Journal of Cell and Molecular Research (2025) 17 (2), 97-108 DOI: 10.22067/jcmr.2025.92644.1108

Research Article

Histological Study of Human Mesenchymal Stem Cells Behavior Cultured on Decellularized Human Gingival Matrix in the Presence of Hyaluronic Acid

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Received 13 March 2025 Accepted 26 September 2025

Abstract

Collagen bioscaffolds show promising results in oral mucosa engineering due to the high biocompatibility and biodegradability of collagen. Because of the high collagen content of gingiva, it can be used to prepare a natural collagen scaffold. Hyaluronic acid (HA) is one of the major components of extracellular matrix that regulates cellular behavior. In the present study, the effects of HA on the behavior of adipose-derived mesenchymal stem cells (Ad-MSCs) cultured on a decellularized gingival matrix were investigated by histological methods. Human gingival tissue (5×5×5 mm) was decellularized using physical methods as well as treatment with sodium dodecyl sulfate and Triton X-100, followed by washing and sterilization procedures. Scaffolds were divided into two groups including soaked scaffolds in HA (0.3% solution) and scaffolds without HA, which served as controls. In the next step, 3×10^5 Ad-MSCs were seeded on each scaffold in both groups, and histological studies were performed after 1, 2, 3 and 4 weeks of culture. Histological studies revealed effective cell removal and also preservation of collagen fibers in the decellularized gingival connective tissue. *In vitro* analysis of cellular behaviors showed adhesion and also migration of human Ad-MSCs towards connective tissue matrix, as well as formation of epithelial-like structures. Overall, statistical analyses indicated significant differences in cell density (P<0.01), migration (P<0.001) and nuclear size (P<0.01) at week one in HA-treated scaffolds versus the control group. The results of the present study demonstrated that the extracellular matrix of decellularized human gingival stroma can induce cellular behaviors such as adhesion, proliferation and migration of human Ad-MSCs.

***Keywords*:** Decellularization, Gingiva, Hyaluronic acid, Mesenchymal stem cells, Scaffold

Introduction

Repair or replacement of injured tissues or organs is one of the major problems in medicine (Gilpin and Yang 2017; Dzobo et al., 2018). Nowadays, tissue engineering, a novel area in the field of regenerative medicine, is considered an alternative solution for tissue or organ transplantation. Tissue engineering combines cells, scaffolds and active molecules to develop biological substitutes (Olson et al., 2011; Gilpin and Yang 2017; Dzobo et al., 2018). Since scaffolds induce and regulate cellular functions such as adhesion, migration, proliferation and differentiation of seeded cells, and guide formation of new tissues, proper design and architecture of the scaffold is very important in this field (Hoshiba et al., 2010). The ideal scaffolds for tissue engineering applications are those that mimic *in vivo* extracellular matrix (ECM) surrounding the cells (Hoshiba et al., 2010; Gilpin and Yang 2017; Eltom et al., 2019). Decellularized ECM is an attractive biomaterial that works well for this purpose (Liang et al., 2023). Depending on some factors such as thickness, density, lipid content and cellularity of tissues or organs, a combination of physical, chemical and/or enzymatic methods are commonly used for decellularization.

The main objective of all decellularization processes is maximal cell elimination while minimizing the alterations in ECM composition and ultrastructure (Crapo et al., 2011; Tapias et al., 2014; Gilpin and Yang 2017). Collagen-based biomaterials have attracted much attention in the field of tissue engineering and regenerative medicine due to abundance of collagen in the ECM and its high biocompatibility, biodegradability and low immunogenicity (Parenteau-Bareil et al., 2010; Copes et al., 2019). Gingiva contains a collagenous

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connective tissue and is a good candidate for preparing natural collagen scaffolds. Moreover, gingival tissue seems to be a rich source of stem cells, with good healing ability for fast regeneration (Peng et al., 2024). Consequently, the use of regulatory signals is another component of the tissue engineering strategies. These include biochemical and/or biophysical stimuli to induce and regulate tissue formation both *in vitro* and *in vivo*. Biochemical stimuli modulate cell signaling processes that regulate cellular adhesion, migration, proliferation, differentiation, and survival (O'Brien 2011; Olson et al., 2011).

Hyaluronic acid (hyaluronan, HA) is a non-sulfated linear glycosaminoglycan composed of repeating units of D-glucuronic acid and N-acetylglucosamine in the ECM that is widely used in medicine and tissue engineering applications due to its unique properties including hygroscopic and viscoelastic properties, biocompatibility, biodegradability and non-immunogenicity (Necas et al., 2008; Menaa et al., 2011). In addition to lubrication of the joints, tissue homeostasis and remodeling, wound healing, tissue repair, regeneration and organization of the ECM, HA plays critical roles in embryogenesis, morphogenesis, angiogenesis, cell adhesion, migration, proliferation, differentiation and cancer invasion (Kogan et al., 2007; Menaa et al., 2011; Solis et al., 2012).

For tissue engineering and regenerative medicine applications, an ideal cell source is required that can be harvested abundantly with minimal invasion, possesses the desired differentiation potential and be safe and efficient after transplantation (Gimble et al., 2007; Frese et al., 2016). Mesenchymal stem cells, derived from different sources are acceptable types of stem cells for tissue engineering and regenerative medicine (Kiarashi et al., 2024). Adipose derived mesenchymal stem cells (Ad-MSCs) are a population of adult multipotent stem cells with higher abundance in adipose tissue compared to bone marrow, while their derivation is less invasive (Baer and Geiger 2012; Miana and Gonzalez 2018). The presence of the HA in the stem cells niche, indicates that it might be a key component in regulating stem cells activities (Solis et al., 2012).
In this study, we attempted to prepare a natural collagen scaffold from human gingival stroma, and the effects of HA were investigated on proliferation and migration of human Ad-MSCs seeded on the scaffolds, *via* histological investigation.

Materials and Methods

Materials

For cell culture, Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS) and trypsin/EDTA were purchased from Gibco, and penicillin/streptomycin was prepared from Biosera. Chemicals used for decellularization including sodium dodecyl sulfate (SDS) and Triton X-100 were bought from CinnaGen, and HA was provided by Sigma-Aldrich (average MW 25,000, Linear Formula: $(C_{16}H_{27}NO_{11})n$). Moreover, for histological studies, hematoxylin and eosin (H&E), picrofuchsin and 4′,6-diamidino-2-phenylindole (DAPI) were purchased from Merck.

Preparing a natural scaffold from human gingival matrix

Human gingival tissue pieces were provided by a dental specialist with informed consents of the patients. The samples were transported in physiological serum to the laboratory and cut into approximate dimensions of 5×5×5 mm. After one-week slow-freezing at 0˚C, gingival pieces were put in cryotubes and frozen in liquid nitrogen for 2 min followed by defrosting at room temperature for 5 min. This snap freeze-thaw, was repeated for 6 times. For chemical decellularization, samples were placed in 1% SDS solution for 24 h and then transferred to 1% Triton X-100 in phosphate-buffered saline (PBS) for 12 h (Mahdavishahri et al., 2012).

Sterilization and soaking of the scaffolds in HA solution

For removing the detergents from the scaffolds and prevention of microbial contamination, the prepared scaffolds were washed several times with sterile distilled water, and then they were placed in sterile distilled water, 70% ethanol and sterile PBS for 1 h, 30 min and 1 h, respectively. The scaffolds were divided into two groups: soaked in HA and those without HA. In control group, 8 scaffolds were put in 12 well-plates (Orange Scientific) containing DMEM supplemented with 15% FBS and penicillin/streptomycin, and 8 scaffolds in the test group were additionally treated with 0.3% w/v HA in PBS solution (0.06 g of hyaluronic acid powder was dissolved in 20 ml of PBS). After that, both groups were incubated at 37˚C in 5% CO_2 in air for 24 h.

Seeding human Ad-MSCs on the scaffolds

After 24 h, 8 samples including 4 scaffolds with HA and 4 scaffolds without HA, were put in a 12 well-plate and seeded with Ad-MSCs; and another 8 samples including 4 scaffolds with HA and 4 without

HA, were maintained in similar conditions without cells. Human Ad-MSCs were derived in our laboratory as described previously (Haddad-Mashadrizeh et al., 2013). The Ad-MSCs were trypsinized and seeded at a concentration of ۳×10^5 cells on each scaffold belonging to the two subgroups containing cells. Then 2 ml culture medium was gently added into each well and all the four subgroups were transferred to CO_2 incubator. The culture medium was changed every 2-3 days.

Histological studies

Histological studies were performed in 4 groups of samples including natural and decellularized gingiva, soaked and non-soaked scaffolds. They were fixed for 24 h in Bouin's and paraformaldehyde solutions. Subsequently they were dehydrated by graded ethanol concentrations in ascending order, embedded in paraffin and cut into 6 µm sections. Slides were then stained with H&E, picrofuchsin and DAPI using standard protocols.

Statistical analysis

Statistical analysis was performed using GraphPad InStat software and one-way analysis of variance (ANOVA).

Results

Efficiency of decellularization protocol

Histological study of sections stained with H&E before and after decellularization process demonstrated the absence of cell nuclei from human gingival connective tissue. Moreover, the cellular elimination from prepared scaffolds was also confirmed with DAPI staining and fluorescence microscopy. In order to assess the collagen content of connective tissue that has inductive effects on the cells, picrofuchsin staining was performed and histological findings indicated that collagen was significantly preserved in the extracellular matrix (Figure 1).

Ad-MSCs behavioral responses in gingival scaffolds in the presence or absence of HA

For assessment of Ad-MSCs behavior in gingival scaffolds with and without HA, 4 different samples were harvested at each time point on days 7, 14, 21 and 28 after culture, and each sample was stained with H&E, picrofuchsin and DAPI, separately. The results related to each time point are explained in the following sections.

Day 7 after culture

Histological analysis of the scaffolds on day 7 in the two groups with and without HA, indicated adhesion of Ad-MSCs to the scaffold and the appearance of single layer and multilayer epithelium-like structures on the scaffolds. Moreover, the cells started to migrate either individually or as colonies into the scaffolds.

Analyzing the scaffolds during the first week of culture revealed higher numbers of Ad-MSCs over the scaffolds and also increased migration of the cells into the scaffolds in the presence of HA as compared with the control group without HA (Figure 2). Furthermore, as shown in Figure 3, in the scaffolds containing HA some cells were observed in different stages of mitosis (Figure 3).

Day 14 after culture

Histological study of the sections in the second week of culture indicated that plenty of Ad-MSCs were still present on the scaffolds and their migration pattern was similar to the first week. Epithelium-like structures of Ad-MSCs were observed in greater surface areas of the scaffolds, however, the cells were being separated from the scaffolds and in places of their penetration, scaffold destruction could be observed (although this detachment might be due to tissue preparation procedure as well) (Figure 4).

Day 21 after culture

During the third week, cell density and cell penetration were decreased so that Ad-MSCs were observed only in some parts of the scaffolds (Figure 5).

Day 28 after culture

Analyzing scaffold sections illustrated that the number of Ad-MSCs was substantially reduced and just limited to one part of the scaffold surface. In this week, cell nuclei were condensed, the size of the cells was decreased and cell migration was minimal (Figure 6).

Statistical results

In order to assess changes in cell density in different groups, cells were counted (per surface unit) and as seen in Figure 7A, there was a significant difference between weeks 1 and 2 with weeks 3 and 4 (P<0.01), and also between week 3 and 4 (P<0.001). Comparing the cell densities in the presence and absence of HA at different time points indicated that a significant difference was evident

(P<0.01) between the two groups only on week 1 (Figure 7A).

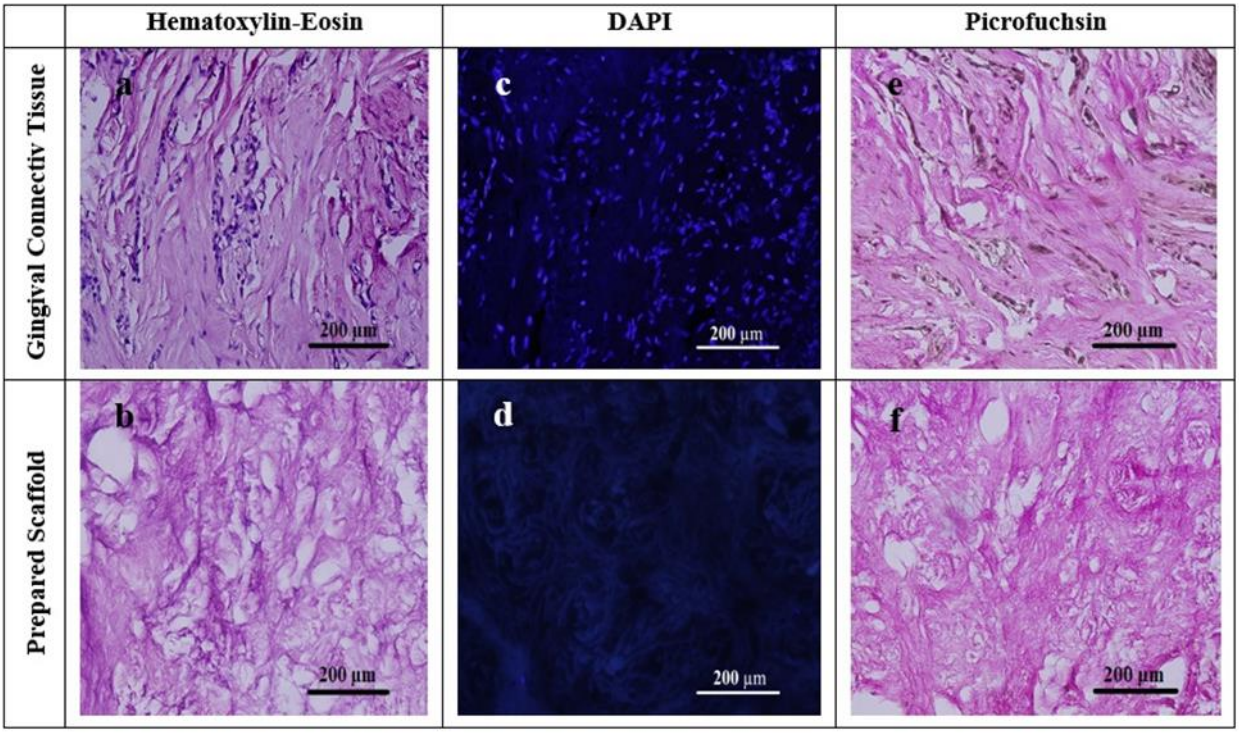

Figure 1. Histological assessment of decellularization efficiency. H&E (**a, b**) and DAPI (**c, d**) staining show the absence of nuclei and cell debris. Picrofuchsin staining (**e, f**) confirms preservation of collagenous structure of human gingival connective tissue after the decellularization process (Scale bars = 200 µm).

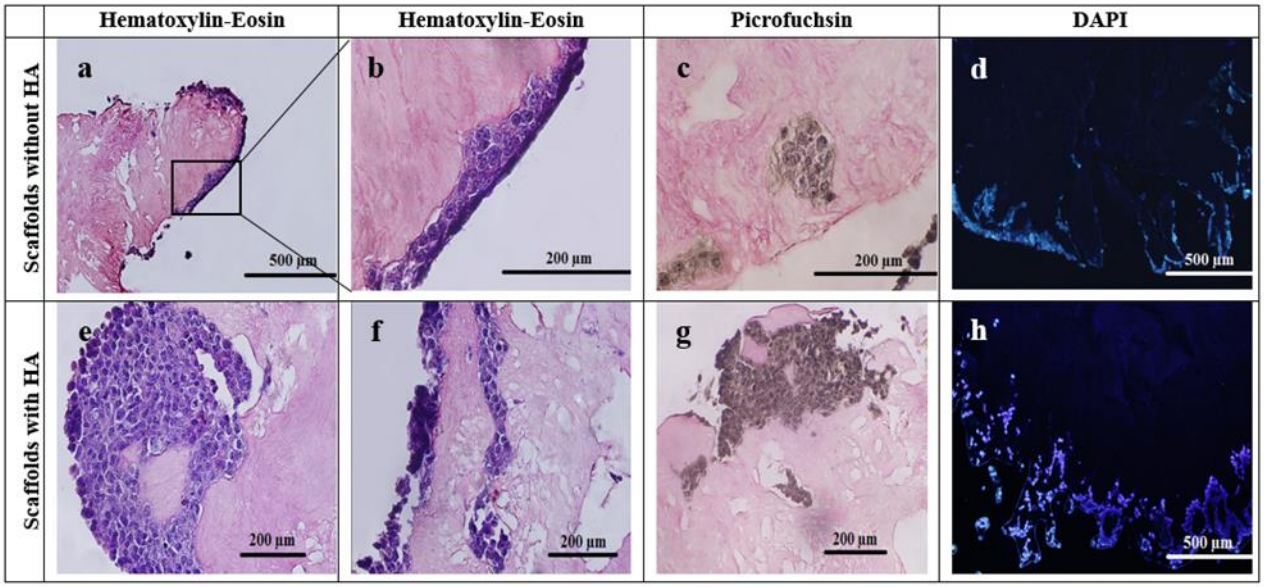

Figure 2. Ad-MSCs behavior after one week of culture on gingival scaffolds without or with HA. H&E (**a, b, e, f**), picrofuchsin (**c, g**) and DAPI (**d, h**) staining show higher number of cells and also increased migration of Ad-MSCs in scaffolds containing HA. **a** and **b** demonstrate formation of epithelium-like structures on the scaffolds. Figure **c** shows penetration of a group of Ad-MSCs into the gingival scaffold. Figure **e, f** and **g** indicate adhesion and migration of mass of cells in scaffolds soaked in HA. Figure **d** and **h** are related to DAPI staining of Ad-MSCs cultured on the scaffolds without or with HA. Scale bars in (**a, d, h**) = 500 µm and in (**b, c, e, f, g**) = 200 µm.

Assessment of cell penetration in gingival scaffolds without HA indicated that Ad-MSC migration at week 2 was significantly higher than week 1 (P<0.001), 3 (P<0.01) and 4 (P<0.001).

Analyzing different samples in which cells were grown on scaffolds containing HA showed that cells had a much better migration on week 1 compared with other three time points i.e. weeks 2, 3 and 4 (P<0.001). Furthermore, comparing the two groups at each time point revealed a significant increase in migration of cells cultured on gingival scaffolds with HA only in the first week of culture (P<0.001) (Figure 7B).

Comparing the changes in nuclear size of Ad-MSCs cultured on the scaffolds without HA at different times indicated significant differences between week 1 with 3 (P<0.05) and 4 and also between week 2 and 4 (P<0.001) and between week 3 and 4 (P<0.01). Ad-MSCs cultured on gingival scaffolds containing HA exhibited a significant decrement in nuclear size from week 1 to weeks 2, 3 and 4 (P<0.001). They also showed significant difference between week 2 and 4 (P<0.01) and between week 3 and 4 (P<0.05). A significant increase in nuclear size was noted at first week in cells cultured on the scaffolds with HA compared with those without HA (P<0.01) (Figure 7C).

Discussion

Application of extracellular matrix from natural sources would be very advantageous for tissue engineering purposes (Eltom et al., 2019). Collagen is one of the most popular biomaterials which is abundant in the ECM (Shahraki et al., 2021).

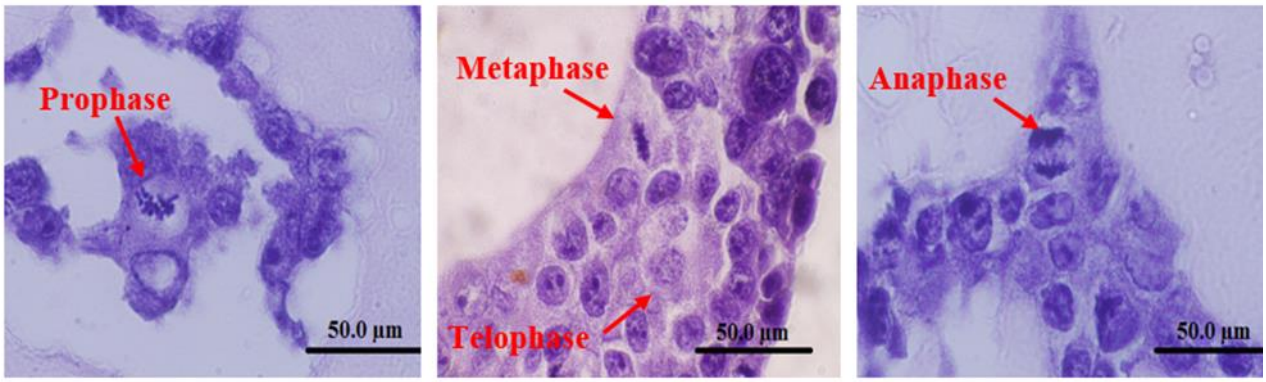

Figure 3. H&E staining representing 4 different stages of cell division in some cells cultured on the scaffolds containing HA for one week (Scale bars = 50 µm).

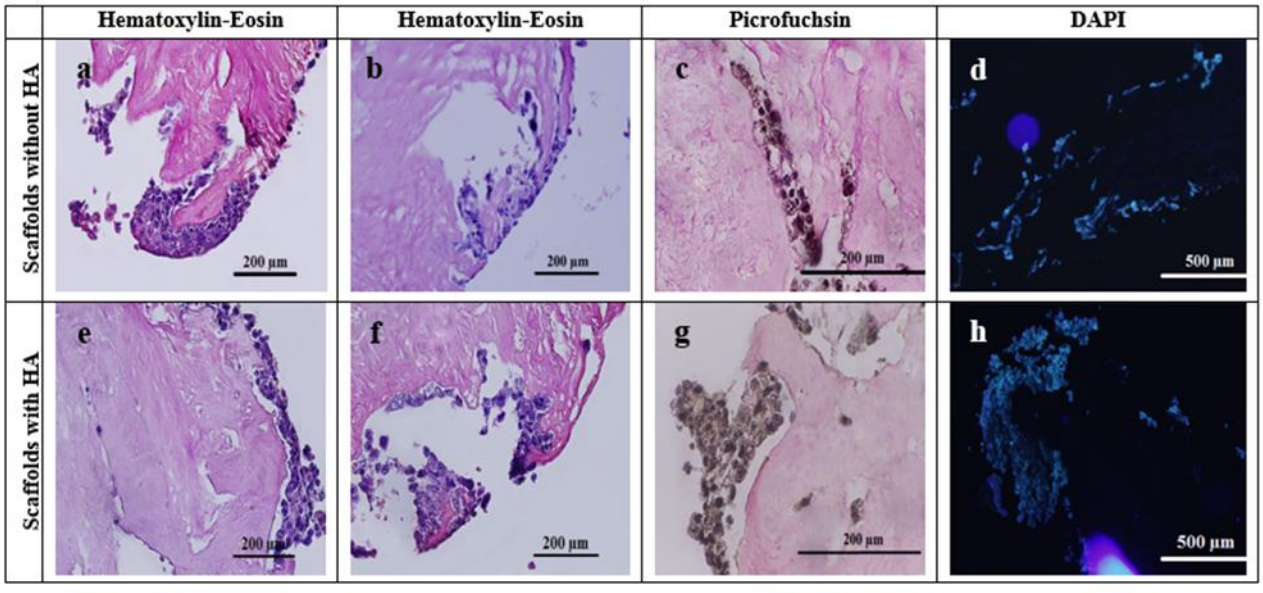

Figure 4. Ad-MSCs behavior after two weeks of culture on gingival scaffolds in the absence or presence of HA. Figures **a-c** indicate cells placement as a multilayer structure on the scaffolds, their migration to inner parts and scaffold destruction in places of Ad-MSCs penetration. Figures **e-g** show the presence of cells on the scaffolds, cell penetration and also scaffold destruction in places of Ad-MSCs penetration, in the presence of HA. Figure **d** and **h**: DAPI staining of Ad-MSCs cultured on gingival scaffolds. Scale bars in (**d, h**) = 500 µm and in (**a, b, c, e, f, g**) = 200 µm.

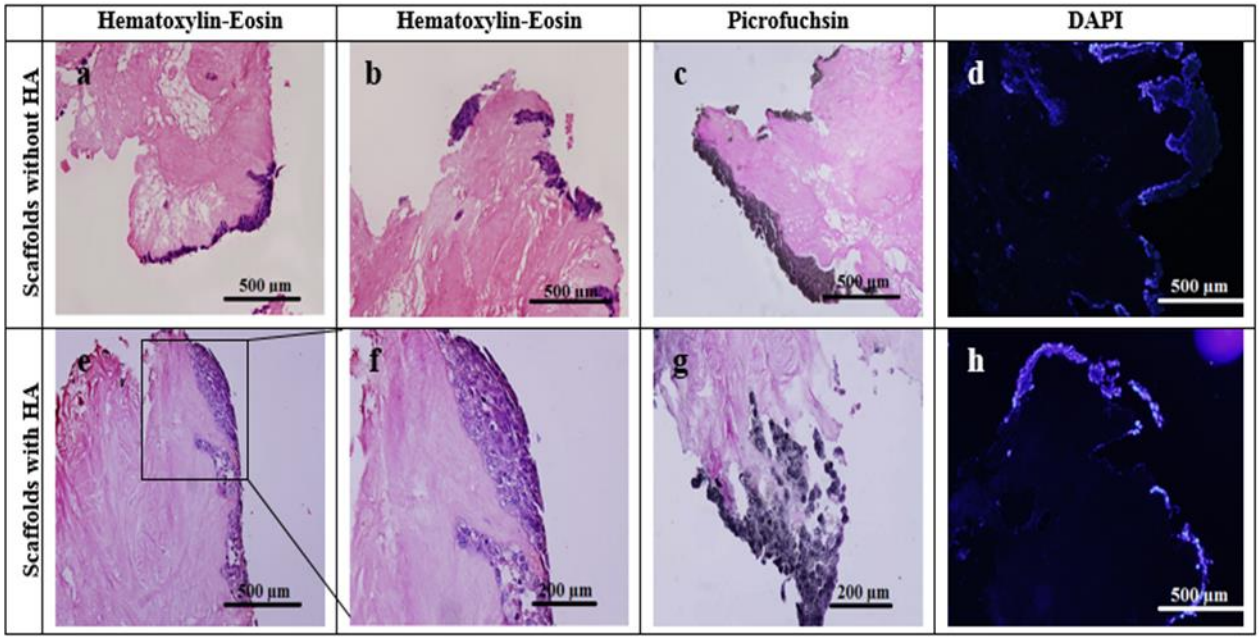

Figure 5. Ad-MSCs behavior after three weeks of culture on gingival scaffolds without or with HA. Figures show a decrease in cell density and cell penetration. Ad-MSCs could be observed only in some places of the scaffolds. Scale bars in (**a, b, c, d, e, h**) = 500 µm and in **f, g** = 200 µm.

Decellularized extracellular matrices have attracted great attention in the last decades due to their high bioactivity and tissue specificity and have been used for successful regeneration of several tissues and organs like aorta, tendon, kidney, heart, liver, and periodontal tissue (Kazemi et al., 2021; Khakpour et al., 2022; Shahraki et al., 2022; Shahraki et al., 2021; Taylor et al., 2018; Zhang et al., 2022; Liang et al., 2023).

In this study, we aimed to investigate the effects of soaking decellularized gingival matrix in HA on proliferation and migration of human Ad-MSCs. Development of 3D porous matrices for tissue engineering has been the focus of intensive research in pervious years (Kamali Baratpoor and Matin, 2025; Zhang et al., 2011; Cai et al., 2012; Dijkman et al., 2012; Sackett et al., 2018). The ECM is a porous network of proteins and glycosaminoglycans (GAGs) that can be altered by cross-linking and enhancing the barrier function of the matrix. In the current study, we first decellularized human gingival tissue to prepare a natural collagen scaffold. We previously showed that decellularized gingival tissue can be used as a natural and suitable scaffold for skin tissue engineering applications because of its capability to support attachment and differentiation of bone marrow mesenchymal stem cells towards keratinocytes (Mahdavishahri et al., 2012). *In vitro* experiments conducted by Naderi et al., demonstrated that decellularized palatal gingival tissue was a useful scaffold for studying cellular behavior (Naderi et al., 2013). The importance of HA scaffolds for wound healing process, coupled with the ability of these scaffolds to provide an open, hydrated structure for the passage of nutrients make them ideal candidates for tissue regeneration and repair (Collins et al., 2013).

Ivanov and colleagues in 2023 reported the effect of ECM containing HA on the differentiation of periodontal stem cells in a collagen I-hydrogel. They demonstrated this ability *via* the expression of osteogenic and odontogenic differentiation markers (Ivanov et al., 2023). These effects should be due to the tendency of HA to interact with surface receptors of stem cells, intracellular signal transduction and affecting cellular activities (Zhu et al., 2017). Meng et al., demonstrated chondrogenesis-inducing capacity of the TCP-COL-HA scaffold on human MSCs. Furthermore, the expression of CD44 was increased, suggesting the interaction between human MSCs and HA (Meng et al., 2014). Zou et al., investigated the effects of different concentrations of hyaluronan on proliferation of porcine BM-MSCs *in vitro*. They demonstrated that hyaluronan stimulated cell proliferation and differentiation and also secretion of some enzymes such as alkaline phosphatase (Zou et al., 2004). The experiments conducted by Choi et al., demonstrated increasing effects of HA oligosaccharides on differentiation of epidermis in skin equivalent models. They also showed an elevation in the expression of skin stem cell marker P63 that promotes basal stem cell

survival (Choi et al., 2012). Another study indicated that MSCs cultured in chitosan-HA membranes formed spheroids that assist to maintain the expression of stemness markers (OCT4, SOX2, and NANOG). By blocking CD44 with antibodies, MSCs could not maintain their spherical form, and stemness gene expression declined (Huang et al., 2011).

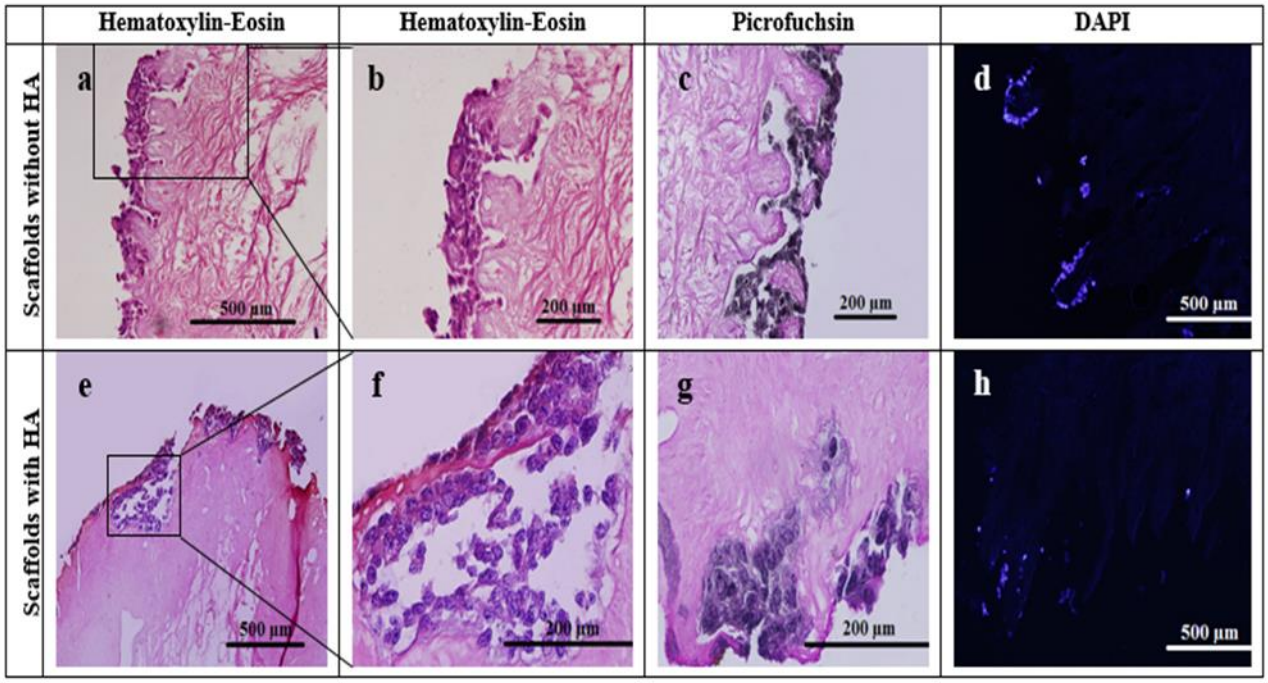

Figure 6. Ad-MSCs behavior after four weeks of culture on gingival scaffolds in the absence or presence of HA. Figures illustrate a drastic decrease in cell density; Ad-MSCs were present in very limited places of the scaffolds. Scale bars in (**a, d, e, h**) = 500 µm and in (**b, c, f, g**) = 200 µm.

A common method to improve the physicochemical properties of hyaluronic acid-based materials is to mix HA with other biopolymers such as chitosan, alginate and chondroitin sulphate (Grabska-Zielińska et al., 2021). Perng et al., compared the angiogenic properties including the rate of vascularization between short-chain and long-chain HA when added to a porous scaffold with cross-linked type I collagen, and showed that collagen scaffolds with short-chain HA revascularize faster than those with long-chain HA and collagen (Perng et al., 2011). In this study, after decellularization treatment of human gingival connective tissue, picrofuchsin staining confirmed preservation of the collagenous structure of the tissue. In the next step, we compared Ad-MSCs behavior in gingival scaffolds in the presence or absence of HA. As mentioned before, results indicated adhesion of Ad-MSCs to the scaffolds and appearance of single layer and multilayer epithelium-like structures. Moreover, the cells started to migrate either individually or as colonies into the scaffolds. Higher numbers of Ad-MSCs over the scaffolds at 1st week of culture was demonstrated with HA as compared with the other group without HA. Intercellular signal transduction of HA *via* interaction with cell surface receptors (hyaladherins) such as CD44 and receptor for hyaluronan-mediated motility (RHAMM), can induce cell survival, proliferation, migration and differentiation (Park et al., 2011; Menaa et al., 2011). Furthermore, in the scaffolds containing HA some cells can be observed in different stages of mitosis, which might be due to the influence of HA on induction of cell division. However, from the third week, cell density and cell penetration were significantly decreased and limited to some parts of the scaffolds. Since most cells need to be attached to a matrix to survive, destruction of this matrix *via* matrix metalloproteinases (MMPs) might have led to induction of apoptosis. Nuclear condensation in the fourth week could be an evidence of apoptosis. MMPs can increase apoptosis through a kind of cell death named “Anoikis” that is triggered by insufficient cell-matrix interactions (Verma and Hansch, 2007). Frisch and Francis reported Anoikis in two different epithelial cell lines due to reduction of cell-matrix interactions (Frisch and Francis, 1994). So, it can be concluded that proteolytic activity of cells and increasing MMPs to open the path of migration into the scaffold, might

have led to destruction of the scaffold, decreasing cell-matrix interactions, induction of apoptosis and finally reducing cell density in the third and fourth weeks of culture. Thus, soaking decellularized scaffolds in HA utilized in this study may have induced the adhesion, migration and proliferation of cultured Ad-MSCs.

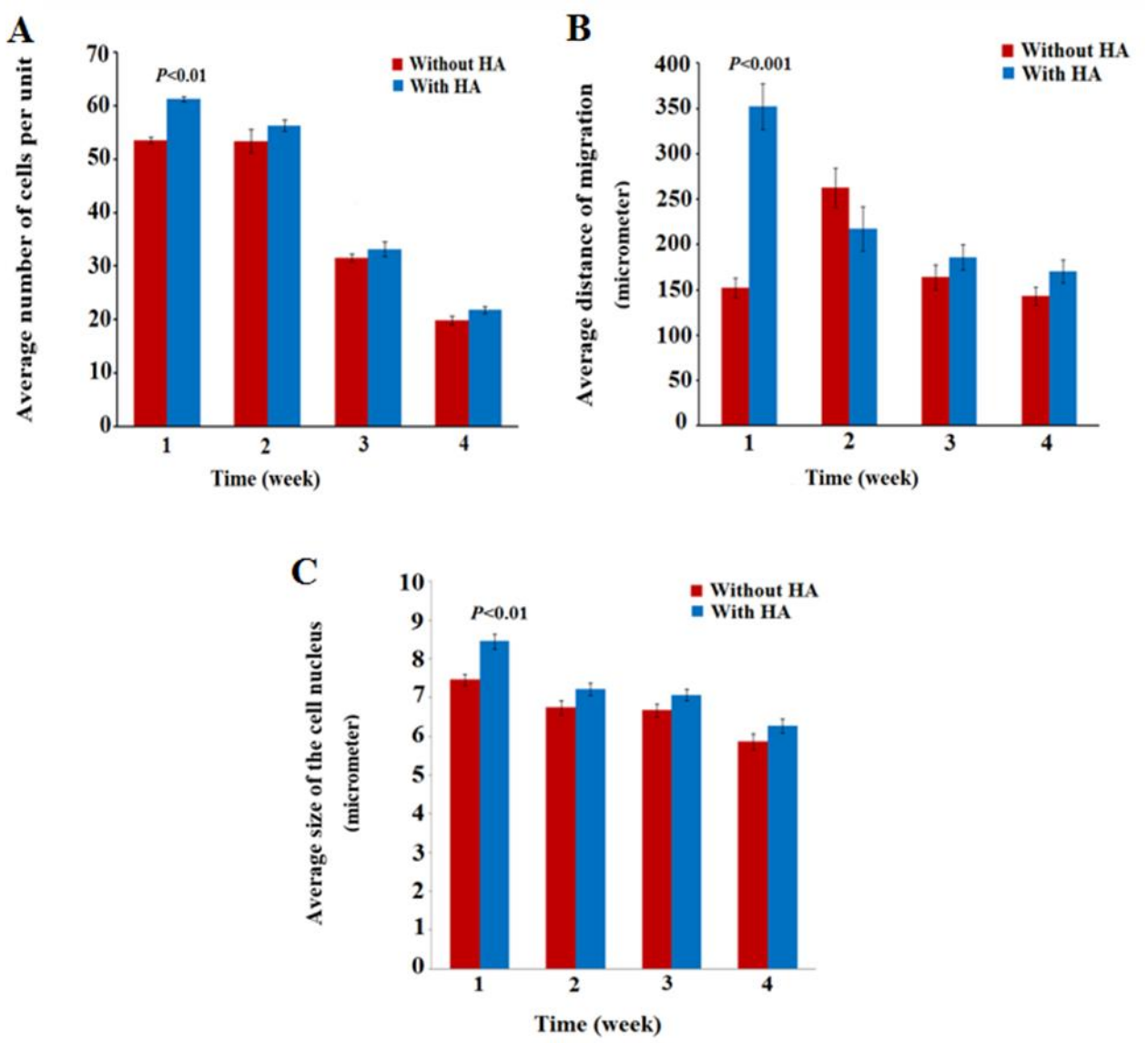

Figure 7. Assessment of cell density, penetration and size of the Ad-MSCs nuclei cultured on gingival scaffolds without or with HA at different time points. **A**. Average number of cells per surface unit in each scaffold at different weeks of culture. **B.** Average distance of Ad-MSCs migration in each group of scaffolds without or with HA at different times of culture. **C.** Average size of Ad-MSCs nuclei in each scaffold on different weeks of culture. Data are analyzed using GraphPad InStat software and one-way analysis of variance (ANOVA).

Limitations of the study

There were some limitations in this research that might be considered in the future studies: For example, decellularization process and the effects of HA could be assessed by both evaluating the DNA content and analyzing the ECM integrity and performance by immunohistochemistry and mechanical tests. Moreover, the residual detergents in the scaffolds were not thoroughly evaluated. Cell proliferation and apoptosis could also be assessed using specific antibodies.

Conclusion

The results of the present study demonstrated that the extracellular matrix of decellularized human gingival stroma can induce cellular behaviors such as adhesion, proliferation and migration of human Ad-MSCs, however immunohistochemical and

molecular analyses are required to confirm these effects. Our findings illustrated that soaking the prepared scaffolds in hyaluronic acid could increase the proliferation and migration of Ad-MSCs especially at the first week. However, various concentrations and molecular weights of HA and different ways to add it to the scaffold will contribute to precise assessment of its impacts on various cellular behaviors.

Author contributions
Conceived and designed the experiments: MA, HM, NMS, MF and MMM; Performed the experiments: MA; Analyzed the data: MA, HM, NMS, MF, RL and MMM; Supervised the experiments: NMS and MMM; Wrote the manuscript: HM, MA and MMM; All authors read and approved the final manuscript.

Ethics approval
The Research Ethics Committee of Ferdowsi University of Mashhad approved the research by approval number "IR.UM.REC.1402.116".

Acknowledgments
The authors would like to thank Ferdowsi University of Mashhad for supporting the study.

Funding
This work was supported by Ferdowsi University of Mashhad (Grant number: 22500).

Conflict of interests
The authors declare that there is no financial or non-financial competing interests.

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