대한성형외과학회지. 제 38 권, 제 4 호
J Korean Soc Plast Reconstr Surg
Vol. 38, No. 4, 345 - 350, 2011

혈소판 농축재제를 이용한 창상치유의 촉진

한형민 · 전여름 · 나동균 · 유대현

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Acceleration of Wound Healing Using Adipose-derived Stem Cell Therapy with Platelet Concentrates: Platelet-rich Plasma (PRP) vs. Platelet-rich Fibrin (PRF)

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Purpose: Although platelet-rich plasma (PRP) potentiate the wound healing activity of adipose-derived stem cells (ADSCs), its effect cannot be sustained for a prolonged period of time due to short duration of action. This led us to design and produce platelet-rich fibrin (PRF), in an effort to develop a tool which lasts longer, and apply it on wound healing.

Methods: Two symmetrical skin defects were made on the back of seven nude mice. ADSCs were applied to each wound, combined with either PRP or PRF. The wound area was measured over 14 days. By day 16, the wound was harvested and histologic analysis was performed including counting of the blood vessel.

Results: The healing rate was more accelerated in PRP group in the first 5 days (p<0.05). However, PRF group surpassed PRP group after 6 days (p<0.05). The average number of blood vessels observed in the PRF group was 6.53 ± 0.51, compared with 5.68 ± 0.71 for the PRP group.

Conclusion: PRF exerts a slow yet pervasive influence over the two-week course of the wound healing process. Thus, PRF is probably more beneficial for promoting the activity of ADSCs for a sustained period of time.

Key Words: Adipose-derived stem cell, Platelet-rich plasma, Platelet-rich fibrin, Wound healing

Received March 19, 2011
Revised May 5, 2011
Accepted May 23, 2011

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* 본 논문은 2009년 제67차 대한성형외과학회 학술대회 구연발표 되었음.

I. INTRODUCTION

Several studies have pointed out that cell therapy with adipose-derived stem cells (ADSCs) enhances the healing process of wounds, which involves bone, tendons, and skin, in animal and clinical studies. The wound-healing effect of ADSCs is mainly mediated by the activation of dermal fibroblasts and keratinocytes via direct cell-to-cell contact or a paracrine mechanism through secretory factors.[3] Platelets, in addition to their well-known function of hemostasis, contain many substances that participate in wound healing. Once activated through aggregation and fibrin formation, they release substances that promote tissue repair and influence the reactivity of vascular and other blood cells in angiogenesis and inflammation from the dense granule and α-granule. With these endogenous properties of platelets, platelet concentrates were developed in the early 1990s for therapeutic purpose in the orthopedic and dental fields. In a report, platelet-rich plasma (PRP), a first-generation platelet concentrate, showed improved wound healing when it applied with ADSCs.[1] While the platelets within PRP do promote the actions of ADSCs through secretion of various growth factors like TGF-1β (transforming growth factor β), platelet-derived growth factor (PDGF), and vascular endothelial growth factor (VEGF), the effect is very short-lived lasting only about a few ours.[2] This shortcoming can be overcome by the arrival of PRF with platelets incorporated within a three dimensional matrix, which in turn releases growth factors for a stable period of time.[2] This study sought to elucidate the long-lasting effects of PRF combined with ADSCs on wound healing and to create a protocol for concurrent chronic wound treatment.

II. MATERIALS AND METHODS

A. Isolation and preparation of human adipose-derived stem cells

To obtain human ADSCs, the adipose aspirate was harvested from a 35-year-old healthy female patient

undergoing liposuction of the abdomen as a body contouring procedure. After the adipose tissue was minced into small pieces (approximately 1 mm^3), the tissue was washed three times with 4°C cold phosphate buffer saline (PBS; Sigma Chemical Co, St. Louis, MO, USA). The specimen then was digested with 0.03% type I collagenase (Worthington Biochemical Corp., Lakewood, NJ) with intermittent shaking in a 37°C water bath for 30 minutes. Digestion was neutralized by the addition of the same amount of fetal bovine serum (Dulbecco's Modified Eagle Medium, 10% fetal bovine serum, penicillin/streptomycin, amphotericin-B, and gentamicin). This cell suspension was centrifuged (1,200 g for 10 minutes at 4°C), the supernatant removed and the cell pellet resuspended in phosphate buffer saline and centrifuged again (1,300 RPM for 3 minutes at 4°C). After adding the basic medium, using a cell strainer, the resultant cell pellet was obtained for culture. The cells were cultured overnight in humidified air with 5% (v/v) CO_2 at 37°C. After washing out non-adherent cells and red blood cells with PBS, ADSCs were acquired.

B. Preparation of platelet-rich plasma

By standard venipuncture using a sterile vacuum tube, peripheral blood was collected from 29-year old healthy male volunteer using anticoagulant, ACDA (acid citrate dextrose) solution. The collected blood sample was centrifuged at 160 g for 10 minutes at 20°C, the upper supernatant (plasma) including the buffy coat, red and white blood cell layers were discarded. Remaining middle layer was centrifuged again at 200 g for 10 minutes at 20°C. The clear supernatant (plasma) was taken as PRP. Three mL of whole blood and 0.6 mL of ACDA solution produced 0.3 mL of PRP fluid, which was applied to each wound admixed with 0.1 mL of PRP activator (5,000 IU thrombin powder plus 3% 16.7 mL $CaCl_2$[1]). PRP was produced from blood drawn from the donor immediately before application.

C. Preparation of platelet-rich fibrin membrane

Whole blood is drawn from the same donor as in PRP using a sterile vacuum tube without anticoagulant. The vacuum tube was centrifuged at 3,000 RPM for 12 minutes and after centrifuge, the blood sample was divided into three layers: red blood cell layer at the bottom, acellular plasma on the top (PPP) and a clot of PRF between the two layers (Fig. 1). After removing the bottom layer of red blood cells and the upper layer of platelet-poor plasma (PPP), PRF was then compressed between two layers of 4 × 4 inch gauze and the fibrin sheet was shaped into a circle using an 8 mm punch (Fig. 1). Three mL of blood produced a 50 mm^2 membrane of 1 mm thickness. PRF was produced from blood drawn from the donor immediately before application.

D. Animal experiment for wound healing model

All animal procedures were performed in accordance with the Guide for the Care and Use of Laboratory Animals (National Institutes of Health [NIH] publication No. 86-23) and approved by the animal experiment committee of our university. As animal experiments, nude mouse (7 week-old, 20~30 g) provided by Orient Bio, INC and raised in the AAALAC-approved experimental animal facility of our university. A total of seven nude mice were recruited for this experiment. Animals were anesthetized by inhaled anesthesia (Isoflurane) and peritoneal cavity anesthesia (Ketamine 30 mg/Kg and Xylazine 3 mg/Kg). After the hair were removed from head to tail, two symmetrical full thickness skin defects including the panniculus carnosus muscle layer were made on the

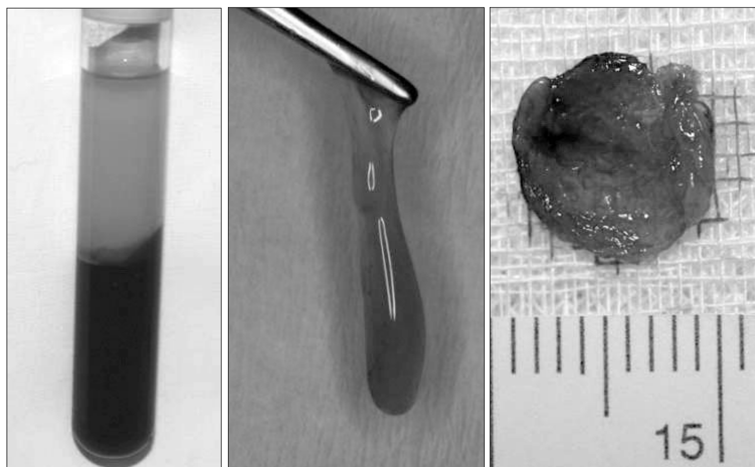

Fig. 1. (Left) The three layers that formed after centrifugation. (Center) The intermediate layer is platelet-rich fibrin. (Right) Platelet-rich fibrin was then compressed between two layers of 4 × 4 inch gauze and the fibrin sheet was shaped into a circle using an 8 mm punch.

dorsum of each mouse using 8-mm punch (Fig. 2). The skin defect was maintained for 24 hours facilitating the retention of tissue fluids using Tegaderm® without drying of the wound.

The experimental group was comprised of the right side symmetrical wound of each mouse, treated with a combination of ADSCs and PRF. We injected 0.1 mL of ADSCs (cell count, 1×10^6) at a total of five sites, at 4 sites subcutaneously along the wound margin of 12, 3, 6, and 9 o'clock position and at the base of the wound intramuscularly using a 26-gauge needle with 1 mL of syringe. A prepared PRF membrane (8 mm in diameter, round shape) was laid on the defect. The control group was comprised of the left side wounds, treated with a combination of the same amount of ADSCs and PRP, which was derived from the same amount of blood as in the PRF group. Adipose-derived stem cells were injected in the same manner. To avoid spilling the PRP fluid over the wound when applied in injection, both wounds were dressed and sealed with adhesive and Tegaderm® (3M Health Care, 3M, St. Paul, MN, USA) dressing first, and 0.3 mL of platelet-rich plasma and 0.1 mL of activator were applied to the wound bed by piercing the normal adjacent skin using 26-gauge needle and shooting beneath the Tegaderm® dressing. The experimental animals were housed individually.

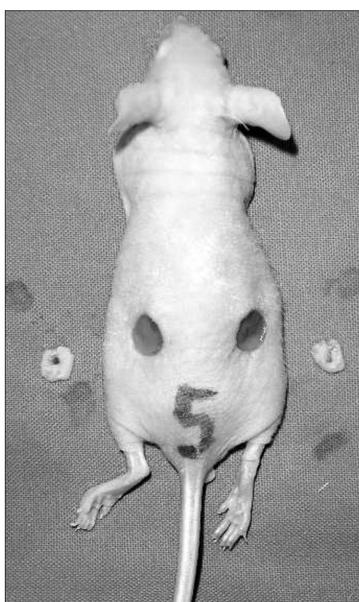

Fig. 2. Full thickness skin defect on the dorsum of a mouse using an 8-mm punch. The experimental group was on right side and the control group on the left.

1) Measurement of the wound healing rate

The wound healing rate was quantitatively assessed

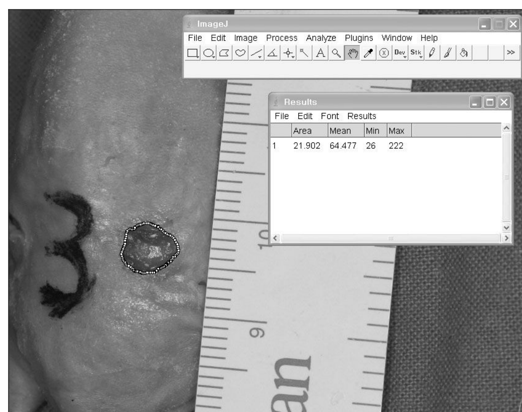

Fig. 3. Measurement of wound area with computer planimetry.

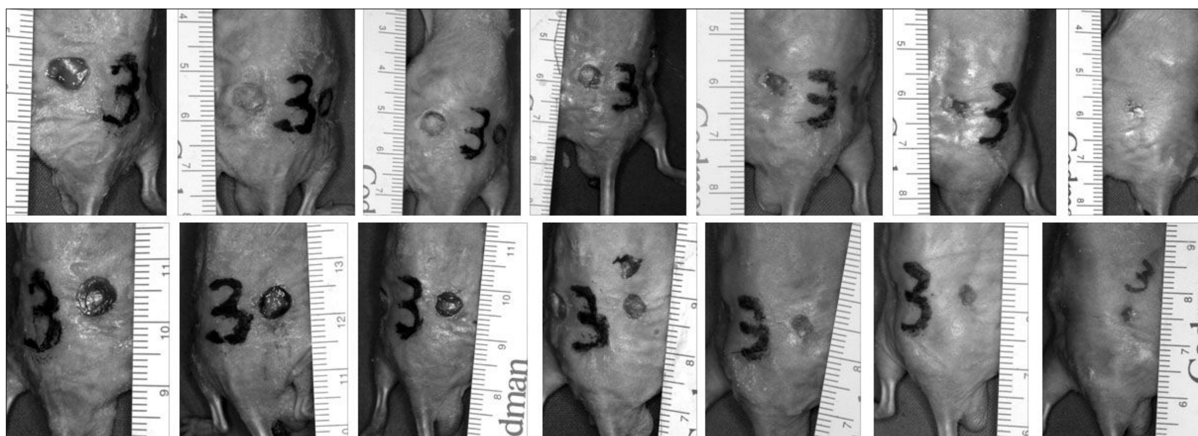

Fig. 4. Serial change of wound area of mouse 3 from postwounding day 2 through day 14. (Above) The left side of symmetrical wound of the back, treated with a combination of ADSCs and PRP. (Below) The right side, treated with a combination of ADSCs and PRF.

by epithelization rate. The dorsal wounds were evaluated every other day starting on postwounding day 2 until the wound was epithelized completely, which took about 15 days (Fig. 4). The subjects were sedated with inhale anesthetic (Isoflurane), followed by cleansing of the normal skin with saline. Qualitative wound evaluation was recorded with metric ruler placed beside the experimental animal and digital photographs (EOS-400D digital SLR camera, macro-lens 50 mm, Canon Inc., Japan) were taken. The wound area was defined as the margin of grossly evident epithelization. The surface area of each open wound was calculated by applying computer planimetry (Image J®; National Institute of Health, Bethesda, MD, USA) and expressed as a percentage of the original wound area on the day of wounding (Fig. 3). The wound was then redressed with adhesive and Tegaderm® dressing.

2) Analysis of vascularization by histologic staining

By day 16, all seven mice were euthanized. The harvested wound was taken for histologic analysis including blood vessel density measuring. Vessel density was determined by counting for vascular structures observed on hematoxylin and eosin stains. The number of lumen-containing vessels was measured using 3 random fields per slide viewed at high-magnification (×100). The images were viewed using Image J® software and the blood vessels in each high-powered field were marked and counted.

3) Statistical analysis

Statistical analysis of all data was performed by the personal computer program SPSS for Windows version 13.0 (SPSS Inc, Chicago, IL, USA), and technical data were presented as the mean ± standard deviation as well as percentages. For the comparison of discontinuous variables, a paired Student t-test was used, and the presence or absence of significance was assessed. *P*-value less than 0.05 were evaluated to be statistically significant.

III. RESULTS

A. Healing rate of the wound

Wound healing was measured by computer planimetry and the healing rate was more accelerated in the PRP group than in the PRF group in the first five days, with statistical significance ($p < 0.05$) (Fig. 5). At postwounding day 4, wound closure of 63.8 ± 9.6% in the control group and 78.2 ± 8.3% in the experimental group were observed. However, as wound healing continued, the PRF group surpassed the PRP group after the postwounding day 6 with statistical significance ($p < 0.05$) (Fig. 5). At postwounding day 10, wound closure of 42.2 ± 13.5% in the control group and 22.7 ± 11.7% in the experimental group were observed. The experiment showed a shorter time to complete wound healing in the PRF group.

B. Differences of vessel density between the two groups

Higher vessel formation was demonstrated in the wound with PRF at day 16 (Fig. 6). The average number of blood vessels observed in the PRF group was 6.53 ± 0.51, compared with 5.68 ± 0.71 for the PRP group, however, the difference was not statistically significant (× 100 high power field).

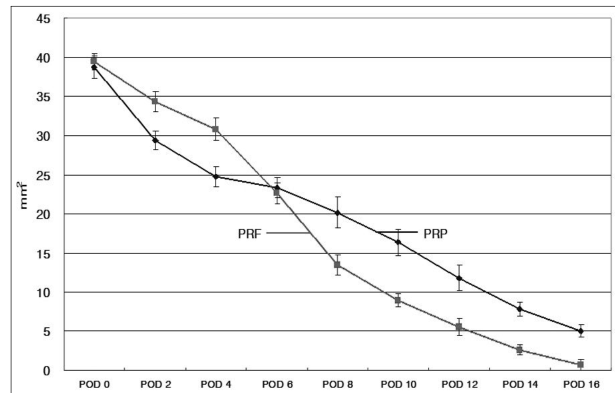

Fig. 5. Measurement of wound healing. The PRF group surpassed the PRP group after postwounding day 6 with statistical significance.

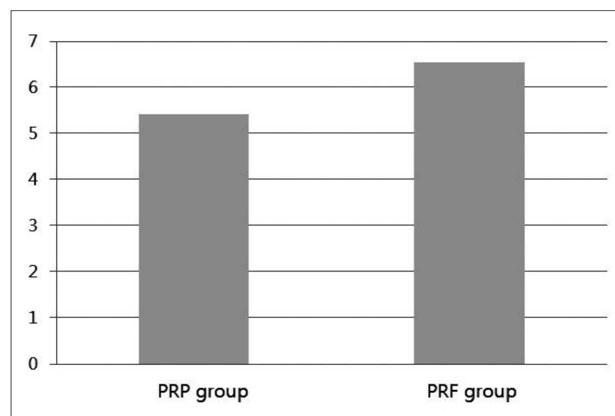

Fig. 6. The vessel density of the wound on day 16 (× 100 high power field). The average vessel density was higher in the PRF group. The average number of blood vessels observed in the PRF group was 6.53 ± 0.51, compared with 5.68 ± 0.71 for the PRP group, however, the difference was not statistically significant.

IV. DISCUSSION

Platelets are specialized secretory cells that release the contents of their intracellular granules in response to activation. Among the stored cytokines mainly involved in wound healing are PDGF with the AB and C isoforms predominating, TGF-β, VEGF, basic fibroblast growth factor (bFGF), platelet-derived epidermal growth factor (PDEGF) and insulin-like growth factor-1 (IGF-1).[4] These molecules promote angiogenesis, cell proliferation and maturation, and matrix formation. With these endogenous properties of platelets, platelet concentrates were developed in the early 1990s for therapeutic purpose in the orthopedic and dental fields. PRP is plasma with more condensed platelets than baseline. Higher than 300,000/μL is considered to be concentrated. PRP can be injected, applied externally or directly to wound. As the amount of released factors increases with the total number of platelets delivered to a site of an injury, application of PRP increases the supra-physiologic response to wound. Advocates of PRP therapy therefore claim its benefits include increased tissue regeneration and a lower rate of infection, pain and blood loss. Recent reviews imply that in the treatment of burns, PRP could offer a certain degree of efficacy by stimulating dermal regeneration, increasing the take rate after skin grafting or speeding-up reepithelization.[5] Also, clinical studies have shown that PRP combined with centrifuged fat tissue successfully treated chronic lower-extremity ulcers.[6] In experimental studies, sustained release of PRP has been found to be effective in restoring blood perfusion to mouse hind limb ischemia because it stimulates angiogenesis, arteriogenesis and vasculogenesis.[7]

The evolved concept that ADSCs and platelet concentrate could be far more beneficial for wound healing than when used alone led us to design the present study. Kakudo et al.[8] confirmed the ability of PRP to enhance the proliferation of human ADSCs and dermal fibroblasts, which proposed the possible basic mechanism of how PRP could promote tissue repair. The wound healing properties of ADSCs are increased when combined with PRP containing a number of wound-healing trophic cytokines. PRP may stimulate ADSCs to interact with and stabilize nascent vessels during ingrowth into the repairing wound.[1] Despite the synergistic effect of ADSCs and platelet concentrates on surgical wound healing, however, there is a shortcoming in that after activation of PRP most of the presynthesized growth factors are secreted within a few hours.[2] This means that PRP is effective only for a few hours during contact with ADSCs. This restriction can be overcome by the advent of PRF first developed by Choukroun et al.[9] in France. The so-called second generation platelet concentrate was devised for use in oral and maxillofacial surgery. It needs neither anticoagulant nor bovine thrombin which as an activator of PRP carries a potential risk of causing a life-threatening coagulopathies.[10] It is merely the resultant product of blood centrifugation, free from the restriction of French law related to blood-derived product reimplantation. Most of all, the powerful advantage of PRF over PRP is its longer duration of growth factor release. In contrast to the burst release of cytokines after platelet activation in PRP, the platelets trapped in the unique three dimensional organization of fibrin mesh in PRF releases cytokines for a stable period of time. Dohan Ehrenfest et al.[2] demonstrated that the PRF membrane sustains a very significant release of key growth factors over a seven-day period.

Our study showed that the effect of PRF on ADSCs in wound healing was superior to that of PRP, as we hypothesized. The group treated with a combination of PRF and ADSCs showed a significantly enhanced rate of wound healing than the PRP group. In the present study, wound contraction may play some role in healing, but the difference in myofibroblast activity between the two groups is under investigation. Enhanced wound healing may be contributed to the long-lasting effect of PRF on ADSCs, dermal fibroblasts and the synthesis of collagen.[11] However, the fine mechanism of how growth factors interact with ADSCs and how they positively influence wound healing needs to be studied further.

One of the important principles in the wound healing process is angiogenesis, which is crucial to sustaining newly formed granulation tissue and surviving of fibroblast and keratinocyte. In the results of our experiment, more vascularity was seen in the PRF group upon histologic analysis. It is possible that the angiogenesis-trophic factor, such as VEGF, PDGF, is higher in the PRF-combined ADSCs group, which may stimulate the transformation of ADSCs into endothelium or recruited vessel from surrounding tissue.[12]

It is unclear how closely the process of wound healing in the mouse models resembles that seen in humans. The wound healing of mouse skin does not perfectly mimic that of human skin because the skin morphology is different. Anatomical differences in subcutaneous layer contribute to some hesitation in comparing mouse skin to human. Mice have a subcutaneous panniculus carnosus layer, which humans do not have. Therefore, an ideal animal wound model, which can investigate the delicate

process of wound repair of is still required.

Although both the platelet concentrates used in this study is made from the same amount of blood of the same donor, there is some possibilities that the amount of platelets and growth factors are different between PRP and PRF. Some investigators estimate the concentration of platelets in PRP up to 3~5 times higher than baseline, there is no general consensus with regard to the number of platelets existing in PRF.[5]

The present study found a potential for the use of PRF and ADSCs in clinical application. Autologous PRF is simple and easy to prepare in an office-based setting. The recent pilot-trial on patients with lower-extremity ulcers demonstrated that autologous PRF membranes have significant benefits for closing ulcers.[13] Together with ADSCs, a paramount wound healing protocol could be created.

V. CONCLUSION

Our experiment supports the evidence that PRF exerts a slow yet pervasive influence on the wound healing process, which is associated with more rapid tissue repair and more vascularity in the healing wound. Thus, although the interaction between PRP and ADSCs in wound healing is not clearly understood, PRF is probably more beneficial for promoting the activity of ADSCs than PRP.

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