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ORIGINAL ARTICLE

Impacts of bone marrow aspirate and peripheral blood derived platelet-rich plasma on the wound healing in chronic ischaemic limb

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Abstract

Platelet rich plasma (PRP) has attracted attention as a safe and cost-effective source of growth factors that stimulate cells to regenerate tissue. Bone marrow cells are also estimated as an effective material for treating chronic ulcers. With the same technique to concentrate PRP from peripheral blood, bone marrow aspirate was processed and marrow cells were concentrated as well as platelets. Impact of PRP derived from bone marrow aspirate (bm-PRP) and that from peripheral blood (pb-PRP) on wound healing of persistent ischaemic rabbits' limbs were observed. Full thickness skin defects were made on the thighs, which had been treated to be persistent ischaemic status 3 weeks previously. Saline, pb-PRP, and bm-PRP were injected into the wound floor, respectively. Skin defected areas on ischaemic limbs were significantly wider than those on non-ischaemic limbs. bm-PRP injected wounds showed a significantly smaller skin defect area compared with pb-PRP and ischaemic-saline wounds at all time points. Fluorescently dyed cells of bm-PRP, injected into the wounds, could be traced 4 weeks after, whereas those of pb-PRP could be traced no more than 2 weeks. Wound healing on an ischaemic limb was accelerated with bm-PRP, whereas pb-PRP could not show any significance from saline. This difference can be attributed to the kind of cells contained in the PRPs. Injection of bm-PRP is a good candidate for treating wounds on ischaemic limbs.

Key Words: Platelet-rich plasma, bone marrow, wound healing, chronic ischaemia, ischaemic limb, skin ulcer

Introduction

Wounds on ischaemic limbs are notorious for retardation of healing. A population of peripheral artery diseases is emerging all over the world. The number of chronic wounds with ischaemic problems is expected to multiply. It is a matter of urgency to seek an effective remedy for this increasing issue.

Bone marrow cells are estimated as an effective material for treating chronic ulcers [1,2]. Those cells are also used to improve the ischaemic status of limbs [3].

Platelet-rich plasma (PRP) is also attracting attention as a good source of several growth factors that accelerate wound healing [4,5]. There are some clinical reports about effectiveness of PRP in treating chronic wounds [6-8]. The authors previously reported a method to concentrate bone marrow aspirate, which is the same procedure to get PRP from peripheral blood [9]. It is a simple and low-cost procedure to obtain working cells and growth factors simultaneously. The procedure does not require expensive and sophisticated separators, and can be done in small community clinics. As well as platelets, bone marrow cells are enriched in the concentrate.

To evaluate the effectiveness of this concentrate, impacts of peripheral blood derived PRP (pb-PRP) and bone marrow derived PRP (bm-PRP) on the wound healing in rabbits' ischaemic limbs were compared.

Materials and methods

Persistent hindlimb ischaemia creation

The animals were housed under standard conditions and treated according to the protocol approved by Hyogo College of Medicine Medical Animal Care and Use Committee (No. 362).

Thirty-two male Japanese white rabbits (weighed around 3 kg) were anaesthetised with intravenous injection of 30 mg/kg pentobarbital sodium through the auricular vein. Hair was clipped from the groin area to medial aspect of the knee. Hindlimb ischaemia was created following a method reported by Pu et al. [10]. Briefly, a longitudinal incision was made in the thigh from the inguinal ligament to above the knee. Under a surgical microscope, the femoral artery and branches were exposed with care not to injure accompanying vein and nerve. The external iliac artery was ligated and severed just above the inguinal ligament. The popliteal and the saphenous arteries were also ligated and severed. All branches, including the deep femoral, inferior epigastric, and lateral circumflex femoral arteries, were cut. The superficial femoral artery was excised and the wound was closed (Figure 1*a*). In our previous study, plantar skin perfusion pressure was confirmed to range 20–63 mmHg, 3 weeks after this operation (Paper presented at the 16th annual meeting of Japan Society of Plastic and Reconstructive Surgery Research Council, 2007, Kobe, Japan). Ischaemia was created on one leg and the other was kept untouched. Twenty-four rabbits were randomly divided into three groups consisting of eight rabbits, respectively: pb-PRP group, bm-PRP group, and ischaemic-saline group. The other eight rabbits were used for a DiI cell tracking study.

Skin defect creation

Three weeks after the primary operations, when the initial inflammation of the operations was supposed to have faded

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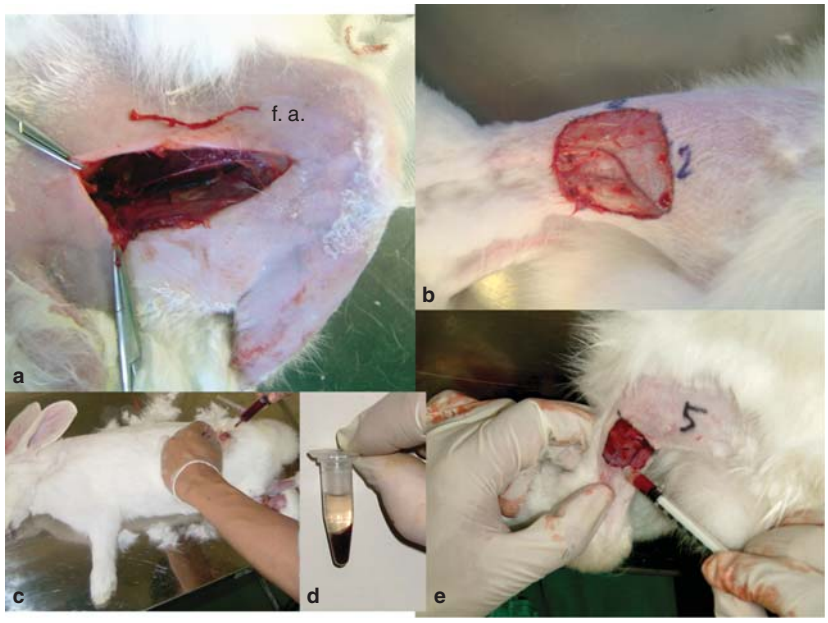

Figure 1. (*a*) A longitudinal wound was made on a rabbit's thigh. Superficial femoral artery was excised to create persistent ischaemic limb. f. a. = excised femoral artery. (*b*) Three weeks after the first operation, a 2 × 2 cm^2 wound was made on the lateral side of the lower thigh. (*c*) The iliac bone of a rabbit was punctured and marrow was aspirated. (*d*) Concentrated bone marrow aspirate. Supernatant was discarded and precipitate was used as bm-PRP. (*e*) 200 µl of saline, pb-PRP, and bm-PRP was injected into the wound floor, respectively.

away, second operations to create skin defects on both legs were done. The animals were anaesthetised and hair was clipped. A plastic template and a skin-marking pen were used to design 2 × 2 cm full-thickness skin defect on the outer side of the lower leg, 5 cm distal from the kneecap. The wound was made with a scalpel (Figure 1*b*). The panniculus carnosus muscle was also excised. An electric cautery was used to stop bleeding.

Processing PRP's

In the pb-PRP group, 2 ml of peripheral blood was drawn from the auricular vein with a 5 ml syringe containing 0.5 ml of ACD-A solution (2.2% sodium citrate, 0.8% citrate, 2.2% glucose) (Terumo, Tokyo, Japan) as anticoagulant. Iliac crests of the bm-PRP animal group were stabbed with a Komiya's bone marrow needle (Kurita, Tokyo, Japan) and aspirated (Figure 1*c*). The aspirate was poured into a 5 ml centrifuge tube and spun at 200 g for 10 minutes. Supernatant, including the buffy-coat and slightly red layer, was aspirated and transferred to the other tubes. Secondary centrifugation was done at 800 g for 10 minutes. Clear supernatant was aspirated out until 200 µl were left. Precipitate was resuspended to get PRP's. The average number of nucleated cells (standard deviation), counted with a veterinary haematology analyzer LC-152 (Horiba, Kyoto, Japan), in bone marrow aspirate, bm-PRP, peripheral blood and pb-PRP was 49.4×10^6 (13.8×10^6), 123.6×10^6 (29.5×10^6), 6.9×10^6 (1.5×10^6), and 13.5×10^6 (5.0×10^6)/ml. Platelets counts were 61.1×10^6 (16.3×10^6), 188.3×10^6 (47.9×10^6), 52.1×10^6 (17.6×10^6), and 159.9×10^6 (37.3×10^6)/ml, respectively.

PRP administration to the skin defects

In the pb-PRP group, 200 µl of PRP processed from autologous peripheral blood was injected into the wound of the ischaemic limb. The injections were done into four sides of the square wound and underlining muscle, 40 µl each with a 27G needle. In the bm-PRP group, 200 µl of bm-PRP derived from autologous iliac bone marrow aspirate was injected (Figure 1*e*). In the ischaemic-saline group, 200 µl of saline was injected into the wound of the ischaemic limb. Two hundred microliters of saline was injected into the wound floor of the non-ischaemic limb as a non-ischaemic control. The data acquisition of this non-ischaemic control limb was done for the first 12 rabbits of 24 created wounds. Since, clinically, as well as epithelialisation, contraction plays a significant part in wound closure, contraction-preventing devices were not applied in this study. In our previous study, the skin on the ischaemic limb could not tolerate mechanical insult by any wound coverage material (e.g. films, gauze, or bandage) and developed hair loss, blisters, and erosion. Thus, in this study, the wounds were kept uncovered to eliminate other factors which may modify wound healing.

Observation

Wounds were observed and pictures of them were taken with a ruler using a digital camera, placed parallel to the wounds with a distance of 50 cm. The images were processed with image processing software, Image-J (NIH, Bethesda, Maryland, USA) on a personal computer, and skin defected areas were measured by one of the authors, who was blinded to which group the image belonged. An image was opened with the software and scale was set with the ruler in the image. The wound edge was traced with freehand selection and the framed area was measured.

Statistical analysis

Statistical analyses were conducted using a two-tailed independent *t*-test to evaluate the significance of the data for skin defected area.

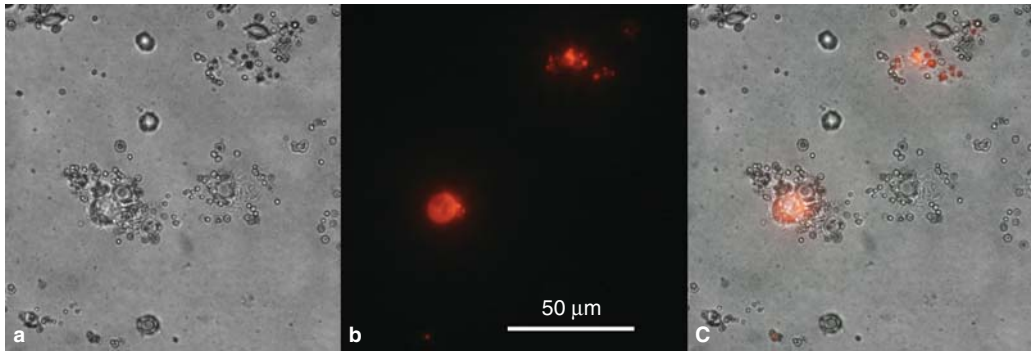

Figure 2. DiI-labelled bm-PRP. Cellular components in bm-PRP were dyed with DiI and smeared on a slide glass. Platelets and marrow cells were labelled. (*a*) Bright view. (*b*) Fluorescent view. (*c*) Overlaid view.

DiI staining

Eight animals were used to track survivability of the injected cells in pb-PRP and bm-PRP. PRP's were suspended in 4 ml of phosphate buffered saline (PBS). They were centrifuged at 1200 g for 5 minutes and supernatant was discarded. Cellular components were washed 3-times and dyed with 5 μg/ml of fluorescent dye Cell TrackerTM CM-DiI (Invitrogen, Eugene, OR). They were incubated at 37°C for 5 minutes followed by 4°C for 15 minutes. Both nucleated cells and platelets were labelled (Figure 2). They were washed with PBS 3-times and suspended in 200 μl of PBS, and injected into the wound floor of skin defects on ischaemia treated legs. The animals were euthanised and the wounded areas were excised after certain periods of time. The specimens were frozen and microsectioned. At least four sections of each specimen were observed under a fluorescent microscope.

Results

The results of femoral artery isolation, atrophy of calf muscles, and ischaemic changes of toes on the treated limbs were observed at the time of skin defect creation.

Macroscopic observation of the wounds (Figures 3 and 4)

In the first few days after the wound creation, the skin defected areas of all animals became larger than the initial defects. After that, they began to contract. At all time-points, the skin defected areas of the saline-injected wounds on the ischaemic limbs were significantly larger than that of non-ischaemic control wounds. The wounds with pb-PRP injection showed no significant difference from saline injected wounds. However, bm-PRP injected wounds revealed significantly smaller skin defected areas than saline-injected and pb-PRP-injected wounds. The wounds with bm-PRP did not show significantly

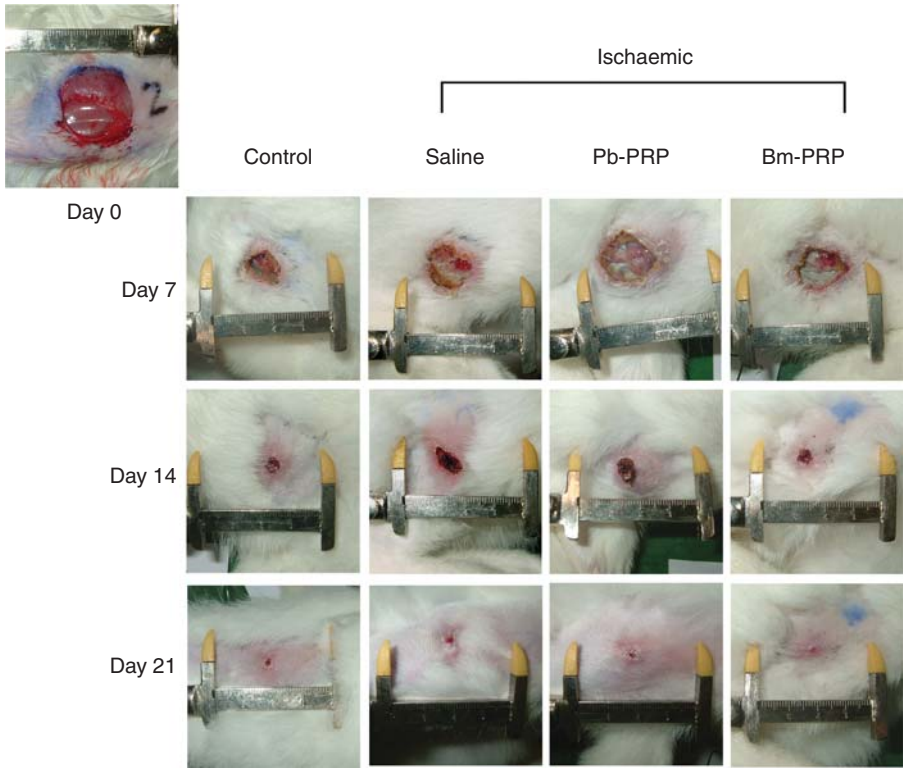

Figure 3. Macroscopic evaluation initially and after wound creation. Mainly, contraction of wound seemed to contribute to the decrease of raw-surfaced area.

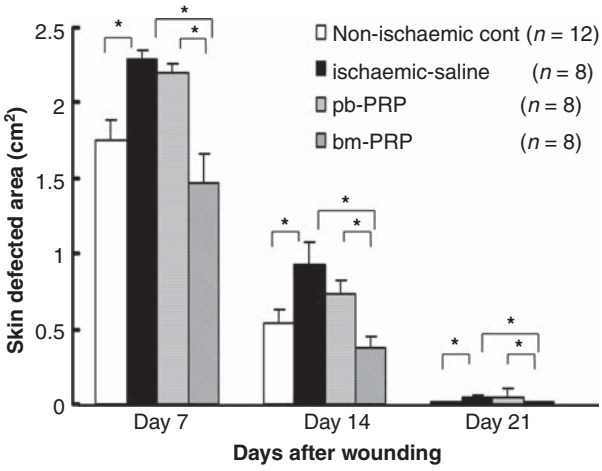

Figure 4. The skin defected area after the 4 cm^2 wound creation. Saline injected wound on untreated limb was smaller than that on ischaemia treated limb at all time-points. The bm-PRP injected wound was smaller than that with saline or pb-PRP injection. No difference could be seen between saline and pb-PRP injection on the ischaemia treated limb. Non-ischaemic cont = control group with saline injection on untreated limbs; ischaemic-saline = ischaemia treated saline injection group; pb-PRP = peripheral blood derived PRP injection into ischaemia treated wounds group; bm-PRP = bone marrow aspirate derived PRP injection into ischaemia treated wounds group. Two-tailed independent *t*-test was done to evaluate the significant difference between groups. * $p < 0.05$. Error bar indicates standard deviation.

different areas from those with non-ischaemic control at all time-points.

Dyed cell tracing

DiI-labelled cells of pb-PRP could be observed in the specimen harvested 1 week after injection. Intensity of fluorescence diminished and could hardly be observed in the 2 and 4 weeks specimen (data not shown). However, clusters of robust fluorescence were observed 4 weeks after injection of bm-PRP (Figure 5). Differentiation of DiI-labelled cells into specific types (e.g. endothelial cell, myofibroblast) was unclear.

Discussion

As the number of patients with peripheral artery diseases is multiplying all over the world, patients with non-healing wounds on ischaemic limbs are increasing. Although it is needless to say that reperfusion of the limbs is the first to be done, not all can resume sufficient circulation practically. Finding treatments for those notorious situations have to be done as soon as possible.

Bone marrow cells are positively estimated to improve ischaemic state of limbs. Numbers of clinical studies have been reported [3,11,12]. Delivery of bone marrow cells to the ischaemic heart is expected to encourage neovascularisation and cardiac muscle preservation [13,14]. Bone marrow cells are also expected to be an effective material for treating chronic ulcers [1,2,15].

Platelet-rich plasma (PRP) has also attracted attention as a good source of growth factors that accelerate wound healing and is considered as one of the good candidates for the solution. Platelets contain various growth factors, such as platelet-derived growth factor (PDGF), transforming growth factor (TGF-beta), and vascular endothelial growth factor (VEGF) in alpha-granules that are expected to have good effects on wound healing [5]. Some clinical reports about using PRP for treating chronic wounds have been done [6-8].

The authors previously reported a method to concentrate bone marrow aspirate, which is the same technique as to make PRP from peripheral blood. It is a simple and low cost technique (as far as consumable supply is concerned, it costs no more than 1500 yen: ~ US$18) of double centrifugation [9]. This can be done within 30 minutes. Both platelets and bone marrow cells are concentrated in bm-PRP. Platelets in bm-PRP contain the same level of growth factors as those in peripheral blood. Among the nucleated cells, relatively large cells (e.g. multinucleated cells) were eliminated [9].

Acceleration of wound healing by bone marrow derived cells injection into excisional dermal wounds of healthy rabbits was reported [16]. Histomorphologically, significantly more neovascularisation, fibroplasia, and collagenation could be seen. They separated nucleated cells from bone marrow aspirate by double centrifuge technique, which is basically the same technique to isolate buffy-coat as ours. Although not mentioned, platelets ought to have also been injected into the wounds.

Not many experimental studies concerning wounds on chronic ischaemic limbs have been reported. In this study, the chronic ischaemic limb model reported by Pu et al. [10] was used. This model has been accepted and recognised as a good rabbit model for hindlimb ischaemia research [17]. Plantar skin perfusion pressure of the animals ranged from 20–63 mmHg at 3 weeks after the femoral artery isolation operation (measured with SensiLase (TM) PAD3000 (väsamed, Eden Prairie, MN, presented at the 16th annual meeting of the Japan Society of Plastic and Reconstructive Surgery Research Council, 2007, Kobe, Japan). It ranged around the data of human critical limb ischaemia [18]. No previous report about skin perfusion pressure and wound repair in rabbits could be found. Calf muscle atrophy and necrosis of the toes were observed, which also indicates a persistent ischaemic state of the limbs. With our observation, wound healing on a femoral artery excised limb was significantly retarded compared with that on an untreated limb, showing the adequateness of the model.

The healing process of wounds on ischaemia-treated and untreated limbs was observed. Wound healing on the artery-excised limbs was delayed compared with that on untreated limbs, which correlates with clinical observations. With macroscopic observation, under any conditions, the hairless area remained no more than 3 mm in diameter after total wound closure, where the initial skin defect was 2 cm^2. Mainly, contraction of the wound seemed to contribute to the decrease of raw-surface area in this study. This observation is similar to human wound healing in large defects (e.g. sacral decubitus).

The pb-PRP could not show any significant difference from saline, which was the same kind of observation reported previously on fresh wounds of normal rabbits' backs [19]. Most of the growth factors contained in the platelets are expected to be released soon after the injection of PRPs to the wounds, when the spontaneous normalisation of calcium level occurs and the platelets are activated with the contact stimulation by extravascular tissue. In this study, PRPs were injected immediately after

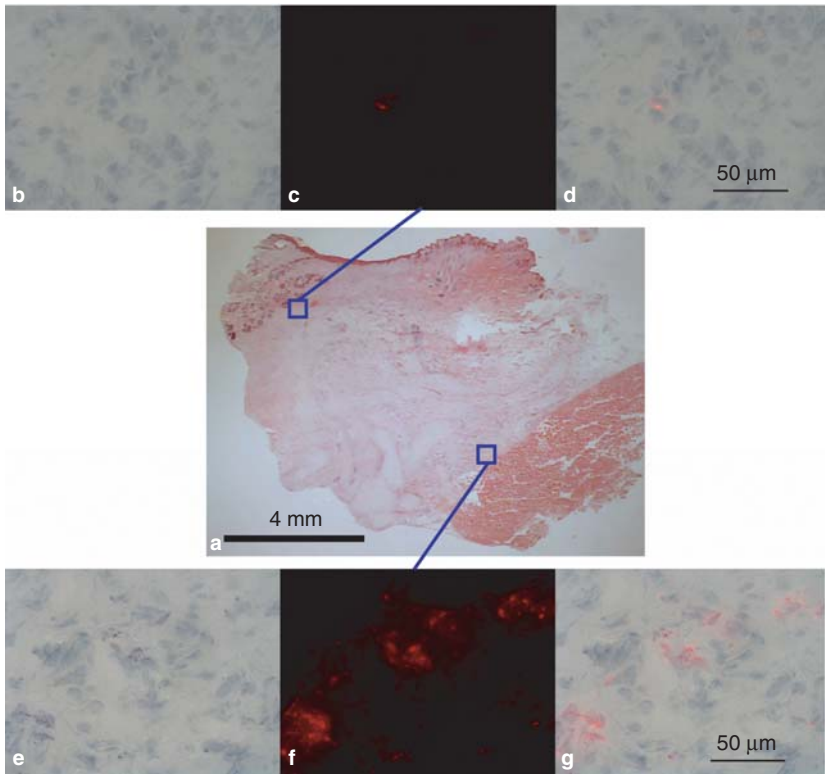

Figure 5. Microscopic images of a wound with dyed cellular components injection of bm-PRP, 4 weeks after the wound creation. Robust fluorescence of labelled cells can be observed at the initial wound edge and above the underlying muscle. Fluorescence could not be detected at the centre of granulation tissue (data not shown). (*a*) Loupe view with haematoxylin and eosin stain, indicating where the fluorescence was observed. (*b*, *e*) Bright view with haematoxylin stain. (*c*, *f*) Fluorescent view. (*d*, *g*) Overlaid view.

the wounds were created. The effects of growth factors released from the platelets act in a short period of time and may have been masked by the inflammatory substances released with the surgical insults. The effects of pb-PRP to clinical chronic wounds, which is under the equilibrium state, may be different from this study.

The bm-PRP revealed a significantly positive effect on wounds on ischaemic limbs. The difference between pb-PRP and bm-PRP is the sort of cells contained; the former mostly lymphocytes [20], the latter small bone marrow cells [9]. Tracing of those cells after implantation demonstrated that the cells in pb-PRP could not survive more than 2 weeks, whereas the cells in bm-PRPcould be traced for 4 weeks. DiI staining has been accepted as a long-term cell tracker. Macrophages might be stained with DiI after phagocytosis of implanted cells. Regarding lifespan of macrophages, it is reasonable to conclude that the cells in bm-PRP survived for a certain period of time.

This study was designed on rabbits, because of the adequate size of the animal to obtain autologous fresh bone marrow aspirate. Since not many antibodies for rabbits' protein can be obtained, immunohistochemical or enzyme-linked immunosorbent assay studies could not be done. Cell typing with surface markers was not done in this study because it was obvious that the bm-PRP was a commixture of various kinds of cells and because antibodies for rabbits' cells were not available. Differentiations of implanted marrow cells into specific types of cells (e.g. endothelial cells or myofibroblasts) could not be clarified with histological morphology.

Robust granulation and earlier closure were observed in bm-PRP injected wounds. Recently, it was speculated that the effects of bone marrow cell delivery to ischaemic limbs were due not only to the vasculogenesis, conversion of cells into endothelial cells or pericytes, but to the angiogenesis, paracrine effects of implanted cells [21]. The same sort of effects can be speculated for the acceleration influence of bm-PRP injection to wound closure in this study. A clinical report with positive effects of intramuscular transplanting bone marrow mononuclear cells on Buerger's disease ulcers was done [22]. It can be speculated that the cell transplantation improved local blood flow, which indirectly encouraged wound healing. Injection of bm-PRP to the wound floor can be expected to have a more direct effect of closing wound by stimulating the local cells. However, it is needless to say that the local skin perfusion pressure and oxygen level have to be good enough for wound healing.

Clinically, separation of nucleated cells from bone marrow aspirate for cell transplantation has been done with complex expensive machines. Hernández et al. [23] compared the outcome of two groups with severe lower limb ischaemia, those receiving injection of bone marrow cells sorted by an automated blood cell separator or by density gradient on Ficoll-Hypaque. They found no significant difference between automated and manually separation and recommended the Ficoll-Hypaque method for the hospitals without sophisticated facilities. Selection of the cells by surface markers or plating and culturing demands high cost and time. A suggestion of eliminating a cell purification process of bone marrow cells for angiogenic

transplantation was done, based on an experimental study [24]. The separation procedure is very simple and costs much less compared with the sophisticated machine procedures. This can be done with ordinal centrifugation machines within 30 minutes. There are many other systems and kits to concentrate PRP from peripheral blood, which are widely used. Using these systems for concentrating marrow cells may be a cost-effective solution for regenerative medicine. Purity may be lower, but proven to be good enough for clinical use [23]. Bone marrow aspirate can be obtained under local anaesthesia, not to mention general or lumbar anaesthesia. According to a report concerning bone marrow transplantation, the incidence of serious complications from marrow donation is low (1.35%) [25].

In conclusion, injection of bm-PRP can be a good candidate for treating wounds on ischaemic limbs, which can be done in local clinics, quickly, and at low cost.

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