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Attenuation of Hindlimb Ischemia after Associated Autologous Transplantation of Bone Marrow Mononuclear Cells and Platelet Rich Plasma

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Abstract

Objective: The purpose of this study was to determine whether autologous bone marrow mononuclear stem cell (BMCs) associated with platelet rich plasma (PRP) transplantation improves peripheral collateralization in a rabbit ischemic limb model.

Methods: Unilateral hindlimb ischemia was surgically induced in New Zealand White rabbits (n=30). A total of 106 BMCs+PRP were intramuscularly implanted into each ischemic limb. Blood flow was monitored by Doppler in the ischemic and control animals. Histological analysis of capillary density in the ischemic limbs was performed 30 days after the ischemia induction.

Results: Histological sections of ischemic gastrocnemius muscle showed that capillary index (capillary/muscle fiber) was greater in the BMCs+PRP implantation group 30 days after the ischemia induction than in the saline group.

Conclusion: This study demonstrated that implantation of BMCs+PRP into ischemic limbs effectively induces collateral vessel formation, suggesting that this cell therapy is useful as a novel strategy for therapeutic peripheral arterial disease.

Keywords: Angiogenesis; Cell therapy; Hindlimb ischemia; PRP; BMCs

Introduction

Obstructive peripheral arterial occlusive disease (PAOD) of the inferior members is a worldwide health problem and its prevalence is estimated in 27 million of people in Europe and North America [1].

PAOD development is a multifactorial process with a variety of severe manifestations as ischemic rest pain, ulcerations, and gangrene increasing the risk of claudication, poor wound healing, limb amputation, and stroke [2]. The therapy for PAOD has increased in the last decades with the introduction of regenerative therapy. The use of stem-cells, vascular endothelial growth factor (VEGF), and fibroblast growth factor FGF significantly improved symptoms and hemodynamics variables in the treated limbs, as reported in the literature [3].

Bone marrow mononuclear cells (BMCs) or platelet rich plasma (PRP) therapies are delivered locally into affected tissues and can contribute to the regeneration of ischemic tissues and enhance the neovascularization of ischemic hindlimbs through both, cellular and paracrine mechanisms [4,5].

Preclinical studies suggest that BMCs transplantation in ischemic limbs increase the number of collateral vessels and are dependent of the supply of endothelial progenitor cells and multiple angiogenic factors [6]. Accordingly, the objective of this study is to analyses the effects of associated transplant of BMCs and PRP on PAOD therapy.

Materials and Methods

Schedule of the study

Time schedule of the study is shown in Figure 1.

Animals

Thirty New Zealand male rabbits (1.5 kg-2.0 kg) were used. The animals were kept in cages under controlled conditions of temperature and light-dark cycle of 12 h, with free access to food and water. The Animal Experimentation Ethics Committee of the Pontificia Universidade Católica do Paraná (PUCPR) approved all experimental protocols used in this study (596).

Experimental design and animal hindlimb ischemia model

The rabbits (n=30) were subjected to unilateral limb ischemia and randomly divided into 3 groups (n=10/group). No rabbit died during the experimentation. The control group received saline, the GPRP group received autologous PRP, and the GBM+PRP group received autologous BMCs and PRP. The saline, PRP alone or BMCs+PRP were administered into the ischemic muscles 7 days after surgery. Animals were anesthetized as described before [7]. The left femoral artery was completely excised from its proximal origin to its bifurcation formed by the saphenous and popliteal arteries, as previously described [8,9]. A total of 5×10^6 BMCs and PRP was injected intramuscularly in treated animals into 3 different sites of the gastrocnemius muscles at the medial thigh of the ischemic hindlimbs [10]. The rabbits were euthanized 30 days after the intramuscular injection by an intravenous overdose of pentobarbital. The hindlimbs were opened and gastrocnemius muscles were isolated.

Preparation of PRP and Doppler vascular analysis

8-15 ml of central auricular artery blood were collected and harvested 7 days after surgery using heparin as anticoagulant, according to the method previously reported [11]. Rabbit GBM was aspirated from the right iliac crest. BMCs were isolated by centrifugation through a Histopaque density gradient Sigma (St Louis, USA) according to Boyum [12]. After centrifugation, cell analysis was conducted by cell counting and fluorescence activated cell sorting (FACS). Cell counting was performed using an automatic blood counter Sysmex XE2100 (Co, Kobe, Japan). BMCs phenotypes were detected by flow cytometry, using FACS Calibur (Becton Dickinson, USA). All antibodies for flow cytometric analysis were purchased from Biolegend (San Diego, CA). The vascular evaluations were performed before hindlimb ischemic surgery and 30 days after saline, PRP alone or BMCs+PRP administration.

Muscle histology and immunohistochemistry (IHC)

Hindlimbs of euthanized rabbits were dissected and muscle tissue specimens were harvested and fixed in formalin. Histopathologic analysis of the gastrocnemius muscle in the medial thigh was performed. Specimens were paraffin embedded and cut into 5 μm slices. The morphometric analyses were performed through the program IN Cell Investigator™ software (GE Healthcare, USA). The angiogenesis was measure by IHC positively stained vessels using anti-human CD31 (Molecular Probes, USA.) antibody conjugated with DAPI (4',6-diamidino-2-phenylindole). The total amount of vascularization was determined as the number of capillaries per fiber in 4 to 6 fields/muscle.

Statistical analysis

Statistical significance was evaluated by the Tukey-Kramer method for multiple comparisons after the confirmation of data variances equality by the Brown-Forsythe test. $p<0.05$ was considered statistically significant. Data are shown as mean ± SE and were performed using GraphPad Prism v.6.0 (GraphPad Software, Inc) software.

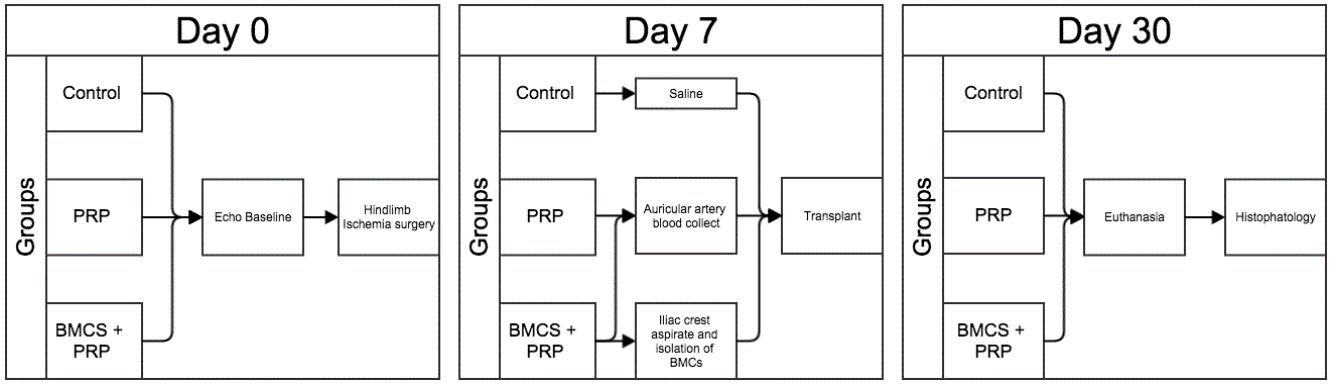

Figure 1: Time schedule of the study.

Results

The rabbits used in the present study showed no adverse effects related to surgery or therapy combined. Within 3 days of femoral excision, rabbits were noted to have mild pallor of the hindlimb compared to the sham operated, as well as small (2 mm) superficial skin ulceration on the plantar surface of the left paw with no evidence of critical limb ischemia, infection or gangrene. Both pallor and ulceration qualitatively improved over the course of the study, with no clear difference among groups.

Analysis of the total nuclei amount per fiber showed a significantly increase in cell number in the GPRP group when compared to the saline group (47.66 ± 18.06 vs. 13.83 ± 7.53, $p<0.05$ and was significantly greater in the GBM+PRP group than in the saline group (93.60 ± 28.63 vs. 13.83 ± 7.53, respectively; $p<0.01$ vs. saline) (Figure 2).

More capillaries were detected in the ischemic muscle of GBM+PRP group when compared with that in the control group at day 30 after cells therapy implantation or saline injection (Figure 3). Capillary density score of the ischemic hindlimb were significantly increase in the GPRP group than in the saline group ($2.3 \times 10^6 \pm 1.0 \times 10^6$ vs. $1.9 \times 10^6 \pm 5.2 \times 10^5$; $p<0.05$ and GBM+PRP group than in the saline group ($3.31 \times 10^6 \pm 1.6 \times 10^6$ vs. $1.9 \times 10^6 \pm 5.2 \times 10^5$, respectively; $p<0.05$ vs. saline).

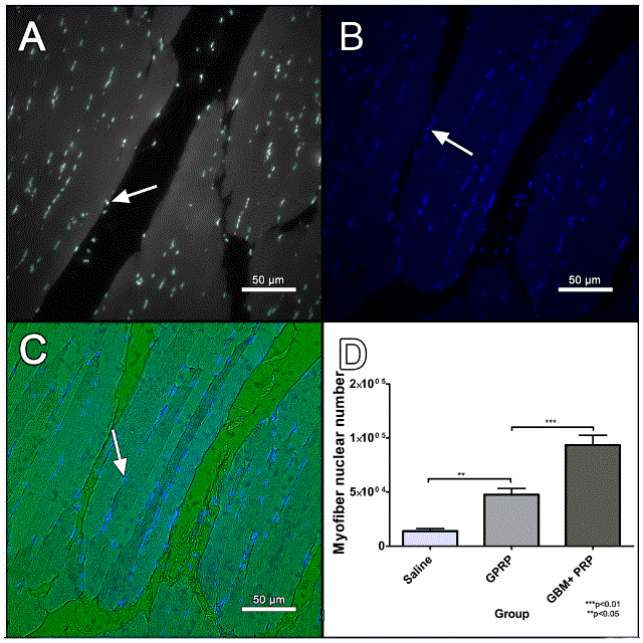

Figure 2: Phase contrast images of the gastrocnemius muscles after injection of saline; **A:** Phase contrast images of the gastrocnemius muscles after injection of bone marrow mononuclear cells (BMNCs) and platelet rich plasma (PRP); **B:** Fluorescence micrographs of the gastrocnemius muscles after injection of (BMNCs) and (PRP) labelled with DAPI (4',6-diamidino-2-phenylindole) marker B and C; **D:** Myofibril nuclei score. Upper panels correspond to day 30 after injection to the gastrocnemius muscle. Bar: 50 µm. ***p<0.01 and **p<0.05.

Discussion

Tissue Engineering provides a variety of strategies to effective therapeutic angiogenesis as the local delivery of cells or bioactive factors that could promote angiogenic mechanisms *in vivo* [13]. Our results show that the combined treatment with BMCs and PRP can enhance collateral vessel formation by improving cell proliferation.

Autologous BMCs, when transplanted in the hindlimb, can differentiate into smooth muscle cells and endothelial cells [14]. Different growth factors in PRP have diverse roles in angiogenesis and restoration of blood flow after hindlimb ischemia and may offer a suitable microenvironment for BMCs to promote proliferation and differentiation [5,15]. According to our data, we can suggest that the combined therapy using GBM+PRP participated in angiogenesis by the secretion of trophic factors.

Yang et al. reported that both types of cells, BMCs and PRP, can divide and proliferate in the transplanted region and differentiate into corresponding cells under local microenvironment so as to replace injured cells [16]. Regarding clinical efficiency, preclinical studies have indicated that a variety of growth factors promote the development of collateral arteries, a concept called therapeutic angiogenesis [15].

The histological findings showed an increase in capillary density after BMSc+PRP therapy in hindlimb ischemia, demonstrated via IHC staining and companied by an augmentation in cell nuclei amount (confirmed via DAPI staining).

Our finding indicate that the combination of BMCs+PRP results in a significantly better healing effect on ischemic hindlimbs when compared to the other 2 groups tested. However, the beneficial effect of PRP therapy alone appears to be transient since the histological assessment of ischemic repair was not significantly different 30 days after cell transplantation, when compared to control group (Figure 2). Several researchers believe that endothelial progenitor cells included in BMCs differentiated into endothelial cells and, after transplantation in the injury region, contributes to cell survival and angiogenesis [17,18].

Autologous BMCs and PRP are a rich source of growth factors that stimulates angiogenesis in ischemic muscle [19]. Moreover, angiogenic factors, including VEGF and epithelial growth factor (EGF), are also released from injured tissue recruiting BMCs to replace injured tissue [20,21].

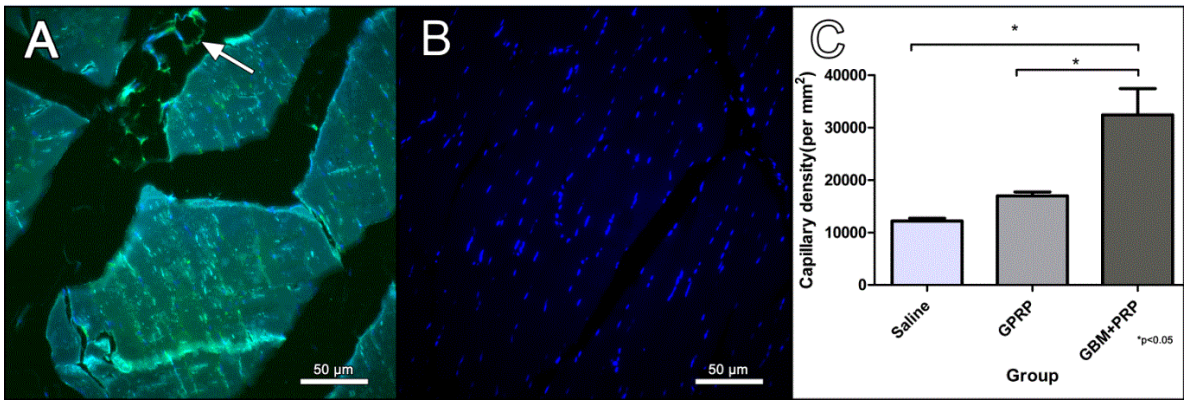

Figure 3: Effects of BMCs+PRP on capillary density in ischemic hindlimb. CD31-positive cells were stained in the gastrocnemius muscle at day 30 after induction of ischemia; A and B: Representative images of DAPI (4',6-diamidino-2-phenylindole) capillary density was identified with capillary per field (× 400 magnification, arrow; C: Capillary score; Values are mean ± SEM; n=10, *p<0.05. Scale bar indicated 50 µm.

Previous studies similarly demonstrated that there is an additional contribution derived from BMCs or PRP progenitors which are mobilized in the setting of limb or myocardial ischemia, migrate to ischemic tissue, and are actively incorporate into new vessels [22]. In preclinical ischemic animal model, it is suggested that collateral vessels might need to be induced for weeks or months before the newly formed vasculatures mature [23].

Our data concurs with the findings mentioned above supporting the hypothesis that PRP, in combination with BMCs, have a modulating effect on angiogenesis through the growth factors involved in wound healing [24].

In the present study, we demonstrated that BMCs+PRP possesses pro-angiogenic properties in hindlimb ischemia in rabbits through the increase in the amount of peripheral collateral vessels and mature vasculatures formed ($p<0.01$) 30 days after the cells implantation.

Of course, other mechanisms involved in collateral vessels function such as migration, muscle formation, and regeneration might also exist. However, it is unclear whether a persistent angiogenic stimulus is required or not to reverse the ischemia in clinical setting.

We believe that there is a direct relationship between the number of transplanted cells via intramuscular route and the functional effect, corroborating the findings of the other authors [25,26].

The increase in the number of cell nuclei in rabbits treated with combined therapy (BMCs+PRP group), when compared with saline group, also indicates a less significative loss of muscle fiber with preservation of capillaries (Figure 2). This finding is in agreement with recent studies that have shown *in vivo* and *in vitro* functional recovery of ischemic diseases promoted by BMCs therapy, revealing their efficiency and safety [6,27,28].

In conclusion, the combined treatment with BMCs and PRP offers a potential option for therapeutic angiogenesis in limb ischemia. This study provides evidence that action between growth factors and cytokines released from BMCs and PRP are capable of inducing mature blood vessel formation.

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