THE EFFECT OF PLATELET-RICH PLASMA ADMINISTRATION ON PROLIFERATION AND DIFFERENTIATION OF ENDOTHELIAL PROGENITOR CELLS IN PATIENTS WITH STABLE CORONARY HEART DISEASE

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ABSTRACT

Endothelial Progenitor Cells (EPCs) are crucial precursors to endothelial cells, playing a key role in regulating blood vessel structure and maintaining homeostasis to protect against inflammation and thrombosis, contributing to stable coronary heart disease (CHD). Growth factors stimulate signal transduction during EPC proliferation and differentiation. Platelet-Rich Plasma (PRP) contains widely recognized growth factors in angiogenesis. Our research aimed to analyze PRP's effects on EPC proliferation and differentiation in stable CHD patients. Using an experimental post-test control group design, mononuclear cells (MNCs) from peripheral blood were cultured with M-199 medium, divided into PRP, Platelet Poor Plasma (PPP), and control groups, and incubated for 14 days. EPC proliferation was quantified with CD34 markers using ANOVA. After 7 days, differentiated cells were counted with von Willebrand Factor (vWF) markers using the Mann-Whitney U test.EPC proliferation significantly increased in the PRP group (1.052 ± 0.16) compared to PPP (0.762 ± 0.19) and the control (0.068 ± 0.05, p=0.000). However, EPC differentiation showed no significant increase in the PRP group compared to PPP (0.00-0.30 vs. 0.00-0.20, p = 0.565) or the control (0.00-0.30 vs. 0.00-0.00, p = 0.064). Additionally, no significant increase in EPC differentiation was observed in the PPP group compared to the control (0.00-0.20 vs. 0.00-0.00, p = 0.144). PRP significantly enhanced EPC proliferation but did not significantly enhance differentiation in the peripheral blood of stable CHD patients compared to PPP and control groups.

Keywords: EPC Proliferation; EPC Differentiation; PRP

INTRODUCTION

Coronary artery disease is globally prevalent, constituting the primary cause of elevated mortality and morbidity, with around 5.7 million new cases reported annually. In the United States, ischemic heart disease affects approximately 5 million individuals, and its incidence continues to rise, with an annual increase of approximately 400,000-600,000 new cases. Noteworthy progress in the management of acute coronary syndrome (ACS) has been made in the last three decades, marked by the widespread utilization of thrombolytic therapy,

percutaneous coronary interventions (PCI), and the development of potent anti-thrombotic medications, resulting in significant advancements (Rosenstrauch et al., 2005).

Despite enhanced survival rates, the growing life expectancy and increased comorbidities contribute to a surge in patients experiencing left ventricular dysfunction due to myocardial cell demise and remodeling processes. This trend ultimately leads to a heightened incidence of heart failure (Jessup et al., 2003). Ischemic heart failure arises from the substantial damage to cardiomyocytes following a reduction in myocardial perfusion. Various therapeutic innovations have been implemented to address heart failure, encompassing pharmacological interventions such as beta-blockers, diuretics, and ACE inhibitors, as well as surgical approaches and left ventricular assist device implantation. However, these strategies fall short of replacing deceased cardiomyocytes, and even heart transplantation encounters hurdles such as donor availability, immunosuppressant complications, and elevated long-term failure rates.

In this context, the potential of stem cell therapy for cardiomyocyte regeneration and neovascularization appears promising, particularly with the advancing understanding of stem cell biology and its application in coronary artery disease (CAD) and heart failure patients (Menasche et al., 2003). In 1997, research disclosed the presence of mononuclear cells expressing CD34 and VEGF-2 (Vascular Endothelial Growth Factor Receptor-2), isolated from human peripheral blood and cultured. These cells, termed Endothelial Progenitor Cells (EPCs), possess the capability to differentiate into endothelial-like cells (Asahara et al., 1999).

Endothelial cells play a pivotal role in regulating the structure of blood vessels to sustain homeostasis, shielding vessels from inflammation and thrombosis. Various risk factors for coronary heart disease (CHD) contribute to endothelial dysfunction, initiating the atherosclerosis process manifested clinically as CHD. Numerous studies emphasize the crucial role of EPCs in re-endothelialization, although the same CHD risk factors diminish EPCs, exacerbating endothelial dysfunction (Shantsila et al., 2007). Endeavors to augment EPC numbers include the administration of growth factors like vascular endothelial growth factor (VEGF). VEGF not only participates in EPC recruitment but also facilitates the homing process, inducing neovascularization (Yancopoulos et al., 2000, and Bir et al., 2009).

Platelets are the initial responders to damaged tissue, actively engaging in the healing process through inflammatory mechanisms (Nachman et al., 2008). Beyond their established role in hemostasis, platelets harbor various growth factors (GF) vital for tissue regeneration and angiogenesis (Borregaard & Cowland, 1997). Platelet Rich Plasma (PRP) contains at least five times more platelets than whole blood (Brass et al., 2010). In clinical practice, PRP has been employed in autologous tissue healing therapy since the 1970s.

This study aims to explore potential disparities in EPC differentiation with the administration of PRP, PPP (platelet poor plasma), and a untreated control group. Our hypothesis posits that PRP administration can augment the differentiation process of EPCs.

METHODS

This study is a true experimental research (in vitro study), single-blind, involving the administration of Platelet Rich Plasma (PRP) and Platelet Poor Plasma (PPP) to the peripheral blood of stable angina pectoris patients using a "Posttest control group design" approach or design.

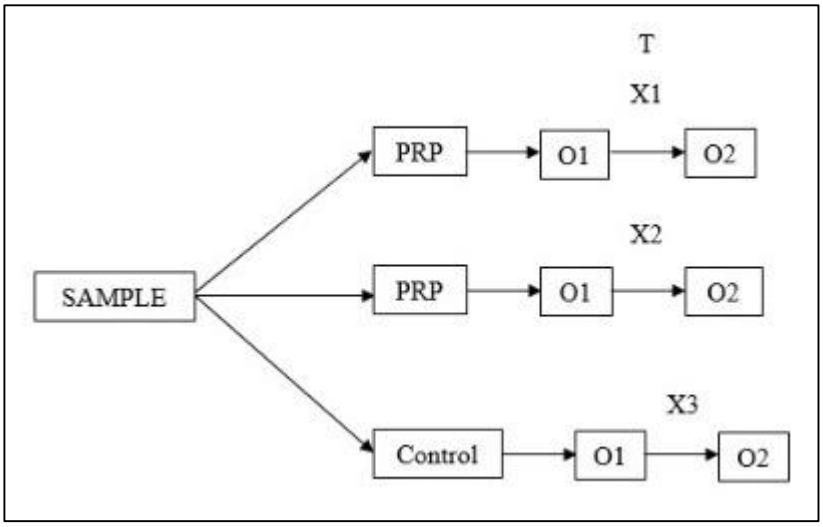

Figure 1. The research design of "Posttest control group design". Observation (O), observation period for 21 days (T), PRP treatment (X1), PPP treatment (PPP) and control (X3).

The experimental unit used in this study is venous blood taken from stable coronary heart disease (CHD) patients, which is then divided into three groups: Group 1, receiving PRP; Group 2, receiving PPP; and the control group, which does not receive any treatment. Observation is conducted for 21 days to observe the EPC differentiation process, assessed with von Willebrand Factor markers and stained with DAB using an electron microscocope.

The data acquired will undergo coding, entry, cleaning, and editing processes. Following that, inferential analysis will be executed to assess hypotheses. The impact of differentiation induced by PRP, PPP, and the control will be investigated through One-way ANOVA (for normally distributed data) and Mann-Whitney U test (for non-normally distributed data). To scrutinize differences between two groups (post-hoc), Least Significant Difference (LSD) will be utilized for normally distributed data, or Mann-Whitney U test for non-normally distributed data. The significance level (α) is established at 0.05, and the power (β) at 80%. The entire data analysis will be carried out using SPSS version 24.

RESULTS

Comparison of EPC Proliferation among groups administered with PRP, PPP, and Control

The assessment of EPC proliferation involved immunocytochemistry (ICC), where the count of cells expressing the CD34 marker, labeled with FITC, was conducted using an immunofluorescence microscope, resulting in cells exhibiting a fluorescent green color (see Figure 2). Subsequently, the normality of the dataset was evaluated through the Kolmogorov-Smirnov statistical test, confirming that the entire dataset followed a normal distribution. The data were then subjected to analysis to detect distinctions among the groups that received PRP, PPP, and the control, utilizing ANOVA. The outcomes were presented in the mean ± standard deviation format, and the significance of differences was assessed

using LSD.

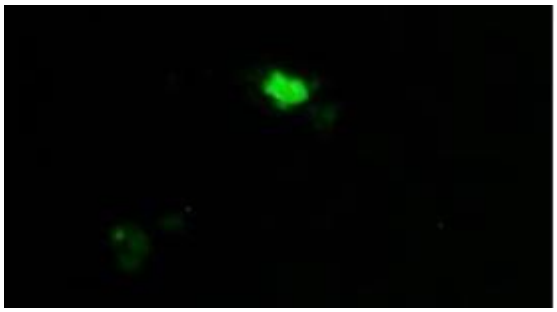

Figure 2. Immunofluorescence Image of CD34 Expression

The proliferation of EPCs in the group treated with PRP was 1.052 ± 0.16, which is higher compared to the group given PPP (0.762 ± 0.19) with a significance value of 0.003. This indicates a significant difference between the two groups.

Table 1. Comparison of EPC Proliferation Between PRP and PPP Groups

PRP Group	PPP Group	p
(Mean ± SD)		
1.052 ± 0.16	0.762 ± 0.19	0.003

There is an increase in EPC proliferation in the PRP group, which is 1.052 ± 0.16, compared to the control group (0.068 ± 0.05), and a significant difference was found with a significance level of 0.000.

Table 2. Comparison of EPC Proliferation Between PRP and Control Groups

PRP Group	Control Group	p
(Mean ± SD)		
1.052 ± 0.16	0.068 ± 0.05	0.000

Similarly, the proliferation of EPCs in the group given PPP (0.762 ± 0.19) is higher compared to the control group (0.068 ± 0.05) with a significance value of 0.000, indicating a significant difference between the two groups.

Table 3. Comparison of EPC Proliferation Between PPP and Control Groups

PPP Group	Control Group	p
(Mean ± SD)		
0.762 ± 0.19	0.068 ± 0.05	0.000

Comparison of EPC Differentiation into Endothelial Cells among groups administered with PRP, PPP, and Control

The determination of EPC differentiation into endothelial cells involved quantifying cells identified by von Willebrand Factor and stained with DAB. This process was observed using an electron microscope. Following this, the Kolmogorov-Smirnov statistical test was applied to assess the normality of differentiation data across all treatment and control groups, indicating a non-normal distribution. Subsequent analysis aimed to detect distinctions among the PRP, PPP, and control treatment groups employed the Mann-Whitney U test. The results were then presented in tabular format, encompassing median, data range (range), and significance values (LSD)**.**

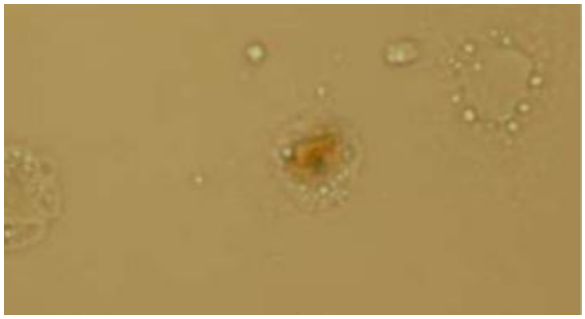

Figure 3. Endothelial Cells Characterized by von Willebrand Factor

The differentiation of EPCs in the group treated with PRP, ranging from 0.00 to 0.30, is higher compared to those receiving PPP, ranging from 0.00 to 0.20, with a p-value of 0.565. This means there is no significant difference between the group treated with PRP compared to PPP (Table 4).

Table 4. Comparison of EPC Differentiation Between PRP and PPP Groups

PRP Group	PPP Group	p
Median (range)		
0.00 (0.00–0.30)	0.00 (0.00–0.20)	0.565

The differentiation results of EPCs in the PRP-treated group are also higher compared to the control group, where no differentiation was observed in the control group (0.00). However, the p-value obtained is 0.064, indicating that there is no significant difference between the group treated with PRP and the control group.

Table 5. Comparison of EPC Differentiation between PRP and Control Groups

PRP Group	Control Group	p
Median (Range)		
0.00 (0.00–0.30)	0.00 (0.00–0.00)	0.064

The differentiation outcomes of EPCs in the group receiving PPP were found to be higher compared to the control group. However, a p-value of 0.144 was obtained, signifying that there is no significant difference between the group treated with PPP and the control group.

Table 6. Comparison of EPC Differentiation between PPP and Control Groups

PPP Group	Control Group	p
Median (Range)		
0.00 (0.00-0.20)	0.00 (0.00-0.00)	0.144

DISCUSSION

An examination was carried out on individuals diagnosed with stable coronary heart disease at the cardiology outpatient clinic of Dr. Soetomo Hospital in Surabaya. Utilizing the centrifugation method, mononuclear cells were extracted from patients' peripheral blood, cultivated in M-199 basal medium, and exposed to different interventions involving PRP, PPP, and a control group. Previous studies have suggested that M-199 medium, supplemented with diverse growth factors and Fetal Bovine Serum (FBS), can enhance the expansion of EPCs more effectively compared to alternative basal media for EPC development (Jianghuo et al, 2010). The presence of EPCs in peripheral blood was identified using various markers, including CD34 (Xu et al, 2008). Immunofluorescence analysis of CD34 revealed cells emitting green fluorescence, confirming the proliferation of EPCs. These EPCs possess the potential to mature into endothelial cells, identifiable through markers such as Von Willebrand Factor (Goon et al, 2006).

Ongoing research aims to elucidate the intricate mechanisms governing the differentiation of pluripotent stem cells into more advanced stages. One notable mechanism is DNA methylation, an epigenetic process intervening in genetic transcription and steering stem cell differentiation. Various theories expound on the transduction pathways involved in the proliferation and differentiation of stem cells, including EPCs, emphasizing the role of growth factors in triggering signal transduction. Growth factors are recognized for their roles in promoting growth, maintaining pluripotency, and instigating differentiation. The role of VEGF in angiogenesis and vasculogenesis, particularly in EPC proliferation, has been underscored in previous studies.

While further research is needed to fully understand the signaling pathways governing EPC proliferation and differentiation, it is established that pathways like Extracellular-Signal Regulated Kinase (ERK) and phosphatidylinositol-3 (PI3) Kinase/AKT play pivotal roles. The ERK pathway is activated by VEGF, mediating EPC proliferation (Rousseau et al, 1997), with activated p38 contributing to vascular permeability and cell motility. Studies have shown that inhibiting ERK results in a significant reduction in angiogenesis sprouting. Additionally, VEGF is known to activate the PI3-Kinase pathway (Qi et al, 1999). Although this study primarily focuses on CD34 marker examination rather than EPC proliferation results, it aligns with prior research, emphasizing the significant impact of platelets, particularly Vascular Endothelial Growth Factor (VEGF), on EPC proliferation. This is notably evident in the PRP-treated group, where platelet enrichment in specific plasma naturally increases the concentration of growth factors.

In contrast to observations on the EPC differentiation process into endothelial cells, counting cells characterized by the Von Willebrand Factor marker did not reveal significant differences between the PRP and PPP treated groups compared to the control group. This study opted for basal medium for EPC expansion, avoiding supplemented media (containing various growth factors and serum) specifically designed to induce EPC differentiation. This choice aimed to evaluate the role of growth factors present in PRP in the EPC differentiation process. Platelet-derived VEGF has been confirmed to play a crucial role in EPC proliferation and migration, influencing capillary sprouting (angiogenesis). Asahara et al. also verified VEGF's role in guiding EPCs from the bone marrow to ischemic areas during postnatal neovascularization. However, the differentiation process into endothelial cells and the formation of blood vessels (vasculogenesis) require various growth factors until the establishment of a stable new blood vessel (Cao et al, 2003).

Studies have reported significant CD34 differentiation results from in vitro isolations of CD34 from Human Umbilical Cord Blood (HUCB) samples treated with fibronectin and Fetal Calf Serum. On the 7th day of EPC culture, endothelial cells positive for Flk-1 and vWF markers were obtained, demonstrating the differentiation of EPCs into endothelial cells compared to the group not treated with fibronectin (Fan et al, 2003). Another study, using HUCB samples, also showed positive results for endothelial cell differentiation on the 14th and 28th days in a group treated with various growth factors, including VEGF, IGF, EGF, FGF, and 10% Fetal Calf Serum. This study recommended HUCB as a superior source for EPC isolation compared to peripheral blood (Eggerman et al, 2003).

Sustained release (SR) administration of PRP has been suggested as an effective method to enhance PRP's potential in vasculogenesis and arteriogenesis processes. In vivo experiments on mice induced ischemia conditions in leg blood vessels, followed by PRP-SR administration through a gelatin hydrogel medium. Observations revealed improved blood vessel conditions and blood perfusion to ischemic tissues. While PRP contains various growth factors contributing to vasculogenesis, arteriogenesis, and angiogenesis processes, these factors are present in limited quantities and are easily degraded. Therefore, sustained release processes are considered highly effective (Bir et al, 2009).

This study possesses several limitations, such as not investigating potential result differences with various PRP doses. Additionally, using peripheral blood samples to isolate EPCs is acknowledged as challenging due to the significantly lower quantity of EPCs in peripheral blood compared to other sources like cord blood. The consideration of specific media for EPC development is also worth exploring, as various serums play a crucial role as a nutrient source for cell growth and development.

CONCLUSION

A significant increase occurs in EPC proliferation when compared to the PPP and control groups when PRP is administered to stable coronary heart disease (CHD) patients. However, conversely, PRP does not provide a significant improvement in the EPC differentiation process compared to the PPP and control groups in patients with stable coronary heart disease.

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