

The Effect of Platelet-Rich Plasma on Survival of the Composite Graft and the Proper Time of Injection in a Rabbit Ear Composite Graft Model

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Background Administration of growth factors has been associated with increased viability of composite grafts greater than 1-cm in diameter. Platelet-rich plasma (PRP) contains many of the growth factors studied. In this study, we evaluate the effect of PRP injection on composite graft viability and the proper time for injection.

Methods A total of 24 New Zealand White rabbits were divided into four groups. Autologous PRP was injected into the recipient sites three days before grafting in group 1, on the day of grafting in group 2, and three days after grafting in group 3. Group 4 served as control without PRP administration. Auricular composite grafts of 3-cm diameter were harvested and grafted back into place after being rotated 180 degrees. Median graft viability and microvessel density were evaluated at day 21 of graft via macroscopic photographs and immunofluorescent staining, respectively.

Results The median graft survival rate was 97.8% in group 1, 69.2% in group 2, 55.7% in group 3, and 40.8% in the control group. The median vessel counts were 34 (per ×200 HPF) in group 1, 24.5 in group 2, 19.5 in group 3, and 10.5 in the control group.

Conclusions This study demonstrates that PRP administration is associated with increased composite graft viability. All experimental groups showed a significantly higher survival rate and microvessel density, compared with the control group. Pre-administration of PRP was followed by the highest graft survival rate and revascularization. PRP treatments are minimally invasive, fast, easily applicable, and inexpensive, and offer a potential clinical pathway to larger composite grafts.

Keywords Platelet-rich plasma / Growth substances / Tissue transplantation / Graft survival

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INTRODUCTION

Composite grafts have been used for reconstruction of soft-tissue defects for several decades. These grafts are composed of tissues that have origins in two or more germ layers, and are commonly used in reconstruction of complex portions of the

integumentary system, including the external ear, alar rim, columella, and the finger tip [1].

Compared to split or full-thickness skin grafts, composite grafts are severely restricted in sizes less than 1 cm in diameter secondary to limitations in re-vascularization from recipient area to the composite graft [2]. To overcome such restriction,

various studies have attempted to modify immunologic response, vascularity, and metabolic rate. Pharmacological agents such as corticosteroid, dimethylsulfoxide, and heparin have been tested [1,3-5]. Cooling and hyperbaric oxygen therapy have also been investigated [6,7]. Angiogenesis was promoted via direct injection of growth factors such as basic fibroblast growth factor and platelet derived growth factor [8,9].

Platelet rich plasma (PRP) contains various growth factors, such as transforming growth factor- β , platelet derived growth factor, vascular endothelial growth factor, and epidermal growth factor. Administration of autologous PRP is known to stimulate angiogenesis and promote reproduction of damaged tissues [10,11].

The aim of this study was to investigate the question of whether PRP administration is associated with increased angiogenesis and survival rate of composite grafts and to determine an ideal time period for PRP administration in the setting of a composite graft transfer.

METHODS

Preparation of platelet-rich plasma

Prior to the grafting procedure, 12 mL of femoral vein blood was collected from each rabbit into a vacuum tube containing 1.2 mL of the anticoagulant (10% sodium citrate). The tube was centrifuged for 10 minutes at 1,000 rpm to prevent displace-

ment of platelets from the bottom of the tube. The initial supernatant plasma was dispensed, and the tube was centrifuged once more at 5,000 rpm for 10 minutes (Tube \times 10 mL, Muhan, Republic of Korea). The supernatant plasma was then removed, leaving 1.2 mL of centrifuged product. The Sysmex XE-200 system (TOA Medical Electronics Co., Kobe, Japan) was used for calculation of the platelet count.

Animal model and surgical procedure

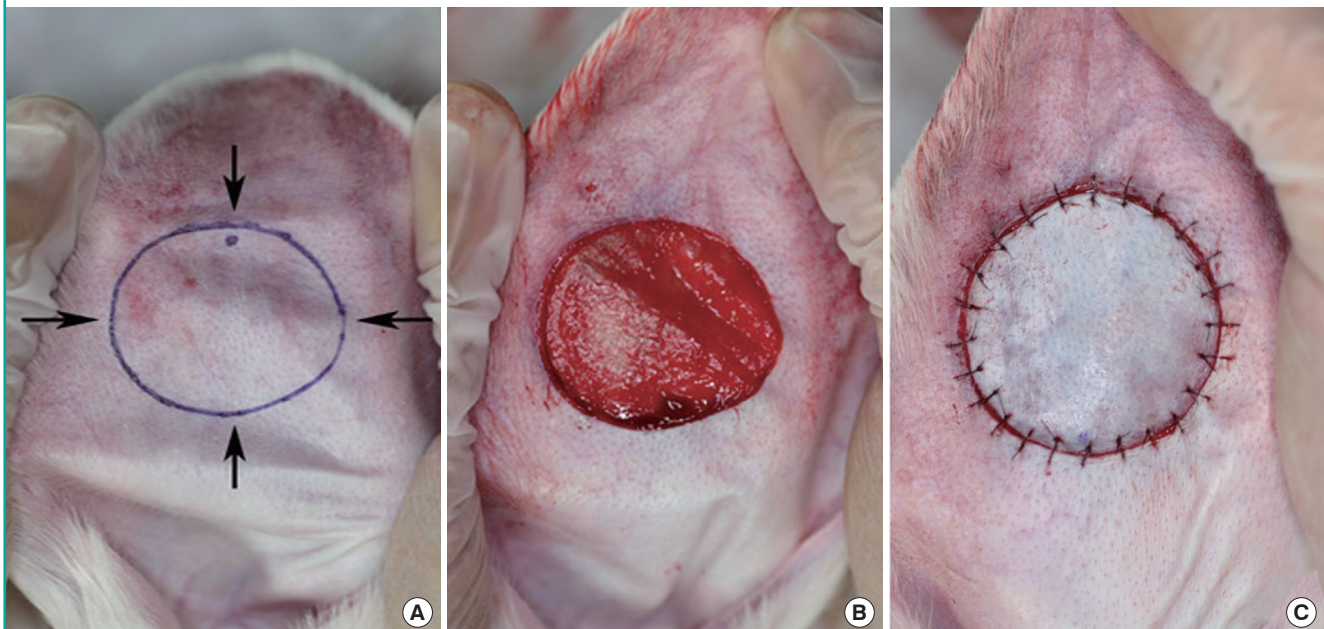
A total of 24 New Zealand white rabbits weighing 2.5 to 3.0 kg were used in the experiment. The same feed and breeding conditions were maintained for all animals. This study was conducted after meeting the approval of the Institutional Animal Care and Use Committee (IACUC No. 12-05).

The rabbits were equally randomized into four groups. Autologous PRP was injected into the recipient sites three days before grafting in group 1, on the day of grafting in group 2, and three days after grafting in group 3. group 4 served as control without PRP administration. In the experimental groups, 0.5 mL of autologous PRP [12] was injected dermally into four quadrants of the recipient area around the composite graft using a 30-gauge needle (Fig. 1A).

Prior to composite graft transfer, each animal was sedated with intramuscular injection of two anesthetic agents (Ketamine 0.25 mL/kg and xylazine 0.05 mL/kg). Both ears were shaved and sterilized with povidone-iodine solution. Skin-cartilage com-

Fig. 1. Composite graft procedure

(A) Each skin-cartilage graft was designed using a 3-cm diameter template. Arrows indicate platelet-rich plasma injection locations. (B) Auricular composite graft was excised while leaving the posterior perichondrium on the recipient site. (C) The graft was rotated 180 degrees and secured with sutures.



posite grafts of 7.1 cm² were designed on ventral surfaces of both ears using a 3-cm diameter template (Fig. 1A). According to previous study findings, the expected viability for the 7.1-cm² control grafts was minimal (less than 40%) [4]. The skin-cartilage composite graft was taken while leaving the dorsal perichondrium intact (Fig. 1B). Each composite graft was rotated 180 degrees and placed back into the wound bed using simple 6-0 nylon sutures (Fig. 1C). Antibiotic ointment was applied to the graft.

Macroscopic evaluation of composite graft survival

Macroscopic graft survival was documented on the 21st day using a digital camera (EOS 550D, Canon, Tokyo, Japan). Each composite graft was evaluated for median area of survival using ImageJ software (National Institutes of Health, Bethesda, MD, USA). Dark zones and zones covered with scabs were defined as necrotic, with the remainder of surface area considered viable. Survival rate was calculated from the viable area as a percentage of the entire graft area. The median graft survival area was calculated using ImageJ software (National Institutes of Health, Bethesda, MD, USA).

Immunofluorescence staining

Microvessel density was evaluated to confirm revascularization via immunofluorescence staining of graft specimens obtained on the 21st day after surgery. A square specimen of 1 cm² was excised from each graft area (both ears per rabbit) to incorporate the suture line. To avoid transection of larger subcutaneous vessels, each specimen was harvested from the lateral margin of each graft. The excised tissue was then immediately fixed in 10% formalin and embedded in paraffin. Four-micrometer serial sections were obtained using a microtome (Leica Co., Buffalo, IL, USA), mounted, deparaffinized, and hydrated.

Primary antibody binding sites were visualized using the ultra-View Universal DAB Detection Kit (Ventana). Nuclei were

counterstained automatically with hematoxylin and bluing reagent (Ventana). Microvessel density was determined using the endothelial cell marker CD31 (mouse anti-rat CD31 antibody, SerotecAbD, Dusseldorf, Germany) on a Ventana Bench Mark XT immunostainer (Ventana, Illkirch, France) in a standardized two-step protocol. First, the tissue area with the highest vessel density was identified at low magnification ($\times 100$) in each CD31-stained specimen. In the second step, three adjacent pictures of this identified area were taken at high magnification ($\times 200$). Microvessel density was then assessed by enumerating the number of CD31-positive vessels. Vessels characterized by thick muscular walls or with lumen greater than 20 μm in diameter were excluded. Digital analysis software (ImageJ) was used for standardized analysis. Reassessment of the results was performed by two pathologists.

Statistical analysis

All quantitative data were analyzed using the Kruskal Wallis test. Statistical outcomes are presented as median (interquartile range, 25th–75th centile). A P-value < 0.05 was considered significant. A multiple comparison test was performed using the Mann-Whitney test. A P-value < 0.008 was considered significant.

RESULTS

Platelet-rich plasma

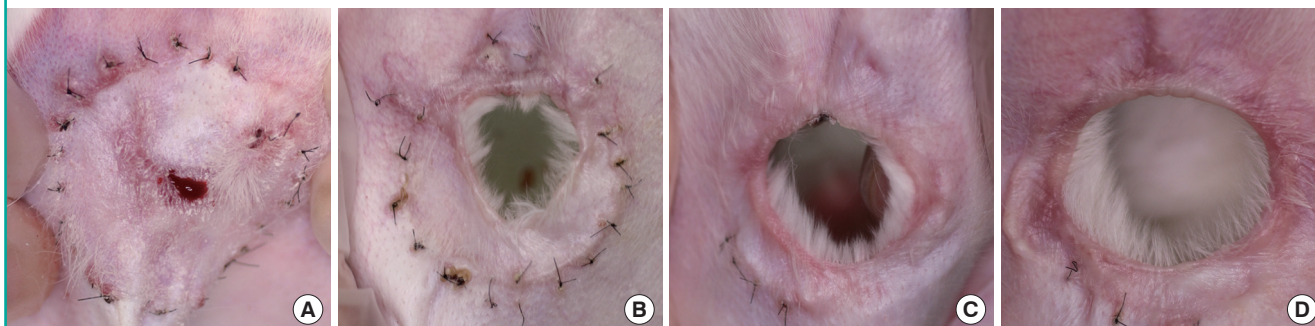
The average platelet count was $0.26 \pm 0.3 (\times 10^6)/\mu\text{L}$ in peripheral blood samples. This same count was $1.37 \pm 0.5 (\times 10^6)/\mu\text{L}$ in the prepared platelet-rich plasma, representing a five-fold increase compared to peripheral blood density.

Survival rate of composite graft

Viable regions of the grafts were clearly demarcated by the 21st postoperative day (Fig. 2). The median (interquartile range, 25th–75th centile) survival rate was 97.8% (93.3–100) in group

Fig. 2. Postoperative photographs of composite grafts

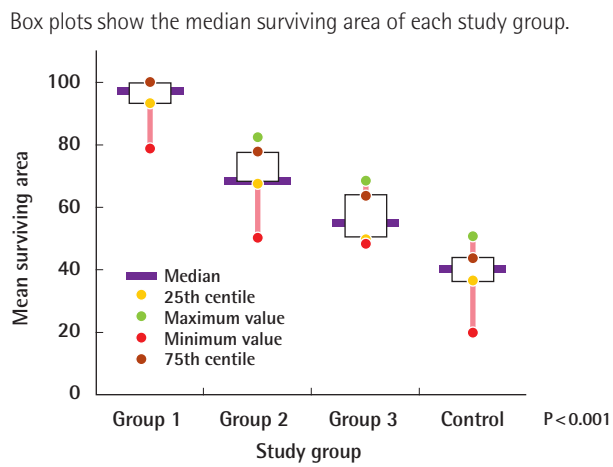
All photographs of composite grafts were obtained 21 days after surgery. Viable regions are clearly demarcated. (A) Group 1. (B) Group 2. (C) Group 3. (D) Control group.



1, 69.2% (67.7–77.9) in group 2, 55.7% (50.2–64.2) in group 3, and 40.7% (36.4–44.2) in the control group (Fig. 3). Survival

rates of all experimental groups were significantly different from that of the control group ($P < 0.001$). Group 1 showed the highest survival rate, compared with that of the other groups ($P < 0.001$).

Fig. 3. Survival rate of composite grafts



Microvessel density

CD31 positive blood vessels lined by light green were observed (Fig. 4). Revascularization (CD31-positive blood vessels) was observed at 34 (31–37)/HPF in group 1, 24.5 (22.3–26.8)/HPF in group 2, 19.5 (15.3–21.8)/HPF in group 3, and 10.5 (8.3–13)/HPF in the control group (Fig. 5). Compared with the control group, higher microvessel density was observed for all experimental groups. In line with the results from macroscopic graft survival analysis, the highest microvessel density was observed for group 1 ($P < 0.001$).

Fig. 4. Immunofluorescence staining

Microvessel density at day 21 was determined using the endothelial cell marker, CD31. Arrows indicate examples of microvessels ($\times 200$). (A) Group 1. (B) Group 2. (C) Group 3. (D) Control group.

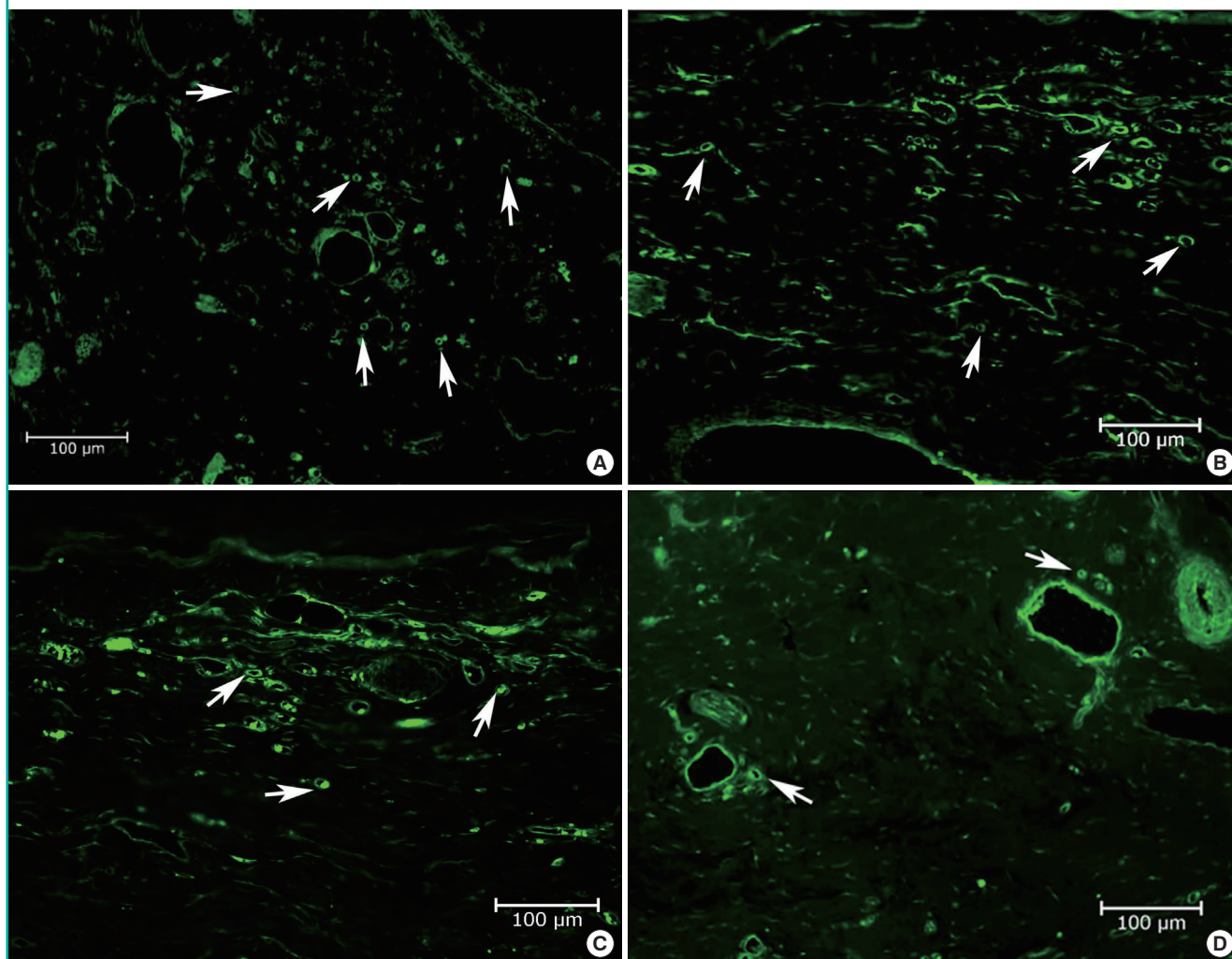
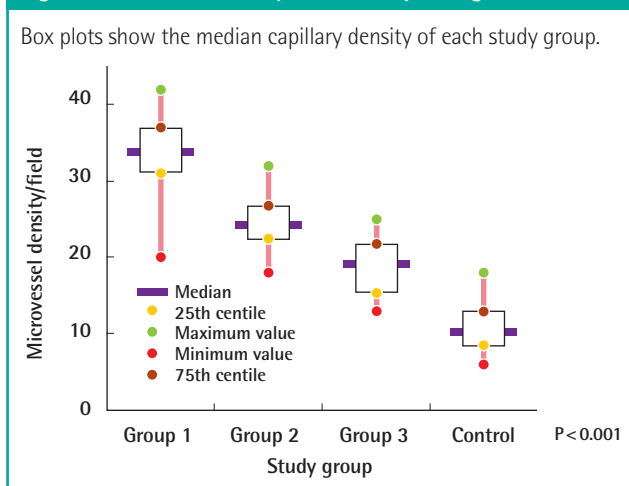


Fig. 5. Microvessel density of the composite grafts

DISCUSSION

Viability of skin-cartilage composite grafts is dependent on a number of factors. Like skin grafts, composite grafts survive via serum imbibition, direct vessel to vessel reanastomosis (inosculation), and neovascularization [13]. Neovascularization is characterized by new vessel ingrowth from the recipient site into the graft [14].

However, unlike skin grafts, composite grafts include the cartilage layer, which may act as a mechanical barrier limiting vascularization from the wound bed [15]. Thus, it is conceivable that revascularization through the dermal-dermal connection at the wound margin is more important for composite graft survival, and in turn limits the size of a composite graft [16].

Brown and Cannon [2] first reported that composite grafts greater than 1 cm in diameter tended to undergo necrosis. Several studies have attempted to increase composite graft survival. Pharmacological treatments such as corticosteroid, dimethylsulfoxide, and heparin have been used to enhance composite graft survival [1,3-5]. Use of cooling methods and hyperbaric oxygen therapy were also reported [6,7].

Since then, many cytokines have been found to stimulate vascularization and eventually came to be classified as angiogenic factors [17]. When locally administered, growth factors such as basic fibroblast growth factor or platelet-derived growth factor (PDGF) have been shown to increase composite graft viability [8,9]; however, the expense and short shelf-life of these biologic factors make for a difficult clinical translation.

Autologous platelet storage via PRP, which contains a high concentration of alpha granules, may be an easy, cost-effective approach to obtaining high concentrations of growth factors [10].

Alpha granules of platelets are known to contain growth factors such as PDGF, vascular endothelial growth factor, trans-

forming growth factor, and epidermal growth factor, which stimulate angiogenesis, cell proliferation, maturation, and matrix formation [18]. Active secretion of these growth factors is initiated by the clotting process of blood and begins within 10 minutes of microvascular injury. More than 95% of presynthesized growth factors are secreted within 1 hour. Therefore, PRP must be processed and stored in an anticoagulated state and should be used on the graft, within 10 minutes of clot initiation. After this initial burst, the platelets synthesize and secrete additional proteins for five to 10 days [10].

Application of PRP can result in increased tissue regeneration and reduced risk of infection, pain, and loss of blood. El-Sharkawy et al. [19] suggested that PRP may suppress cytokine release, limit inflammation, and thereby promote tissue regeneration. Pallua et al. [20] reported that application of PRP for burn patients could accelerate re-epithelization. In another study, subcutaneous injection of PRP in rabbit skin flap was found to promote arteriogenesis and increased flap survival [21].

Angiogenesis via PRP-secreted factors is known to require three to five days [13]. Previously, Yoo et al. [9] described the composite graft engraftment process using the terms primary revascularization and secondary revascularization. The said primary revascularization is presumed as inosculation, and the said secondary revascularization as neovascularization. They suggested that neovascularization following composite grafting occurs too late for the composite tissue to survive [9]. They also suggested that a composite graft can survive only when neovascularization occurs between the graft and subdermal plexus of the recipient site, identifying inosculation as the most important process in graft survival [9]. Since creation of new blood vessel takes 3 to 5 days, we hypothesized that more inosculations are possible because local injection of PRP three days prior to grafting induces angiogenesis of the recipient site as much as possible before inosculation after grafting.

Immunofluorescent staining of vascular endothelial cells was performed using an antibody against CD31 in order to visualize vascular density. CD31 is present on the surface of platelets, monocytes, macrophages, and neutrophils and is a constituent of the endothelial intercellular junction. It plays a major role in the adhesion cascade between endothelial cells and inflammatory cells during inflammation in facilitating leucocyte migration and between endothelial cells during angiogenesis. It has been recognized for its angiogenic role [22]. Von willebrand factor (vWF), another endothelial cell marker, appears to be expressed exclusively on endothelial cells, where it shows a granular pattern of reactivity. Although it is commonly used as an immunohistochemical marker for endothelial cells, little is known about the relative level of expression of vWF in endothelial cells

of the same or different vascular beds *in vivo* [23].

Alpha smooth muscle actin, an isoform typical of smooth muscle cells, which is present in high amounts in vascular smooth muscle cells, was detected in the cytoplasm of pericytes of various organs by means of immunocytochemistry [24]. Immunofluorescent staining of vascular smooth muscle cells can be performed using an antibody against alpha smooth muscle actin for determination of mature and functional vessels.

Although the single immunohistochemical staining method enables analysis of morphological aspects of angiogenesis, these criteria might not be sufficient for assessment of vascular function. Double immunohistochemical staining using an endothelial cell marker and a pericyte marker can be used for evaluation of the morphology and maturity of microvessels.

The relationship between angiogenesis at the recipient site and inosculation with a composite graft had already been determined through microangiogram in the study by Yoo et al. [9]. Thus, we did not perform an experiment for mature vessel density since the microvessel density and engraftment difference were sufficient to support the hypothesis that pre-administration of PRP increases revascularization. Conduct of further studies would be needed in order to correlate these findings.

In this study, graft survival rate was the highest with pre-administration of PRP three days prior to the grafting procedure, possibly due to the process of angiogenesis already taking place prior to the harvest of grafts. Most revascularization was observed among experimental groups through immunofluorescence staining. In the case of injection of PRP before grafting, we supposed that the amount of the inosculation was less than that of group 1. We supposed that the amount of inosculation was the least when PRP was injected three days after grafting in group 3.

In our study, dorsal perichondrium and dorsal skin, left after harvesting a composite graft, had good vascularity and thus were expected to survive, however, groups 2, 3, and 4, except group 1, showed a full thickness defect at the center of the composite graft. Breuing et al. [25] reported that wound desiccation may cause tissue damage. Dorsal perichondrial skin flap, left after harvesting a composite graft, is very thin and weak, and is therefore vulnerable to tissue damage due to graft necrosis and subsequent desiccation, apparently causing defects across all layers.

In conclusion, this study showed that pre-administration of PRP was followed by an increased composite graft survival rate using the rabbit ear composite graft model. In addition, results of the study experiments demonstrated that PRP can increase vascularity on a composite graft. It can be supposed that pre-administration of PRP was associated with the most significant amount of inosculation, resulting in improved graft survival rate. We supposed that PRP administration can induce angiogenesis

at the recipient site as much as possible prior to grafting. It is possible that PRP administration still increases the composite graft survival rate even when administered a day or two prior to grafting. Thus, conduct of additional experiments will be necessary for establishment of the most effective preoperative injection time. PRP treatments are minimally invasive, fast, easily applicable, and inexpensive, and offer a potential clinical pathway to larger composite grafts.

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