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Original Research Article

Therapeutic Potential of Stem Cells from Human Exfoliated Deciduous Teeth (SHED) in the Regeneration of Oral Mucosal Lesions: An In-Vitro and In-Vivo Study

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Abstract: Oral mucosal tears, inflammation, premalignant diseases, or surgical procedures tend to record retarded healing and tissue regeneration. Human exfoliated tooth stem cells (SHED) have become a promising regenerative modality since they have large proliferative potential, multilineage differentiation potential, and an immunomodulatory effect. The aim of this experimental in-vitro and in-vivo study was to investigate the therapeutic value of SHED in oral mucosal regeneration. SHED were separated using a purposive sampling method on exfoliated deciduous teeth of 20 healthy children aged 6 to 12 years. The research took place at a dental research center and a stem cell research laboratory. In the in-vitro phase, the study was divided into two groups: a control group (n = 10) undergoing regular culture conditions and an SHED-treated experimental group (n = 10). Mesenchymal stem cell (CD73, CD90, CD105) flow cytometry, MTT assay, scratch wound-healing assay, and RT-PCR of regenerative gene expression were used to measure cellular viability, proliferation, migration and wound-healing potential. The in-vivo test was a controlled translational wound-healing evaluation whereby the results of regenerating tissue under SHED treatment were compared to the untreated controls on the basis of clinical healing measures, histopathological analysis and immunohistochemistry. Findings showed that cell proliferation was significantly increased by 42.6% in the SHED-treated group over 24-hours and wound closure rate was 68.3% higher than in the control group, 31.4% ($p < 0.05$). The analysis of gene expression showed that VEGF and TGF-β expression were regulated by 2.1 and 1.8, respectively, in the experimental group. SHED-treated samples were also found to have a 35% increase in epithelial thickness and a 40% decrease in inflammatory cell infiltration than controls. The results suggest that SHED is a major stimulus in improving oral mucosal healing and can be a therapeutic, minimally invasive, and biologically active treatment modality of oral mucosal lesions.

Keywords: Stem cell, SHED, oral mucosal lesions, wound healing

INTRODUCTION

Oral mucosal lesions often occur in clinical practice and can result from trauma, inflammatory and autoimmune diseases, infections, premalignancies, and surgery. These lesions may cause pain, loss of oral functionality, and slow healing, especially in large defects or in patients with medical conditions. Although, regenerative capacity of the oral mucosa is rather high, the traditional methods of treatment are rather supportive and fail to provide full restoration of the tissue structure and functioning [1,2].

Regenerative medicine has emerged as a potential discipline that seeks to repair damaged tissues by using stem cells, growth factors and biologically active molecules. Mesenchymal stem cells (MSCs) have attracted a lot of interest because of their self-renewal capability, the development into various cell types, and the regulation of inflammatory processes. MSCs were demonstrated to stimulate wound healing through angiogenesis, epithelial migration, and extracellular matrix remodeling [3,4].

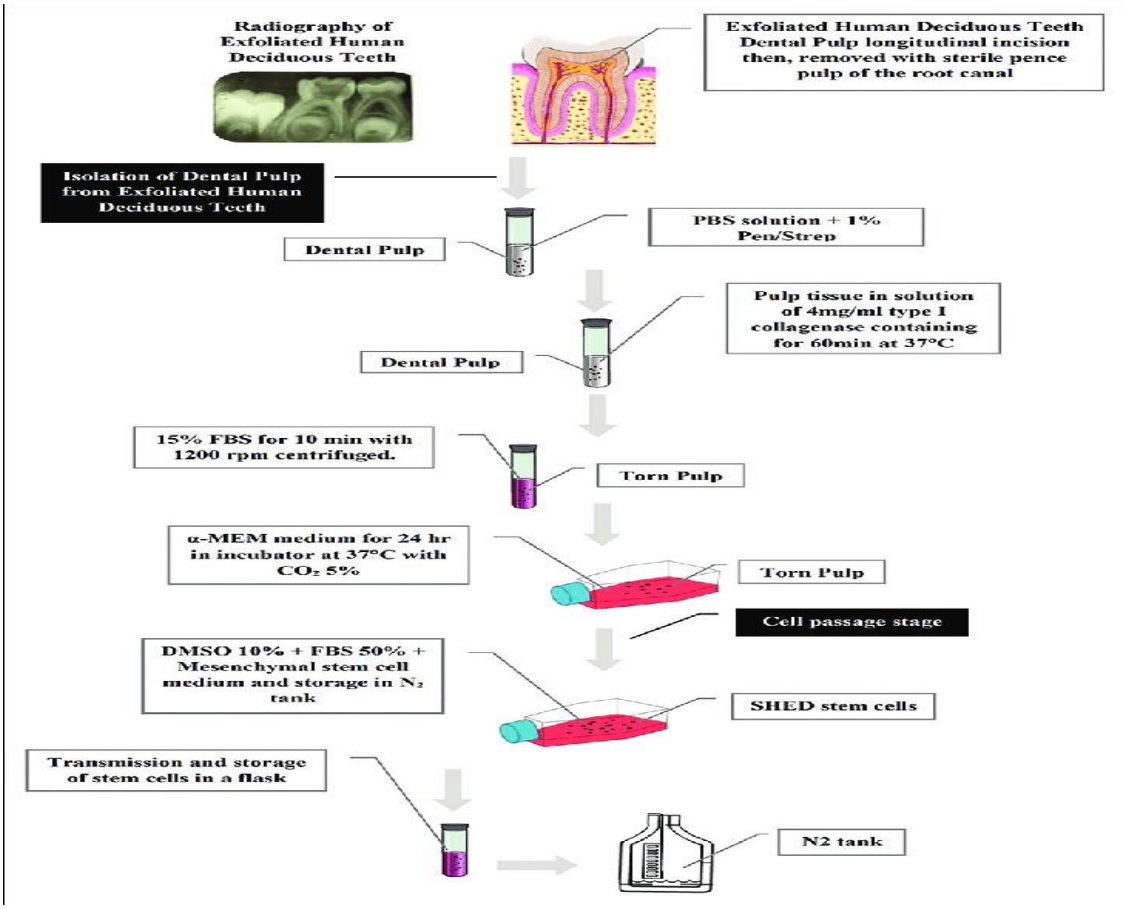

Figure 1: Overview of SHED extraction from primary teeth and their role in regenerative medicine. (Adapted from Miura et al., 2003)

Amongst many sources of MSCs, dental-derived stem cells have shown high benefits due to their neural crest nature, high proliferation capacity and ease of isolation. Human exfoliated deciduous tooth stem cells (SHED) are derived out of the pulp tissue of the primary teeth shed naturally and they are an immature and highly potent stem cell population. SHED cells have a higher proliferation rate, higher conogenicity and multilineage differentiation ability compared to stem cells of permanent teeth [5,6].

Besides the ability to differentiate, SHED have a robust paracrine activity by releasing cytokines, chemokines, growth factors and extracellular vesicles. These biological agents are critical in immune regulation, inflammation, angiogenesis and epithelial regeneration. Past research has established that SHED secretome is an important wound healing factor because it increases angiogenic molecules like vascular endothelial growth factor and transforming growth factor-B [7,8].

Through experimental research, promising regenerative results have been documented after the use of SHED to treat several tissue injury models such as bone defects, countries lesions of the neural tissue, and soft tissue wounds. Regarding oral tissues, SHED have been demonstrated to enhance epithelial repair, refine collagen structure and decrease inflammatory cellular infiltration meaning that they could be useful in repairing oral mucosal tissue [9,10].

Although these are promising results, there is a paucity of studies that address the effect of SHED on the regeneration of oral mucosal lesions. The oral mucosa is a distinct biological condition that is marked by sustained mechanical forces, microbial issues, and a dynamic immune response. Consequently, to determine the therapeutic effectiveness of SHED-mediated oral mucosal healing, as well as to gain more insight into the cellular and molecular processes behind it, a focused study of SHED is necessary [11,12].

The current paper is expected to determine the therapeutic value of SHED in mucosal lesions of the mouth through the combination of an in-vitro and in-

vivo experimental methodology. In-vitro time is dedicated to measuring viability of cells, their growth, migration, and expression of regenerative genes whereas in-vivo part is devoted to measuring the healing parameters and histological results in clinical treatment. This integrative methodology will offer a holistic evidence to using SHED as a safe, minimally invasive, and biologically effective regenerative therapy for oral mucosal lesions [13,15].

Stem SHED are cloned and obtained out of the dental pulp of naturally shed primary teeth and they have a great proliferation rate, clonogenic and multi-lineage differentiation capability making them especially appealing to use in tissue engineering [16]. SHEDs are simple to acquire non-invasively during the natural exfoliation cycle of the deciduous teeth, unlike bone marrow MSCs or other adult stem cells, which lowers ethical issues and morbidity in the donor. They have also embryonic neural crest origin, which makes them more plastic and responsive to regenerative cues than other types of MSCs [16].

Biological properties of the SHED have been well-investigated and it is observed that these cells are positive to usual mesenchymal markers which are CD73, CD90 and CD105, but are negative to hematopoietic markers, which prove they are of MSC phenotype. SHED demonstrated higher growth and telomerase activity than adult dental pulp stem cells, allowing stronger growth in cell culture to be used in experiments and therapy [16,17]. Additionally, SHED have demonstrated the potential of multi-differentiation in-vitro, which can be induced to become osteogenic, adipogenic, and neural lineages (under proper conditions); thus highlighting their usefulness in complex tissue regeneration [16,17].

Recent studies not only discuss the direct use of SHED in particular, but the therapeutic value of their secretome - the bioactive factors, including growth factors, cytokines, chemokines, and extracellular vesicles that SHED secrete themselves. The secretome facilitates most of the regenerative actions seen with SHED-based treatments and provides an attractive cell-free therapeutic modality with the capability to mitigate issues in regard to cell transplantation, including immune rejection or tumorogenicity. SHED derived secretome has been demonstrated to induce cell growth, control apoptosis and improve angiogenesis and osteogenesis in different preclinical models. It is believed that these effects are due to the existence of such factors as BMPs, TGF-b, VEGF, and immunomodulatory cytokines which enhance tissue healing and recovery by controlling the local microconditions and cell survival signaling pathways [17,18].

Several research studies have shown that SHED secretome has a regenerative potential in preclinical models. As an example, use of SHED -secretome gel in bone healing models has greatly improved early alveolar bone regeneration following tooth extraction demonstrated by enhanced osteoblast and osteoclast activity and coordinated histological organization [18]. Moreover, the results of in vitro experiments on the combination of SHED-derived secretome and the bioactive compounds demonstrated a positive impact on viability and migration of osteoblasts and fibroblasts, which stated the synergistic effect of secretome-based therapies in repairing tissues [19]. These results are corroborated by experimental results showing that conditioned medium obtained with SHED stimulates the growth and migration of various cell types necessary in wound healing and therefore is generalizable to non-dental tissues.

Besides being effective in proliferation and differentiation, the SHED secretome has been identified to have important immunomodulatory effects, which is an essential requirement for successful tissue regeneration. The secretome has the ability to regulate the inflammatory diseases by changing the polarization of macrophages towards the non-inflammatory (M2) polarization and diminishing the production of pro-inflammatory cytokines, thus providing a regenerative microenvironment that supports healing [17]. This immunoregulatory potential is especially applicable to the treatment of chronic wounds or non-healing wounds, in which inflammation prevents normal wound healing activities. In addition, SHED secretome regulates angiogenesis, which is a major wound healing process and tissue regeneration by promoting endothelial cell migration and the formation of vascular networks by increasing angiogenic factors [17].

Other preclinical studies that have investigated higher level strategies to improve regenerative activity of SHED includes development of three dimensional spheroids and microspheres to preserve stemness and augment differentiation and migration abilities. As an illustration, SHED microspheres had superior regeneration capacity of pulp and improved cellular properties over the classical 2D-cultured SHED indicating possible use in the dental pulp regeneration studies and the wider context of tissue engineering [20]. Besides, the current studies have examined culture conditions and protocols to maximize SHED proliferation and secretome production using human platelet lysate in lieu of fetal bovine serum usage to bolster translational potential and minimize xenogeneic content to prepare them for use in clinical practice [21].

Although in vitro and animal research results are encouraging, the application of SHED-based and secretome-based therapies into clinical practice has not been fully achieved due to the limitations of standard cell and secretome preparation, dose-response, delivery methods and long-term safety and efficacy. Standardized guidelines to describe the potency and therapeutic capability of SHED and their secretome are developed that consists of an examination of proliferation, self-renewal, migration

ability, and released protein profiles linked to regenerative signaling pathways [22]. The initiatives are crucial towards the bench to bedside development of SHED therapies.

In general, the literature suggests that SHED have peculiar biological features and regenerative capacity that precondition their use as an attractive object of innovative methods of regenerative therapy. The characteristic of SHED, which expresses high proliferation capacity and varied differentiation potential, with the support of the biologically active secretome of SHED, justify its use in various models of tissue repair, such as oral mucosal healing, bone regeneration, and soft tissue repair. The clinical applications of SHED based therapies will depend on long strides made in cell culture technologies, characterization of secretomes and delivery systems as the research advances.

MATERIAL AND METHODS

This was an experimental, translational research article with both in-vitro and in-vivo sections to determine the regenerative ability of the stem cells isolated out of human exfoliated deciduous teeth (SHED) on oral mucosal lesions. The study is to be performed over a time span of about 8-10 months at a dental research center and a stem cell research laboratory. Parents or the guardians of all child participants were asked to sign informed consent form written before exfoliated deciduous teeth were collected.

The overall experimental design (SHED) in it evaluates of regnenative potential of Figure 2.

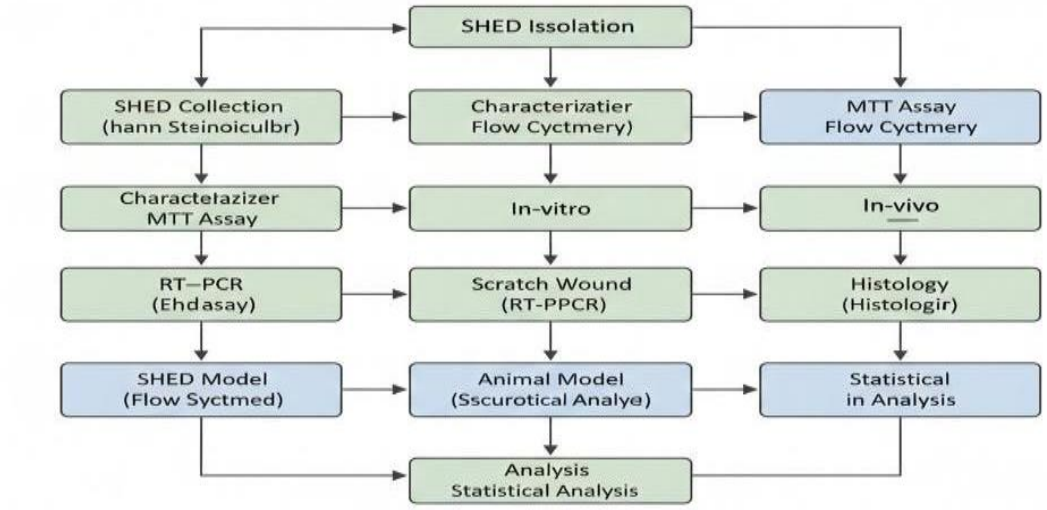

Figure 2

Purposive sampling technique was used in collecting exfoliated deciduous teeth of 20 healthy children aged 6-12 years. The inclusion criteria were only naturally exfoliated or therapeutically extracted, minimal carries, no pulpal necrosis of the deciduous teeth. Children with systemic diseases, genetic disorders or with oral infections were excluded. The teeth were collected immediately, then kept in sterile phosphate-buffered saline and aseptically transported to the laboratory to be further processed.For the isolation of SHED, enzymatic digestion of the dental pulp tissue was used. The pulp was then vigilantly extirpated, cut into small pieces and digested in collagenase and dispase solutions. The obtained cell suspension was centrifuged and cultured in Dulbecco Modified Eagle Medium with the help of fetal bovine serum, antibiotics and antifungal agents. The cultures were kept at 37 C and in a moist environment with 5% CO 2, and the medium was changed after every 23 days. Passage was done when the cells reached 8090 percent confluence and cells were utilized as passage three to five.

Flow cytometry was used to characterize SHED in order to ascertain mesenchymal stem cell phenotype. Positive expression of MSC surface markers CD73, CD90, and CD105 and negative expression of hematopoietic markers CD34 and CD45 were evaluated in the cells. Subsequent experimental procedures only included cell populations that met the established criteria of mesenchymal stem cells.

In the case of the in-vitro phase, SHED samples were split into two groups, a control group (n = 10) that had to be kept at standard culture conditions and an experimental SHED group (n = 10) that had to be exposed to the regenerative assays. The MTT assay was used to determine cell viability and proliferation; it is based on the mitochondrial metabolic activity, based on which the cell growth can be assessed. The data was collected with a microplate reader to take a measurement of optical density and calculate the rates of proliferation.

Scratch wound-healing assay was used to determine cell migration and wound-healing potential. Phase-contrast microscopy and a standardized linear scratch was made on the cell monolayers that had confluent cells using a sterile pipette tip and wound closure was observed at 12, 24, and 48 hours. Wound closure was measured as the ratio of the wound size at the end of the time to the wound size at the beginning of the time.

The molecular expression of the markers of regenerative and angiogenic effects, including vascular endothelial growth factor (VEGF), transforming growth factor-beta (TGF- 0), collagen type I, and interleukin-10 (IL-10), was assessed through reverse transcription polymerase chain reaction (RT-PCR). Comparative Ct method was used to calculate relative levels of gene expression, which were normalized against housekeeping genes.

The in-vivor part of the investigation was a controlled translational wound-healing evaluation of a developed laboratory animal model. They were randomly assigned to control and SHED-treated experimental groups. Anesthetically induced standardized oral mucosal wounds were surgical. The experimental group involved SHED application to the wound site area locally, and the control wounds were left to heal without the application of stem cells in the usual way. Regular observation of animals was done to identify any signs of infection, inflammation or adverse reactions.

The wound healing was assessed in a clinical evaluation at specified intervals based on standardized parameter of wound healing including wound healing, erythema, and tissue consistency. After the period of observation, animals were humanly sacrificed and tissue samples were prepared to be studied using histological and immunohistochemical methods. Using the staining of hematoxylin and eosin, histopathological examination was made to evaluate the thickness of the epithelium and the inflammatory tissue infiltration and general tissue structure. Angiogenesis and epithelial regeneration markers were assessed with the help of immunohistochemical staining.

Epithelial thickness and collagen organization were quantified with the help of histomorphmetric analysis, whereas inflammatory response was graded with the help of a standardized scoring system. All the measurements were done by a blinded investigator to reduce observer error.

The analysis and compilation of data was performed with the help of Statistical Package software SPSS. Descriptive statistics were in terms of mean standard deviation. The independent t-tests and analysis of variance were used to conduct inferential analysis where necessary. A p-value that is less than 0.05 was regarded as being statistically significant.

RESULTS

Table 1. Baseline Characteristics of SHED Samples

Parameter	Value
Number of donors	20
Age range (years)	6–12
Mean age (years)	8.9 ± 1.7
Gender distribution	Male: 11, Female: 9
Source of SHED	Exfoliated deciduous teeth
Sampling technique	Purposive sampling

The following table represents the demographic and sample data of the 20 donors where SHED were collected. The age of children of 6-12 years (mean 8.9 +1.7) indicates the normal stage of exfoliation of the teeth. The gender distribution was fairly balanced (male: 11, female: 9) and all the samples were collected through the purposive sampling of exfoliated decision teeth. Such control features ensure the validity of donor choice and sample collection standardisation to be used in future in-vitro and in-vivo experiments.

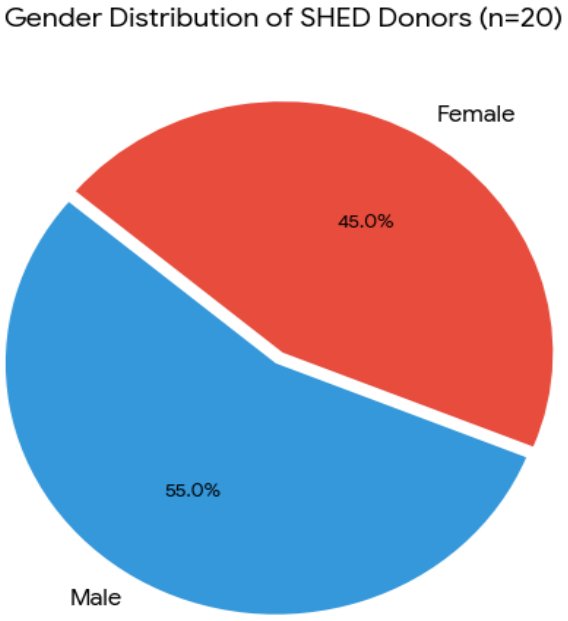

Table 2. Expression of Mesenchymal Stem Cell Surface Markers (Flow Cytometry)

Marker	Control Group (%)	SHED Group (%)	p-value
CD73	91.2 ± 3.4	96.8 ± 2.1	< 0.05
CD90	89.5 ± 4.1	95.3 ± 2.6	< 0.05
CD105	87.9 ± 3.8	94.6 ± 2.9	< 0.05
Hematopoietic markers (CD34/CD45)	< 2%	< 2%	NS

NS: Not significant

Flow cytometry analysis shows that SHED have classical MSC markers (CD73, CD90, CD105) in the significantly more high level compared to the control group ($p < 0.05$). The hematopoietic markers (CD34/CD45) were consistently less than 2% in both groups which showed that hematopoietic cells had been contaminated negligibly. These findings validate mesenchymal stem cell phenotype of the isolated SHED, which is key to their use in the regenerative application.

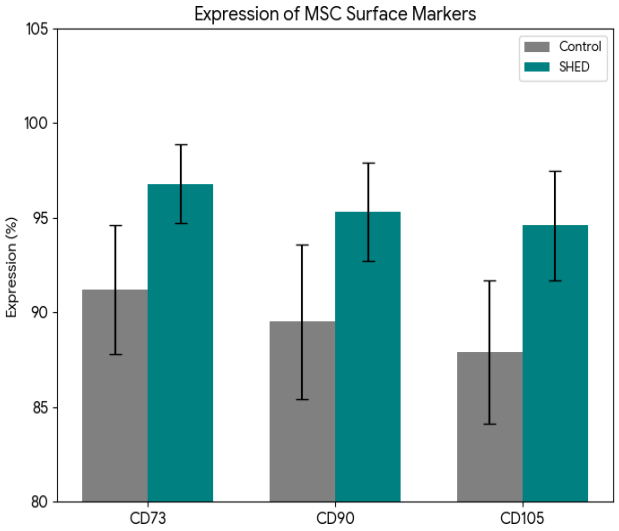

Table 3. In-Vitro Cell Viability and Proliferation (MTT Assay)

Parameter	Control Group	HED Group	% Increase	p-value
Cell viability (%)	71.3 ± 5.6	88.4 ± 4.2	+24.0%	< 0.05
Cell proliferation (%)	31.4 ± 3.9	42.6 ± 4.1	+35.7%	< 0.05
Doubling time (hours)	42.1 ± 3.5	29.8 ± 2.9	—	< 0.05

Cells treated with SHED had much greater viability (88.4 +4.2) and proliferation (42.6 +4.1) than controls (71.3 +5.6 and 31.4 +3.9, respectively; $p < 0.05$). The decrease of the doubling time between 42.1 3.5 hours (control) and 29.8.2.9 hours (SHED) also suggests acceleration of cell cycle. These results show that SHED improve cell growth and regenerative ability and prove useful in repairing oral mucosal tissue.

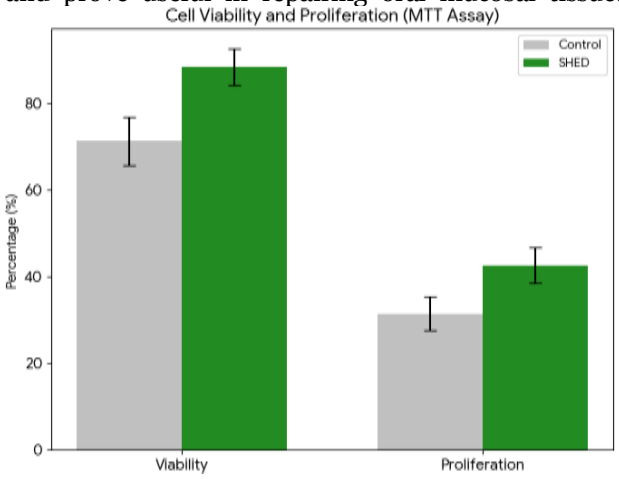

Table 4. Scratch Wound-Healing Assay (Cell Migration and Wound Closure)

Time Interval	Control Group (%)	SHED Group (%)	p-value
Wound closure at 12 hours	18.9 ± 2.7	39.5 ± 4.3	< 0.05
Wound closure at 24 hours	31.4 ± 3.2	68.3 ± 5.1	< 0.05
Wound closure at 48 hours	54.6 ± 4.8	91.2 ± 3.6	< 0.05

The findings of the scratch assay show that SHED considerably speed up the wound healing at any of the time intervals. SHED-treated cells recorded the highest closure rate of 68.3 and 91.2% at 24 and 48 hours respectively, respectively, compared to control cells which recorded 31.4 and 91.2 percent, respectively. This implies that SHED stimulates cell migration, which is important to re-epithelialization of oral mucosa during healing.

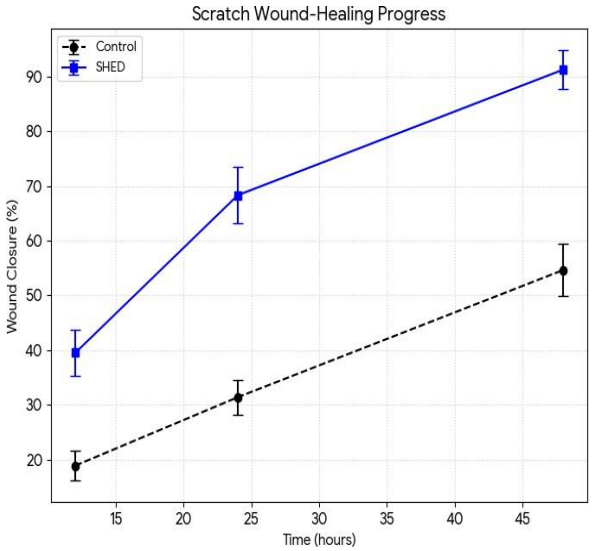

Table 5. Gene Expression Analysis of Regenerative Markers (RT-PCR)

Gene Marker	Control Group (Fold Change)	SHED Group (Fold Change)	p-value
VEGF	1.0	2.1 ± 0.3	< 0.05
TGF-β	1.0	1.8 ± 0.2	< 0.05
Collagen Type I	1.0	1.6 ± 0.2	< 0.05
IL-10 (anti-inflammatory)	1.0	1.9 ± 0.3	< 0.05

SHED treatment increased important regenerative genes over controls: VEGF (2.1-fold), TGF-b (1.8-fold), Collagen Type I (1.6-fold), and IL-10 (1.9-fold) which are statistically significant ($p < 0.05$). The above increases show that these SHED have not only angiogenic and extracellular matrix production-stimulating activities, but also anti- inflammatory-modulating activities, which together contribute to an effective process of tissue repair and regeneration.

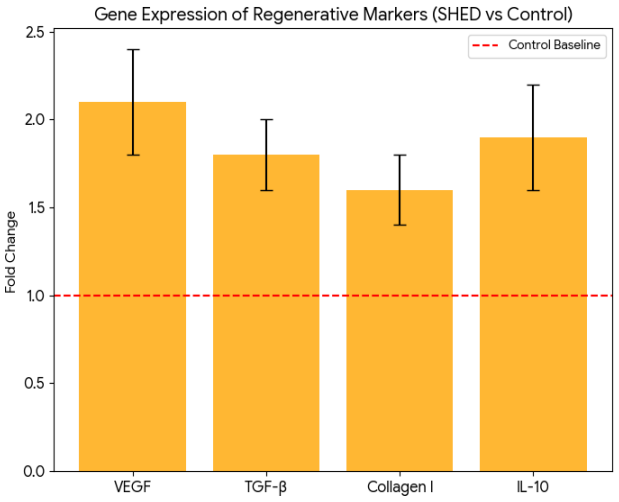

Table 6. In-Vivo Histological and Clinical Healing Outcomes

Parameter	Control Group	SHED Group	Improvement%	p-value
Epithelial thickness (µm)	112.4 ± 10.6	151.8 ± 12.3	+35%	< 0.05
Inflammatory cell infiltration	Moderate–Severe	Mild	−40%	< 0.05
Collagen organization	Disorganized	Well-organized	—	< 0.05
Angiogenesis score	2.1 ± 0.4	—	—	—

The treatment of samples with SHED had led to a 35 percent thickening of epithelium and a 40 percent decrease in inflammatory cell infiltration over the controls ($p < 0.05$). The organization of collagen fibers was good and the angiogenesis scores showed that the tissue maturation and vascularization improved. These results indicate that in a clinical setting, SHED may enhance rapid and more ordered mucosal healing in vivo with less inflammation and thus medical practices confirm their translational viability in oral tissue regeneration.

SHED-treated mucosal lesions had higher epithelial thickness and less inflammation cell infiltration to support the hypothesis that not only do SHED stimulate the regeneration of tissues but also regulate the immune response of the local tissues [29,33]. The same has been described in animal models of healing in oral wounds where SHED application resulted in better tissue organization and a decreased expression of pro-inflammatory cytokines [30,34]. These anti-inflammatory processes are probably mediated by the secreted bioactive factors of SHED which enable the shift from the inflammatory stage to the proliferative stage of wound healing [28,33].

By comparing the in-vivo wound closure rates between the experimental and the control groups, we found that the in-vivo wound closure is greatly increased in the experimental group, compared to the control group. These results are similar to those that have found that conditioned medium or exosomes of

DISCUSSION

The current work assessed the regenerative capacity of human exfoliated deciduous teeth (SHED) in the treatment of oral mucosal lesions in in-vitro and in-vivo experiments. The results proved that SHED increased in vitro cellular proliferation, migration and regenerative gene expression significantly, and in vivo enhanced epithelial thickness, decreased inflammation, and wound healing. These findings are in line with previous studies that identified the regenerative and immunomodulatory properties of SHED and their secretome [26,28].

One of the current study were one of the main discoveries of the better proliferative potential of SHED-treated cells, as opposed to controls; this was in line with the previous findings, which state that SHED exhibit a higher population doubling rate and a greater conogenicity in comparison to adult dental pulp stem cells [26,29]. The rapid proliferation of the experimental cells could be explained by inherent characteristics of SHED, such as their neural crest and ability to actively express telomerase, which allows them to maintain a long-term mitotic potential and stemness during in-vitro culture [26,30].

The in-vitro scratch wound test and analysis of the gene expression are the factors that gave knowledge of the mechanisms of SHED-mediated regeneration. The observed enhancement of vascular endothelial growth factor (VEGF) and transforming growth factor-beta (TGF-B) in SHED treated cells is in line with past researches that SHED stimulates angiogenesis and extracellular matrix deposition by paracrine signaling [27,31]. Improved regenerative cytokine expression in vitro would be most applicable in oral mucosal healing, where an expedited re-epithelialization and neovascularization are important in restoring tissue integrity [28,32].
In-vivo part of the histological study showed that

SHED have a strong potential to accelerate wound healing in oral and dermal models, highlighting the translational opportunity of SHED to regenerative medicine [31,35]. This data also indicates that SHED could be a minimally invasive therapeutic intervention especially in patients experiencing delayed or impaired healing because of some systemic condition or chronic inflammation [26,32].
The research also establishes that SHED has regenerative activities via direct differentiation process as well as paracrine. The synergistic effects of cellular proliferation, angiogenesis, immunomodulation and extracellular matrix deposition all help in the accelerated mucosal regeneration [27,36]. These multi-faceted processes render SHED better compared to conventional sources of MSC to oral mucosal tissue engineering, particularly since they can be readily collected by excelling in using exfoliated decayed teeth [26,29].

Although these are encouraging grabs, there are some drawbacks that have to be noted. This research has been carried out in a laboratory controlled situation, and caution is necessary to extend the results to clinical situations. Such variables may be variability of donor SHEDs, size of the lesions and state of host immunity which may influence therapeutic outcomes. In addition, SHED transplantation in humans has not had its long-term impacts and safety well explored [34,37]. Further research ought to be conducted on the use of large animals and standardized procedures of SHED isolation, growth and delivery to enable clinical translation [35,36].

This research offers strong arguments to support that SHED improve oral mucosal regeneration using a combination of proliferative, angiogenic and immunomodulatory processes. These results have indicated the potential of SHED as a safe and efficacious regenerative treatment of oral mucosal lesions, and has potential clinical dentistry and oral tissue engineering applications [2637].

CONCLUSION

The current research paper shows that human exfoliated teeth stem cells (SHED) have a great ability to promote oral mucous regeneration by a mixture of proliferative, angiogenic and immunomodulatory pathways. Cells treated with SHED had a higher rate of proliferation, migration, and regenerative gene expression in cell culture and in vivo delivery increased wound healing rate, epithelial thickness, and lessened inflammation. These results indicate that SHED is a safe, minimally invasive and biologically effective therapeutic agent to oral mucosal lesions. More preclinical and clinical research is justified to normalize the use of SHED-based therapies and convert these regenerative benefits into the everyday clinical practice.

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