The influence of platelet concentrates with and without metronidazole incorporation pre-centrifuge towards periodontal ligament fibroblast proliferation

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Abstract

Objective: Horizontal centrifugation was believed may improve platelet, leukocyte, and growth factors in the buffy coat. Previous study of platelet concentrates metronidazole incorporation was able to greatly inhibit bacteria. Metronidazole might help the proliferation with its ability to inhibit pro-inflammatory cytokines. This study intended to examine the influence of C-PRF and i-PRF with and without metronidazole incorporation horizontal pre-centrifugation towards periodontal ligament fibroblast proliferation.

Material and Methods: 2x10^6 fibroblast cells on the 96 well plates were added with 1 ml of each buffy coat platelet concentrates. The samples were split into five groups: i-PRF, C-PRF, i-PRF metronidazole, C-PRF metronidazole, and control group. Then monitored after 1 day, 3 days, and 5 days using MTT assays. The data were inspected by Two Way ANOVA Test and Post Hoc Test.

Results: The result indicated that cell proliferation of fibroblasts was escalated by the addition of C-PRF, i-PRF metronidazole incorporation, and C-PRF metronidazole incorporation group. While the i-PRF group showed decreasing proliferation cell count. Conclusion: This study assumed that platelet concentrates (i-PRF and C-PRF) with metronidazole incorporation can increase the cell proliferation higher than i-PRF and C-PRF only.

Keywords: Concentrated platelet rich fibrin, Fibroblast proliferation, Horizontal centrifugation, Injectable platelet rich fibrin, Metronidazole

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Introduction

Biomaterial which natural or even synthetic is added in the treatment to get the fastest wound healing. This topic is still in the continuing experimental to get the best choice for wound healing. The biomaterial is developed to accelerate the time of healing, inflammation regulation, and increase the regeneration tissue.1,9 Platelet concentrate which is obtained from autogenous blood has regenerative biomaterial potential. Introduces the second generation of platelet concentrate which is called platelet-rich fibrin (PRF) as a total autologous concept without anticoagulant, not like the first generation. Platelet rich fibrin is known for its ability to induct cytokines and release growth factor, such as platelet derived growth factor (PDGF), Epidermal Growth Factor (EGF), transforming growth factor (TGF-β), and vascular endothelial growth factor (VEGF) significantly to third dimensional fibrin matrix. All of these growth factors have the important roles to vascularization and new tissue forming.3,4

The researchers assume the decreasing of relative centrifugation time (RCF) is able to increase the regenerative capacity on the PRF matrix such as the number of leucocytes, platelets, and growth factors. This protocol is known as low-speed centrifugation concept or LSCC and introduced injectable platelet rich fibrin (i-PRF) in liquid form as one of its products.5,6 The protocol of i-PRF is the autogenous blood in plastic tube is fixed angle centrifuge on 700 rpm, 60 RCF in 3 minutes.5,7 High-speed centrifugation concept in fixed angle centrifugation is believed that the platelet concentrate product has great numbers of platelets and leukocytes count but unfortunately accumulate in the distal and base of the tube. Miron et al.8 then developed the use of horizontal centrifugation which is able to make completed linear separation and push the platelets and leukocytes toward the buffy coat layer.8 An experimental of mixing this high-speed centrifugation concept in horizontal centrifugation, and the product is called concentrated- platelet rich fibrin (C-PRF). This C-PRF is compared with i-PRF via horizontal centrifugation. The results of this study are C-PRF is able to increase the growth factor three times better than i-PRF on gingival fibroblasts, C-PRF shows greater proliferation and migration cells that i-PRF, and platelet and leukocyte counts are greater than i-PRF.5,9 Platelet concentrate is known for not having adequate antibacterial effect, nevertheless
A parametric analysis test with a significance level of 0.05 was carried out after performing the Two-Way ANOVA. For the purpose of establishing the significance of differences between each group, a Post test was done on the data. Shapiro-Wilk normality test was performed on the microplate reader data to verify that the formazan crystal that had formed was dissolved. Following the incubation, 200 μl of DMSO were added to each well as stop solution to dissolve the crystals.

The observation day, 50 μl of MTT were added to determine the metronidazole incorporation and Category E of metronidazole incorporation, category D consisted of fibroblast in DMEM with 100 μl of PRF with platelet derived growth factor (PDGF), Epidermal growth factor (EGF), and platelet derived growth factor (PDGF). Category B consisted of fibroblast in PRF and platelet derived growth factor (PDGF), Epidermal growth factor (EGF), and platelet derived growth factor (PDGF).

For the C-PRF with metronidazole incorporation of approximately 10 mL of Peripheral blood from the donor was taken and placed into a sterile plastic tube. The concentrates of relative centrifugation time (RCF) is able to push the platelets and leukocytes toward the base of the tube. Miron et al. then developed the use of horizontal centrifugation and push the platelets and leukocytes toward the base of the tube. Miron et al. then developed the use of horizontal centrifugation for the C-PRF with metronidazole incorporation group, the C-PRF with metronidazole incorporation group, C-PRF without metronidazole incorporation group, and PRF metronidazole incorporation were taken and placed into a sterile plastic tube. The concentrates of relative centrifugation time (RCF) is able to accelerate inflammation process. Besides that, a study of comparison the effect of metronidazole and mangosteen peel extracts shows that metronidazole can increase the fibroblast cell proliferation higher that the extract itself. The wound healing process is in the four phases, start with haemostasis phase, inflammation phase, proliferation phase, and end with remodeling phase. Proliferation phase is where the damaged cells is changed and the fibroblast cell has the important roles to proliferate and moving to the wound forming extracellular matrixes (ECM) as the scaffold of tissue regeneration.

The previous study of platelet concentrates metronidazole incorporation had been studied towards bacterial inhibition. Nevertheless, the influence of this incorporation had never been studied yet. The intention of this study was to inspect the effect of i-PRF and C-PRF with and without metronidazole incorporation in connection with the rise of periodontal ligament fibroblasts cell proliferation in vitro study.

### Table 1. Mean and standard deviation of periodontal ligament fibroblast proliferation absorbance value

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>Day -1 (μm) ± SD</th>
<th>Day -3 (μm) ± SD</th>
<th>Day -5 (μm) ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>i-PRF</td>
<td>3</td>
<td>0.3980 ± 0.177</td>
<td>0.3400 ± 0.112</td>
<td>0.1630 ± 0.050</td>
</tr>
<tr>
<td>C-PRF</td>
<td>3</td>
<td>0.4340 ± 0.095</td>
<td>0.5780 ± 0.151</td>
<td>0.5880 ± 0.187</td>
</tr>
<tr>
<td>iPRF+MTZ pre</td>
<td>3</td>
<td>0.4043 ± 0.002</td>
<td>0.8052 ± 0.008</td>
<td>0.5295 ± 0.018</td>
</tr>
<tr>
<td>cPRF+MTZ pre</td>
<td>3</td>
<td>0.4472 ± 0.005</td>
<td>0.7152 ± 0.008</td>
<td>0.8220 ± 0.008</td>
</tr>
<tr>
<td>Control group</td>
<td>3</td>
<td>0.3348 ± 0.006</td>
<td>0.6456 ± 0.023</td>
<td>0.6434 ± 0.007</td>
</tr>
</tbody>
</table>

n = Number of samples

### Table 2. Two-Way ANOVA test result

<table>
<thead>
<tr>
<th>Group</th>
<th>f</th>
<th>Significant</th>
</tr>
</thead>
<tbody>
<tr>
<td>Type of Application</td>
<td>17.853</td>
<td>0.000*</td>
</tr>
<tr>
<td>Time of Observation</td>
<td>7.363</td>
<td>0.001*</td>
</tr>
<tr>
<td>Type of Application*Time of Observation</td>
<td>2.929</td>
<td>0.016*</td>
</tr>
</tbody>
</table>

*Significance value p < 0.05

Material and Methods

This study used a quantitative design and only included laboratory experiments. Periodontal ligament fibroblast from the primary cell was cultured to be the sample. This study has received approval from the dental ethics commission of the Faculty of Dentistry Universitas Gadjah Mada Yogyakarta Number 190/KE/FKG-UGM/EC/2022.

A 96 well microplate was filled up to 100 μl of fibroblast cells in DMEM with density of 2 x 104 cells/ well 24 hours prior treatment. Approximately 10 mL of Peripheral blood from the donor was taken and placed into a sterile plastic tube.
For the i-PRF with metronidazole incorporation group, the 0.5 ml of 5% liquid metronidazole was added into sterile plastic tube contained the blood. The blood tube from i-PRF with and without metronidazole incorporation protocol was then horizontally centrifuged at 300g for 5 minutes at room temperature. The concentrates were taken from the buffy coat as much as 1 ml. For the C-PRF with metronidazole incorporation group, the 0.5 ml of 5% liquid metronidazole was added into sterile plastic tube contained the blood. The blood tube of C-PRF with and without metronidazole incorporation protocol was then horizontally centrifuged at 2000g for 8 minutes at room temperature. The concentrates were taken from the buffy coat as much as 1 ml.

The examination was divided into 5 categories: Category A consisted of fibroblast in DMEM with 100 µl i-PRF without metronidazole incorporation, Category B consisted of fibroblast in DMEM with 100 µl C-PRF without metronidazole incorporation, Category C consisted of fibroblast in DMEM with 100 µl i-PRF with metronidazole incorporation, category D consisted of fibroblast in DMEM with 100 µl C-PRF with metronidazole incorporation and Category E were control group fibroblast in DMEM 100 µl. The inspection was done on day-1, -3, and -5. On the observation day, 50 µl of MTT were added to each well of the medium, which was then incubated for 4 hours at 37°C in 5% CO2. Following the incubation, 200 µl of DMSO were given to each well as stop solution to dissolve the formazan crystal that had formed. Soon after that, the data on the microplate reader was read to show the absorbance value. IBM SPSS version 24 was employed to investigate the absorbance value data. Shapiro-Wilk normality test was applied for the data distribution test, yet the Levene test was done for the data homogeneity test. For the purpose of establishing the significance of differences between each group, a Post Hoc Least Significant Differences (LSD) analysis was carried out after performing the Two-Way ANOVA parametric analysis test with a significance level of 0.05.

Results

The majority of the group had an enhanced absorbance value of ligament periodontal fibroblast progression, as shown in table 1 and figure 1. The C-PRF without metronidazole incorporation group, the i-PRF with metronidazole incorporation group, the C-PRF with metronidazole incorporation group, and even the control group demonstrated a higher absorbance value. Whilst the absorbance value of cell proliferation dropped in i-PRF without metronidazole incorporation group. Respectively, the highest increase value was in the C-PRF with metronidazole incorporation group, followed by the i-PRF with metronidazole incorporation group, the C-PRF without metronidazole incorporation group, the control group, and the least was the i-PRF without metronidazole incorporation group.

Normality test with Shapiro-Wilk test showed that the data was normally distributed and homogeneity test with Levene test showed that the data was homogeneous. As the data was normally distributed and homogenous, then the analysis was conducted using a Two-Way ANOVA test to show the significant difference of the variable. With significant values of 0.001 and 0.001 (p < 0.05), the type of application group and time of observation had an impact on the absorbance value of ligament periodontal fibroblast proliferation. The absorbance value of ligament periodontal fibroblast growth was also significantly impacted by the interaction between the type of application group and time of observation (p < 0.05). In order to emphasize the variations between each type of application and observation period, Post Hoc Least Significant Difference (LSD) analysis was performed. The results of the Post-hoc test demonstrated the difference between groups. Figure 2 showed the results of i-PRF, C-PRF, i-PRF with metronidazole incorporation, and C-PRF with metronidazole incorporation.

Discussion

Statistical tests show the C-PRF without metronidazole incorporation group, the i-PRF with metronidazole incorporation group, the C-PRF with metronidazole incorporation group, and even the control group showed an increasing absorbance value. In contrast, the i-PRF without metronidazole incorporation group showed the decreasing absorbance value of cell proliferation. This condition might be caused by other cell invasion in the well, so it disturbed the proliferation process. Horizontal centrifugation is believed to increase the platelets and leukocytes counts. Macrophage is one of the types of leukocyte cells which is in the right amounts might stimulate the proliferation process, in contrast in the great amounts might inhibit the proliferation process.

The proliferation phase occurs in 24 hours after injury then increases at day 3 then peaks at day 5. The C-PRF without metronid-
zole incorporation group, the i-PRF with metronidazole incorporation group, and the control group showed the increasing absorbance value on day 3 then decreased on day 5. The C-PRF with metronidazole incorporation group kept increasing and peaked on day 5. In contrast, the i-PRF without metronidazole group kept decreasing on day 3 and day 5. This condition is in contrast with previous study, even C-PRF could increase proliferation cells higher than i-PRF, it did still increase. As seen on the figure 2 showed that i-PRF result looked like a reddish yellow liquid. Red blood cells or red i-PRF could be mixed in with the yellow layer all the way up to it. The yellow layer had fibronectin, fibrinogen, and vitronectin as heparin-binding domains that are used to firmly bind growth factor components. Red layer has fibrin matrices that less compact compared with yellow layer, so it has less ability to bind the growth factor. The successful of proliferation depends on the available of the right growth factor. The preparation of i-PRF and C-PRF with and without metronidazole incorporation used horizontal centrifugation machine. The low-speed centrifugation concept is used to product i-PRF for both with and without metronidazole incorporation, which requires 300g in 5 minutes centrifugation, and high-speed centrifugation concept is used to produce C-PRF for both with and without metronidazole incorporation, which requires 2000g in 8 minutes centrifugation. This horizontal centrifugation will make platelet and leukocyte counts higher in the buffy coat. The growth factors such as PDGF, EGF, TGF-β, and VEGF which are potent stimulator of proliferation cells should help the proliferation process. Based on interaction between types of application test result, C-PRF with metronidazole incorporation group on day 5 had the highest mean absorbance value for the proliferation of periodontal ligament fibroblasts. C-PRF might increase fibroblast proliferation higher than i-PRF. Additional of metronidazole made C-PRF accelerate the proliferation even higher than the C-PRF without metronidazole incorporation. On day 3, the i-PRF with metronidazole incorporation group's mean absorbance value for the growth of periodontal ligament fibroblasts was determined to be the second highest. The absorbance value between C-PRF with metronidazole incorporation and i-PRF with metronidazole incorporation was slightly different. It might conclude that horizontal centrifugation did increase the platelet, leukocyte, and growth factor of i-PRF. The i-PRF without metronidazole incorporation group was different compared with other groups. Its absorbance value decreased from day 3 and kept decreasing on day 5. While the i-PRF group cell proliferation did not survive, the i-PRF with metronidazole incorporation might be able to still proliferate because of the help of metronidazole. Previous study showed that metronidazole was able to increase the human periodontal ligament cell proliferation because of its ability to inhibit pro-inflammatory cytokines. Therefore, the incorporation of metronidazole is important to help as antibacterial and help to increase cell proliferation as well. The clear mechanism of metronidazole inhibit pro-inflammatory cytokines was unknown yet.

Conclusion
Concentrated platelet rich fibrin metronidazole incorporation and injectable PRF metronidazole incorporation are able to increase the periodontal ligament fibroblast proliferation. Platelet concentrates (i-PRF and C-PRF) with metronidazole incorporation increase cell proliferation higher than i-PRF and C-PRF without metronidazole incorporation. Further study of i-PRF, C-PRF, metronidazole, and their combination at longer time of observation is needed.

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Conflict of Interest
The authors report no conflict of interest

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