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Research Article

The Aggrecan Expression Post Platelet Rich Fibrin Administration in Gingival Medicinal Signaling Cells in Wistar Rats *(Rattus novergicus)* During the Early Osteogenic Differentiation *(In Vitro)*

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Abstract

Platelet Rich Fibrin (PRF) is rich for growth factors which can improve the Gingival Medicinal Signaling Cells' (GMSCs) osteogenic differentiation. Aggrecan is chondrogenic differentiation marker which has a significant role in the early stage of GMSCs' osteogenic differentiation. This study aimed to analyze the expression of Aggrecan post PRF administration on the osteogenic differentiation in vitro. This research is a true experimental study using the post-test only control group design with a simple random sampling. GMSCs were isolated from the lower gingival tissue of healthy male Wistar rats (Rattus novergicus) (n=4), weighted around 250 g, a month old, then cultured for 2 weeks and passaged for 4-5 days. GMSCs in the passage 3-5 were cultured in five M24 plates (N=54; n=6/group) for 7 days, 14 days, and 21 days in three different culture mediums, they were negative control group which included α Modified Eagle Medium; positive control group which contained High Glucose-Dulbecco's Modified Eagle Medium (DMEM-HG) combined with osteogenic medium; and at last, treatment group which were DMEM-HG combined with both osteogenic medium and PRF. A one-way Analysis of Variance (ANOVA) test ($P<0.05$) was performed. The treatment group showed the highest Aggrecan expression of 16.15±2.15 on the 7th day. The lowest Aggrecan expression with a value of 3.67±0.76 on the 21th day occurred in the negative control group. There was a significant difference of Aggrecan expression between groups ($P<0.05$). PRF administration unexpectedly stimulates Aggrecan expression of GMSCs during the osteogenic differentiation that useful to accelerate the bone remodeling or neo-cartilage formation.

Keywords: *Aggrecan, Gingival medicinal signaling cells, Osteogenic differentiation, Platelet rich fibrin*

Erken Osteojenik Farklılaşma *(In Vitro)* Süresince Wistar Sıçanlarda *(Rattus novergicus)* Gingival Medicinal Signaling Hücrelere Post Trombositten Zengin Fibrin Uygulamasının Agrekan Ekspresyonuna Etkisi

Öz

Trombositten Zengin Fibrin (TZF) büyüme faktörlerince zengin olup Gingival Medicinal Signaling Hücreleri (GMSH)'nin (GMSH) osteojenik farklılaşmasını geliştirebilir. Agrekan, kondrojenik farklılaşma markırı olup GMSH'nin osteojenik farklılaşmasının erken evrelerinde önemli bir rol oynamaktadır. Bu çalışma, TZF uygulaması sonrasında in vitro osteojenik farklılaşmada Agrekan ekspresyonunu araştırmayı amaçlamaktadır. Bu araştırma, post-test sadece kontrol grup dizayn kullanan rastgele örneklemeli gerçek bir deneysel çalışmadır. Yaklaşık 250 gram gelen bir aylık sağlıklı erkek Wistar sıçanların (Rattus novergicus) (n=4) alt gingival dokularından GMSH izole edildi, sonrasında 2 hafta kültüre edildi ve 4-5 gün pasajlandı. 3-5. pasajlarda GMSH, beş M24 besiyeri içerisinde 7, 14 ve 21 gün boyunca üç farklı kültür medyumunda (α Modified Eagle Medyum içeren negatif kontrol grubu, osteojenik medyum katkılı High Glucose-Dulbecco's Modified Eagle Medyum (DMEM-HG) içeren pozitif kontrol grubu, hem osteojenik medyum hem de TZF katkılı DMEM-HG içeren uygulama grubu) kültüre edildi. Tek yönlü varyans analizi (ANOVA) testi uygulandı ($P<0.05$). Uygulama grubu 16.15±2.15 ile 7. günde en yüksek Agrekan ekspresyonu gösterdi. En düşük Agrekan ekspresyonu, 3.67±0.76 ile 21. günde negatif kontrol grubunda meydana geldi. Gruplar arasında Agrekan ekspresyonu bakımından anlamlı derecede farklar bulunmaktaydı ($P<0.05$). TZF uygulaması osteojenik farklılaşma süresince GMSH'nin Agrekan ekspresyonunu umulmadık bir şekilde uyarmaktadır ve bu nedenle kemik remodelleme veya neo-kartilaj oluşumunun hızlandırılmasında faydalı olabilir.

Anahtar sözcükler: *Agrekan, Gingival medicinal signaling hücreler, Osteojenik farklılaşma, Trombositten zengin fibrin*

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INTRODUCTION

Mesenchymal Stem Cells (MSCs) are immature and unspecialized cells which possess a potential ability to differentiate the various cell lineages [1]. MSCs based on their regenerative secretome and capacity for differentiation toward multiple mesenchymal lineage show a promise for a wide range of regenerative medical applications and tissue engineering [2]. In 2017, Caplan changed the name of MSCs into Medicinal Signaling Cells because it more accurately reflects the endogenous stem cells in defect, injury or disease area. These cells secrete the bioactive factors which are immunomodulatory and regenerative tropically, thus, make MSC as therapeutic and medicinal drugs. Site-specific and tissue-specific endogenous stem cells that revive the new tissue formation as stimulated by the bioactive tropical factors are secreted by MSCs exogenously [3].

Mesenchymal Stem Cells have an important role to improve innovative technologies for tissue engineering such as to regenerate or replace damaged, defect or missing tissues by in vitro cell manipulation and extracellular niche design [4]. Stem cell and tissue engineering therapies are expected to be the regenerative medicine strategies in dentistry that provide a novel capability to restore various tissues in orofacial region such as alveolar bone or condylar cartilage of temporomandibular joint [5]. The oral tissues, which are easily accessed by dentists, are a rich source of MSCs. MSCs from the oral cavity such as Gingival Medicinal Signaling Cells (GMSCs) possess an ability to induce the endogenous stem cell to differentiate into various types of cells; for example, osteoblast and chondroblast [3,4].

The ability of GMSCs' osteogenic differentiation can be accelerated by Platelets Rich Fibrin (PRF) administration in the osteogenic culture medium [5]. PRF contains with abundant and various beneficial growth factor for GMSCs to differentiate and proliferate optimally. PRF as a natural biomaterial also serves and acts as a bio-scaffold to support GMSCs. PRF increases the early indicator of osteogenic differentiation such as Bone Alkaline Phosphatase (BALP) and Runt-related Transcription Factor 2 (RUNX2) /Core-Binding Factor Subunit Alpha-1 (CBF-alpha-1) in 7th day and late marker of osteogenic differentiation such as Osteocalcin in 21st day [5,6]. Our previous study showed that PRF administration in GMSCs' osteogenic culture medium decreases Sox9 expression which is the master gene of chondrogenic differentiation [6]. During the early stage of osteogenic differentiation and pre-osteoblast, some chondrogenic differentiation markers play have important role. Even though the osteogenic and chondrogenic differentiation are considered as two separate processes during endochondral bone formation. The previous study mentioned that there is a correlation between them as a continuous developmental lineage which defines the biological process [6,7]. Furthermore, Aggrecan as the early chondrogenic differentiation marker also has an important role during the osteogenic differentiation for osteoblast formation. It is because RUNX2 as the master gene transcription for osteogenic differentiation is not sufficient enough to mature osteoblasts which cannot be induced by activation of RUNX2 alone. The other transcription factors are needed to activate the genetic pathways controlling GMSCs osteogenic differentiation [8]. The Aggrecan expression is used to evaluate the chondrogenesis for any potential endochondral ossification. In the previous study, the aggrecan expression between MSCs osteogenic culture medium and control medium did not alter significantly different [9].

Thus, the hypothesis of this study is that the PRF administration in GMSCs osteogenic culture medium can increase the Aggrecan expression during the early osteogenic differentiation. Furthermore, this study aimed to analyze the Aggrecan expression post PRF administration to GMSCs culture *in vitro* during an osteogenic differentiation.

MATERIAL and METHODS

Ethical Clearance

This research has been granted an approval of animal research ethical clearance with the reference number 289/HRECC.FODM/XII/2017 from the Faculty of Dental Medicine, Universitas Airlangga, Surabaya, East Java, Indonesia.

Study Design and Animal Model

This study was a true experimental research with a post-test only control group design. Sample groups were selected by using a simple random sampling. The minimum sample size was determined using Lameshow's formula, which was (n=4) for GMSCs isolation. The subjects consisted of male Wistar rats *(Rattus norvegicus)*. GMSCs were isolated from the lower gingival tissue of four male rats aged a month old, healthy, with the weight of 250 g each. In minimizing the suffering of animal model used rodent anesthesia with an intramuscular (IM) injection at the dosage of 0.05-0.1 mL/10 g body weight, they were ketamine, xylazine, acepromazine, and a sterile isotonic saline from Sigma Aldrich, USA. It followed the method of Nugraha et al.[5], GMSCs was passaged every 4-5 days also based on the culture method of Nugraha et al.[5] in Gingival Mesenchymal Stem Cells (MSCs) [6]. The GMSCs in passage 3-5 were cultured in five M24 plates from Sigma-Aldrich with (N=54) and (n=6) per group until 7th, 14th, an 21st day in three different culture mediums, which were control negative group, control positive group and treatment group (see below for details) [5,6]. The study was conducted at an experimental laboratory within the Stem Cell Research and Development Centre in Universitas Airlangga, Surabaya, Indonesia for stem cell culture and animal model.

Platelet Rich Fibrin Preparation

Platelet Rich Fibrin was isolated and extracted from whole blood of different rat population. PRF isolation was done with (n= 36), 36 months-old; with the mean weight of 250 g each. The rats terminated using the rodent anesthesia with the dosages of 60 mg/body weight of ketamine and a 3 mg/body weight of xylazine from Sigma Aldrich intraperitoneally (IP). Next, the whole blood (6 mL) was aspirated using a 10 mL disposable syringe and inserted in a non-coagulant vacutainer tube then centrifuged at 3000 rpm/min for 10 min (Kubota, Tokyo, Japan). Thus, PRF obtained mince and it was inserted into each culture plate of the treatment group [5,6].

Osteogenic Differentiation in a Combination of Platelet Rich Fibrin and Gingival Medicinal Signaling Cells

The analysis was conducted for three groups which consisted of two control groups and anexperimental group. In the treatment group, GMSCs were cultured with PRF which contained with 2 mM L-glutamine, 100 µg/mL sodium pyruvate, 0.2 mM ascorbic acid-2 phosphate, dexamethasone 10-7 M 10 ng/mL TGF-β3 and a high dosage of glucose-Dulbecco's Modified Eagle Medium (DMEM-HG) from Sigma Aldrich, USA. While, in the positive control group, GMSCs were placed on an osteogenic culture plate medium with 2 mM L-glutamine, 100 µg/mL sodium pyruvate 0.2 mM ascorbic acid-2 phosphate, dexamethasone 10-7 M from Sigma Aldrich, USA. Furthermore, in the negative control group, GMSCs were cultured with α Modified Eagle Medium (αMEM) also from Sigma Aldrich, USA.

Osteogenic differentiation was analyzed on the 7th, 14th, 21st day of culture cells groups. It employed an immunocytochemical staining by indirect technique using a 3.3'-diaminobenzidine stain kit by Pierce DAB Substrate Paint Kit 34002 from Sigma Aldrich, USA and monoclonal antibodies by Abcam, Cambridge, MA, USA. An anti-Aggrecan (mouse monoclonal; ab-3773) was performed to analyze all samples. The Aggrecan expression was read using a light microscope using the CX22 Binocular from Olympus at 200x magnification. Every cell expressing Aggrecan in five field was examined three times by three experts which were WR, EH and FAR; and then, the mean was then calculated.

Data Analysis

All data were recapitulated and then Statisitical Package for Social Science (SPSS) version 20.0 by IBM SPSS, Chicago, USA was used to analyze the data. The experiments were replicated three times with (n=54). The data was then duplicated for (n=108) using an estimation formula and SPSS. Saphiro-Wilk normality test and a Levene's variance of homogeneity test (P>0.05) were performed then Analysis of Variance (ANOVA) test (P<0.05) was conducted to analyze the different between groups.

RESULTS

All data were normally distributed and homogeny (P>0.05). The positive expression of Aggrecan was detected in all groups *(Fig. 1)*. The treatment group showed the highest Aggrecan expression with the value of (16.15±2.15) on the 7th day. While, the lowest Aggrecan expression with the value of (3.67±0.76) on the 21st day was seen in the

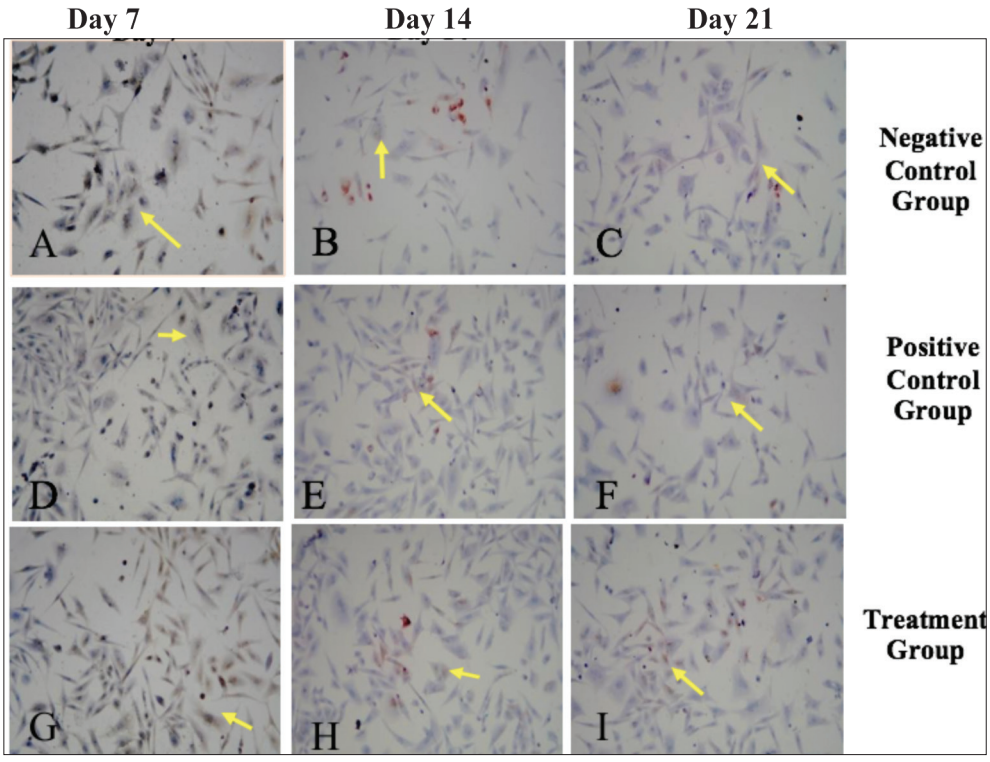

Fig 1. Aggrecan expression in GMSCs of Wistar rats *(Rattus novergicus)*. A, B, and C show the Aggrecan expression in the negative control group; D, E, and F exhibit the Aggrecan expression in the positive control group; and G, H, and I present the Aggrecan expression in the treatment group. Aggrecan expression in GMSCs is observed on the 7th, 14th and 21st day. A positive Aggrecan expression is marked by a yellow arrow in the cell and examined through an Olympus CX22 Binocular, at 200x magnification

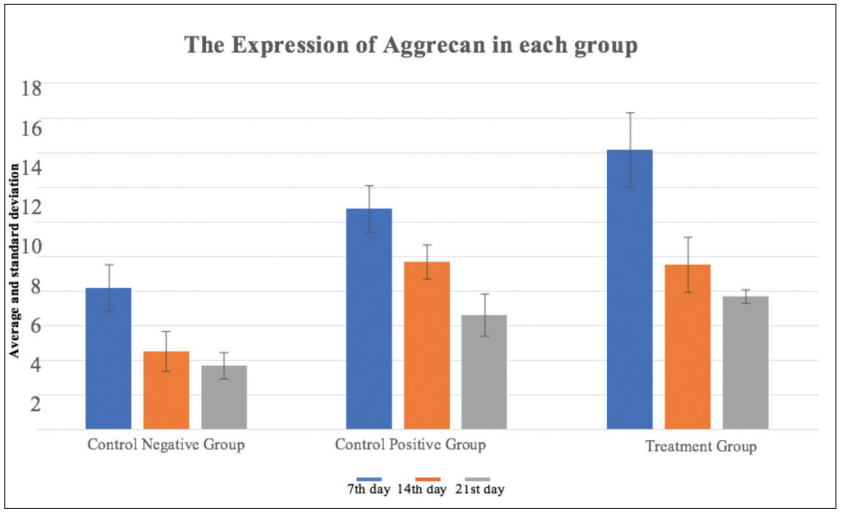

Fig 2. The average and standard deviation of Aggrecan expression on the 7th, 14th and 21st day in each group

Table 1. *Mean±Standard deviation (SD) and one-way anova test result of aggrecan*

Time	Aggrecan Expression Negative Control Group	Aggrecan Expression Positive Control Group	Aggrecan Expression Treatment Group	One-way ANOVA P–value
Day 7	8.17±1.37	12.74±1.35	16.15±2.15	
Day 14	4.5±1.16	9.67±0.99	9.49±1.59	0.001*
Day 21	3.67±0.76	6.59±1.22	7.67±0.39	

** Information: significant at P<0.05*

negative control group *(Fig. 2)*. There was a significant difference Aggrecan expression between groups with the value of (P< 0.05) *(Table 1)*.

DISCUSSION

The large Chondroitin Sulphated Proteoglycan Aggrecan or Aggrecan (ACAN) is the most plentiful non-collagenous protein in cartilage and essential for its structure and function [10]. Aggrecan is the founding member of lectican protein family. Aggrecan includes versican, brevican and neurocan. Aggrecan consists of a 250 kDa protein core with around 100 chondroitin sulphate glycosaminoglycan and also 30 keratan sulphate chains attached to a large domain and located between three globular domains. Aggrecan comprises an N-terminal domain, two globular domains (G1 and G2), two inter-globular domains, a selectin-like domain (G3) and a C-terminal domain [11]. The aggrecan expression in the osteogenic differentiation or bone is lower than in the chondrogenic differentiation or cartilage. The function of aggrecan in bone is to help endochondral ossification. Aggrecan relatively exists in low concentration, but it has an effect on growth plate (cartilage) calcification, rather than having a direct effect on bone. Thus, it is very substantive and important for growth plates [12,13].

Endochondral ossification is a fundamental biology process in forming hard tissue when bone replaces the cartilage. During endochondral ossification, abundant bones are formed, for example it is the primary way that long bones increase in length. The cartilage and the underlying bone are linked through the deepest layers of the hypertrophic chondrocytes, which are surrounded by a mineralized matrix [10,11,13]. Aggrecan expression increases during the endochondral ossification. In line with that, the previous study conducted by Namkoong et al.[9] showed that Aggrecan does not show any expression differences between the control and the osteogenic mediums. In this study, the Aggrecan expression increases on the 7th day in the treatment group with a significant different between group. PRF administration in osteogenic culture medium unexpectedly stimulates the Aggrecan expression during the early osteogenic differentiation of GMSCs. This result of study is consistent with the research of Sumarta et al.[14] which showed that Aggrecan expression increases significantly with PRF administration in culture medium. Furthermore, the triad tissue engineering consists of 3 elements, they are MSCs, natural scaffold and niche. Growth factor contained in PRF plays an important role to enhance MSCs differentiation capability and acts as advantageous bio-scaffold. Biodegradable polymerized fibrin matrix forms networks that support and stimulate the beneficial MSCs secretome [5,6,14].

Platelets Rich Fibrin is abundant with growth factor such as Insulin Growth Factor (IGF-I), Transforming Growth Factor-β1 (TGFβ-1), Vascular Endothelial Growth Factor (VEGF), Insulin Growth Factor (IGF-I), and Platelet Derived Growth Factor-β (PDGF-β) [6,14]. IGF stimulates aggrecan

proteoglycan synthesis and suppresses catabolism of proteoglycans. While, TGF-β increases Aggrecan expression in MSCs. PDGF is a strong mitogen and plays an important role in the proliferation and maintenance of MSCs. Moreover, FGF-2 enhances the Aggrecan expression from MSCs [15]. Ten percent concentration of PRF stimulates the synthesis of proteoglycan (ACAN) [16]. The highest result is shown in the treatment group due to PRF administration in GMSC. GMSCs combined with PRF will fulfill all mandatory factors that complete the key of tissue engineering, for example cells, GFs, and scaffold [17]. In addition, it is unexpected that in the treatment group shows the highest Aggrecan expression in MSCs osteogenic culture medium compared to the other groups. The beneficial result of this study surprisingly can be useful as the references for the further research to accelerate bone remodeling or neo-cartilage formation especially PRF administration as a new generation of platelet-derived concentration for bone or cartilage healing in human.

Platelets Rich Fibrin unexpectedly stimulates and increases the Aggrecan expression of GMSCs during osteogenic differentiation. With this the beneficial result, the combination of PRF and GMSCs is recommended to be an alternative to accelerate bone remodeling or neo-cartilage formation as this will support all important substance of tissue engineering which are the scaffold, the growth factor and the cells. We would like to suggest that further research is needed to study combined GMSC and PRF on *in vivo* model.

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Conflict of Interest

No conflict of interest was associated with this work.

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