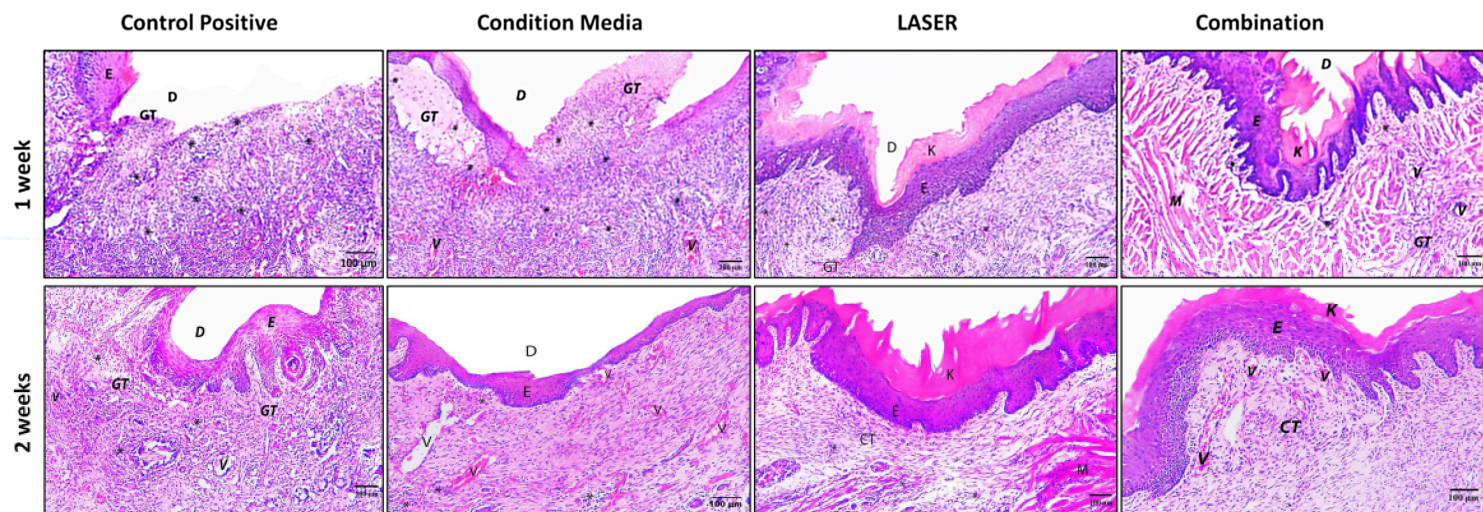


Figure 7

Histological assessment of tongue healing at 1 and 2 weeks. (H&E staining, scale bar = 100 μm). After 1 week, the control positive group exhibits moderate granulation tissue (GT) with scattered inflammatory cells (*), and a deep defect (D). The Condition Media group shows early re-epithelialization with notable granulation tissue and neovascularization (V). The LASER group displays a thicker epithelium with a keratin layer (K). The Combination treatment group demonstrates significantly increased epithelial thickening, bundles of regenerating muscles (M), and early restoration of mucosal architecture.

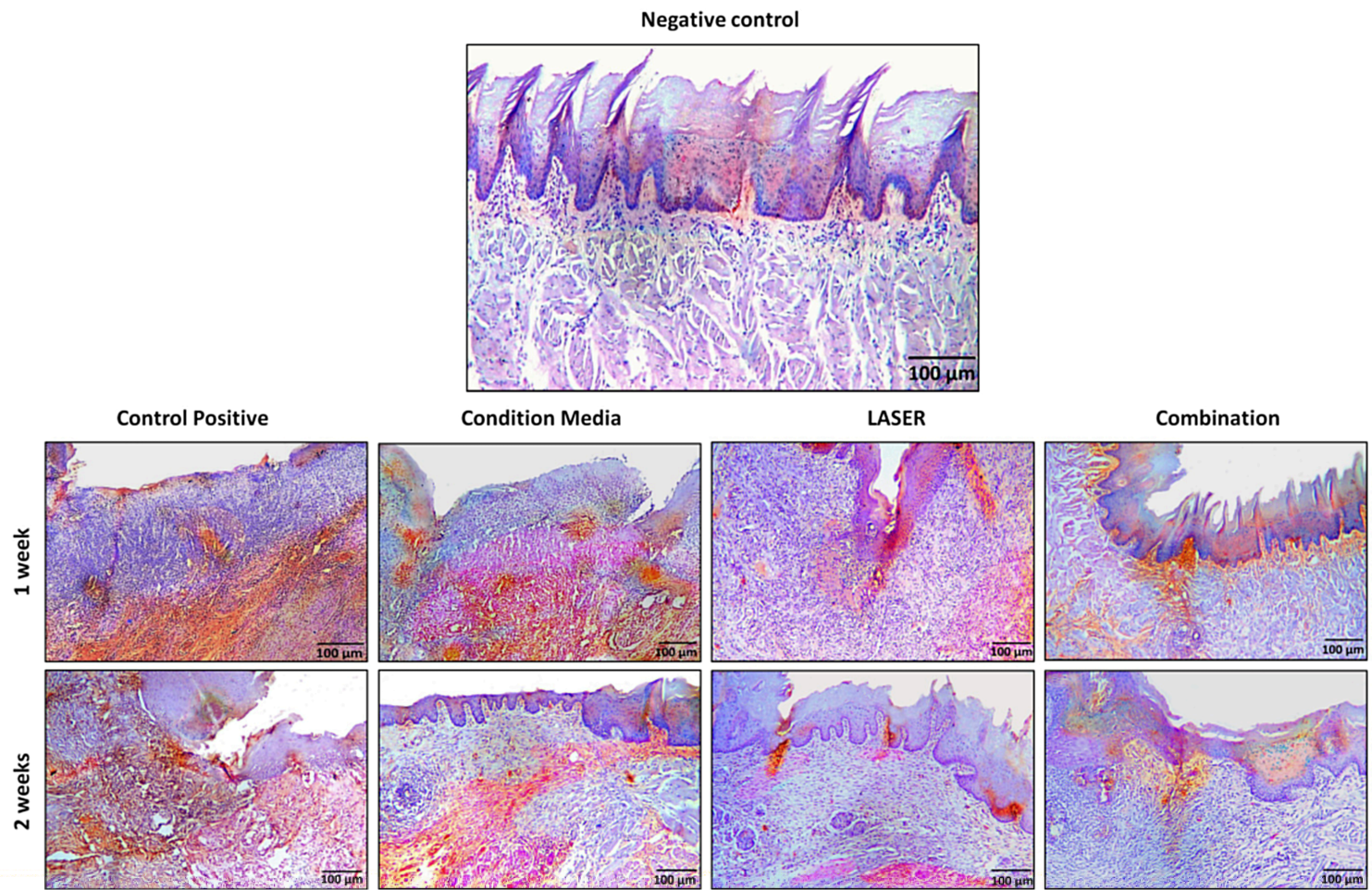


Figure 8

IL-6 immunohistochemical staining at one and two weeks. The intact mucosa in the negative control group lacks IL-6 expression. Strong IL-6 immunoreactivity is seen in the positive control group, especially in the ulcerated epithelium and inflammatory cells. Decreased IL-6 expression is seen in the conditioned media group. The LASER group display moderate IL-6 expression while combination group show the lowest expression. (Scale bar = 100 μ m).

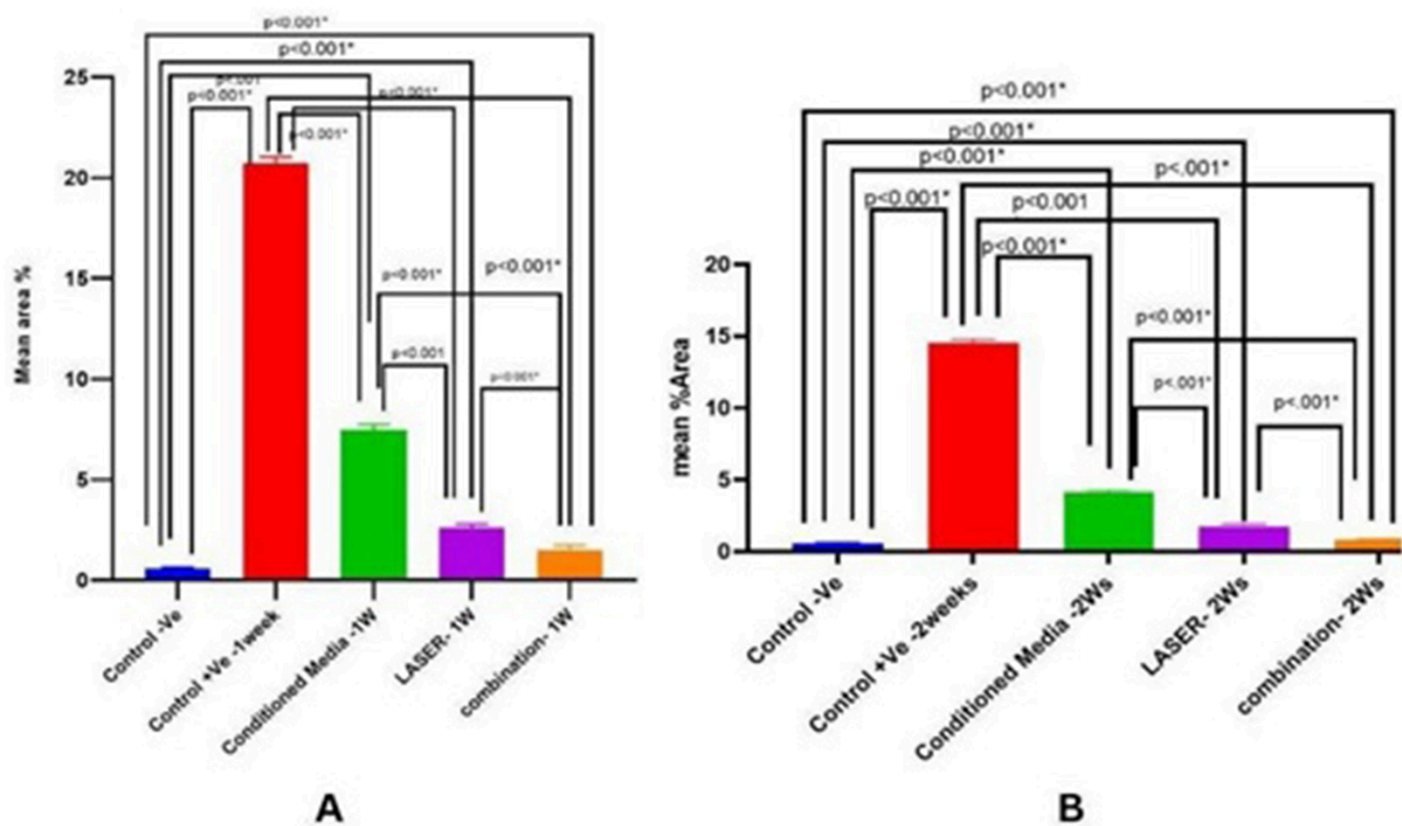


Figure 9

Bar chart represents the mean % area of IL-6 between different studied groups at 1 week (A) & 2 weeks (B).