Effect of Platelet Rich Plasma on Ischemia-reperfusion Injury in Mice Flap Model

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Directed by Professor Dong Kyun Rah

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Young Woo Cheon

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Special thanks goes to my co-supervisor, Hogeun Kim and Hyun Ok Kim, who are most responsible for helping me complete the writing of this article as well as the challenging research that lies behind it. Professor Kims had been a friend and mentor. They taught me how to write academic papers, made me a better doctor, had confidence in me when I doubted myself, and brought out the good ideas in me.

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June, 2016

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ABSTRACT

Effect of Platelet Rich Plasma on Ischemia-reperfusion Injury in Mice Flap Model

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Ischemia-reperfusion injury is a leading cause of flap compromise and organ dysfunction. Therefore, reducing ischemia perfusion injury in the necrotizing flaps has long been a clinical challenge. Platelet-rich plasma (PRP) is a reserve of a much amount of various growth factors. Platelet-rich plasma can be collected autologously and the cost of making is not expensive. Activated platelet play a role in endothelial damage during ischemia-reperfusion injury, however, exogenous PRP could reduce platelet recrution and inhibit the production of reactive oxygen species. The goal of this study is to investigate the effect of PRP to the ischemia reperfusion flap model in mouse. Four groups of mice were used and each group has 30 mice. Group A received flap elevation and repositioning. Group B injected PRP just after flap elevation

and repositioned immediately. Group C has 4 hour of ischemia and reperfused. Group D injected PRP after flap elevation and has 4 hours of ischemia then reperfused. The survival area of flap, blood perfusion of flap was assessed with digital photography and Doppler flowmeter. PRP enhanced the survival area and perfusion of flap. The flap specimens were harvested and histologic evaluation was pwerfomed by neutrophil count and vessel count. PRP reduced the neutrophil accumulation and enhance angiogenesis especially with ischemia-reperfusion.

We measured nitric oxide (NO), myeloperoxidase (MPO), Malondialdehyde (MDA), and superoxide dismutase (SOD) to evaluate the ischemia-reperfusion injury. The decreased SOD activity and increased MPO, NO and MDA content prove the redox imbalance and high reactive oxygen species level in ischemia-reperfusion flaps. However, PRP showed protective effect against reperfusion injury with decrease of MPO, NO and MDA. We also confirmed that PRP effectively suppresses proinflammatory cytokines, such as monocyte chemotactic protein-1 (MCP-1), TNF-α, IL-1β, IL-6 by RT-PCR. As an antioxidant, PRP reduced reactive oxygen species and proinflammatory cytokines. In addition to its direct antioxidant action, PRP also effect as an apoptosis regulatory messenger. PRP reduced the expression of apoptosis signaling regulating kinase-1(ASK-1), in the contrast, p-p38 did not affected by PRP.

In this study, we demonstrate that PRP acts as a protective factor during flap

ischemia-reperfusion by reducing reactive oxygen species level and proinflammatory cytokines, also by inhibiting the apoptosis signal-regulating kinase 1 pathway.

Keywords: Platelet rich plasma, Ischemia, Reperfusion, Angiogenesis,

Effect of Platelet Rich Plasma on Ischemia-Reperfusion Injury in Mice Flap Model

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I. INTRODUCTION

Surgical skin flaps have been increasingly used in reconstructive surgery for the closure of various surgical defects. Partial or complete flap necrosis is a common problem after reconstructive flap surgery. Although axial flap is more preferred by surgeons because of its confidential pedicle, however, an axial flap is more vulnerable to ischemic reperfusion injury. [1] Management of the necrotizing flap usually needs time-consuming and repetitive dressing changes or even a secondary surgical procedure. [2] Inadequate blood perfusion and ischemia-reperfusion injury are thought to be the major factors that cause several detrimental changes in the tissue and vasculature, resulting in flap

necrosis.[3] Therefore, reducing ischemia perfusion injury in the necrotizing flaps has long been a clinical challenge. It also has a common feature with a organ transplantation surgery. Ischemia-reperfusion injury is a complex process in which all steps of the inflammatory cascade may take part. Most of the damage is inflicted via leukocyte endothelium interaction, reactive oxygen species, complement, mast cell, and immune complexes. The role of molecular mediators has been shown by many studies.[4] Many investigations had performed to reduce ischemia-reperfusion injury with small molecules, proteins, cytokines and drugs. [5] [6] One possible way to prevent reactive oxygen species-mediated cellular injury is to augment endogenous oxidative defenses with dietary intake of antioxidants such as vitamins A, C, or E. Recently, attention has been focused on a variety of non-vitamin antioxidants such as phenolic compounds, which may also contribute to cellular antioxidative defense mechanisms and can be found in many plant species including green tea, edible fruits, or vegetables. [7] However, these methods are could not reach the clinical applications, because they have limited function, expensive, complicated, and hard to handle.

Platelet-rich plasma is a reserve of a much amount of various growth factors. [8] Platelet-rich plasma can be collected autologous and the expense of making is not expensive. The autologous platelet-rich plasma is biocompatible and safe if there are no contaminations during the process of making. Thus, for clinical use, no special considerations concerning antibody formation and the

possible risk of infection from donor are needed. [9] Lots of clinical devices are currently available to automatically prepare platelet-rich plasma. [10] For a long time, autologous platelet-rich plasma has been used intraoperatively to clinically enhance wound healing, bone regeneration, reduce inflammation, and decrease blood loss in the fields of orthopedics and plastic surgery.[11,12] Collectively, these studies provide strong evidence to support the clinical use of platelet-rich plasma.

However, the evaluation of platelet-rich plasma quality remains controversial, and treatment with poor-quality platelet-rich plasma results in nonbeneficial effects. [13] Therefore, the common definition of platelet-rich plasma is established by Marx et al. [14] The regenerative potential of platelet-rich plasma depends largely on the amounts of secretory cytokines released upon platelet activation, including vascular endothelial growth factor (VEGF), epidermal growth factor (EGF), platelet-derived growth factor (PDGF), transforming growth factor (TGF)-1, TGF-b2, and insulin-like growth factor (IGF)-1. The release of these cytokines occurs through platelet activation or physical disruption of the platelet–a-granule structure. [15] The most common method of platelet-rich plasma activation involves the addition of thrombin and calcium chloride (CaCl2). Thrombin directly activates platelets, and the calcium ions from CaCl2 replenish those bound by acid citrate dextrose type A anticoagulant. Although this method is often used to activate platelet-rich plasma clinically, the activation that occurs during clot formation does not

necessarily lead to complete release. [16] Platelets play a role to damage an endothelium during ischemia reperfusion injury with thrombosis. However, exogenous PRP showed to reduce reactive oxygen species and mitochondrial depolarization *in vitro*. During myocardial ischemia reperfusion, PRP could improve electrical and mechanical function of heart via altered mitochondrial function & reduced apoptosis. [17] However, The protective effect of platelet-rich plasma to ischemia-reperfusion injury in flap model is poorly understood.

The author will investigate the effect of PRP to ischemia-reperfusion injury in mouse axial pattern flap model. We will measure the survival of flap, tissue perfusion of flap, reactive substances, and proinflammatory cyrokines to reveal the effect of PRP.

II. MATERIALS AND METHODS

1. Preperation of platelet-rich plasma

Platelet-rich plasma was produced from full blood of 10 weeks old C57BL/6N mice. (Oriental bio, Seoul, Korea). An intracardiac blood volume of 1.2 ml was obtained from the mice, mixed with 120μl of anticoagulant citrate dextrose solution formula A (ACDA), and mixed by inversion. It was centrifuged at 160*g* for 15min to separate the plasma in the superior layer from the red blood cells in the inferior layer and the white blood cells in the

intermediate layer. In a second step, using a sterile syringe, plasma and buffy coat were transferred to a new tube without anticoagulant and centrifuged for 10 min at 400*g*, yielding mean platelet-rich plasma at a concentration of 90,400/μl.

2. Surgical procedure for axial pattern flap model

All animal experiments were conducted in accordance with the guidelines of the Korean animal protection statute and approved by the institutional review committee. C57BL/6N mice (Oriental bio, Seoul, Korea) of 8 weeks of age were used in this study. The animals were housed in a general, temperature and humidity controlled, pathogen-free environment on a cycle of 12 h of light and 12 h of darkness, and were allowed free access to food and water. After elevation of axial flap based on lateral thoracic artery, those mice that had irregular vessel anatomy were excluded for study. A total of 120 mice were used. These were placed randomly into four groups (n = 30 per group): group A - Control (flap elevation only), group B - platelet-rich plasma (flap elevation with platelet-rich plasma injection), group C - ischemia (flap elevation with 4 hours of clamping), and group D - ischemia and platelet-rich plasma (flap elevation with platelet-rich plasma injection followed by 4 hours of clamping).

For the surgical procedure, mice were anaesthetized by 30 mg/kg pentobarbital sodium by intraperitoneal injection after light anaesthesia with

isoflurane. After anaesthesia, the mouse placed on a heating pad to maintain constant body temperature throughout the surgery. After surgical cleansing of whole dorsal area, a lateral thoracic artery pedicled 1.5 x 3.5 cm island skin flap was raised from caudal to cranial by careful dissection with direct visualization of pedicle. This island flap contained skin, subcutaneous tissue and panniculus carnosus muscle. In group A (control group), the flaps, were injected 120μl of PBS, inset to the original position and sutured using 6-0 polypropylene sutures without microclamp ischemia. In group B (platelet-rich plasma group), the flaps were injected 120μl of platelet-rich plasma. After injection, the flaps were inset to the original position and sutured using 6-0 polypropylene sutures without microclamp ischemia. In group C (ischemia group), the flap was elevated, 120μl of PBS was injected, and the pedicle was ligated with microclamp (Fine Science Tools, Foster City, CA) for 4 hours. After ischemia the microclamp was removed to make reperfusion injury. [18] The reperfusion of the flaps was checked using a laser doppler imager (Moor LDI2-HR, Moor Instruments, Axminster, UK) and the flaps were inset to the original position and sutured. In group D (ischemia and platelet-rich plasma group), the flap was elevated and injected 120μl of platelet-rich plasma. After injection, the pedicle was ligated with microclamp for 4 hours and followed by removal of microclamp to make reperfusion injury. Then the flaps were inset to the original position and sutured. Medical silicone sheets were laid on the muscle bed in all groups. (Fig. 1)

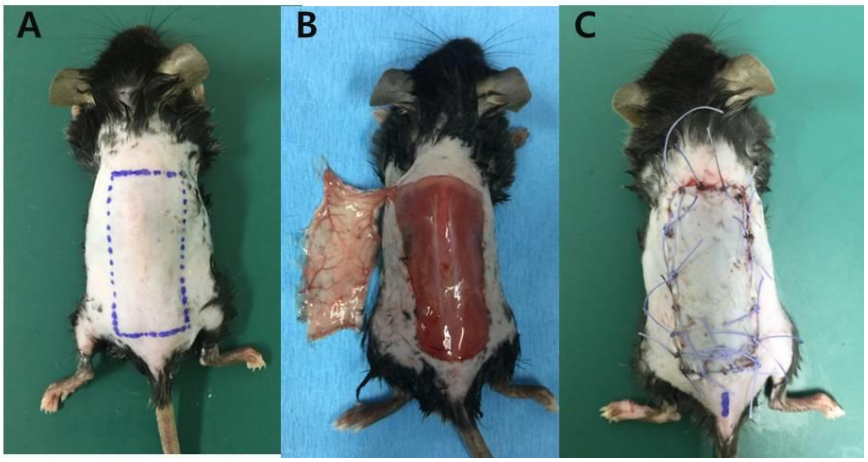


Fig. 1. Surgical flap elevation procedure (A) A design of lateral thoracic artery based axial island flap sized 1.5 x 3.5 cm (B) The flap was elevated with preservation of left lateral thoracic artery. The pedicle was observed at undersurface of flap (C) The flap was insetted to its original position with 4-0 vicryl sutures.

3. Assessment of survival areas

On days 1, 3, 5, 7 and 10 after the operation, the surviving area of the flap was measured by digital image analysis. Pictures of the flaps at the same distance were taken by a digital camera. (Nikon D70s; Nikon corporation, Tokyo, Japan) The surviving area of the flap was defined by independent observers watching the gross appearance, color, and consistency of skin, elasticity, eschar and the texture of the skin. The defined surviving area was measured using Image-Pro Plus Software (version 5.0; Media Cybernetics LP,

Silver Spring, Md.). The results were expressed as percentages of the surviving areas comparative to the total flap surface areas that was defined by the surgical borders.

4. Hemodynamic assessment of the flaps

In all mice from each group, tissue blood perfusion of the skin flap was measured with laser doppler flowmetry (Peri-Flux System 5000; Perimed, Inc., Stockholm, Sweden) on postoperative day 1, 3, 5, 7, and 10. The probe was placed on the median line of the flap and the testing points were fixed on proximal, median, and distal portions, respectively. The room temperature was maintained at around 21°C during the measurement of blood flow. For consistency, every measurement lasted at least 30 seconds. The results were expressed using the ratios of the postoperative blood perfusion units (BPU) to the preoperative BPU.

5. Histopathologic analysis

After the interventions described, full thickness specimens were taken from the center of each flap 12 hours after the onset of reperfusion. They were placed in 10% formalin and stained with hematoxylin–eosin stain for histological examination to determine the infiltration of neutrophils to flap tissue. Five-micrometer-thick sections were evaluated at 200 magnification, and the neutrophils per 75 random, nonoverlapping fields were recorded. The

mean number of neutrophils was calculated. Histologic changes for hematoxylin and eosin staining were evaluated by analyzing tissue damage that presented hyperemia, neutrophil aggregation, and intravascular microthrombosis. The flap tissue was harvested after postoperatively 10 days for measuring capillary density. The CD31 immunohistochemical stain was performed as previously described. [19] The capillary density was assessed by measuring the number of capillaries in 10 fields of each rat (total of 50 fields/group) and expressed as the mean number of capillaries per mm^2. The analysis was performed under 100 magnification by three independent reviewers in a blinded fashion.

6. Measurement of ischemia-reperfusion injury

We measured nitric oxide (NO), myeloperoxidase (MPO), malondialdehyde (MDA), and superoxide dismutase (SOD) to evaluate the ischemia-reperfusion injury. [20] For biochemical examination, 1x1 cm sized specimens were taken from the center of the flaps, 12 hours after reperfusion. Specimens were stored at 80°C immediately within individual containers. Since tissue nitrite (NO_2) and nitrate (NO_3) levels can be used to estimate NO production, we measured the concentration of these stable NO oxidative metabolites. Quantitation of NO_2 and NO_3 was based on the Griess reaction. [20] Results are expressed as mmol/g tissue. Myeloperoxidase (MPO) was measured by the procedure with myeloperoxidase mouse ELISA kit (Abcam, Cambridge, UK).

Samples were homogenized initially in 50 mmol per liter potassium phospate buffer and were centrifuged at 1,500 *g* for 10 minutes. A total of 500μl of homogenate was then centrifuged at 40,000 *g* for 15 minutes at 4°C. The supernatant was used for measuring the proteins.

Malondialdehyde (MDA) was measured by measuring the presence of thiobarbituric acid reactive substances. [21] A total of 100 mg per milliliter tissue was homogenized in buffer at a pH of 7.4. Artifactual production of additional MDA during processing was eliminated by the addition of 2% butylated hydroxytoluene to homogenized tissue. To this was added 20% trichloroacetic acid in 0.6 N hydrochloride. The mixture was centrifuged at 10,000 *g* for 10 minutes at 4°C. A total of 0.12 mol per liter thiobarbituric acid in buffer (pH, 7.0) was added to the supernatant. Pigment was measured spectrophotometrically at 532 nm.

SOD enzyme-activity determination was based on the production of H_2O_2 from xanthine by xanthine oxidase and reduction of nitroblue tetrazolium. The measurement was performed with superoxide dismutase assay kit. (Abcam, Cambridge, UK) The product was evaluated spectrophotometrically. Results are expressed as U/ml.

7. Real-time RT-PCR

The flap sample was harvested in each group to analyze the mRNA levels of expression in ischemia-reperfusion injury. Total RNA was harvested using

TRIzol reagent (Invitrogen, Waltham, MA, USA) and was subjected to reverse transcription using a SuperScript III First-Strand Synthesis System for RT-PCR (Invitrogen) according to the manufacturer's instructions. Quantitative RT-PCR was performed with the SYBR system (Applied Biosystems, Foster City, CA) using ABI 7300 real-time PCR instrumentation (Life Technologies). SYBR probes and primers for monocyte chemotactic protein (MCP)-1, tumor necrosis factor (TNF)-α, Interleukin (IL)-1β, IL-6 and 18S were purchased from Takara Bio Inc. (Otsu, Japan). As an internal control, levels of 18S were quantified in parallel with target genes. Normalization and fold changes were calculated using the comparative Ct method.

8. Western blot

The protein expression of ASK-1(apoptosis signaling regulating kinase 1), p-p38MAPK, p38MAPK, (all from Cell Signaling Technology, Danver, MA, USA) in proximal portion of the skin flap was visualized by western blot. Total protein was extracted mouse skin flap using a RIPA lysis buffer (Thermo Scientific, Rockford, IL,USA) containing protease and phosphatase inhibitor cocktail (Roche, Indianapolis, USA) following the manufacturer's protocols.

The protein concentrations of extracts were determined using a BCA protein assay reagent kit (Pierce, Rockford, IL, USA). 30 μg protein was then loaded onto a 12% SDS-PAGE gel. Proteins were separated electrophoretically and

transferred onto PVDF membranes (Merck Millipore, Bedford, MA,USA). For immunoblotting, membranes were blocked with 5% nonfat dried milk in Tris-buffered saline. For 1h and then incubated with primary antibodies specific for PI3K, pERK1/2, p-p38MAPK, p38MAPK, pAKT, AKT and b-actin overnight at 4˚C.

All antibodies were diluted 1:1000 except for pERK1/2 (at a dilution of 1:2000). The membrane was washed in Tris-buffered saline with 0.1% Tween 20 and incubated for 1h with secondary Goat anti-rabbit IgG peroxidase-conjugated antibody (Enzo Life Science, Farmingdale, NY, USA). b-actin (Cell Signaling technology, Danver, MA, USA / 1:5000) was used as internal control. The blots were developled with west-Zol PLUS kit (Intron biotechnology Co.,Ltd. KOREA) Immunoreaction was visulalized by chemiluminescence.

9. Statistical analysis

The data was expressed as a mean ± standard deviation. Flap tissue NO, Data were analyzed by one-way ANOVA. Post-hoc comparisons were done using Tukey test. Differences were regarded as statistically significant for two-tail values of $p < 0.05$. All data were analyzed using SPSS for Windows version 12.0 (SPSS, Inc., Chicago, Ill.)

III. RESULTS

1. Survival areas of the flaps

At 1 day after the operation, we could observe group C (ischemia group) showed less survival than the other 3 groups. However, the difference could not reach statistical significance. The differences of survival area were getting larger at 3 days after the operation. Group B (platelet-rich plasma group) and group D (ischemia and platelet-rich plasma group) showed more surviving area compared with the other 2 groups. The differences are started at postoperative 3 days and continued to 10 days. ($p < 0.05$) Group B showed more surviving area compared with group D at postoperative 10 days, however, it did not achieve statistical significance. Group A (control group) showed more surviving area compared with group C (ischemia group) with statistical significance. ($p < 0.05$) The difference of survival area between group A and group C initialized at postoperative 5 days and the differences getting larger to postoperative 10 days. For all groups, the decline of survival area was most prominent from postoperative 1 day to 3 days. (Fig. 2 and 3)

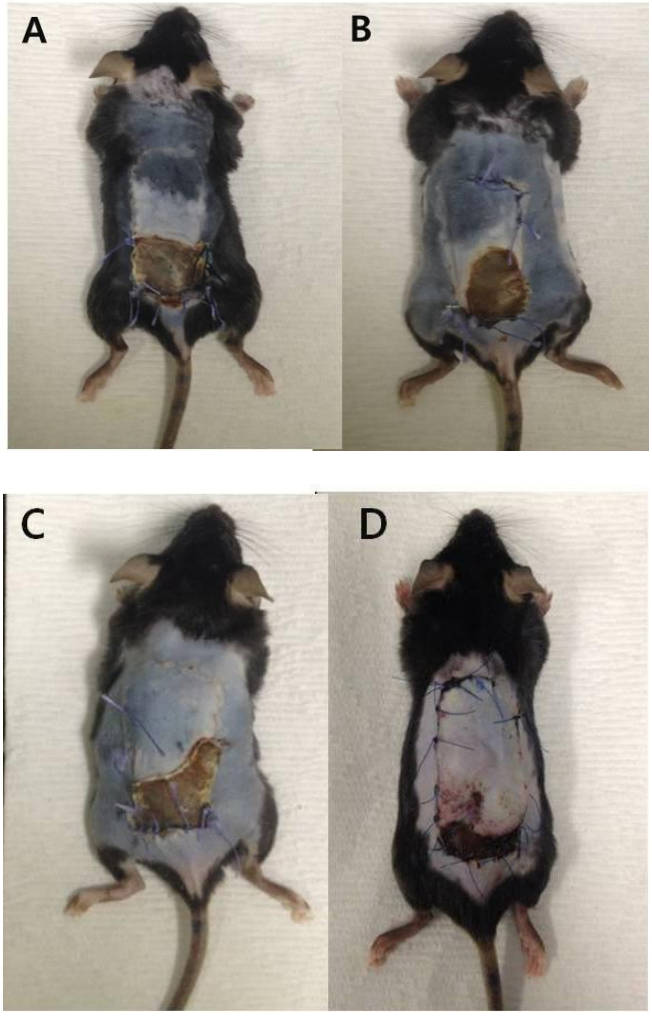


Fig. 2. The photo of flap survial area at POD#10 (A) Control group (B) Platelet rich plasma group (C) Ischemia group (D) Ischemia and platelet rich plasma group. Group D showed more survial area than group C with statistical significance. ($p < 0.05$)

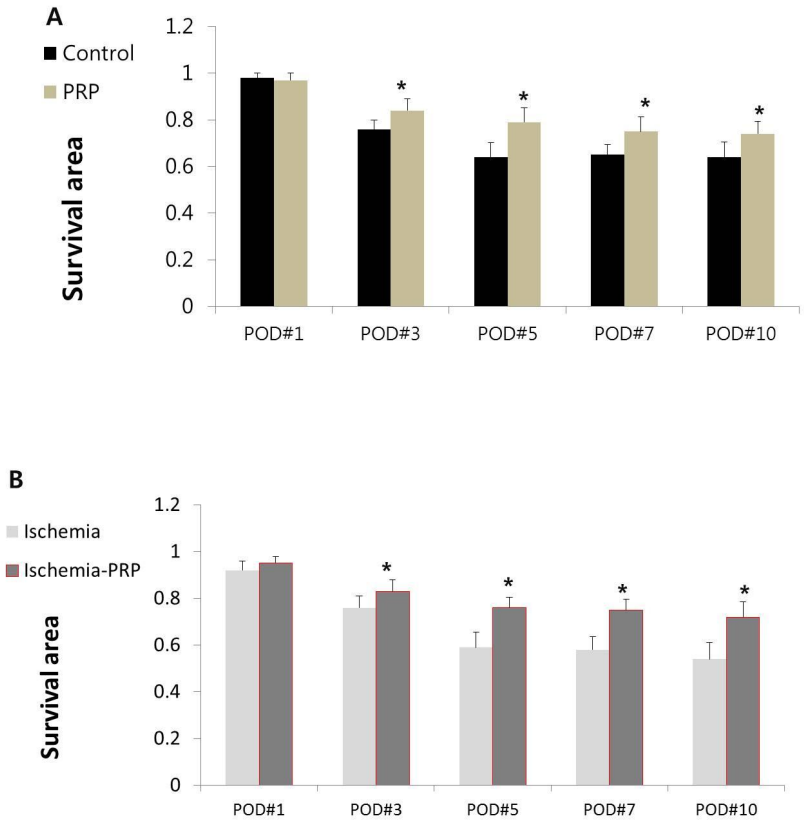


Fig. 3. The flap survival area of four groups according to postoperative day. (A) Control and PRP group. PRP group showed more flap survival than control group from 3rd postoperative day to 10th postoperative day.

* means statistical significance ($p < 0.05$) (B) Ischemia and ischemia-PRP group. We observed more flap survival of ischemia-PRPgroup than ischemia group. The differences were getting larger after 5th postoperative day. * means statistical significance ($p < 0.05$)

2. Regional blood perfusion in the flaps

We could observe the main vascular network of the lateral thoracic artery and vein on the undersurface of elevated flap. A microsurgical free flap procedure was simulated by occlusion of the main source artery and vein with microclamp. According to the clinical operation procedure, the 4 hour of ischemia time was applied to all flaps. We could observe all the flaps in group C (ischemia group) and D (ischemia and platelet-rich plasma group) were cyanotic with adequate ischemia. After the removal of microclamp, the flaps showed hyperemic immediately with reperfusion injury. The regional blood perfusion (expressed as the ratio of postoperative to preoperative blood perfusion units) was different between the four groups when measured at postoperative day one. (Fig. 2B) At this time, group B (platelet-rich plasma group) showed more blood perfusion than the other three groups, however, except comparing with group C, it could not achieve statistical significance. ($p < 0.05$) We could observe group D showed more blood perfusion than group C with statistical significance. ($p < 0.05$) At postoperative day 5, the perfusion was significantly greater in groups B than group A (control group). Also it was revealed that the perfusion in groups D was greater than group C with statistical significance. ($p < 0.05$) Before the postoperative day five, group A and group B does not show significant difference, however, at the postoperative day five the difference became statistical significance. We could observe that the largest perfusion in group D at the postoperative day 10.

However, comparing with group B, it could not reach statistical significance. The perfusion of group D showed less than group B during postoperative day 7, however, it was reversed at postoperative day 10. (Fig. 4)

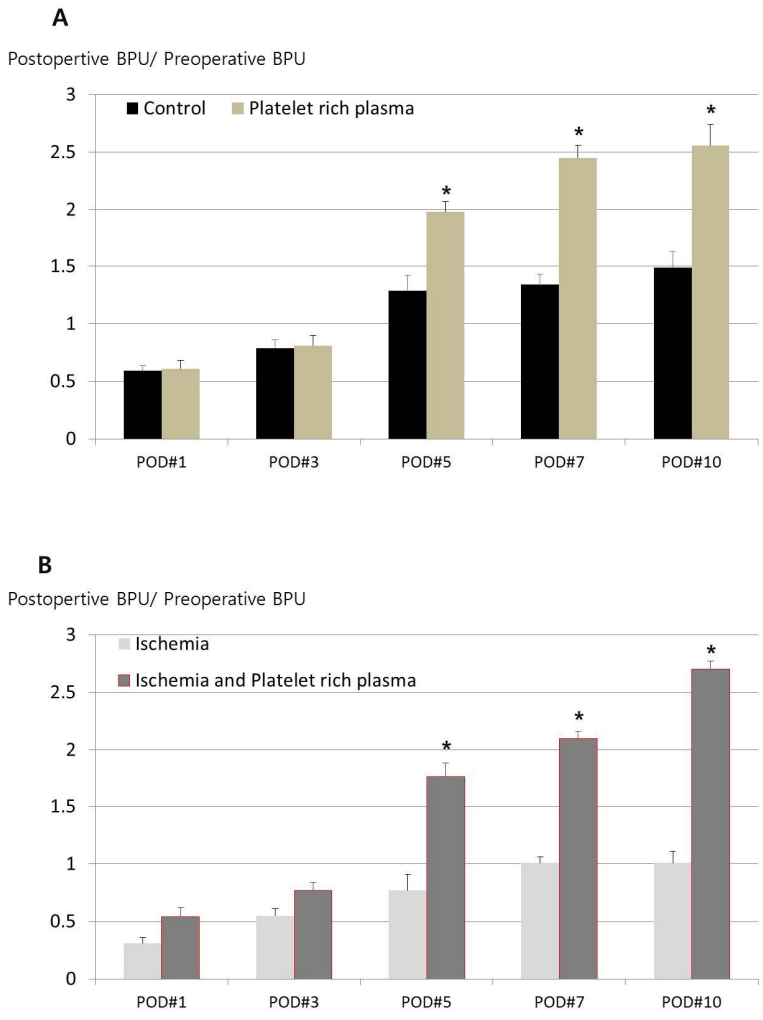


Fig. 4 (A) The perfusion of flap in control and PRP group according to the postoperative day. The perfusion of each group showed increased according to

the postoperative time. At postoperative day 7, the perfusion in group B was significantly greater than all the other groups. ($p < 0.05$) (B) The perfusion of flap in ischemia and ischemia-PRP group according to the postoperative day. The perfusion of flap according to the postoperative day. We could observe group D (ischemia and platelet-rich plasma group) showed more blood perfusion than group C with statistical significance. ($p < 0.05$)* Also, we could observe that the largest perfusion in group D at the postoperative day 10 comparing with all the other groups. * means statistical significance. ($p < 0.05$)

3. Histopahologic assessment (Neutrophil count and capillary density)

We could observe group C (Ischemia group) showed highest neutrophil count than the other three groups. However, PRP could decrease the neutrophil count more than 2 fold even though ischemia-reperfusion injury. Group D (ischemia and platelet-rich plasma group) showed more neutrophil count than group A and B. Non ischemia-reperfusion groups (group A and B) showed lower neutrophil count compared to ischemia-reperfusion injury groups. (Fig 5.A and B)

Group C showed extensive hyperemia, neutrophil aggregations, and intravascular microthrombus in H&E stain. However, we could observe less neutrophil aggregation in group D. (Fig. 5. B)

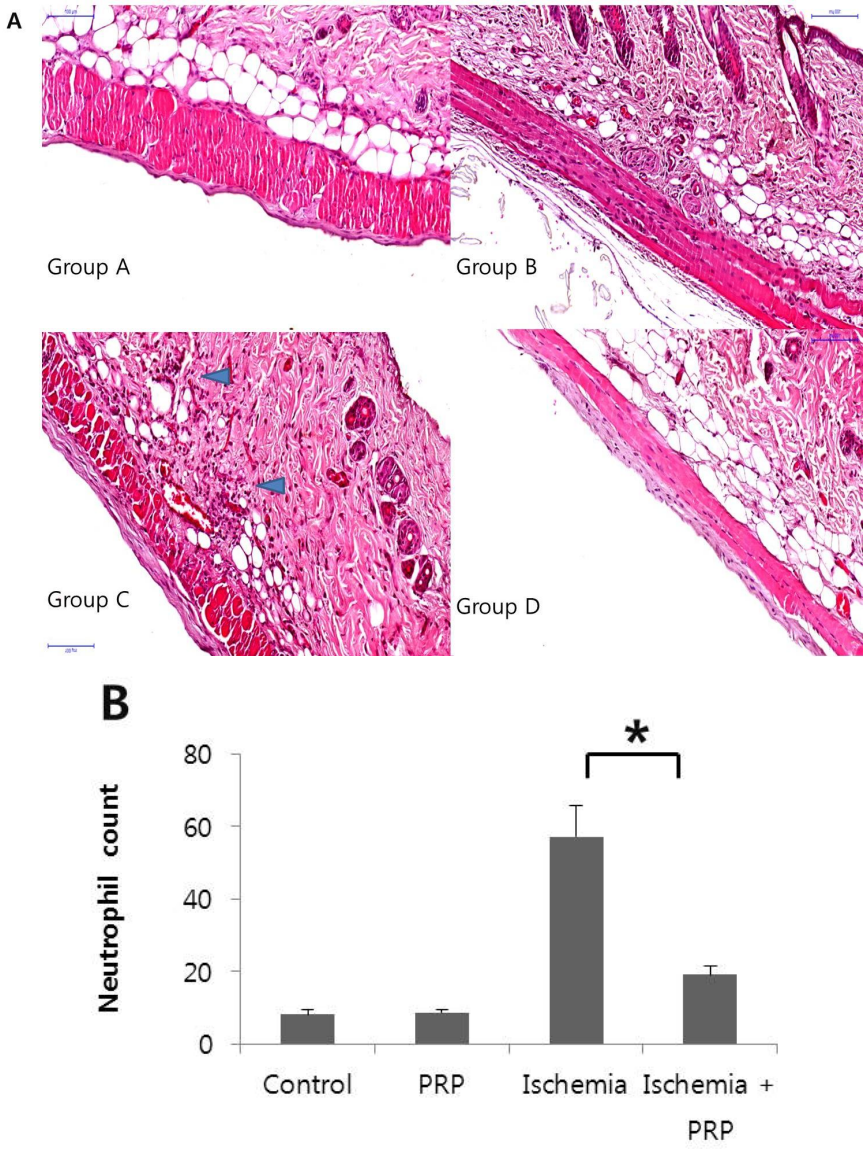


Fig. 5. (A) Histopathologic analysis of tissue damage in mouse skin flaps. Hematoxylin and eosin staining (× 200) showed extensive hyperemia, neutrophil aggregation, and intravascular microthrombi in group C. In group A and B, neutrophil aggregation and microthrombi could not be found. In

group D, we could observe minimal neutrophil infiltration. We could observe much more neutrophil infiltration in ischemia group, however, PRP could decrease the neutrophil count after 4 hours of ischemia. Arrow indicates neutrophil infiltration. (B) Neutrophil count of flap specimens. Group A and B showed lower neutrophil count than ischemic groups. We observed more than 5 fold increase in group C compared to non-ischemia-reperfusion groups. However, PRP reduced the neutrophil count with statistical significance, even though higher than non-ischmia-reperfusion groups

Quantitative analyses revealed that the capillary density in group D was significantly higher than those in group C. The mean capillary density of group C was significantly higher than that of non ischemia-reperfusion groups. When comparing group B and C, we could confirm that PRP promote angiogenesis especially in ischemic condition. (Fig. 6A and B)

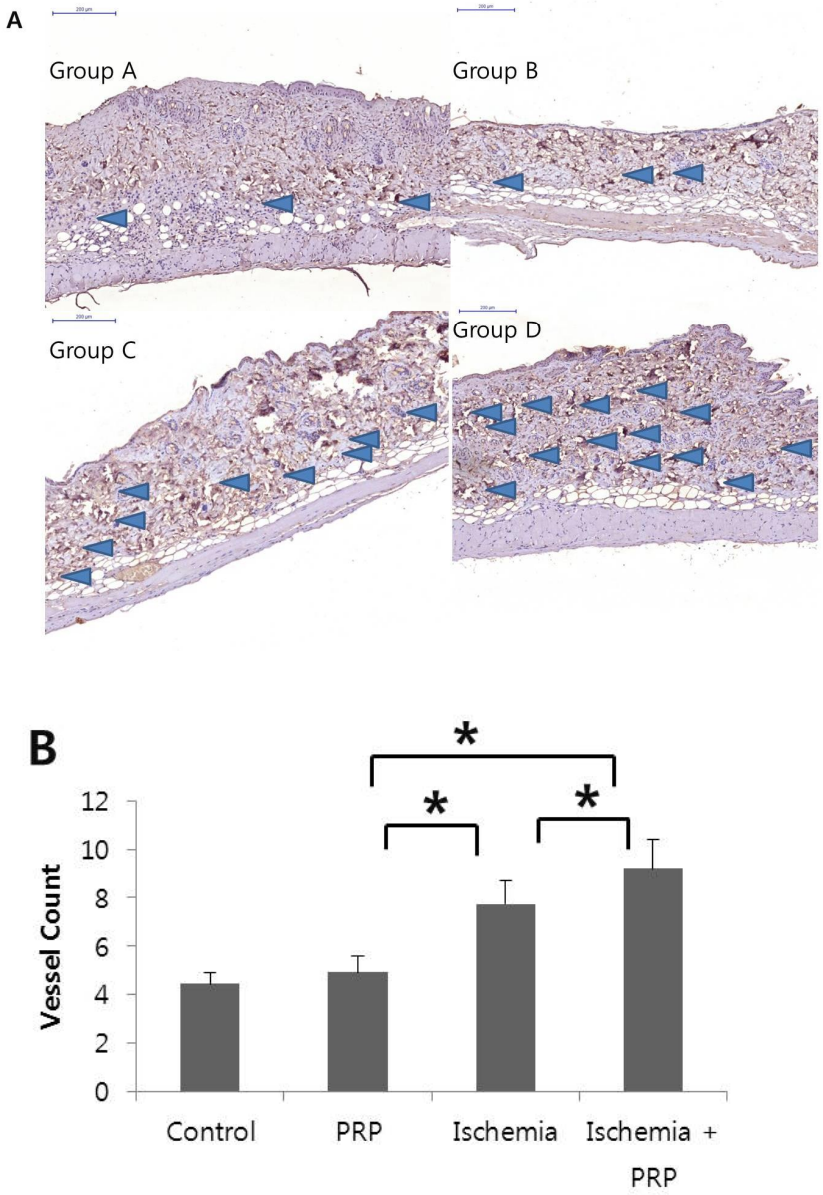


Fig. 6 Vessel analysis of flap(A) Histologic specimens with CD31 immunostain of each group at 10th postoperative days. Arrowhead indicates vessels. Group D showed more vessels than group C. (× 100) (B) Vessel count of each group at 10th postoperative days. Group B showed more vessel

count than group A, however, it could not achieve statistical significance. The capillary density in group D was significantly higher than those in group C. Platelet rich plasma promote angiogenesis in ischemic condition. The mean capillary density of group C was significantly higher than that of non ischemic groups.

4. Measurement of ischemia-reperfusion injury

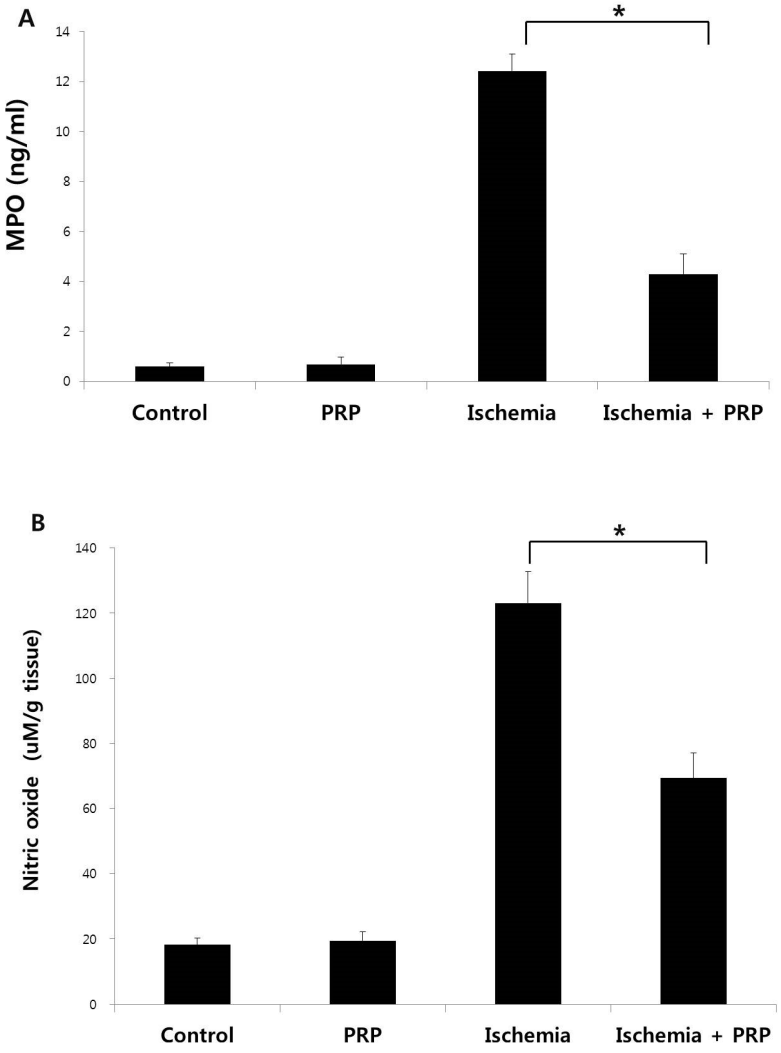
We measured nitric oxide (NO), myeloperoxidase (MPO), malondialdehyde (MDA), and superoxide dismutase (SOD) to evaluate the degree of ischemia-reperfusion injury in axial flap.

Nitric oxide level was highest in group C (ischemia) than other 3 groups. ($p < 0.05$) Group A (control group) and group B (platelet rich plasma group) showed low level of NO than group C, however, the difference between two groups were could not reach statistical significance. Group D (ischemia and platelet rich plasma group) showed significant lower level of NO compared to group C. When comparing group A and B, we could reveal PRP could not decrease reperfusion injury without a time of ischemia. However PRP could reduce reperfusion injury effectively with 4 hours of ischemia. (Fig. 7A)

Tissue myeloperoxidase (MPO) in group C showed significant increasement than the other three groups. (Fig. 7B) In group A and group B showed lower level of MPO than group C and group D with statistical significance. ($p < 0.05$) This effect does not observed without ischemia.

Tissue malondialdehyde (MDA) in group D (ischemia and platelet rich plasma group) showed higher level than group A (control group) and group B (platelet rich plasma group). However the level of MDA in group D was significantly lower than group C (ischemia group). The two groups without ischemia showed lower level of MDA than the two groups with ischemia with statistical significance. (Fig. 7C)

In group C (ischemia group), superoxide dismutase (SOD) showed lower levels than the other three groups. ($p < 0.05$) We observed higher SOD level in group A (control group) and group B (platelet rich plasma group) than group D (Ischemia and platelet rich plasma group). However, group D showed higher level of SOD comparing to group C. (Fig. 7D)



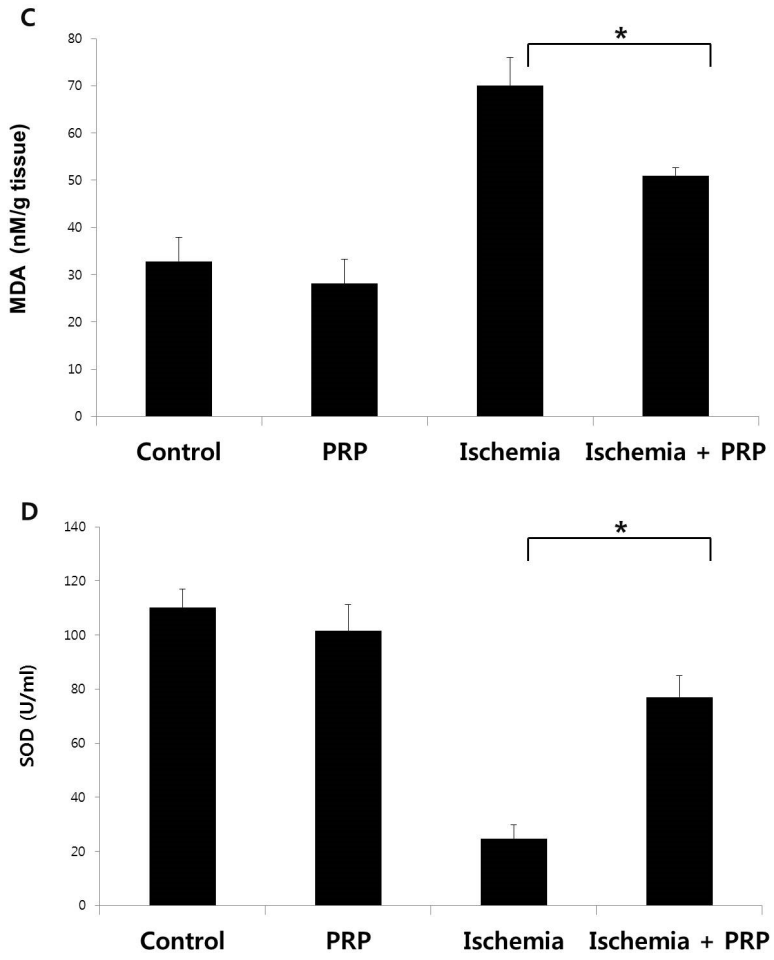
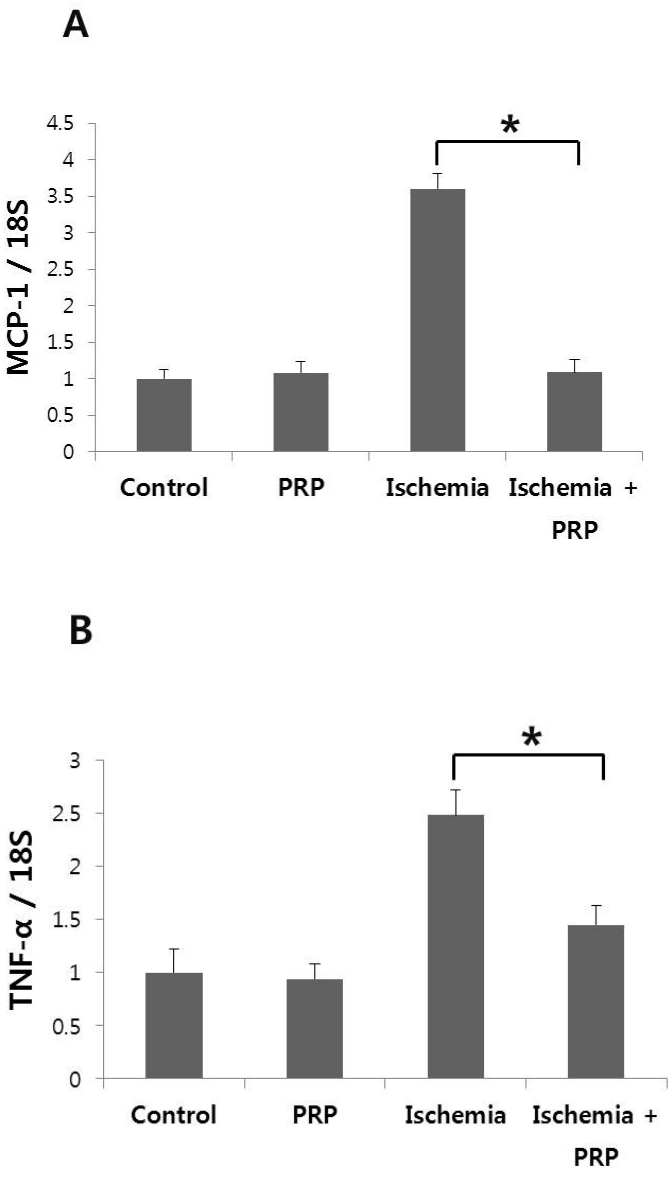


Fig 7. Measurement of nitric oxide (NO), myeloperoxidase (MPO), malondialdehyde (MDA), and superoxide dismutase (SDO) (A) Nitric oxide level was highest in group C (ischemia) than other 3 groups. ($p < 0.05$) Group D (ischemia and platelet rich plasma group) showed significant lower level of NO compared to group C, however higher level compared to group A and B. PRP alone does not reduce reperfusion injury according to group B, however platelet rich plasma decreased reperfusion injury with 4 hours of ischemia. (B)

Tissue myeloperoxidase (MPO) in group C showed significant higher level than the other three groups. PRP decreased MPO when combined with ischemia according to group D. This effect does not observed without ischemia. (C) Tissue malondialdehyde (MDA) in group D showed higher level than group A and group B, however it was significantly lower than group C. The two groups without ischemia showed lower level of MDA than the two groups with ischemia with statistical significance. (D) We observed higher SOD level in group A (control group) and group B (PRP) than group D (ischemia ischemia and PRP). However, group D showed higher level of SOD comparing to group C. ($p < 0.05$)

5. Proinflammatory cytokines

We could observe that platelet rich plasma suppressed mRNA levels of proinflammatory cytokines and kemokines. The tissue samples were harvested at postoperative day one. In group D (ischemia and platelet rich plasma group), the level of MCP-1 was lower than group C (ischemia group) with statistical significance. Interestingly, the level of MCP-1 in group D did not show statistical differences with group A (control group) and group B (platelet rich plasma group). The expression level of TNF-α, IL-1β, IL-6 were significantly decreased in group D compare to group C. However, the expression level of TNF-α, IL-1β, IL-6 in groups without ischemia showed lower level than groups with ischemia. (Fig 8. A-D)



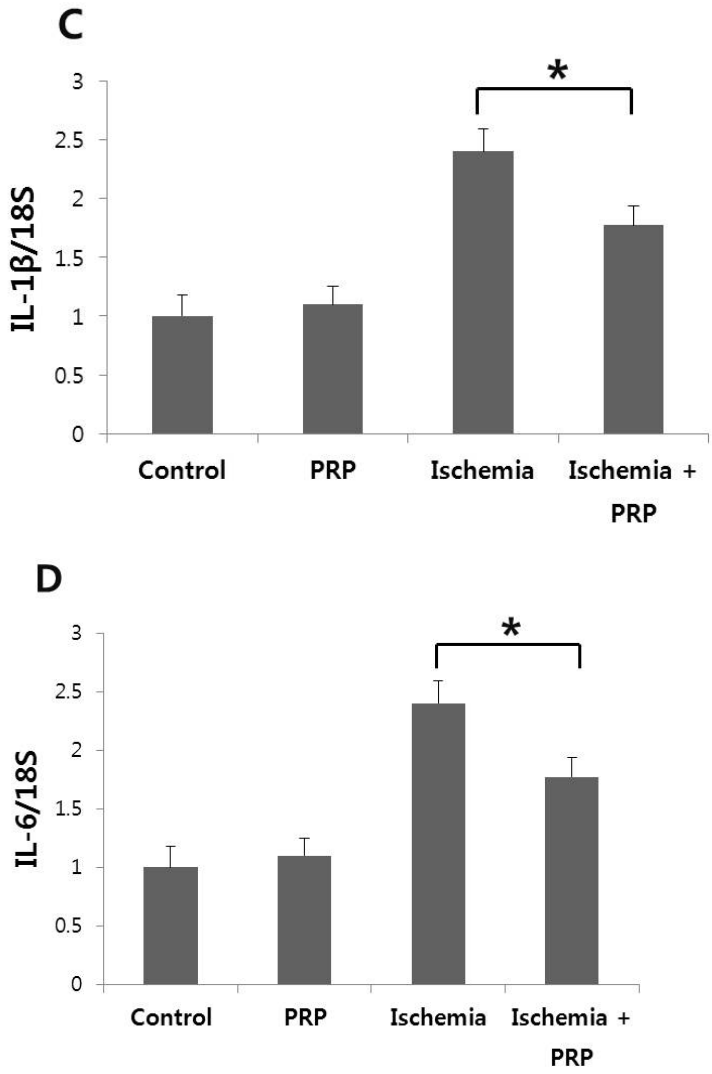


Fig 8. Quantification of mRNA levels of monocyte chemotactic protein-1 (MCP-1), TNF-α, IL-1β, IL-6, mRNA levels in control mice were assigned values of 1. (A) Group D showed decreased expression of MCP-1 compared to group C. ($p < 0.05$) The level of MCP-1 between group A, B, D did not show statistical differences. (B) The expression of TNF-α was higher in group

C than group D. Non-ischemia groups showed lower level of expression compared to group D. ($p < 0.05$) (C) Group A and group B did not show statistical differences in the level of IL-1β. Platelet rich plasma significantly suppressed the expression of IL-1β compared to group C. ($p < 0.05$) (D) IL-6 in group C was higher than non-ischemic groups, however was lower than group C. ($p < 0.05$)

6. Apoptosis signal pathway

The expression of pASK-1 (phospho apoptosis signal-regulating kinase 1), p-p38 were evaluated in the ischemia-reperfusion flaps by Western blot analysis after 12 hours of reperfusion. Group C showed increased apoptosis pASK-1 expression and promoted the phosphorylation of p38. In contrast, platelet-rich plasma significantly reduced apoptosis signal-regulating kinase 1 expression, indicating that platelet-rich plasma may protect ischemia-reperfusion flaps by suppressing apoptosis signal-regulating kinase 1. (Fig 9. A) However, the phosphorylation of p38 showed slight decrease without statistical significance in the ischemia-reperfusion with platelet-rich plasma (Group D). Group B (platelet-rich plasma group) also showed slight decrease of phophorylated p38 compared to control group. However, it could not reach statistical significance. Non ischemic groups showed lower level of phophorylated p38 compared to ischemic groups. (Fig. 9 B)

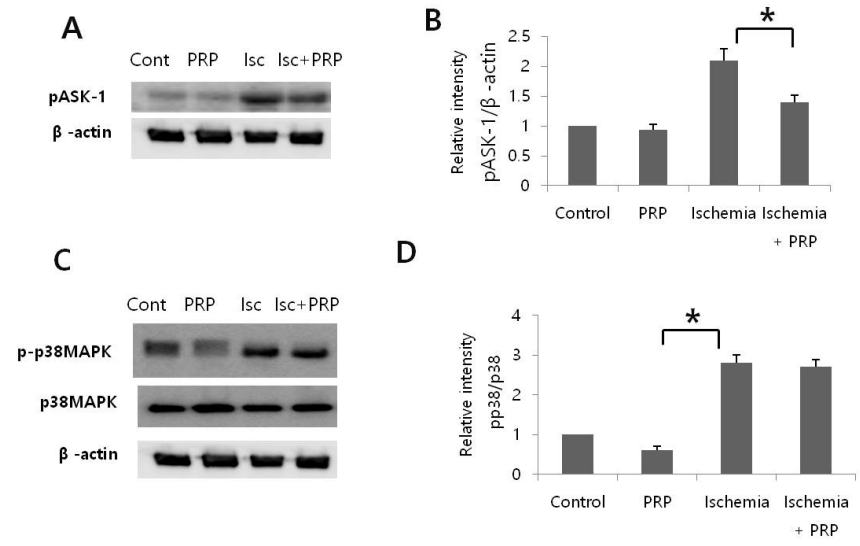


Fig 9. Relative expression of ASK-1 and p38MAPK. Expression levels of phospho apoptosis signal-regulating kinase 1, p38 of skin flaps after reperfusion for 12 hours were detected by Western blot analysis. (A) Representative blotting of pASK-1 (B) Quantification of protein levels of pASK-1. Ischemia-reperfusion increased apoptosis pASK-1 expression, however, platelet-rich plasma significantly reduced pASK-1. *$p < 0.05$, (C) Representative blotting of p38 (D) Quantification of protein levels of p38. Group C and D showed more expression of phosphor-p38 expression than group A and B. The difference between group C and D is not significant. Non ischemic groups showed lower level of phophorylated p38 compared to ischemic groups. *$p < 0.05$

IV. DISCUSSION

This is a study to investigate the role of platelet-rich plasma in ischemia reperfusion injury in mouse dorsal pedicled flap model. Ischemia-reperfusion injury is inevitable during microsurgical free flap transfer, organ transplantation, and other major surgeries. [22] This can lead to organ compromise or even infarction. It is of great importance to clarify the mechanisms and search for effective treatment of ischemia-reperfusion. In this study, we determined that PRP significantly increase the survival area of flap with or without ischemia-reperfusion. Interestingly, the survival area of group D (ischemia and platelet rich plasma) was greater than the control group without ischemia. (Fig 1) Also, the difference between group C (ischemia) and group D was greater than that of group A (control) and B. This phenomenon could be explained that prolonged ischemia could open choke vessels and increase angiogenesis. [23] In this way, ischemia itself could be beneficial to flap surgery with increasing angiogenesis.[24] We could confirmed more perfusion in group D at the end of the study. On the contrast, group C showed the lowest perfusion compared to the other three groups. (Fig. 2) This is because ischemia has beneficial effect with angiogenesis, however, the reperfusion induced tissue damage and apoptosis.[25] Therefore, in terms of free flap surgery and organ transplantation, minimizing the reperfusion injury is essential to complete tolerable result.

Platelet rich plasma (PRP) has been used in various clinical applications, including periodontal and oral surgery, maxillofacial and aesthetic plastic surgery, spinal fusion, cardiac bypass surgery, and treatment of soft tissue ulcers. [13,26] PRP administered during surgical procedures under sterile conditions is easily performed and safe to use. Moreover, PRP lacks surface immunogenic antigens, and thus potential allergic reactions are avoided. The secreted growth factors induced by PRP immediately bind to the external surface of cell membranes of cells in the graft, flap, or wound via transmembrane receptors.[16] Recently, PRP has been reported to activate anti-oxidant response element in tenocyte cluture model through Nrf3-ARE pathway in dose-dependent manner. [27]

Some procedures improve skin flap survival after an ischemia-reperfusion injury. These include the use of restoration of high levels of energy-rich phosphate compounds such as ATP-MgCl2, the maintenance of oxygen supply with the synthetic haemoglobin substrate Fluosol-DA, together with thromboxane synthetase inhibitors like dazoxiben hydrochloride and UK-38. [20, 28, 29] However, these procedures are expensive, not easy to use, and need clinical trials to expand indications. PRP is easy to use, cheap, ready to made, and stable without any rejection when used as autologous manner. In this study, PRP increased survival of ischemia-reperfusion flaps. We thought that this improvement may be resulted from its anti-inflammatory properties, and its protective effects against ischemia-reperfusion injury.

In the present study, MPO, NO, and MDA levels were decreased in group D. On the contrary, SOD enzyme activities were increased in group D compared to group C. (Fig. 4) MPO is a characteristic constituent of neutrophil granules and it is used as a biochemical marker for tissue invasion of neutrophils.[18] Preventing or decreasing neutrophil invasion to reperfused tissues by blocking any step of neutrophil activation has shown to decrease tissue MPO activity. [30] The decreased SOD activity and increased MPO, NO and MDA content in group C prove the redox imbalance and high reactive oxygen species level in ischemia-reperfusion flaps. However, PRP showed protective effect against reperfusion injury. MDA is an end product of lipid peroxidation and has been known as an index of tissue injury. In both non-ischemic groups an interesting finding was the low MDA levels. In the current study, MDA levels were effectively suppressed by PRP in ischemia group. PRP did not reduce MDA level in non-ischemic group.

The improved tissue survival is accompanied by decreased neutrophil recruitment and resulting tissue lipid oxidation along with decreased inflammatory cytokine levels. (Fig. 3 & 5) These findings indicate a decreased inflammatory response resulting from treatment with PRP. A macrophages infiltrate in the early phase of response to ischemia-reperfusion injury, and involved in inflammation by secreting proinflammatory mediators, including MCP-1, NO, IL-1, and IL-6 [31]. We investigate that PRP effectively suppresses inflammatory cytokines, such as monocyte

chemotactic protein-1 (MCP-1), TNF-α, IL-1β, IL-6. Interestingly, PRP could reduce MCP-1 at the level of non-ischemia group. (Fig. 5A) MCP-1, by its chemotactic activity, causes diapedesis of monocytes from the lumen to the subendothelial space where they become foam cells, initiating fatty streak formation that leads to atherosclerotic plaque formation. Inflammatory macrophages probably play a role in plaque rupture and the resulting ischaemic episode as well as restenosis after angioplasty. There is strong evidence that MCP-1 plays a major role in myocarditis, ischaemia/reperfusion injury in the heart and in transplant rejection. [32] We assume that the protective effect of PRP injury might be associated with both suppression of inflammation and promotion of angiogenesis.

Apoptosis is one of most important event and a main form of cell death during skin flap ischemia-reperfusion. The reactive oxygen species during early reperfusion is known as the trigger to activate the apoptosis cascades. [22]As an antioxidant, PRP reduced reactive oxygen species. In addition to its direct antioxidant action, PRP also effect as an apoptosis regulatory messenger. Because the signal transduction involving PRP is rarely documented in skin flaps, we investigate the expression the apoptosis signal-regulating kinase 1. ASK-1, a typical member of the mitogen-activated protein kinase kinase kinase family, is a crucial component in the progression of reactive oxygen species–induced apoptosis. [33] In this study, the expression

level of ASK-1 was higher in ischemia groups compared to non-ischemia groups. (Fig. 6) However, PRP reduced the expression of ASK-1 in group D. In the contrast, p-p38 did not affected by PRP.

V. CONCLUSION

In this study, we investigate the effect of PRP to ischemia-reperfusion flap model. We confirmed the angiogenic activity of PRP when combined with ischemia-reperfusion. Our results demonstrate that PRP acts as a protective factor during flap ischemia-reperfusion by reducing reactive oxygen species level and supressing the apoptosis signal-regulating kinase 1 pathway.

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ABSTRACT (IN KOREAN)

쥐 피판 모델에서 혈소판 농축 혈장이
허혈-재관류 손상에 미치는 영향

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전 영우

허혈-재관류 손상은 피판의 실패와 장기 기능 부전의 주요 원인으로 이를 줄이기 위한 많은 연구가 진행되어 왔다. 활성화된 혈소판은 허혈 재관류 손상에서 혈전과 함께 내피세포의 손상을 일으키지만 체외에서 투여된 다량의 혈소판은 오히려 혈소판의 모임을 저하함이 심근 경색 모델에서 규명되었다. 본 연구의 목적은 쥐의 피판 모델에서 허혈-재관류 손상에 대해서 혈소판 농축 혈장의 영향을

규명하고 그 기전을 밝히는데 있다. 4개의 군을 무작위로 나누었으며 군별로 30마리의 마우스를 사용하였다. A군은 피판을 거상 후 아무 처치 없이 제자리에 봉합하였으며 B군은 혈소판 농축 혈장을 피판 봉합전 주입하였다. C군은 피판 거상 후 미세클램프를 이용하여 4시간의 허혈 후 재관류 손상을 주었으며 D군은 허혈-재관류 손상 후 혈소판 농축 혈장을 피판에 주입하였다. 피판 거상 1,3,7,10일 후 생존 면적과 혈류량을 측정하였다. 혈소판 농축 혈장이 피판의 생존 면적과 혈류량을 유의하게 증가 시켰으며 특히 허혈-재관류 손상이 있을 때 그 효과가 증대되었다. 피판의 생존부분을 채취하여 조직 샘플을 광학 현미경으로 관찰한 결과 혈소판 농축 혈장이 혈관 신생을 촉진시키며 또한 호중구의 모임과 혈전을 감소시켜 허혈-재관류 손상으로부터 조직을 보호하였다. 허혈 재관류 손상의 정도를 측정하기 위하여 재관류 12시간 후 조직에서 일산화 질소, 마이엘로퍼옥시다아제 (Myeloperoxidase), 말론디알데하이드 (Malondialdehyde), 과산화물제거효소 (Superoxide dismutase)를 측정하였다. 허혈-재관류 손상을 입은 군과

비교하였을 때 혈소판 농축 혈장은 일산화 질소, 마이엘로퍼옥시다아제, 말론디알데하이드(Malondialdehyde)를 감소시키는 작용을 보였으며 과산화물제거효소는 증가가 확인되었다. 또한 전염증사이토카인(proinflammatory cytokines)의 변화를 알기 위하여 실시간 중합효소 연쇄반응 (RT-PCR)응 이용하여 단핵세포 화학주성 단백질(monocyte chemotactic protein-1), 종양괴사인자 (tumor necrosis factor-α), 인터루킨 (IL-1β, IL-6)를 측정하였다. 혈소판 농축 혈장은 허혈-재관류 손상에 있어서 전염증사이토카인을 모두 감소시켜서 조직을 보호하는 효과를 보였다. 이러한 혈소판 농축 혈장의 작용은 세포사멸신호조절 카이네이즈(Apoptosis signaling regulating kinase 1)의 표현 저하와 연관이 있을 것으로 사료된다.

본 연구에서 혈소판 농축 혈장은 쥐의 피판 모델 허혈-재관류 손상을 감소시키며 조직을 보호하는 것으로 판명되었다. 이를 이용하여 임상에 적용함으로 보다 얻기 쉽고 저렴한 보호제로 이용될 수 있을 것이다.

핵심되는 말: 혈소판 농축 혈장, 허혈-재관류, 혈관생성