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Preparation of three-dimensional matrices of human gingival tissue and comparison of induction of mesenchymal and blastema stem cell behaviors in prepared scaffolds

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ABSTRACT

This study aimed to compare the behavior of rat bone marrow mesenchymal stem cells and rabbit auricle blastema cells implanted in non-cellular gingival tissue scaffold of human. In this regard, the tissues obtained from gingival surgeries in the dental clinic were de-cellulated using two detergents of sodium dodecyl sulfate and triton 100-X. After washing and sterilization, they were used as a scaffold for culture with bone marrow mesenchymal stem cells of rats. Using light and electron microscopy, these scaffolds were examined before and after 1, 2, and 4 weeks of cell culture. Also, the prepared three-dimensional scaffold was placed in the blastema ring obtained from the rabbit earlobe punch. The samples were evaluated 1, 2, and 4 weeks after culture based on histology techniques. The results showed that the study of scaffolds by electron microscopy showed preserving the epithelium matrix and the collagen fibers in the tissue. Structures similar to the epithelium were created in both samples. In addition, induction of cellular secretion was observed in scaffold cells migrating to the scaffold. In general, scaffolds made from human gums can be a good platform for studying cellular behaviors. Of course, further experiments to determine the nature of differentiated cells can help advance our knowledge of matrix cell interactions.

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Introduction

Each tissue in the body comprises cells and an extracellular matrix that acts as a three-dimensional scaffold. The cells are located on the surface of this scaffold and are constantly interacting with it (1). Synthetic and natural scaffolds are scaffolds used in tissue engineering (2). Because biological scaffolds are derived from decellularized tissues and organs, cellular antigens are removed, and many of the structural and functional proteins of the extracellular matrix are conserved, these scaffolds have been used successfully by engineers (3).

The decellularization process aims to remove cellular and nuclear material while maintaining the integrity and minimizing any damage to the composition and biological activity of the extracellular matrix (4). So far, many studies have been done on scaffolding preparation methods, and various tissues, including bladder, vascular system, heart valves, knee cartilage, ligament, and tendon, have been used to produce tissue substitutes cell replacement. In many of these studies, sodium dodecyl sulfate (SDS) or triton has been used for tissue decellularization (5, 6).

The oral mucosa comprises two components: the stratified squamous epithelium and the connective tissue beneath it (7). In connective tissue, two main types of collagen (primarily types I and III) and elastin are observed. This tissue has cellular and extracellular components that include fibers and substrate material. This substrate fills the space between cells and fibers and contains a large amount of water, proteoglycans (hyaluronic acid and chondroitin sulfate), and glycoproteins (mainly fibronectin) (8). Yamada *et al*. (9) cultured gingival fibroblasts on sponges of recombinant human type I

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and III collagen and placed the sponge in a culture medium. After a few days in type III collagen-containing scaffold, the proliferation of fibroblasts was higher than that of type I. Izumi *et al*. (10) cultured epithelial cells taken from the patient's oral mucosa on a cell-free allogeneic dermal matrix (alloderm) four weeks before surgery and used it for intraoral transplantation.

One of the cells used in various tissue engineering is mesenchymal stem cells (7, 11). Initially, it was thought that the differentiation potential of these cells was limited to mesenchymal cell lines. However, it was later found that they can also differentiate into cells of non-mesenchymal origin under different culture conditions. Therefore, mesenchymal stem cells have been used to treat spinal cord injuries, myocardial ischemia, bone injury, neurodegenerative diseases, and skin (12, 13). Cells undergo cytoskeletal and morphological changes during keratinocyte differentiation. The main morphological difference is the progressive expansion of the cell. Also, various membranous bodies, including keratohyalin granules, appear inside the cell. In addition, desmosomal junctions are formed between cells, and as the differentiation process progresses, most of the macromolecules and intracellular organs are destroyed (14). When a wound is created, the keratinocytes in the vicinity of the wound and the keratinocytes in the skin appendages migrate to the wound bed, forming small islets and participating in the regeneration of the epithelium (15). Plasma tissue cells, which form circularly in the perforated area of the rabbit's earlobe, are stem cell-specific cells that can differentiate in different directions under the influence of environmental factors (16).

This study aimed to investigate the interaction between mesenchymal stem cells and earlobe blastema tissue cells of rabbits in a natural 3D scaffold obtained from human gingival tissue.

Materials and methods

Human gingival specimens were prepared by a specialist and a gingival surgeon in a specialized dental clinic and complied with the relevant ethical principles. The samples belonged to men and women between 20 and 40 years old that did not have systemic diseases and were non-smokers referred to the clinic for restorative-prosthetic treatments and impacted wisdom tooth surgery. Gingival fragments from the surgeries were stored in physiological saline at 0°C.

After removing the extra pieces, the size of the tissues reached 3×6 mm. To decellularize the tissues, the samples were immersed in liquid nitrogen for 2 minutes for rapid freezing and then dipped in distilled water for 5 minutes for rapid thawing. This freezing-thawing process was repeated six times, followed by using 1% sodium dodecyl sulfate (Merck, Germany) for 24 hours and then 1% Triton 100-X (Merck, Germany) for 12 hours. To remove detergents from the tissue, the samples were placed in Phosphate-buffered saline (PBS) (Bio-Gene, UK) for 2 hours (17). To sterilize the scaffolds, the samples were placed in 70% ethanol for 30 minutes, and after washing with sterile distilled water, they were placed in sterile PBS for 1 hour.

Adult male Wistar rats were anesthetized using chloroform to prepare bone marrow mesenchymal stem cells (BM-MSCs), and the femur was isolated under completely sterile conditions. The two bone heads were first removed, and the contents were injected in Dulbecco's Modified Eagle's Medium (DMEM) (Gibco, USA). Also, 10% fetal bovine serum (FBS) (Gibco, USA) and ten microliters of penicillin/streptomycin (Biosera, UK) were added to DMEM. The suspension was then transferred to a culture flask and stored at 37°C and 5% CO_2 in an incubator. Twenty-four hours after initial culture, the supernatant was removed and the cells adhered to the bottom of the flask (18). The culture medium was changed every 3 to 4 days, and the cells were used after the fourth passage.

In group A, to culture cells on decellularized gingival scaffolds, they were first trypsinized to separate them from the flask. After centrifugation and determining the number of cells with neobar slide, a cell suspension with a density of 2×10^5 cells in 50µl was prepared. After 24 hours in the culture medium, the scaffolds were placed in 24 house culture dishes, and 2×10^5 bone marrow mesenchymal stem cells were cultured on them (Figure 1A). One hour after adding the cell suspension to the scaffolds, 1ml of culture medium was added to each well, the culture vessel was transferred to the incubator, and the culture medium was changed every 3 to 4 days.

In group B, New Zealand white rabbits aged 6 to 8 months with a weight of approximately 2.5 kg were used to prepare blastema tissue. At all times of use, these animals were placed in a cage individually on an elemental diet with a controlled temperature of 20°C and light of 12 hours a day. In all stages of this research, approvals related to the principles of working with laboratory animals were observed. After lidocaine anesthesia of the rabbit's ear, a 2 mm diameter hole was made in the rabbit's earlobe using a punching machine. After removing the blastema ring, the rings were washed seven times with sterile physiological serum and then under the laminar hood for decontamination. The prepared scaffolds were placed among the ready blastema rings (Figure 1B). This study used a DMEM culture medium and 37 ° C with 5% CO_2.

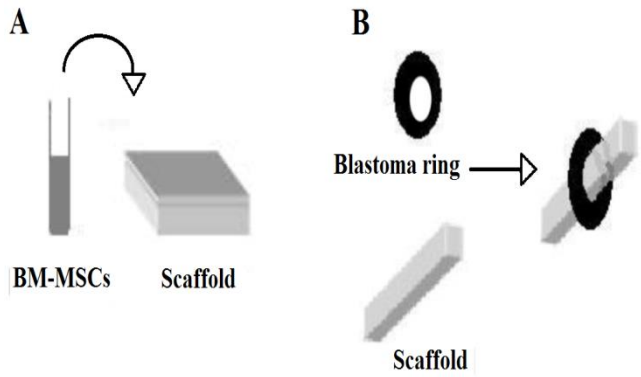

Figure 1. (A) Culture of bone marrow mesenchymal stem cells on a scaffold. (B) Placement of the scaffold inside the blastema ring.

In histological studies, to study the migration of cells to the scaffold, the tissue passage steps were performed according to the usual method, and Hematoxylin-eosin (H&E) staining, picrofuscin, indigo carmine peak, and PAS-peak indigo staining were performed. In picrofuscin staining (100 ml mixture of saturated picric acid + 0.1 g of fuchsin acid powder), collagen turns fiery red, and the epithelium turns yellow or cream. Indigo Peak Color (mixture of 100 ml of saturated picric acid + 0.1 g of indigo carmine powder) is a specific dye with different acidophilic intensities. With this color, the background, the connective tissue matrix, and collagen turn light green with the epithelium, and the nuclei become pale brown with hematoxylin. Periodic acid-Schiff reagent (PAS) is used to search for glycoproteins. In the PAS technique, mucus compounds containing sialic acid and hexoses turn a glossy pink or reddish-purple color. In scanning electron microscope (SEM) studies (Leo-VP1450, Germany), the samples were fixed with glutaraldehyde and osmium tetraoxide for specimen imaging and dewatered with ascending degrees of ethanol. After drying, the samples were placed on a grid and covered with gold-palladium and photographed. In the examination of transmission electron microscopy (TEM) (Leo-910, Germany) to prepare microscopic sections, samples were fixed with glutaraldehyde and osmium tetraoxide dehydrated with elevated ethanol. After impregnation with resin, they were molded. Then, thin sections with a thickness of 80nm were prepared, observed, and photographed by TEM.

Results and discussion

In this study, human gingival tissue was decellularized during different stages and used as a scaffold and inducers to differentiate rat BM-MSCs and blast cells. Rapid freezing in liquid nitrogen, 1% SDS for 24 hours, and 1% triton for 12 hours were used for descaling. Cellular components were completely removed from the gingival tissue (Figure 2).

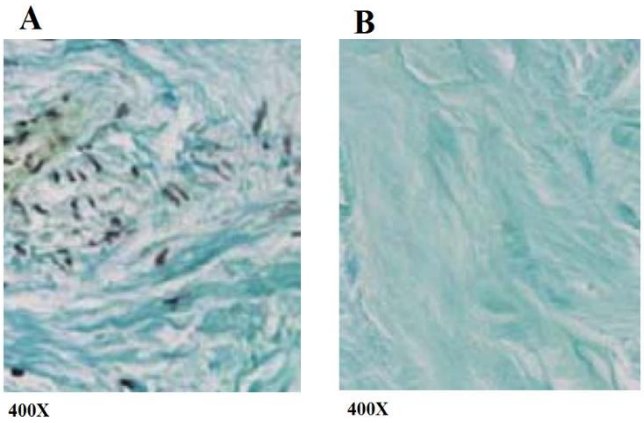

Figure 2. Human gingival tissue before (A) and after (B) the process of decellularization; staining was indigo carmine.

The prepared scaffold was considered using SEM to study the structural changes of gingival tissue during the preparation processes. As Figure 3 shows, the overall structure of the gingival tissue was preserved after the decellularization process (Figure 3A), and the gingival epithelium as an empty cell

network (Figure 3B) was attached to the remaining connective tissue. In addition, the connective tissue collagen fibers also remained intact (Figure 3C).

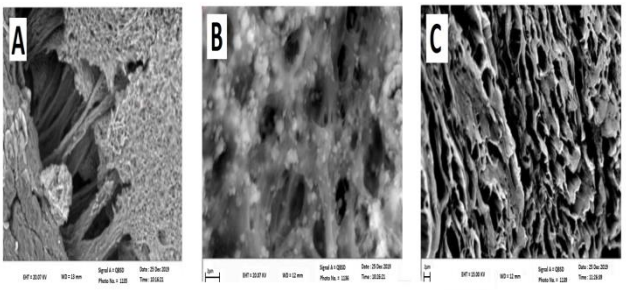

Figure 3. Human gingival tissue after decellularization with scanning electron microscopy; (A) preservation of the overall structure of the human gingival tissue after decellularization; (B) the epithelium as an empty cell network; (C) Collagen fibers after decellularization

In group A, mesenchymal stem cells taken from Wistar rat bone marrow were used for culture on the gingival scaffold. According to Figure 4, the cells were able to form a layer on the epithelium in the first week. In the second week, these cells proliferated and developed an epithelium-like structure. The cells were elongated and spindle-shaped in the fourth week and formed more stable structures. In the first week of transplantation, rats' bone marrow stem cells could settle on the scaffold and form Pseudopodia. In Figure 5, the pseudopodia of the cells are identified by SEM and TEM. Figure 6 shows the cells in the second week of transplantation under an electron microscope formed desmosomal junctions. In group B, after culturing the scaffold with blastema in the first week, cells from the blastema tissue were observed penetrating the scaffold. In the second week after culture, more cells infiltrated the scaffold, and the number of cells present in the scaffold increased. In the fourth week, epithelial-like structures were formed on the scaffold surface (Figure 4). In addition, in group B, from the second week, secretory behaviors (positive pass response) were observed in cells migrating to the scaffold (Figure 7 and Table 1).

Table 1. Observed cellular behaviors during the study period

Cell Type	Cellular Behavior			
	Adhesion (Desmosomal Junction)	Migration (Pseudopodia Formation)	Cell Division	Cell Secretion
Mesenchymal Stem Cells	Observed	Observed	Observed	Not observed
Blastema Cells	Observed	Observed	Observed	Observed

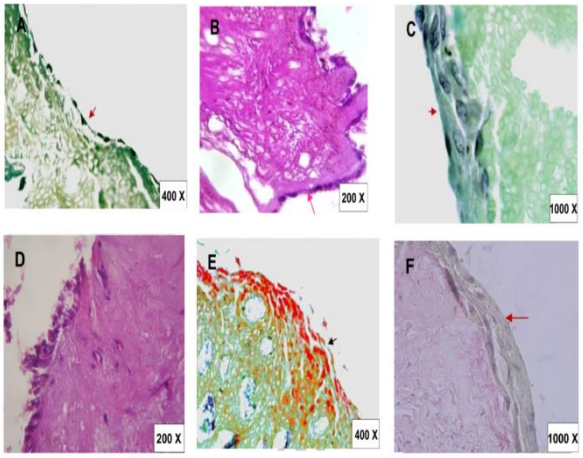

Figure 4. Comparison of the interaction of mesenchymal stem cells and blastema cells with the extracellular matrix of the human gingival; New epithelium-like layer in the first week (A), in the second week (C) (indigo peak staining), and in the fourth week (E) (carmine staining); Infiltration of blastema cells into the scaffold in the first week after culture (B), two weeks after culture (D) (hematoxylin-eosin staining) and epithelium-like layers formation in the fourth week (F) (picrofuscin staining)

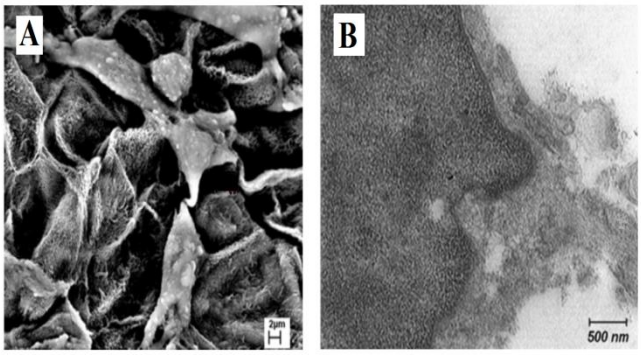

Figure 5. Formation of cell pseudopodia in the first week of mesenchymal stem cell culture; (A) Cells from SEM view, (B) Cell pseudopodia from TEM view

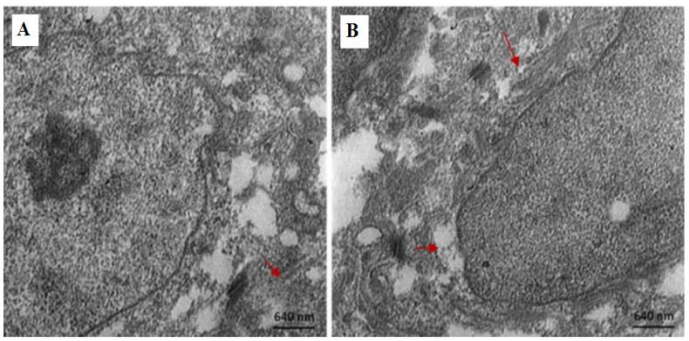

Figure 6. The formation of desmosomal junctions by mesenchymal stem cells in the second week of culture indicates a possible differentiation into keratinocyte-like cells (arrow sign indicates desmosomal junctions)

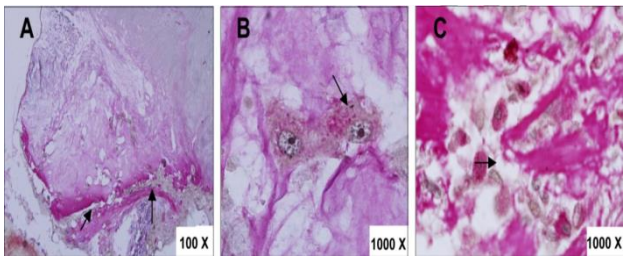

Figure 7. Cross-section of blastema ring with scaffold on different days after culture; (A) Arrows indicate discoloration of the scaffold margin due to cell infiltration two weeks after culture. (B) Scaffold-positive blast cells infiltrated the scaffold in the second week after transplantation. (C) Scaffold-positive pass blast cells infiltrated the scaffold four weeks after culture (PAS-peak indigo staining)

Human gingiva was first decellularized using physical and chemical methods in this study. This tissue was then used as a scaffold to inoculate mesenchymal stem cells from Wistar rat bone marrow (group A) and rabbit earlobe blast cells (group B).

In recent studies, decellularized cartilage matrices, bone, ureter, umbilical cord, and dermal tissues have been used as scaffolds in tissue engineering (19, 20). In this study, the decellularized matrix of the human gingiva was used as a scaffold to induce migration and cell differentiation due to its high levels of collagen and the presence of various cytokeratins in its connective tissue. Multiple studies have reported the destructive effects of decellularization and sterilization methods on tissue structure. However, according to electron microscopy studies, the gingival tissue maintained its overall structure after preparation, and the epithelium matrix remained attached to the underlying connective tissue. In addition, the collagen fibers in the connective tissue remained intact. Wu *et al*. (21) showed that BM-MSCs improved wound healing in diabetic and non-diabetic mice by increasing epidermal regeneration, cell permeability, and angiogenesis.

An essential part of these cells expressed cytokeratin subunits and formed granular structures similar to the sweat or sebaceous glands (22). In the present study, BM-MSCs flattened and formed a single layer after being placed on the anterior epithelium matrix. In the second week, the cells were divided and created a multilayered structure. TEM studies showed that these cells formed desmosomal junctions. These observations suggest that BM-MSCs may have begun to differentiate into keratinocytes by induction of human gingival scaffold. However, additional studies such as molecular methods or immunocytochemistry are necessary to determine the fate of keratinocytes. Studies showed that the gingival connective tissue matrix induces keratinization in cells (23, 24). In studies that used collagen hydrogels as dermal equivalents, poor epithelial stability on the scaffold, incomplete differentiation, and scaffold degradation were observed in the early stages (25). In contrast, the results of this study showed that the matrix remaining from the epithelium could be a suitable substrate for the strong attachment of cells to it.

A group of researchers cultured human gingival and dermal fibroblasts in a three-dimensional collagen matrix to study matrix changes and examined the secretion of extracellular matrix by these two cell types (26, 27). By looking at matrix metalloproteinases (MMPs), they found that the expression of these factors was first concentrated in the cellular cytoplasm of fibroblast cells and then distributed in extracellular compounds (28). Qi *et al.* (29) also showed differentiation of mesenchymal stem cells derived from rat synovium in alginate scaffold. They used several inducers of chondrogenic differentiation, including BMP-2, TGF-β3 proline, pyruvate, ascorbate-2-phosphate, and dexamethasone.

In the case of blast cell culture and the formation of epithelial-like structures, one of the points observed from the second week and intensified in the following days was the change in scaffold composition due to interaction with blast cell cells (Table 1). Areas of the scaffold that allowed infiltration and establishment of blastema cells showed a positive response to indigo PAS-peak color. Therefore, it can be concluded that in the present study, blastema cells started synthesizing and secreting compounds into the scaffold simultaneously with the penetration into the scaffold (Figure 7).

Conclusion

The decellularization matrix of the human gingival was able to cause the establishment of cells and the formation of epithelial-like structures in both groups A and B. Based on our findings in this study, it can be stated that cells derived from blastema tissue may have been more viable than mesenchymal stem cells because according to histological studies, in the fourth

week after culture, observable blastema cells had large and clear nuclei. In contrast, in the case of mesenchymal stem cells, the nuclei were observed to be necrotic. Therefore, blastemic tissue can probably be a more suitable source for studying the process of cell migration and induction of cell secretions.

Acknowledgments

None.

Conflict interest

The authors declare no conflict of interest.

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