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Effect of platelet-rich plasma on fibroblasts induced by lipopolysaccharide: in vitro study for wound healing

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Abstract

Platelet-rich plasma (PRP) is expected to be an alternative therapy in wound healing by accelerating the inflammatory process and increasing the healing factors so that the healing process or inflammation is faster. The purpose of this study was to examine the effect of calcium chloride ($CaCl_2$)-activated PRP on the viability, migration, and cytokine levels of interleukin-6 (IL-6) and vascular endothelial growth factor (VEGF) of dermal fibroblast cells in lipopolysaccharide (LPS)-induced inflammatory conditions, as a model in wound healing in vitro. Fibroblast cells were grown in DMEM medium induced with LPS, and then $CaCl_2$-activated PRP treatment was added. Measure fibroblast cell viability using CCK-8 kit (cell counting kit-8) was evaluated using a microplate reader, and the cell migration was evaluated using scratch-assay and TScratch software. Expression of IL-6 and VEGF using ELISA kit. All data were analyzed using software SPSS version 26 by performing a one-way analysis of variance (ANOVA), Kruskal Wills, and Mann-Whitney tests. The results showed that PRP significantly increased fibroblast cell viability in the 10% PRP treatment group. This study shows that PRP does not reduce IL-6 cytokine levels but can increase VEGF growth factor in fibroblast cell cultures. PRP increased cell migration so that the healing process was faster. In conclusion, the CaCl2-activated PRP on LPS-induced fibroblast cells can increase viability and accelerate cell migration; it can't decrease IL-6 but can increase VEGF expression. PRP is expected to be an alternative therapy in wound healing.

Keywords: human dermal fibroblast | IL-6 | migration | VEGF | viability | platelet-rich plasma

Introduction

Wound healing is a complex process involving interactions between cell migration and proliferation and is regulated by many cytokines and growth factors (GFs). Wound healing phases include hemostasis, inflammation, proliferation, and remodeling. Some GFs that play a role in the wound healing process include epidermal growth factor (EGF), fibroblast growth factor (FGF), insulin-like growth factor (IGF), keratinocytes growth factor (KGF), platelet-derived growth factor (PDGF), transforming growth factor (TGF), and vascular endothelial growth factor (VEGF) (Primadina *et al.*, 2019; Canedo-Dorantes & Canedo-Ayala, 2019; Vaidyanathan, 2021).

Dermis fibroblasts are the most important cells in the wound healing process, with growth factors producing extracellular matrix, collagen biosynthesis, wound contraction, re-epithelialization, and tissue remodeling (Wei *et al.*, 2021). Lipopolysaccharide (LPS) is the main component of the cell wall of Gram-negative bacteria, which can cause an acute inflammatory response and trigger the release of large amounts of inflammatory cytokines in fibroblast cells. This response is by activating Toll-Like

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Receptor 4 (TLR4) signalling in dermal fibroblasts via NF-κB, producing pro-inflammatory cytokines, which will ultimately cause inflammation. LPS can stimulate inflammation in vitro in wound healing by inducing fibroblast cells with LPS (Huang & Kraus, 2016; Shi *et al.*,2021).

Platelet-rich plasma (PRP) is autologous platelets in plasma that are concentrated by centrifugation. PRP contains 2 to 6-fold higher platelet concentrations with a low plasma volume. PRP contains many growth factors and other bioactive proteins, including VEGF, PDGF, TGF-beta, chemokines, cytokines, and other plasma proteins essential in hemostasis or wound healing. PRP functions as an anti-inflammatory and pro-regenerative, making the wound healing process faster. PRP also contains adhesion molecules such as fibronectin, fibrin and vitronectin. These cell adhesion molecules affect cell migration (Damayanti & Wathon, 2017; Wen *et al.*, 2018; Vaidyanathan, 2021; Shams *et al.*, 2022).

Platelet activation is the main process in using PRP. The α-granules in platelets in PRP are still inactive, their growth factors are not yet functional because they have not been activated. To initiate the release of this growth factor, PRP must be activated. This activation can be done using calcium chloride ($CaCl_2$) to cause platelet aggregation. After PRP is activated, platelet degranulation as results in the release of growth factors from α-granules, and fibrinogen cleavage occurs to initiate the formation of extracellular matrix. Over 95% of the GFs in platelets will be secreted within 1 hour and undergo degranulation in 3 to 5 days. After $CaCl_2$ activation, GFs remains active for 7 to 10 days. Calcium chloride catalyzes the conversion of fibrinogen into fibrin, forming platelet aggregation. Approximately 70% of growth factor release occurs within 10 minutes of activation, and almost 100% of release occurs within 1 hour (Cavallo *et al.*, 2016; Roh *et al.*, 2016; Kikuchi *et al.*, 2019).

Methods

Procedure PRP

The process of making PRP uses one bag of whole blood (500 ml) obtained from the Indonesian Red Cross (PMI), which has been added with an anticoagulant citric acid dextrose (ACD). The blood was then centrifuged two times. The first centrifugation was carried out for 4 minutes at a speed of 2000 G, which aimed to separate the blood into 3 layers, namely the base layer (erythrocytes), the middle layer (buffy coat), and the top layer (plasma). The supernatant plasma containing platelets is separated with a plasma extractor and then transferred into a sterile bag without anticoagulant. The supernatant taken was subjected to a second centrifugation for 7 minutes at a speed of 4000 G at a temperature of 22°C to obtain a high concentration of platelets. The upper 2/3 was found to be platelet-poor plasma (PPP), while the lower 1/3 was PRP, where platelet concentrate was deposited at the bottom of the bag. PPP was separated from PRP using a plasma extractor to obtain 60 ml of PRP (platelet level 1.3 million/μL). All procedure-making PRP was conducted at the PMI.

Cell thawing and culture

Human dermal fibroblast cells were obtained from the biorepository of the YARSI University Stem Cells Research Center. A 9 ml of DMEM medium was added to 1 ml of dermal fibroblast cell solution in a 15 ml tube then centrifuged for 7 minutes at 1200 rpm. The supernatant was discarded, and then DMEM consisting of 10% fetal bovine serum (FBS) and 1% antimycotic antibiotics was added to the cell pellets, with a density of 5×10^3 in flash. Next, the cells were incubated at 37°C and 5% CO_2. The culture medium was changed every 2 days until the cells were confluent, and 2.4 million cells were used for this study.

Lipopolysaccharide induction and PRP treatment

Fibroblast cells were grown in 12-well plate culture dishes with a complete medium and then induced by 10 µg/mL LPS for 24 hours. PRP treatment was given at doses of 2.5%, 5%, and 10%, activated by $CaCl_2$, and incubated for 24 hours.

Evaluation and data analysis

This research divides into 5 groups, namely: Group K- as control negative (fibroblast without LPS and PRP), Group K+ as control positive (fibroblast + LPS without PRP), Group PRP 2.5% (fibroblast + LPS + PRP 2.5%), Group PRP 5% (fibroblast + LPS + PRP 5%), and Group PRP 10% (fibroblast + LPS + PRP 10%). Viability test using 10,000 cells in 96-well plates, test in each well by adding CCK-8 reagent, then incubating for 1 hour, then measuring the absorbance using spectrophotometry. The higher the graph is, the higher the cell viability. Test cell migration by performing tests *scratch* (TScratch) by making scratches on the bottom wall of each well, then assessing the acceleration of cell closure every 2 hours starting from 0 hours to 24 hours at the scratch location using TScracth. Interleukin-6 (IL-6) and VEGF levels were tested using ELISA, this examination used a human IL-6 ELISA kit (RAB-3-6-IKT) and human VEGF-A ELISA kit (RAB0507-IKT) from Sigma MCLS.

Data analysis used Shapiro-Wilk to assess whether a data set is normally distributed and Levene's test to assess homogeneous data variance. Next, parametric analysis of variance was carried out using one-way ANOVA, Kruskal Wallis, and Mann-Whitney.

Results

Effect of PRP on cell viability

Fibroblast cells giving PRP vary in cell viability. The results showed that PRP 10% had the ability to increase cell viability better than the other groups (**Figure 1**).

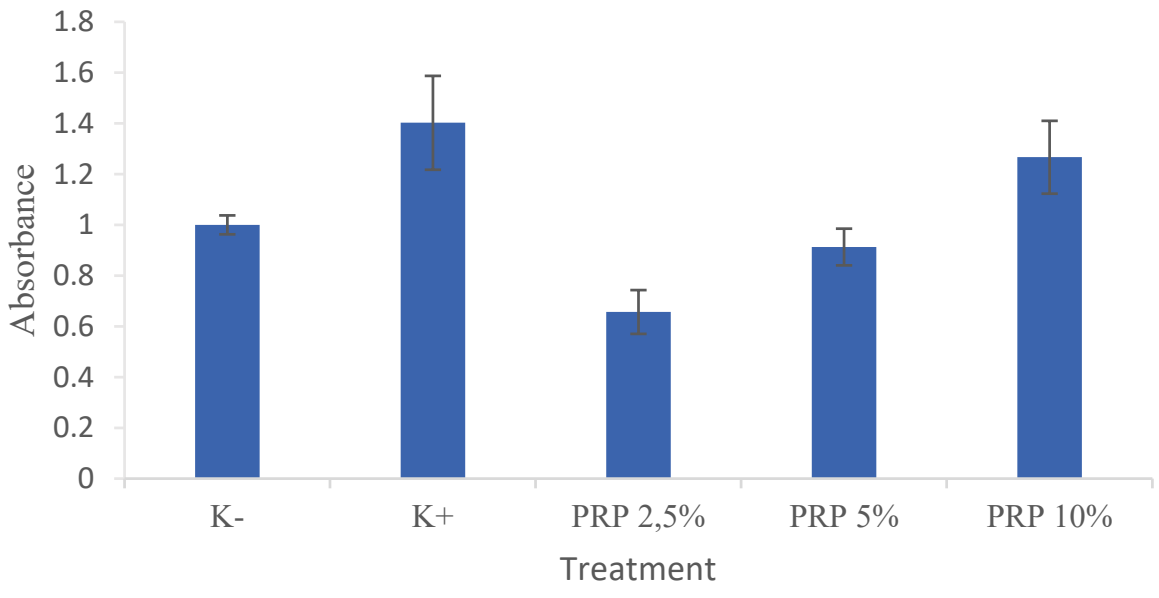

Figure 1 Fibroblast cell viability in the treatment group after giving PRP for 24 hours. The viability increased after being given treatment with the PRP 10% group, which showed a better increase in cell viability than the other groups. Post hoc test results showed that there was a significant difference in cell viability in the positive control (K+) group compared to the PRP 2.5% and PRP 5% treatment groups ($P<0.05$). Still, there was no significant difference in the positive group compared to the PRP 10% treatment group ($P>0.05$).

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Effect of PRP on cell migration

The effect of $CaCl_2$-activated PRP on fibroblast cells showed that cell migration occurred in all groups. **Figure 2** shows that cell migration occurred in all treatment groups, and the scratches closed almost completely within 24 hours.

The PRP 10% treatment group showed faster closure of the scratch area compared to other treatment groups within 24 hours. The lower the graph of the percentage of open area, the less open area remains in areas that have been previously scratched, or the open area has completely closed (**Figure 3**).

Effect of PRP on IL-6 levels

The effect of $CaCl_2$-activated PRP on lipopolysaccharide-induced fibroblast cells showed increased IL-6 levels (**Figure 4**). The results showed PRP did not reduce interleukin-6 levels in LPS-induced cells. The higher the PRP dose given, the higher the interleukin-6 levels.

Effect of PRP on VEGF levels

The effect of $CaCl_2$-activated PRP on LPS-induced fibroblast cells showed increased VEGF levels (**Figure 5**). The results showed PRP could increase VEGF levels in LPS-induced fibroblast cells. The higher the dose of PRP given, the higher the concentration of VEGF levels.

Discussion

The research results show that administration of PRP can accelerate wound healing using an inflammatory wound model in human dermal fibroblast cells. Cell viability is the ratio between living cells and the total number of cells. PRP can increase cell viability in inflammatory conditions, proven by the CCk-8 results, which showed a maximum dose-dependent increase in cell viability at 10% PRP. This result supports previous research that found increased fibroblast cell viability with PRP administration (Hadi *et al.*, 2019; Hassan *et al.*, 2022). Fibroblasts are inactive under normal

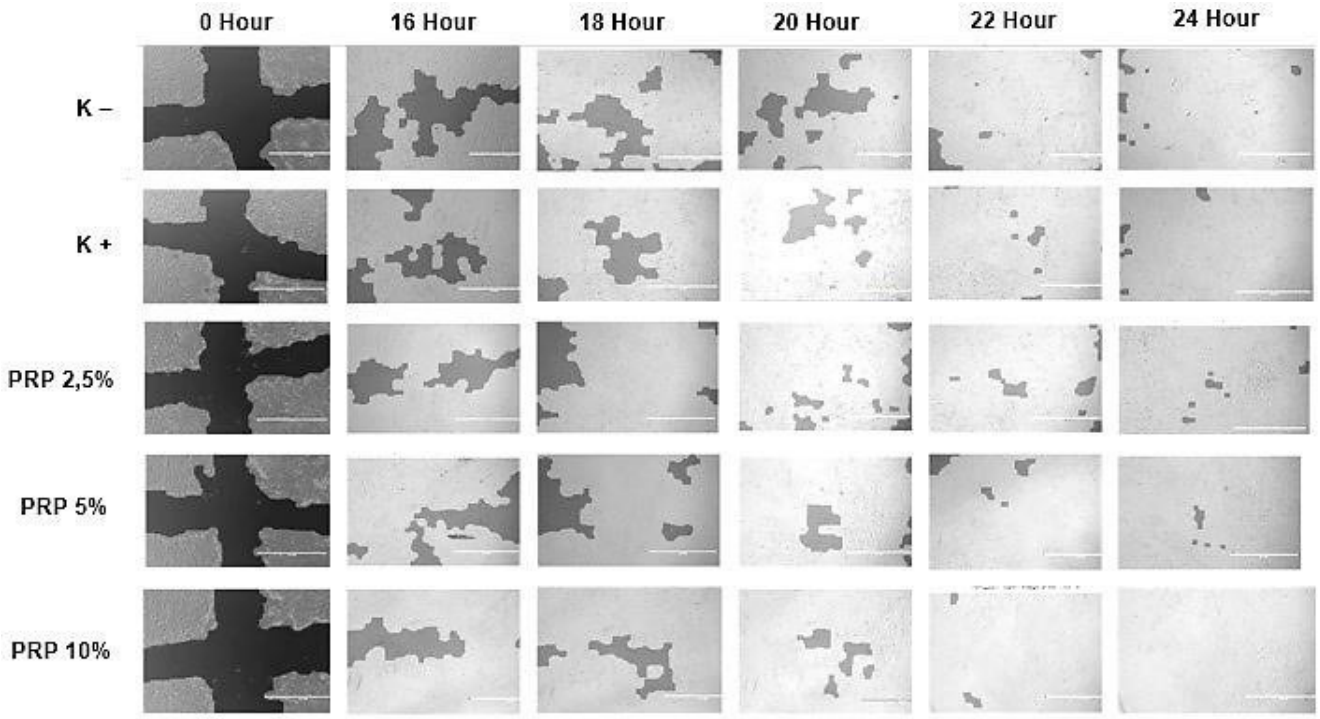

Figure 2 Fibroblast cell migration in treatment groups. The cell migration occurred in all treatment groups, and the scratches closed almost completely within 24 hours.

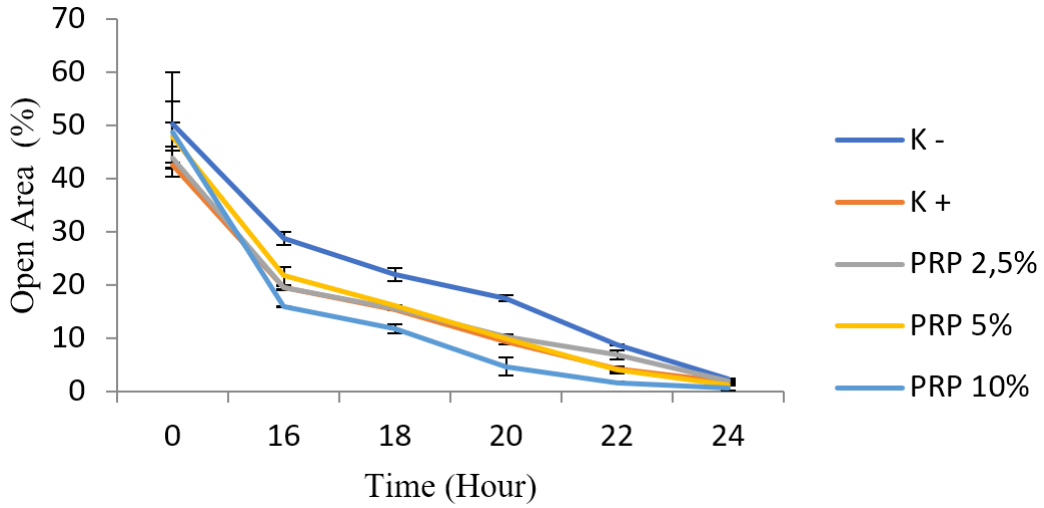

Figure 3 Percentages of closuring area by fibroblast cell migration during the PRP treatment group in 24 hours. Fibroblast cell migration occurred in all groups, but the closure of the open area was fastest in the PRP 10% group, which was almost completely closed within 24 hours.

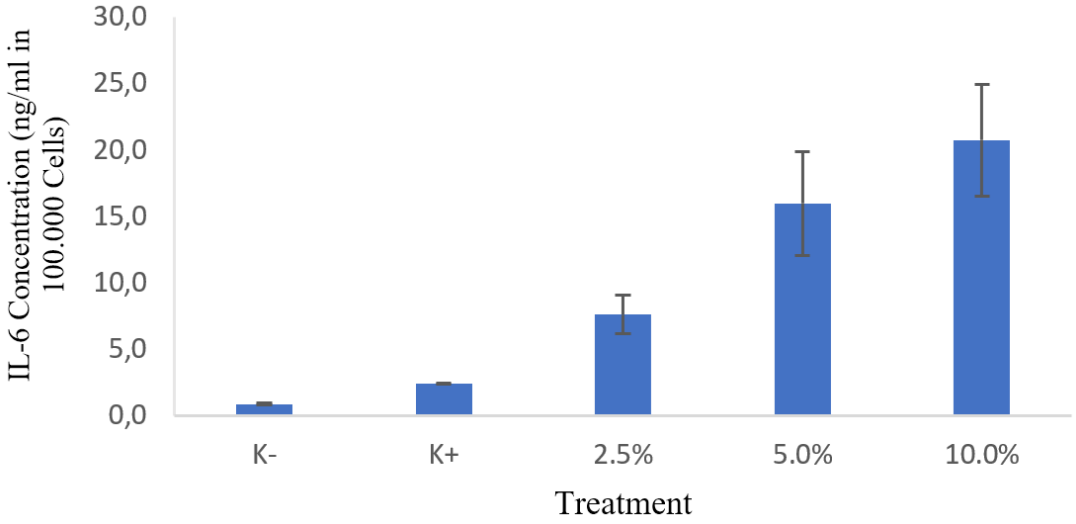

Figure 4 Average of IL-6 levels after treatment. PRP does not reduce interleukin-6 levels in lipopolysaccharide-induced fibroblast cells. The PRP 10% showed the highest mean compared to other groups.

conditions; however, fibroblast cells will be active and carry out chemotactic growth factors when injury or inflammation occurs, which will cause angiogenesis.

Cell migration is the process of moving cells from one location to another; this is an important biological mechanism in assessing cell movement (Grada *et al.*, 2017). The results showed that the scratch area closed more quickly in the PRP treatment group compared to the positive control group. The 2.5%, 5%, and 10% PRP treatment groups showed faster cell migration acceleration than the positive control group at 0 to 24 hours. However, the results of statistical tests for the 2.5%, 5% and 10% PRP treatment groups did not show significant differences in fibroblast cell migration compared to the positive control.

Fibroblast cells produce an extracellular matrix that regulates fibroblast migration activity, angiogenesis, granulation tissue, and epithelialization. According to previous research, fibroblast cell migration in the wound area is stimulated by TGF-β produced by

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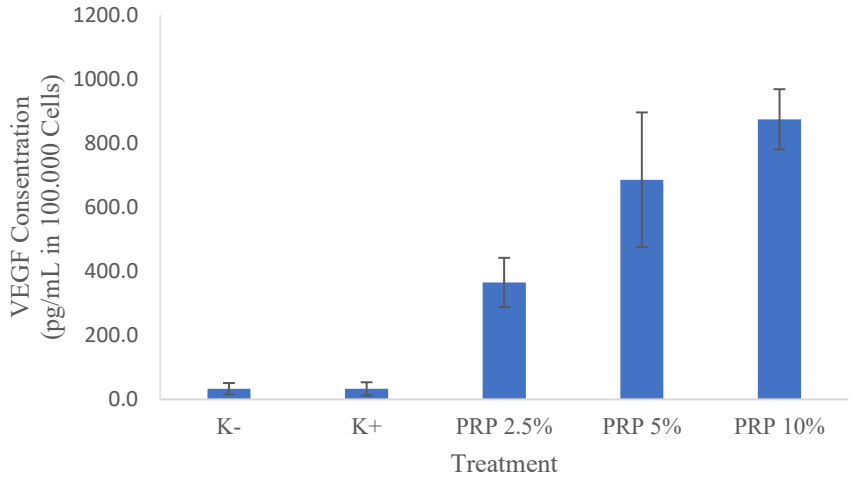

Figure 5 Average of VEGF levels in fibroblast cells after treatment. The graph shows that PRP can increase VEGF levels in fibroblast cells induced by LPS. Kruskal Wallis Test. Mann-Whitney post-hoc test: K+ vs PRP 2.5% p=0.021 (p<0.05); K+ vs PRP 5% p=0.021 (p<0.05); K+ vs PRP 10% p=0.248 (p>0.05).

granulation tissue formed during the inflammatory process. Platelets in PRP contain adhesion molecules such as fibronectin, fibrin, and vitronectin. These adhesion molecules play a role in cell migration. In platelets, there are more α-granules. Alpha-granules contain various growth factors such as PDGF, TGF-β, FGF-2, IGF-1, EGF, and VEGF (Tao *et al.*, 2017; Nguyen & Pham, 2018; Vaidyanathan, 2021).

VEGF is a factor that participates in the migration process of fibroblast cells through the induction of components of the wound healing cascade, such as angiogenesis, collagen deposition, and epithelialization. Epithelialization starts when epithelial cells around the wound's edges begin to migrate into the wound area. As migration progresses, cells in the basal layer begin to proliferate to form additional epithelial cells. Migration continues until the epithelial cells connect with other cells until they fuse (Cen *et al.*, 2022).

Bioactive proteins include growth factors in α-granules which will be released after platelets are activated by calcium chloride ($CaCl_2$). PRP has an effect in accelerating the migration of fibroblast cells. The higher the PRP concentration, the faster the cell migration process. This finding aligns with research by Saputro *et al.* (2022), who reported that if platelet levels are 3 to 5 times greater than the general value, it will induce an inflammatory process more quickly, and the wound will heal faster. Previous research using an in vitro model research revealed if the number of platelets is $0.6–1.3\times10^{10}$ mL, it can stimulate cell migration and differentiation (Saputro *et al.*, 2022; Szwedowski *et al.*, 2021).

LPS stimulates in vitro inflammation to activate and release proinflammatory cytokines, including IL-6. Fibroblast cells have IL-6, which functions to stimulate fibroblast growth. Interleukin-6 functions as a pro-inflammatory and anti-inflammatory cytokine secreted by T cells and macrophages to stimulate immune responses during inflammation. The release of the cytokine IL-6 contributes primarily to the induction of inflammation. PRP contains many growth factors and cytokines, one of which is the cytokine IL-6. The study showed that IL-6 levels did not decrease after being given PRP treatment, because in the acute inflammatory phase, it will secrete IL-6 as a defence mechanism or immune response in the wound healing process so that IL-6 levels increase. This is similar to previous research stating that IL-6 levels increase sharply after infection. In cell culture conditions, it cannot be

determined whether IL-6 appears because it is pro-inflammatory or anti-inflammatory. IL-6 levels were higher in the PRP treatment group, which is the same as previous research that IL-6 concentrations increased in cultures with PRP treatment (Sundman *et al*., 2013; Kovacs *et al*., 2014; Masfufatun *et al*., 2018; Zongfei *et al*., 2020).

PRP initiates wound repair through the release of growth factors such as VEGF. Growth factors stimulate migration, proliferation, healing, and cell differentiation. After $CaCl_2$ activates PRP, growth factors derived from platelets exit the α-granule and function in the recruitment and activation of fibroblast immune cells. So, this study shows an increase in VEGF levels in fibroblast cells that were given PRP treatment. This result is similar to research by Saputro *et al*. (2022) that showed PRP can increase VEGF levels more than treatment without PRP.

Conclusion

PRP activated by $CaCl_2$ can increase lipopolysaccharide-induced fibroblast cells' viability, the highest in the 10% PRP treatment group. Compared to the positive control group, PRP did not increase the migration speed of fibroblast cells induced by lipopolysaccharide. Administration of $CaCl_2$-activated PRP did not reduce IL-6 cytokine levels in lipopolysaccharide-induced fibroblast cells. PRP can increase VEGF levels in fibroblast cells that are induced by lipopolysaccharide.

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Author contributions: IK and MI conceived and designed the experiments; MI analyzed the data; MI, IK, and J performed the experiments and wrote the paper.

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