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Combination Effect of Collagen-Chitosan Hydrogel and Injectable Platelet-Rich Fibrin Fractionation on Osteoblast Migration and Proliferation

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ABSTRACT

Injectable Platelet Rich Fibrin (i-PRF) is the one of concentrate-platelets which is used to periodontal regenerative therapy. Centrifugation of i-PRF result in the fractionation into the yellow and red layers. The red layer contains a greater number of growth factor carrier cells than yellow layer. The i-PRF has a liquid consistency so it easy to combine with other biomaterials, there is collagen-chitosan with hydrogel preparations. This study aimed to determine the effect of a combination of collagen-chitosan hydrogel with injection of i-PRF fractionation on osteoblast migration and proliferation. The sample of this research was divided into 3 groups, group A (combination of the collagen-chitosan hydrogel and red layer), group B (combination of collagen-chitosan hydrogel and yellow layer), and group C (collagen-chitosan hydrogel). Hydrogel was prepared with collagen/chitosan (25/75), then mixed by i-PRF fractionation, ratio of 1:1. The migration test used scratch wound healing, while the proliferation test used Cell Counting Kit-8. Data were tested with One way ANOVA for migration and Two way ANOVA for proliferation, followed by a Post Hoc test. The results showed that there was significant difference in migration of osteoblast cells after 24 hours between group A and other groups ($p<0.05$). For osteoblast cell proliferation on days 1, 3, and 5 in the group A were significant difference with the other group ($p<0.05$). This study concluded that the combination of collagen-chitosan hydrogel with red layer had a more effective on increasing the migration and proliferation of osteoblast cells than the other groups.

Keywords: Collagen, chitosan, hydrogel, injectable platelet-rich fibrin.

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I. Introduction

Loss of periodontal ligament attachment and bone destruction are signs of untreated chronic periodontitis. Periodontal treatment is expected to provide good regeneration. This depends on the availability of cell types and the signals needed to stimulate cell regeneration. One of the ingredients that can be used as a regenerative treatment for damaged periodontal tissues is the use of platelet concentrates [1]. Platelet-Rich Fibrin (PRF) is the second generation of autologous platelets that do not use anti-coagulants and have many leukocyte cells as the body's defense system [2]. The new formulation of PRF is injectable platelet-rich fibrin (i-PRF) which has better regenerative abilities than PRF [3].

Centrifugation in i-PRF produces fractionation of 2 layers namely yellow layer and red layer with buffy coat. The yellow layer results in good formation and strength of the fibrin clot, while the red layer contains more cells and growth factors. The concentration of growth factors is in accordance with previous studies that the red layer with buffy coat, contains more platelets and cells, releases more vascular endothelial growth factor (VEGF) and platelet derived growth factor (PDGF), which are strong stimulators of cell proliferation, while the yellow layer stimulated the differentiation of osteogenic periodontal ligament stem cell (PDLSC) earlier than the red layer [4]. Previous studies have reported that fractionation of i-PRF red layer with buffy coat affects osteoblast cell proliferation more than i-PRF without fractionation. This is because yellow layer has a dense fibrin network, so the release of growth factors is slower than that of the red layer [5]. There is a difference in the rate of growth factor release and differences in soft tissue regeneration between PRF and i-PRF. The results showed that i-PRF is better but has not shown significant result in hard tissue regeneration such as bone [6]. This is due to the weak mechanical strength and too fast degradation, so that the addition of other biomaterials is needed [7].

Another biomaterial that can be combined with i-PRF is collagen-chitosan as a scaffolding material for the formation of new tissue [8]. The use of collagen with i-PRF is a safe and effective combination to regulate the rate of release of growth

factor by i-PRF [9]. One of the limitation of collagen is rapid degradation, which makes it easy to lose its shape and size. Collagen requires the addition of chitosan which has a slower degradation rate so it will increase mechanical strength when combined [10]. Both of these materials will be more easily applied to tissues if they are in hydrogel form. The hydrogel form has shown its potential as a microenvironment provider for tissue regeneration and drug delivery systems. Previous study showed that the combination of collagen-chitosan hydrogel was better in resistance to enzymatic degradation, denaturation, and compressive strength. Its potential as a scaffold is good that it can support regenerative treatments [7], [11]. The ratio of the collagen-chitosan hydrogel combination that can be used according to previous studies is 25/75. According to [8], this ratio showed an increase in the ratio of collagen and a decrease in the ratio of chitosan in hydrogels so that it will slow down the kinetics of gelation and decrease in mechanical properties. A cell culture that has characteristics similar to primary osteoblast cells is the human osteoblast cell line MG63. MG63 cells are cells that are available in unlimited quantities and do not need long isolation or ethical approval with the advantage of more reliable reproducibility [12].

II. Method

This research is a laboratory experimental research conducted at the Advanced Pharmaceutical Laboratory and Pharmaceutical Technology Laboratory, Faculty of Pharmacy, Universitas Gadjah Mada with Ethical Clearance from the ethical committee of the Faculty of Dentistry, Universitas Gadjah Mada, no. 162/KE/FKG-UGM/EC/2022. The research subjects used were MG63 cells and donors for i-PRF with the inclusion criteria of patients aged 20-30 years without systemic diseases, no long-term drug use, no blood disorders with platelet levels of 150,000-450,000, and willing to sign an informed consent.

A. Preparation of Culture Medium

The culture medium consisted of 88% DMEM medium supplemented with 10% fetal bovine serum (FBS), 1% penicillin, and 1% fungzone. After being inserted into the tube, the media ingredients were homogenized using a table shaker, filtered using a sterile syringe filter into an empty bottle, and stored in the refrigerator [12].

B. Osteoblast Cell Culture

Osteoblast cells were taken from cell stock as much as 100 µl using a micropipette, then put into a centrifugation tube and added to 10 mL of Phosphate Buffer Saline (PBS) and then centrifuged at 2000 rpm at 24 °C for 10 minutes. The results of centrifugation will produce 2 parts of the liquid, namely the supernatant and the pellet. The pellet was then redissolved in the culture medium and made homogeneous. The solution was transferred to a petri dish and culture medium was added until the cup volume reached 7 mL. The Petri dishes were put into the incubator and incubated at 37 ºC and 5% CO_2 for 24 hours. Cells multiply to reach a confluent state, then osteoblasts can be harvested [12].

C. Preparation of Collagen-chitosan Hydrogel

The hydrogel made at the Pharmaceutical Technology Laboratory, Faculty of Pharmacy, Universitas Gadjah Mada, is a mixture of chitosan and collagen (fish collagen), both of which are pharmaceutical grade. Chitosan was put into a beaker glass containing 20 ml of water, then stirred using an ultraturax homogenizer at 3000 rpm for 15 minutes. Next, prepare HPMC and collagen powder by weighing them on a digital scale. Enter the collagen powder first into the chitosan after that put the HPMC powder into the solution then stir again using the ultra turax homogenizer until it is homogeneous. After being homogeneous, the preparations were allowed to stand for 15 minutes at room temperature and then put into the cooling machine. The comparison of collagen chitosan was 25/75 [13].

D. Retrieval of i-PRF Fractionation

Donor blood was taken by the nurse according to the inclusion criteria in the cubital diffosa vein as much as 10 ml, then centrifuged at 700 rpm for 3 minutes at room temperature. After centrifugation, 2 types of i-PRF were collected, namely the upper yellow layer and the lower red layer. The red layer along with the buffy coat and yellow layer was collected using a small syringe with an 18G hypodermic needle. The bevel edge is used as a reference point [4].

E. Mixing the Combination of Collagen-chitosan Hydrogel with i-PRF Fractionation

Combine collagen-chitosan hydrogel and i-PRF according to the group with a ratio of 1:1. Group A combined 1ml i-PRF red layer and 1 ml collagen-chitosan hydrogel 25/75, group B combined 1 ml i-PRF yellow layer with 1ml collagen-chitosan hydrogel 25/75, group C 1 ml collagen-chitosan hydrogel [2], [13].

F. Observation of Osteoblast Cell Migration

24 well plates were used with a density of 10^4. After 24 hours and 80% confluent cells, wash the PBS 1x then replace the media with 0.5% FBS. Re-incubate for 24 hours. Next, make a scratch with a yellow tip, then wash the PBS once and document the results of the scratch. Discard the PBS and insert the treatment medium and then incubate again for 24 hours. Document back according to the time of observation using a microscope [2].

G. Observation of Osteoblast Cell Proliferation

96 well plates were used with a cell density of $2.5x10^3$. 24 hours after incubation at 37 °C in a 5% CO_2 incubator, the culture medium was added with 10 µL of CCK-8 solution to each well, then incubated again for 2.5 hours at 37 °C in a 5% CO_2 incubator. Osteoblasts were measured using a 450 nm microplate reader on days 1, 3, and 5 [2].

III. Result

First, a physical parameter test was performed on the collagen-chitosan hydrogel formulation with a ratio of 25/75 which met the criteria in previous studies. In this study, the pH was 6.6 and the gel viscosity was 2365 cps. Second, a physical parameter test of the collagen-chitosan hydrogel

formulation was carried out according to the criteria that had been added with i-PRF with a ratio of 1:1 [8].

The results of the observation of migration and proliferation testing of MG63 cells were divided into 3 groups, which were the red i-PRF + collagen-chitosan hydrogel group (A), the yellow i-PRF + collagen-chitosan hydrogel group (B), and the control group collagen-chitosan hydrogel (C). Migration observations were carried out after 24 hours using the scratch wound healing assay method, while proliferation tests were carried out on days 1, 3 and 5 which were determined using the Cell Counting Kit-8 (CCK-8), and measured using a 450 nm microplate reader.

A. Migration

Scratch wound healing assay migration test was performed to determine the effect of the combination of collagen-chitosan hydrogel and i-PRF fractionation on osteoblast cell migration. The parameter used in the migration test analysis is the percentage (%) of osteoblast cell coverage. The osteoblast cell migration test was carried out with an observation time after 24 hours and was measured based on the reduction of 0 hours with 24 hours can be seen in Fig. 1.

Photos of the osteoblast cell migration test at the 0 and 24th hours obtained from observations using an inverted microscope with a magnification of 100x were analyzed using the ImageJ software. The data obtained was in the form of an area not overgrown with cells as a result of reduced observations between 0 hours and 24 hours and then converted into percentage (%) data of osteoblast cell cover. The average difference in the results of the percentage (%) of closing osteoblast cells can be seen in Table I.

The parametric statistical test requires is data that is normally distributed and homogeneous. To find out whether the data were normally distributed, a normality test was performed using Shapiro-Wilk with a significance value of 0.803 ($p>0.05$). This test was indicating that the average migration of osteoblast cells in each group had a normal distribution. The results of the homogeneity test with Levene's test showed a significance value of 0.078 ($p>0.05$), which means that the data between the time of observation and the treatment group was homogeneous and met the requirements for the One-way ANOVA parametric test. One-way ANOVA test was conducted to compare migration rates between groups. The results of the One-way ANOVA test obtained a significance value of <0.001 ($p <0.05$), indicating that there was an effect of differences in group treatment on the closure percentage (%) of osteoblast cell migration at 24 hours of observation.

The next test is the Post Hoc Least Significant Difference (LSD) Test. Based on this test, it was found that there were significant differences between all treatment groups, between group A with group B ($p=0.000$), group A with group C ($p=0.000$), and group C with group B ($p=0.003$) as shown in Table II.

B. Proliferation

Table II showed that the average absorbance value of MG63 cells increased in each group based on incubation time on day 1, 3, and 5. On day 1, 3 and 5, MG63 cell proliferation was lowest in the group C, then the group B, and the highest in the group A. The difference in the average absorbance value can be seen in Fig. 2.

The requirement to perform a parametric statistical test is that the data must be normally distributed and homogeneous. The results of the normality test using the Kolmogorov-Smirnov showed a significance value of 0.200 ($p>0.05$) indicating that the mean osteoblast cell proliferation in each group was normally distributed. The results of the homogeneity test with Levene's test showed a significance value of 0.129 ($p>0.05$), which means that all data is homogeneous based on days in each treatment group and meets the requirements for the Two-way Anova parametric test. A two-way ANOVA test was performed to compare the average proliferation between groups showed that the treatment, day, and interactions between treatments and day had a significant effect on MG63 cell proliferation with a significance value ($p<0.05$). The next test is the Post Hoc Least Significant Difference (LSD) Test. This test was conducted to determine the difference between the treatment interactions and the time of observation. The results of the LSD Post Hoc test can be seen in Table III.

Based on Table III, there were significant differences in almost all treatment groups, the time of observation, and the interaction between the two groups, except on day 3 (the control group) did not have a significant difference from day 1 group B ($p=0.461$), then on day 5 group B with the 3rd day of group A ($p=0.461$), and the 5th day of the control group with the 1st day of group A ($p=0.133$).

TABLE I: MEAN AND STANDARD DEVIATION OF OSTEOBLAST CELL MIGRATION CLOSURE AT 24 HOURS OF OBSERVATION

Incubation Time	Samples	Group A	Group B	Group C
24 hours	9	13,289 ± 1,790	10,533 ± 1,020	8,480 ± 0,880

TABLE II: MEAN AND STANDARD DEVIATION OF PROLIFERATIVE ABSORBANCE VALUES BASED ON INCUBATION TIME AND TREATMENT

Incubation Time	Samples	Group A	Group B	Group C
Day 1	9	0.444 ± 0,043	0.363 ± 0,039	0.276 ± 0,060
Day 3	9	0.740 ± 0,055	0.548 ± 0,060	0.381 ± 0,071
Day 5	9	0.995 ± 0,043	0.722 ± 0,034	0.482 ± 0,056

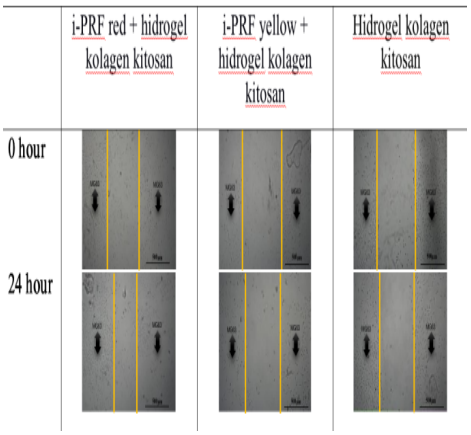

Fig. 1. Migration of osteoblast cells with 100x magnification using an inverted microscope. The yellow mark is the border of % cell closure.

TABLE III: LSD POST HOC TEST RESULTS FOR MG63 CELL PROLIFERATION

		Day 1			Day 3			Day 5		
		A	B	C	A	B	C	A	B	C
Day 1	A	-	0.002	<0.001	<0.001	<0.001	0.014	<0.001	<0.001	0.133*
	B			<0.001	<0.001	<0.001	0.461*	<0.001	<0.001	<0.001
	C				<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
Day 3	A					<0.001	<0.001	<0.001	0.461*	<0.001
	B						<0.001	<0.001	<0.001	0.010
	C							<0.001	<0.001	<0.001
Day 5	A								<0.001	<0.001
	B									<0.001
	C									-

Note: A: i-PRF red + collagen-chitosan hydrogel, B: i-PRF yellow + collagen-chitosan hydrogel, C: collagen-chitosan hydrogel, *: group that is not significant

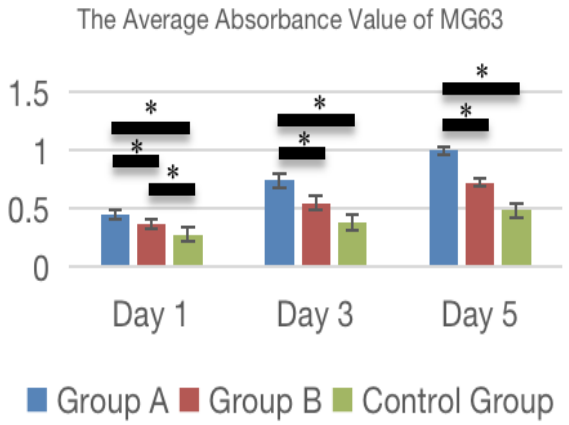

Fig. 2. Graph of the average absorbance value of MG-63 cells. *indicates a significant difference p<0.05.

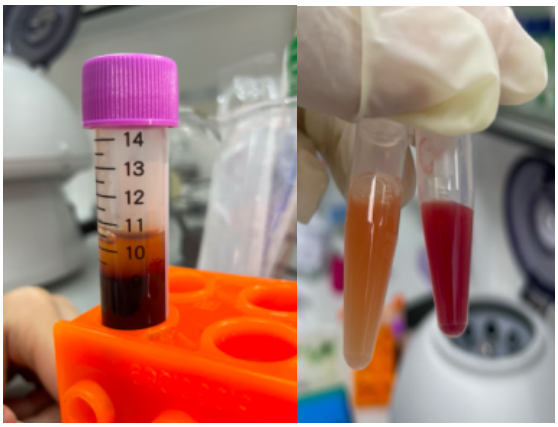

Fig. 3. Centrifugation and Fractionation of i-PRF.

IV. DISCUSSION

The requirements for making an appropriate collagen-chitosan hydrogel formulation are in accordance with previous studies with an appropriate pH in the oral cavity between 6.2-7.6 and gel viscosity values between 2000-4000 cps. In this study, the pH was 6.6 and the gel viscosity was 2365 cps. The first step was to test the physical parameters with a pH of 6.6 and a gel viscosity of 2365 cps. The second step was the addition of Collagen-chitosan hydrogel with i-PRF with a ratio of 1:1 [8].

Observation and data analysis regarding the combination of collagen-chitosan hydrogel and injectable platelet-rich fibrin fractionation on the migration and proliferation of osteoblast cells have been carried out. Injectable platelet-rich fibrin is divided into 2 types after the centrifugation process, which were the red i-PRF layer and the yellow i-PRF layer. The yellow i-PRF layer was taken from above the buffy coat while the red i-PRF layer was taken together with the buffy coat. Another biomaterial that can be added with i-PRF is collagen-chitosan hydrogel. Research conducted by [14] explained that a hydrogel containing chitosan would increase the occurrence of osteogenic differentiation by increasing mineralization, while the addition of collagen would promote greater cell proliferation [15].

From the observation of osteoblast cell migration after 24 hours, the three groups showed significant results, so the three groups showed osteoblast cell migration. This is due to the presence of a collagen component as a motivating agent which can affect cell migration. These matrix proteins also facilitate the production of new ECM and supports cell growth which is essential for cell repair processes. Through chemical, mechanical, and morphological cues, the ECM regulates cell migration.

From the observation of each group, namely the red i-PRF + collagen-chitosan hydrogel group showed a significant difference with the yellow i-PRF + collagen-chitosan hydrogel and collagen-chitosan hydrogel groups. These results indicated that the migration of osteoblast cells in the red i-PRF + collagen-chitosan hydrogel treatment group was faster than the other two groups. This is due to the addition of red i-PRF fractionation to the collagen-chitosan hydrogel so the release of various growth factors such as platelet-derived growth factor (PDGF) and transforming growth factor (TGF) in larger amounts will increase cell migration [14]. The yellow i-PRF + collagen-chitosan hydrogel group showed faster osteoblast cell migration than the collagen-chitosan hydrogel group. This is because the yellow layer on i-PRF has components such as fibronectin, a fibrin-rich glycoprotein. Fibronectin can promote cell growth and will spread and adapt to the wound area [16].

The migration of osteoblast cells is a very important process in tissue repair. Osteoblast cells develop and migrate to the wound area, synthesize new extracellular matrix, and play a role in wound healing. Growth factors promote wound healing by promoting cell migration and proliferation [17]. In accordance with the research of [6], i-PRF has platelets, leukocytes, collagen type 1, osteocalcin, and growth factors which are the best choices for healing soft and hard tissues [14].

Observation of cell proliferation on days 1, 3, and 5 in the three groups had significant results so each group experienced an increase in osteoblast cell proliferation. This is in accordance with the research of [18] who stated that the addition of collagen to the chitosan hydrogel increased the

porosity of the hydrogel. This porosity makes the cells in the collagen-containing hydrogels show increased proliferation. The growth factor produced by i-PRF will also increase cell proliferation [17].

On days 1, 3, and 5, the red i-PRF + collagen-chitosan hydrogel group with the yellow i-PRF + collagen-chitosan hydrogel group and the collagen-chitosan hydrogel group showed significant differences. This result showed that the red i-PRF + collagen-chitosan hydrogel group experienced the highest proliferation of osteoblast cells among the other groups. Furthermore, on days 1, 3, and 5 the yellow i-PRF + collagen-chitosan hydrogel group with the collagen-chitosan hydrogel group also showed significant differences. This indicated that the yellow i-PRF + collagen-chitosan hydrogel group had higher osteoblast cell proliferation than the collagen-chitosan hydrogel group.

On day 3, the yellow i-PRF + collagen-chitosan hydrogel group showed no significant difference with the red i-PRF + collagen-chitosan hydrogel group on day 1, but there was a significant difference with the collagen-chitosan hydrogel group. This showed that the proliferation in the yellow i-PRF + collagen-chitosan hydrogel group was higher than the collagen-chitosan hydrogel group, but the results were the same as the red i-PRF + collagen-chitosan hydrogel group on day 1.

On day 5, the yellow i-PRF + collagen-chitosan hydrogel group showed no significant difference with the red i-PRF + collagen hydrogel group on day 3, but there was a significant difference with the collagen-chitosan hydrogel group, while the collagen-chitosan hydrogel group showed no significant difference with the red i-PRF + collagen hydrogel group on day 1. This indicated that the proliferation in the red i-PRF + collagen hydrogel group on day 1 was the same as the proliferation in the collagen-chitosan hydrogel group on day 5.

These results indicated that fractionation of i-PRF red collagen-chitosan hydrogel affects osteoblast proliferation more than yellow collagen-chitosan hydrogel and collagen-chitosan hydrogel, then fractionation of i-PRF yellow collagen-chitosan hydrogel influences osteoblast proliferation more than collagen-chitosan hydrogel. According to [19] many factors influence osteoblast proliferation, one of which is the growth factor. In the research of [4], the growth factor produced by red i-PRF is more than yellow i-PRF. The release of these growth factors can stimulate cell proliferation. The cell proliferation phase started on day 1 and continued to increase on day 3 and 5 [20].

The results of this study are in accordance with previous research conducted by [4], that compared the effect of the yellow i-PRF layer and the red i-PRF layer on human periodontal ligament cells. The researchers reported higher cell proliferation and migration for the red i-PRF-coated group compared to the yellow i-PRF-coated group ($p = 0.046$ and $p = 0.024$) [21].

In this study, the observation of osteoblast cell migration still used the 2D cell cult technique, so that the morphology and phenotype, and cell polarity could easily change. The use of 3D cell culturing techniques can describe the actual tissue conditions and cell microarchitecture, so it is necessary for further research as a basis for clinical use.

V. Conclusion

Based on the research results, it was found that the combination of collagen-chitosan hydrogel and i-PRF fractionation had an effect on increasing osteoblast cell proliferation, and the combination of collagen-chitosan hydrogel and injectable platelet-rich fibrin fractionation on the red layer had a more effect on increasing osteoblast cell proliferation than the yellow layer.

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Conflict of Interest

Authors declare that they do not have any conflict of interest.

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