ORIGINAL RESEARCH

Effect of Adipose Stem Cells Injection on Type VII and VIII Collagen Expression of Wistar Rat's Gingiva

Yolenta Alina Hale[1], Nurin Sobrina[2], Mohammed Ahmed Aljunaid[3], Agung Krismariono[4]

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Abstract

Aims: This study investigated the effect of injection of adipose stem cells (ASCs) on the expression of type VII and VIII collagen in Wistar rat's gingiva. Adipose stem cells can modulate the immune system, angiogenesis, wound healing, and extracellular matrix (ECM) remodeling.

Materials and methods: Ten Wistar rats aged three months were divided into two groups: the treatment group and the control group. The Wistar's gingival sulcus between the two incisor teeth was wounded with curettage. In the control group, PBS 1×10^6 was injected, meanwhile, the treatment group was injected with ASCs 1×10^6, then the Wistar rats were terminated on the 14th day and the expression of type VII and VIII collagen was observed, and examined using the immunohistochemical method.

Results: In Wistar rats injected with ASCs, collagen VIII increased more than type VII collagen.

Conclusion: Adipose stem cells can increase the expression of collagen VIII compared to collagen VII.

Clinical significance: Adipose stem cells can influence collagen VII and VIII expression because ASCs release growth factors to restore damaged tissue. Collagen VIII increased more than type VII collagen because type VIII collagen contains integrin receptors, which aid in extracellular protein matrix interactions. Adipose stem cells have multiple signal recognition molecules on the cell membrane, which can be used as potential carriers for drug delivery. So ASCs can be used as an effective and promising method for periodontal treatment.

Keywords: Collagen type VII, Extracellular matrix, Immune system, Stem cells, Wound healing.

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Introduction

Tissue engineering involves developing procedures for the creation and regeneration of connective tissues or organs by combining cells and scaffolds to form functioning organs.[1] The basic principle of tissue engineering is that cells, genes, and proteins are delivered via degradable materials, called scaffolds that function to regenerate tissue. In general, scaffolds have osteoconductive properties for cell attachment to support the process of new bone growth. However, as a good scaffold, it not only provides osteoconductive elements but also has osteoinductive properties which will stimulate osteogenic factors to accelerate the process of new bone formation.[1]

Collagen is one of the most commonly used scaffold materials for bone tissue engineering due to its excellent biocompatibility and biodegradability.[2] Collagen not only acts as a physical support for cells to attach to and to grow on but also influences cell behavior and fate through receptor-mediated interactions.[3] Collagen-based materials can be used for suturing, implants, wound dressings, and drug delivery systems, and can be developed into scaffolds with or without bioactive proteins and cells that have the potential to regenerate soft tissue and hard tissue such as skin and bone.[4] Collagen is found in various places throughout the body, including tendons, ligaments, bone layers, and the dermis.[5]

About 30% of all the protein in a mammal's body is composed of collagen, which is the most prevalent structural protein.[6] There are 28 distinct varieties of collagen were identified.[6,7] The morphology of collagen is identical with the literature as reported by previous research which mentions a protein fibril presence of the collagen. The shape of the fibril structure in the collagen is also appropriate as reported by other research.[8] Collagen can be categorized into multiple groups according to its properties: membrane collagen, collagen VI, VII, XXVI, and XXVIII, fibril-forming collagen, fibril-associated collagen with interrupted triple helices, tissue-forming collagen, and multi-plexin (collagen XV and XVIII).[9] The first and most prevalent category is fibrous collagen uninterrupted helices, especially collagen I, II, III, V, and XI. The second category consists of high molecular weight collagens that are linked to the basement membrane and include numerous non-helical intervening sequences, especially collagen IV and VII. The short-chain, non-domain helix makes up the third group, which includes types VI, VIII, IX, X, XII, and XIII.[10] Ligaments include trace amounts of type IV, V, VI, and VII collagen. The majority of the protein fraction in the basal lamina of blood vessels, neurovascular bundles, and the remaining PDL epithelium is composed of collagen types IV and VII.

Stem cells are specific cells with diverse potentials that can self-renew and develop into different kinds of cells.[11] Stem cells are classified into two groups according to where they came from

[1,2,4]Department of Periodontics, Faculty of Dental Medicine, Airlangga University, Surabaya, Indonesia

[3]Department of Dental Medicine, Faculty of Medicine, Taiz University, Taiz, Yemen

Corresponding Author: Agung Krismariono, Department of Periodontics, Faculty of Dental Medicine, Airlangga University, Surabaya, Indonesia, Phone: +082146474590, e-mail: agungkr@yahoo.com

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mesenchymal stem cells (MSCs) and embryonic stem cells (ESCs). One of the cells characterized by MSCs was from adipose tissue. Subcutaneous fat is the most relevant source of adipose tissue. Adipose stem cells (ASCs) can isolated from the subcutaneous adipose tissue of the arms, thighs, and belly. Adipose stem cells can enhance, maintain, or repair different kinds of networks.[11,12]

Adipose stem cells are thought as mediators in tissue regeneration by secreting soluble substances. Adipose stem cells release multiple growth factors, including platelet-derived growth factor (PDGF), transforming growth factor (TGF)-β1, vascular endothelial growth factor (VEGF), and fibroblast growth factor (FGF).[12] However, mast cells also contribute to angiogenesis, tissue remodeling, and fibrosis. They also create type VIII collagen in both pathological and normal conditions.[13] Where type VIII collagen functions as an attachment promoter factor for DAT cells and epithelial cells so that it can inhibit the loss of DAT cell attachment to the tooth surface, as well as inhibit the migration of junctional epithelium towards the apically so that ASCs can be used as an effective and promising method in periodontal treatment to speed up the wound healing process. Based on this description, the purpose of this study is to know the effect of ASC injection on the expression of type VII and VIII collagen in male Wistar rats gingiva.

Materials and Methods

The type of research used is experimental laboratories. The design of this research is a post-test control group design. The Independent Ethics Committee (IEC) number is 144/HRECC.FODM/VIII/2017. This research was carried out for a duration of three months, from July to October 2017 at the Faculty of Veterinary Medicine, Airlangga University using 10 Wistar rats. The inclusion criteria of the Wistar rats are male rats aged three months and weighing 200–300 gm and healthy rats, characterized by active movements without defects, eyes not pale, fur not dull. The exclusion criteria of Wistar rats that had been used in previous studies and whose condition declined or died during the study. The Wistar rats were divided into two groups. Five Wistar rats were assigned to the control group and five Wistar rats to the treatment group. The control group and the treatment group were curettaged. Curettage is done under anesthesia on the gingival sulcus between the two incisor teeth. Curettage is carried out using a curette with the cutting side placed on the mesiobuccal gingiva and then pushed apically. The curette tool is moved until the attachments are separated. Curettage was repeated three times.

After curettage, PBS 1×10^6 was injected into the gingival sulcus between the two incisors in the control group. In the treatment group, ASCs 1×10^6 were injected into the gingival sulcus between the two incisors. Adipose stem cells were taken from the thawing process of Wistar rat's fat tissue in the visceral area which had undergone cryopreservation. Thawing ASCs 1×10^6 is carried out by first thawing the frozen sample (−80°), then placing it in a 15 ml conical cup and washing it in a growth medium, then centrifuging. The results from the upper centrifuge are discarded because they contain DMSO which can be toxic, while the lower part is resuspended 2–3 times. After that, culture in α-MEM media, or split to increase the number of ASCs. Soak with trypsinization to remove the cells from the petridice, until get a monolayer, then take 1×10^6 which is counted by painting with trypan blue and reading with a hemacytometer. After that, culture in α-MEM media, or split to increase the number of ASCs. Soak with trypsinization to remove the cells from the petri dish, until get a monolayer, then take 1×10^6 which is counted by painting with trypan blue and reading with a hemacytometer. Adipose stem cell characterization was carried out by immunohistochemical examination using monoclonal antibodies CD 73 (positive), CD 90 (positive), CD 105 (positive), and CD 45 (negative). Labeling ASCs using fluorescence staining.

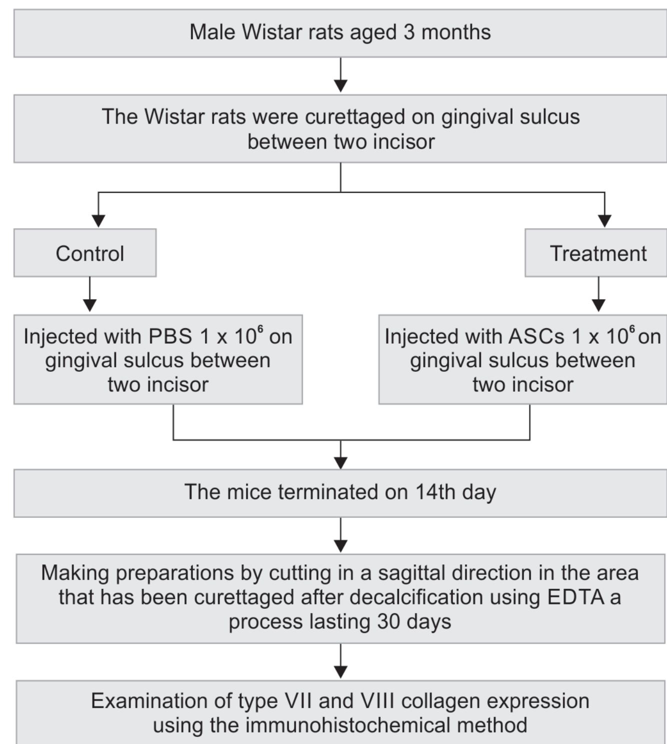

Fig. 1: Research flow

After injection of PBS 1×10^6 and ASCs 1×10^6 in the control and treatment group, on the 14th day, the Wistar rats were terminated. Then, preparations are made by cutting in a sagittal direction in the area that has been curettaged after decalcification using EDTA a process lasting 30 days. Next, the expression of type VII and VIII collagen in the preparation was examined using the immunohistochemistry examination. The method for reading immunohistochemical preparations is carried out on the 14th day. The research method is briefly explained in the flow diagram (Fig. 1).

The data obtained from the observation of type VIII collagen expression were analyzed using a non-parametric test with the Mann–Whitney test because the data was ordinal. To facilitate statistical calculations, statistical product and service solution (SPSS) version 20 is used. If the value of significance (significance or and close to significance) is obtained which is greater than the price of $\alpha = 0.05$, then the null hypothesis (Ho) is accepted and if no value of significance (significance or and close to significance) is obtained which is less than the price of $\alpha = 0.05$, then the hypothesis is rejected.

Results

The control group and treatment group were curetted between two incisor teeth. After curettage, the control group was injected with PBS 1×10^6 and the treatment group was injected with ASCs 1×10^6. Collagen VII and VIII expression is a number obtained by counting collagen VII and VIII cells that express collagen

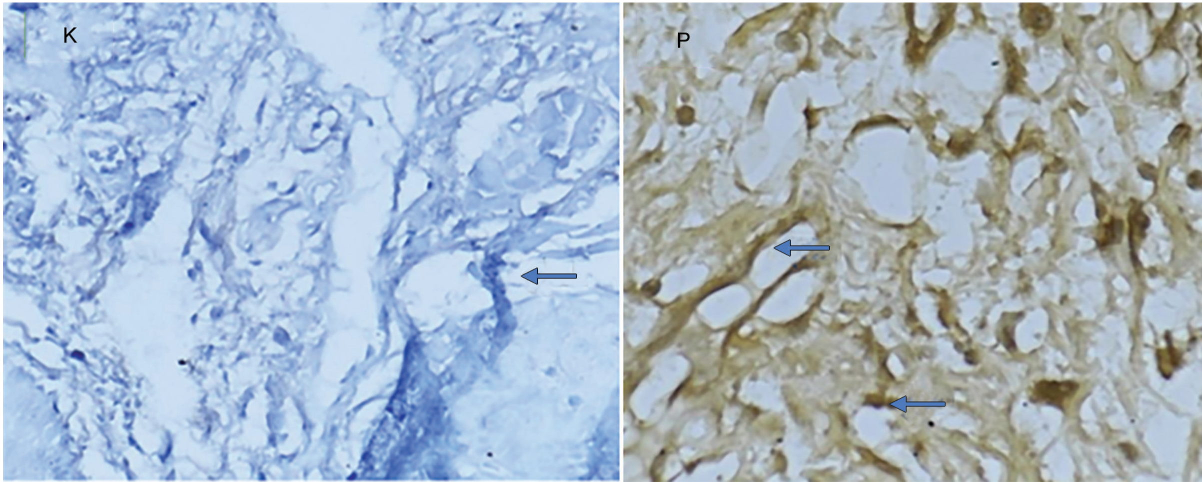

Fig. 2: Image of collagen VII expressions in Wistar rat's gingiva on IHC examination with 400× magnification. (A) Image K (control group) shows the absence of collagen VII expression injected with PBS; (B) Image P shows the expression of collagen VII injected with ASCs, characterized by the presence of brown chromogen (arrow) in the gingival fibers which appear as fibers that fill the intercellular space in the injury area located between the two incisor teeth

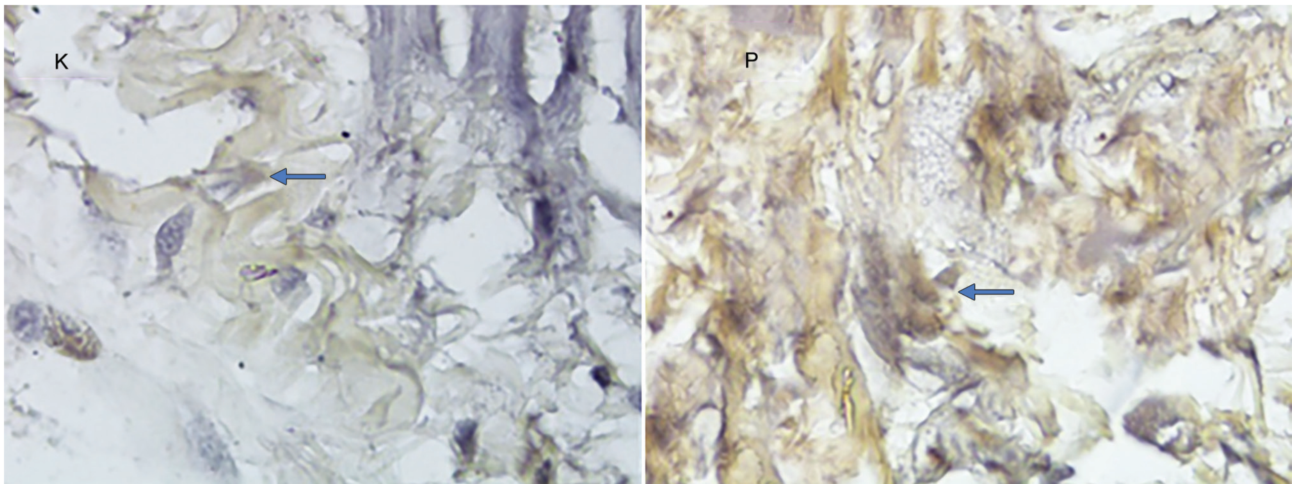

Fig. 3: Image of collagen VIII expressions in Wistar rat's gingiva on IHC examination with 400× magnification. (A) Image K (control group) shows chromogen dark color expression (arrow) injected with PBS; (B) Image P (treatment group) shows the expression of type VIII collagen is stronger than the control group (chromogen dark brown color) in the gingival fibers injected with ASCs, which appear as fibers that fill the intercellular space in the injury area located between the two incisor teeth

types VII and VIII based on color after immunohistochemical staining (Figs 2 and 3). Examination using immunohistochemistry staining using type VII and VIII collagen antibodies.

To facilitate statistical calculations, SPSS version 20 is used. Results were analyzed by non-parametric tests. Variations between groups showed an average increase in collagen VII was 3.90 in the control group and 7.42 in the treatment group. Meanwhile, the average increase in collagen VIII was 10.60 in the control group and 23.80 in the treatment group. The Mann–Whitney test is used to determine if there are not differences between two independent samples. The Mann–Whitney test is used because the data is in ordinal form (Table 1). The results show significance $p = 0.029$ and $p = 0.043$ ($p \leq 0.05$), so there is a significant difference between the control group and the treatment group in type VII and type VIII collagen. Collagen VII and VIII showed increased expression, but collagen VIII showed more increased expression than collagen VII expression. Thus, ASCs have a greater effect on increasing collagen VIII than collagen VII (Table 1).

Table 1: The expression of type VII and type VIII collagen in each group

	$\bar{x} \pm SD$		
Groups	*Control*	*Treatment*	*The Mann–Whitney test*
Collagen VII	3.90 ± 1.06	7.42 ± 1.10	$p = 0.029$
Collagen VIII	10.60 ± 2.04	23.80 ± 3.71	$p = 0.043$

Discussion

This research was conducted in 2017 but has clinical relevance today. Clinical relevance in this research is carried out with the hope that it can provide insight into materials that can accelerate the

regeneration and repair of periodontal tissue, thereby accelerating the healing of periodontal tissue. Adipose stem cells are MSCs with a range of functions, including immunomodulation, angiogenesis, wound healing, and extracellular matrix (ECM) remodeling.[14] Adipose stem cells can differentiate into distinct cell types, including keratinocytes, fibroblast-like cells, and endothelial cells. These cell types secrete growth factors and cytokines that facilitate angiogenesis, fibroblast development, fibroblast migration, and the synthesis of collagen and fibronectin.[15] Type VIII collagen (Col8) is a short-chain nonfibrillar collagen found in the gingival epithelial cell layer and at the functional epithelial-substratum epithelial interface. This collagen functions in the extracellular space as a factor that stimulates the attachment of gingival epithelial cells to the tooth surface.[16] Type VIII collagen is a macromolecular component of the ECM associated with the cell surface and has a role in angiogenesis, tissue remodeling, and fibrosis. In a variety of tissues, type VII collagen (Col7) takes the form of anchoring fibrils, a unique attachment complex at the epithelium/mesenchyme interface. This collagen is described as a long molecule because it comes from long-chain collagen.[17] Type VII collagen is distributed in the skin, dermal–epidermal junctions, and oral mucosa, and was identified as a major protein component that protects the basement membrane and underlying connective tissue.[18]

The effect of ASC injection on rat gingiva was observed after 14 days and a microscopic examination with 400 × magnification. It was found that the expression of type VIII collagen was higher in rats injected with ASCs when compared with the expression of type VII collagen. After curettage, it forms a blood clot, then an inflammatory reaction occurs and there is a mast cell in it. Mast cells synthesize type VIII collagen (functions in angiogenesis). Synthesis of type VIII collagen increases drastically after injury because ASC injection releases several growth factors that can stimulate the repair or restoration of damaged tissue. In this research, labeling, and characterization were also carried out. Labeling stem cells is done using coloring fluorescence. This is useful to know if there are stem cells around the target cell. Meanwhile, the characterization of stem cells is used to determine whether the stem cells used are MSCs. The results show some red light luminescence in areas of green light (fluorescence) in the area around the target cells when viewed under a fluorescence microscope.[12]

Adipose stem cells can influence collagen VII and VIII expressions because ASCs release growth factors like keratinocyte growth factor (KGF), VEGF, transforming growth beta factor 1 (TGF-β1), FGF, dan PDGF. These growth factors affect the growth and differentiation of fibroblasts so that fibroblasts increase. Fibroblasts and KGFs influence the growth of mast cells. Next, type VIII collagen is produced by the mast cell. Collagen proliferation and production significantly increase by the mast cell. The function of type VIII collagen is to promote adhesion in DAT and gingival epithelial cells.[19] DAT cells are the epithelial cells that adhere to the surface of the tooth (directly attached to the tooth cells). DAT cells adhere to the tooth surface via an internal basal lamina in a hemidesmosome. There is a dominant adhesive protein that is laminin-5. This protein plays an important role in the attachment of junctional epithelium to the tooth surface. Laminin-5 also has a role in cell adhesion and migration.[20] Inflammation on the junctional epithelium can cause loss of attachment from DAT cells against the tooth surface, which can also cause the junctional epithelium migration to the apical direction.[21] Type VIII collagen helps the repair of injured tissue because type VIII collagen contains integrin receptors, which aid in extracellular matrix (ECM) protein.[19]

Different protein components found in the ECM can control cell phenotypic using integrins, focal adhesions, and cytoskeletal remodeling. These components can control cell behaviors like migration, proliferation, and differentiation.[22] Collagen promotes cell proliferation, and cell survival under stress and promotes high cell adhesion to the culture surface. Collagen is the main protein constituent of the ECM which plays an important role in shaping the periodontal tissue architecture.[23] Culturing MSCs on collagen demonstrates that collagen enhances osteogenic differentiation of cells, which is associated with enhanced activation of RAS homology gene family member A (RHOA). Mesenchymal stem cells can protect against inflammation-induced tissue lesions.[24] The cell adhesion factor poly-L-lysine is typically used by MSCs grown on collagen and fibronectin to form cells and function as a scaffold in regenerative medicine.[25] Type VIII collagen is associated with cells of junctional epithelium which is directly attached to the tooth (DAT Cell) forming EAA and by culture of gingival epithelial cells.[26] This resulted in that after injection of ASCs, the expression of collagen VIII increased more than collagen VII. Thus, ASCs are better at increasing collagen VIII expression.

Besides the ability of ASCs to secrete growth factors, ASCs also secrete cytokines and other paracrine factors in large quantities in the target environment which can be used for regeneration therapy applications. Adipose stem cells therapy shows great advantages and potential in the promotion of healing. In addition, recent studies have found that ASCs have multiple signal recognition molecules on the cell membrane, which can be used as potential carriers for drug delivery. So, based on this research, ASCs can be used as an effective and promising method for periodontal treatment to improve the wound healing process.[27]

However, there are limitations in this study, namely the limited number of samples and the variable timing of ASCs application to increase the expression of collagen which was not observed. Thus, it is necessary to carry out further research in the future to observe the effect of the length of time ASCs are applied on increasing collagen expression with a larger number of samples.

Conclusion

Adipose stem cells affect type VII and type VIII collagen. After injection of ASCs, the expression of type VIII collagen is more increased than type VII collagen in the gingival of Wistar rats.

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