Effect of Collagen-Activated Platelet-Rich Plasma in The Fibroblast Migration of The Periodontal Ligament

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Abstract

Platelet-Rich Plasma (PRP) has been found to be a delivery system of essential growth factors. The collagen is an activator that causes clotting of the PRP and stimulates growth factor release from the platelets and granulocytes. The effectiveness of growth factors in PRP can enhance the migration of fibroblast in the phase of proliferation, which are involved in the healing of periodontal tissues. The research aim was to investigate the activation effect of PRP using collagen for the migration of fibroblast in the periodontal ligament. The PRP from the human donor was activated using collagen (collagen-activated PRP) and compared with inactived PRP (control group). The migration of fibroblast was measured after a half, one, two, and three days. The collagen-activated PRP resulted in a more significant migration of fibroblast compared with the inactivated PRP. Substantial differences in fibroblast migration were observed after a half, one, two, and three days. Collagen-activated PRP can be used as an alternative to stimulate platelet-associated growth factors and result in greater fibroblast migration. Polypeptide growth factors (PDFs) are biological mediators in periodontal regeneration. Platelet-derived growth factor and transforming growth factor-β are the most potent stimulators of PGF for the migration of fibroblast. It is vital in the process of wound healing. The outcome of this research is Platelet-Rich Plasma could improve the activity of the fibroblast migration, and the addition of a Collacure collagen on the PRP affected the migration of periodontal ligament fibroblast.

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Introduction

Periodontal disease is a pathological condition characterized by destruction of periodontal tissue which includes the gingiva, alveolar bone, periodontal ligament and cementum. According to Gurav and Jadav (2011), periodontal diseases can caused by accumulation of bacteria attached to the tooth surface, that expand until sub gingiva and reduce the alveolar bone.[1] The objective of periodontal surgery as part of regenerative procedure is to improve the structure and function of the periodontium tissue. Regeneration means replacement and reconstruction of body parts that are damaged or missing, with cells and tissues which has the same form and function. Periodontal regenerative procedure is intended to improve the structure of gingiva, alveolar bone, cementum and periodontal ligament so it is strong enough to support the teeth.[2,3] Wound healing is a dynamic process of homeostasis, inflammation, proliferation, and remodeling tissue or resolution in sequent. Regenerative process mediated by the presence of fibroblasts, new blood vessels and chronic inflammatory cells in the areas of injury. Proliferation and differentiation of the cells is an important process for the formation of new tissue and lead to wound closure. Increasing the number of fibroblasts in the wound area is the result of the combination between proliferation and migration process. The overlapping process is affected by cytokines released by macrophages and lymphocytes. The process of migration and proliferation of fibroblasts is an essential process in the formation of granulation tissue and wound closure. The rapid migration of cells on the root surface will play an important role in the reattachment of the periodontal ligament during

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repairs.[4] Collagen formation is one of key component for wound healing. Immediately after the injury, collagen fibers enter the blood clot causing platelet aggregation and activation, then release chemotactic factors which initiate the wound healing process. Fragments of collagen release leucocytic collagenase to attract the fibroblasts to the area of injury. Consequently, the collagen became the foundation for the formation of a new extracellular matrix.[5]

Platelet-Rich Plasma (PRP) is an autologous growth factor with dense platelet obtained from several centrifugations process of blood.[6] Platelets release Platelet Derived Growth Factor (PDGF), Transforming Growth Factor-β (TGF-β), Vascular Endothelial Growth Factor (VEGF), Basic fibroblast growth factor (bFGF), Epidermal Growth factor (EGF), Insulin Growth Factor (IGF), Platelet-Derived Endothelial Cell Growth Factor (PDECGF), Platelet Derived Angiogenesis Factor (PDAF) and Fibroblast Growth Factor (FGF). Those growth factors play an important role in the process of migration, proliferation and differentiation.[7]

Platelet Rich Plasma could stimulate Polypeptides Growth Factor (PGF) release. The PGF's role to regulate biological event in the wound healing process such as cell adhesion, cell migration, proliferation, differentiation and morphogenesis of tissues. It also regulates the synthesis of extra cellular matrix as a mediator of biological process in periodontal regeneration. Moreover, PGF is a stimulator for fibroblast in cell migration, mitogenesis and proliferation as well as in the synthesis of matrix.[8]

Growth factor of PRP will be much removed from the granules during platelet activation. Platelet activator are widely used is thrombin. However, cow thrombin reported has some side effects, which is the formation of antibodies against thrombin, prothrombin, factor V and cardiopilin. It could cause bleeding after surgery clinically and an autoimmune syndrome similar lupus disease in animal study.[8] Cow thrombin also inhibits cell proliferation and viability in vitro studies, in addition, its serine proteases could degrade the growth factor. In vitro studies reported that cow thrombin inhibits the migration of fibroblasts and reduces clot strength.[5]

Collagen is an alternative to replace thrombin as the activator of PRP since it partakes in the intrinsic clotting cascade and widely used as a biomaterial. Fufa et al. uses collagen type I as a clotting agent and activator of platelets in PRP instead of bovine thrombin, by measuring the level of TGF-β1, PDGF and VEGF release of both activators for few days. The cumulative release of TGF-β1 on collagen activated PRP was higher. It showed sustained release of cytokines during few days. Collagen activating process started when platelet contacted with collagen through receptor GP VI. In wound healing cascade, collagen often acts as initial platelet activators, by forming a monolayer of platelets to collagen injured area.[5]

In this study, collagen used as PRP growth factors activator, since it is the natural protein involved in the release of growth factor from platelet granules in the human.

Materials and Methods

The research was a pure experimental and has been approved by the Ethics Committee of Dentistry Faculty of Universitas Gadjah Mada Indonesia with registration number 0052/KKEP/FKG-UGM/EC/2014. The variables were: (a). Effect Variable: This type of treatment: Platelet Rich Plasma (PRP) and PRP activation using collagen at 1, 2, 3 and 7 days and time Observations 12, 24, 48 and 72 hours (b). Affected Variable: periodontal ligament fibroblast cell migration. Periodontal ligament fibroblast cell culture was taken from primary cell from freshly-extracted premolar tooth from subjects who prior to underwent orthodontic procedure. The primer cells was taken from periodontal ligament in the middle third of the root. The PRP as prepared by double centrifuges methods.[9] The samples were divided into six groups: control, PRP and PRP activation using collagen for 1 day, 2 days, 3 days and 7 days groups, the migration of fibroblasts were then calculated at 12 hours, 24 hours, 48 hours and 72 hours. Calculations using the Inverted Microscope (4 times magnification objective lenses) and Optilab by calculating the periodontal ligament fibroblast cells that migrate to the area of incision in percent.

Fibroblast culture

The periodontal ligament fibroblast taken from middle third the root surfaces of the teeth using a blade # 15. Cells taken then cultured using Dubelco's Modification of Eagle's Medium (DMEM) added with supplement of 10% FBS,

penicillin-streptomycin 2%, 0.5% fungizone then put in a CO_2 incubator at a temperature of 37 ^oC.[10]

PRP preparation

Tube contained 100 mL whole blood then added by 1 ml of 3.8% sodium citrate. Each tube centrifuged for ten minutes at 2400 rpm. This centrifuge produced two layers, the top layer and bottom layer contained PPP and red blood cell respectively. PPP layer was taken using a long canula + water-intake canula inserted into tubes without anticoagulant. Then the tube was centrifuged again for 15 minutes at 3600 rpm. This second centrifugation produced two layers, the top layer is a PPP 2/3 and 1/3 bottom layer is the PRP. Platelet-rich plasma separated from PPP with an isolation kit.[11]

PRP activation

PRP obtained then mixed with collagen, in 1:1 volume ratio.[12]

Cell migration assessment

Fibroblast culture was added to the culture medium in 24 wells microplate and cell migration assessed with the injury in the test cell. Briefly, if fibroblast with a density of 10,000 cells/35-mm, then, the culture medium was replaced with medium DMEM serum-free, and made scratches in a monolayer of cells using a pipette tip sterile, then 200 uL PRP was added to the treatment group 1 as many as 10 wells microplate 24 and PRP were activated collagen in the treatment group 2 as much as 10 microplate 24 wells. As control, ten 24 wells microplates filled with fibroblasts without stimulation was put in the incubator. After 12 hours, 24 hours, 48 hours and 72 hours, cells were washed with FBS then stained using HM. Periodontal ligament fibroblast cell migration observed using an inverted microscope equipped with an optical viewer mounted on the ocular lens through six random fields at the cut point. The rate of wound closure was calculated by Adobe Photoshop program presented as percentages at each time point measured.

Results

The mean of periodontal ligament fibroblast cell migration was observed lowest in the control group (13.26 ± 1.03). The highest average fibroblast was the group stimulated with PRP + Collagen for 7 days (48.48 ± 1.27). Observations average percentage of fibroblast migration of the periodontal ligament corresponding activation time and observation time stated in Table 2. The mean percentage of fibroblast migration of the periodontal ligament observed lowest in the control group since this group did not stimulated. The migration increased in the PRP and collagen activated PRP groups. The migration peaked in group of collagen activated PRP after 24 hours incubation, then decreased after 2 and 3 days. The mean of periodontal ligament fibroblasts increase back in 7 days with an incubation period of 72 hours. Data obtained were homogenous and normally distributed confirmed by Levene and Shapiro-Wilk (p > 0.05) respectively.

Parametric test with ANOVA showed a significance value of p < 0.05, both among the treatment group and the mean time (Table 2). It showed statistically significant differences of the percentage of cell migration between the control and treatment group, both PRP and collagen activated PRP were activation using collagen. Significance level showed the value of p = 0.000 at comparison across time and between group. It was indicated a significant difference of fibroblast cell migration rate between groups and over time. It was followed by Post Hoc test of Least Significant Difference (LSD) to determine the differences of periodontal ligament fibroblast cell migration between each group in percentage (Table 3).

Table 3 showed comparison between control and treatment groups on 12 hours incubation activated using collagen for 1, 2 and 3 days and showed significant difference (p < 0.05). The group treated with PRP activation using collagen for 7 days showed no significant difference with p > 0.05. Table 4 showed significant difference (p < 0.05) between control group with treatment groups for 1 day, 2 days, 3 days and 7 days. PRP treatment groups activated with collagen for 1 day and 2 days to 3 days showed no significant difference (p > 0.05). Table 5 showed significant differences (p < 0.05) between control and treatment groups after 1 day, 2 days, 3 days and 7 at 48 hours incubation, except between control group and 7 days PRP activation which showed no significant difference (p > 0.05). Table 6 showed significant differences (p < 0.05) between control and the treatment group after 72-hour

incubation.

Variables	n	Observation Time			
		12 hours	24 hours	48 hours	72 hours
Control	10	13,26 ± 1.03	17.18 ± 0.99	19.00 ± 0.90	28.27 ± 1.11
PRP	10	14.60 ± 1.46	16.22 ± 1.04	25.21 ± 0.70	38.47 ± 1.17
PRP+C 1 day	10	18.02 ± 1.17	20.11 ± 0.98	28.36 ± 1.63	44.38 ± 1.44
PRP+C 2 day	10	16.53 ± 0.76	22.93 ± 1.28	28.35 ± 1.57	37.29 ± 1.15

Table 1. The mean and standard deviation of the percentage of migration periodontal ligament fibroblast after the PRP activation using Collagen.

	Degree of freedom	F value	Significance level
Group	23	690.336	0,000
Time	1	101724.416	0,000
Group*Time	23	690,336	0,000

Table 2. Test Results two ways ANOVA between groups and times.

Treatment Group	PRP	PRP+C 1 day	PRP+C 2 day	PRP+C 3 day	PR+C 7 day
Control	0,012	0,000	0,000	0,000	0,051
PRP		0,000	0,000	0,000	0,559
PRP+C 1 day			0.005	0,127	0,000
PRP+C 2 day				0,191	0,000
PRP+C 3 day					0,000

Table 3. Test Results Least Significant Difference (LSD) between groups after 12 hours Incubation.

Treatment Group	PRP	PRP+C 1 day	PRP+C 2 day	PRP+C 3 day	PRP+C 7 day
Control	0,000	0,000	0,000	0,000	0,257
PRP		0,000	0,000	0,000	0,003
PRP+C 1 day			0.005	0,205	0,000
PRP+C 2 day				0,191	0,000
PRP+C 3 day					0,291

Table 4. Test Results Least Significant Difference (LSD) between groups after 24 hours Incubation.

Treatment Group	PRP	PRP+C 1 day	PRP+C 2 day	PRP+C 3 day	PRP+C 7 day
Control	0,000	0,000	0,000	0,000	0,123
PRP		0,000	0,000	0,000	0,000
PRP+C 1 day			0.988	0,000	0,000
PRP+C 2 day				0,000	0,000
PRP+C 3 day					0,000

Table 5. Test Results Least Significant Difference (LSD) between groups after 48 hours Incubation.

Treatment Group	PRP	PRP +C 1 day	PRP+C 2day	PRP+C 3day	PPR+C 7day

Control	0,012	0,000	0,000	0,000	0,000
PRP		0,000	0,025	0,000	0,000
PRP+C 1 day			0.005	0,000	0,000
PRP+C 2 day				0,000	0,000
PRP+C 3 day					0,000

Table 6. Test Results Least Significant Difference (LSD) between groups after 72 hours Incubation.

Discussion

The application of PRP activation using collagen generate expenditure growth factor that is required in the process of cell migration and can improve the ability of cells to migrate into the wound area. These results are consistent with the results of research cell migration test 10. With the result that the cells stimulated with growth factor rich PRP showed increased levels of migration at 12 hours and 24 hours as compared to cells not stimulated by PRP.

These results did not describe the difference between each treatment groups at the same time. To see the significance of treatment groups, Post hoc Least Significant Difference (LSD) conducted to determine which groups are capable of stimulating cell migration of periodontal ligament fibroblast.

The percentage of cell migration in the periodontal ligament fibroblasts stimulated group with PRP and PRP activation using collagen was higher than the unstimulated group. It means PRP caused the release of growth factors needed to accelerate the migration of the periodontal ligament fibroblast in wound healing. This is in line with Fufa et al. (2008) which stated that the release of growth factors of PRP activated with collagen increased along with days it is incubated[7]. The collagen continues to stimulate the release of a greater growth factor at 3 to 7 days 5. It also stated that the PRP can regulate biological events such as cell migration, cell proliferation, cell adhesion, and the synthesis of extracellular matrix. Platelet Rich Plasma is the supernatant with a high concentration of platelets that can release various types of growth factor. Signaling molecules Growth factor is critical in many cellular processes, each growth factors have a regulatory function at the cellular level and involved in the process of tissue regeneration. The use of PRP stimulates the release of polypeptides Growth Factor (PGF) which can regulate biological events in wound healing processes such as cell adhesion, cell migration, proliferation.

The number of growth factors in PRP lysate after one, two and three days of incubation was sufficient to stimulate cell migration activity of periodontal ligament fibroblasts. Collagen, when used as a growth factor activator, could release the growth factor on an ongoing basis during the seven days of observations. In this study, PRP can stimulate the migration of fibroblast cells after being stored for seven days at a temperature of $4^{o}C$[5,8]. The process of platelet activation induced by collagen begins to bond with the collagen receptors glycoprotein VI, this binding induces a series of intracellular signals, on the one hand, activates integrin α2bβ3 which then binds to another collagen molecule. Collagen bonds to two important receptors then induce a cascade of signals intercellular. Then it leads to calcium release and activation of C protein kinase, which both are responsible for the platelet response to a series of such signals. The response of platelets is aggregated and release the contents of granules through a process of exocytosis so that the activation PRP with collagen needs time to issue a growth factor.[13]

During the process of wound healing, fibroblast migrates and proliferate 2 - 3 days after the injury that would later become the main cell into the collagen matrix in the wound area. Fibroblasts from normal tissue migrate into the wound area, and fibroblast precipitate the basic substance which would occupy by the collagen. Fibroblast is critical for the formation of scar tissue, and collagen provides tensile strength to the healing of soft tissue. When the inflammation occurs in a certain tissue, the fibroblasts will soon migrate towards the wound, proliferate and produce collagen matrix to repair damaged tissue.

Conclusion

It can be concluded that the addition of collagen in platelet-rich plasma (PRP) can improve periodontal ligament fibroblasts cell migration. There are differences in the percentage of cell migration in the periodontal ligament fibroblasts with collagen PRP activation after 1, 2 and 7 days of incubation at 72-hour observation.

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Declaration of Interest

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