Effect of autologous transplant of peripheral blood mononuclear cells in combination with proangiogenic factors during experimental revascularization of lower limb ischemia

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Abstract
Peripheral blood mononuclear cells (PBMCs) contain a cell fraction of mononuclear progenitor cells (MPCs), which own significant angiogenic potential. Autologous transplant of PBMC and/or platelet-rich plasma (PRP) promotes endothelial cells differentiation in experimental lower limb ischemia, which is considered a safe and effective strategy to support revascularization, either in animal models or clinical trials. In addition, thrombin has been proposed to enrich biological scaffolds, hence increasing MPC viability after intramuscular administration, whereas proangiogenic mediators such as vascular endothelial growth factor (VEGF), tumor necrosis factor alpha (TNF-α), inhibitor of the plasminogen activator-1 (PAI-1), and chemokine (CXCL1; GRO-α) participate in the endothelial response to ischemia, through their proangiogenic effects over endothelial cells proliferation, survival, migration, endothelial integrity maintenance, and physiologic vascular response to injury. In the present study, we describe the effect of autologous PBMCs transplant and PRP, either with or without thrombin, over proangiogenic mediators (measured by enzyme-linked immunosorbent assay) and revascularization response (angiographic vascular pattern at 30 days after vascular occlusion) in a rat model of lower limb ischemia. The group treated with PBMC + PRP significantly induced PAI-1, an effect that was prevented by the addition of thrombin. Furthermore, treatment with PBMC + PRP + thrombin resulted in the induction of VEGF. GRO-α showed a sensitive induction of all proangiogenic mediators. All treatments significantly stimulated revascularization, according to angiographic assessment, whereas higher effect was observed with PBMC + PRP treatment (p < .0001). In conclusion, autologous PBMC transplant stimulates revascularization during experimental ischemia of the lower limb, whereas...
1 | INTRODUCTION

Acute and chronic limb ischemia are vascular diseases that show several differences. Acute limb ischemia relates to an embolus causing arterial occlusion, rapidly manifested by pain pallor, pulselessness, parasthesia, poikilothermia, and loss of function, whereas in chronic limb ischemia, thrombotic occlusion may be evident after hours or days, usually manifested as ischemic rest pain, longer than 2 weeks, and other manifestations like chronic claudication, bruits, and/or ulcers. Their recognition is important due to their specific therapeutic approaches. (Cronewett, 2014).

Particularly, for chronic limb ischemia, it has been estimated that 20% of the population older than 70 years old develops atherosclerotic damage and chronic arterial occlusion of the lower limbs (Rowlands & Donnelly, 2007). Revascularization procedures (arterial derivation or endovascular reperfusion) may reestablish blood flow in the affected vessels; however, 30% of the population with severe lower limb ischemia would not obtain any benefit from a vascular procedure. The outcome for these patients is denoted by increasing pain and development of ulcers, gangrene, and sepsis that eventually increase the risk of limb amputation and mortality rate (Dormandy & Rutherford, 2000).

A better understanding of the biological mechanisms underlying revascularization process would facilitate the development of new and more effective therapeutic strategies (Rafii, 2003). Nowadays, regenerative medicine offers therapeutic approaches that promote revascularization. In this regard, tissue engineering strategies supporting vascular structures and function require at least three biologic components: cells, scaffold material, and cytokines that stimulate cell migration, proliferation, and differentiation (Asahara et al., 1997; Benoit, O’Donnell, & Patel, 2013; Padilla et al., 2009).

Peripheral blood mononuclear cells (PBMCs) contain a cell fraction of mononuclear progenitor cells (MPCs), which own significant angiogenic potential. PBMC and MPCs have shown to promote endothelial cell differentiation in models of ischemic tissues (Musilli et al., 2015). Likewise, platelet interactions with endothelium have demonstrated a crucial role in vasomotor function, chemotaxis, inflammation, and atherosclerosis, as well as maintenance of vascular health. Activated platelet-rich plasma (PRP) is a source of platelets, cytokines, and growth factors that regulate proliferation and differentiation to endothelial cells (Bir et al., 2009; González et al., 2013; Yu et al., 2009). PRP-containing factors may interact with cell receptors related with the activation of endothelial cells, mesenchymal cells, and fibroblasts, which play a key role within the angiogenic response (Wahlberg, 2002). PBMC and MPC autologous transplants have been a secure and effective strategy to support revascularization, either in animal models or clinical trials of patients with advanced vascular disease (Aranguren, Verfaillie, & Lettun, 2009; Franz et al., 2009; Padilla et al., 2007, 2003). Benoit et al. (2013) reported a meta-analysis of 45 studies of transplanted autologous MPCs in population with lower limb critical ischemia. Notably, seven of these studies were randomized, placebo-controlled trials that found a significant preventive effect for major amputation (odds ratio = 0.36, \( p = .0004 \)).

The design of biological scaffolds has been thought to increase PBMC and MPC viability after intramuscular administration. Therefore, tissue engineering strategies include synthetic gels, collagen, and fibrin gels, as well as thrombin-containing microenvironments (Allender, Scarborough, Peto, & Rayne, 2008; Faisal et al., 2008; Tsopanoglou & Maragoudakis, 1999). Thrombin is the main effector of the coagulation cascade, which interacts with membrane surface phospholipids and accelerates the transformation from fibrinogen to fibrin and fibrinopeptides (Caunt et al., 2006). Thrombin also induces platelet aggregation and activation of several cells like endothelial cells, smooth muscle cells, inflammatory cells, neurons, astrocytes, osteoblasts, and tumoral cells (Dupuy et al., 2003). Notably, thrombin plays a role in the angiogenic cascade by modulating the mitogenic effect of endothelial growth factors over endothelial cells (Caunt et al., 2006; Katsanos et al., 2009) and it owns a strong antiapoptotic effect on the same cells, providing vascular protection and maintenance (Katsanos et al., 2009).

Several cytokines participate in the endothelial response to ischemia. Vascular endothelial growth factor (VEGF) exerts strong angiogenic effects over endothelial cells by a direct control of endothelial cell proliferation, migration, specialization, and survival. It plays an important role in maintaining endothelial integrity, survival, and physiologic function (Li et al., 2017). In fact, it has been shown that the only presence of VEGF is enough to start angiogenesis (González et al., 2013), whereas lack of VEGF inhibits cellular proliferation and promotes apoptosis of endothelial cells (Peng et al., 2016).

Tumor necrosis factor alpha (TNF-\( \alpha \)) regulates leukocyte activation, cytokines and chemokines release, and production of oxygen/nitrogen reactive species. TNF-\( \alpha \) has been postulated to promote cellular migration and angiogenesis through its interaction with the inhibitor of the plasminogen activator-1 (PAI-1), which regulates the degradation of extracellular matrix. Then, both factors maintain a necessary base for endothelial cell’s migration and vascular formation. Finally, the chemokine CXCL1, also known as GRO-\( \alpha \), may regulate macrophage activation, blood coagulation, and endothelial cell’s expression of proteins related with angiogenesis, particularly that induced by thrombin (Bechara, Chai, Lin, Yao, & Chen, 2007; Ciszek et al., 2007; Figure 1).

particular effects over proangiogenic and fibrinolytic mediators may be attributed to PBMCs and its combination with PRP and thrombin.

KEYWORDS
angiogenesis, lower limb ischemia, PBMC, proangiogenic factors, revascularization
Several angiogenic factors have been described during the angiogenic adaptive response; however, a main challenge is to elucidate which factors are more related with revascularization after ischemic injury (Katsanos et al., 2009; Li et al., 2017). In the present study, we describe the effect of autologous PBMC transplant and PRP, either with or without thrombin, over proangiogenic mediators and revascularization response in a rat model of lower limb ischemia.

2 | MATERIALS AND METHODS

An experimental study was performed, with prospective and comparative analysis.

2.1 | Ischemic lower limb animal model

Male Wistar rats, 350–400 g in weight, with free access to water and feeding, 12/12 hr light controlled, were used. The model involves the induction of subacute ischemia on the right lower limb by performing two surgical procedures. The first surgical procedure includes a medial laparotomy and ligation of the right external iliac artery using 8-0 suture and preserving the internal iliac artery. Then, the second surgical procedure was performed 7 days after the first procedure and consists of a vascular approach with the ligation of the deep femoral artery, where a complete excision of the superficial femoral artery was carried out (Padilla et al., 2012). During this latter procedure, 2 ml of blood were extracted from the cava vein to determine plasma angiogenic factors. All animals were handled according to the guidelines of the Research, Ethics and Biosafety Committee of Centro Médico Nacional “20 de Noviembre” ISSSTE, in accordance with the Surgery Department, UNAM, and the Mexican General Health Law, regarding experimental studies in animals. Animal handling and experiments were approved by Ethics Committee (ID No. 005.2011).

2.2 | Treatments

All treatments were obtained from healthy rats and were intramuscularly administered during the second surgical procedure of the experimental lower limb ischemia. The preparation of the different treatments was as follows.

2.2.1 | Platelet-rich plasma

Briefly, 6 ml of blood were obtained through medial laparotomy and cava vein puncture. Blood was placed into a vacutainer tube containing calcium citrate. Pure PRP (according to standard nomenclature, Dohan Ehrenfest, Rasmusson, & Albrektsson, 2009) was obtained after centrifugation at 400 RCF, 10 min, with further transfer of buffy coat layer and platelet-poor plasma to another tube and centrifugation at 1000 RCF, 10 min. Then, pure PRP was activated through the addition of 10% (v/v) 10% calcium gluconate (Sigma-Aldrich, USA).

2.2.2 | Peripheral blood mononuclear cell

Briefly, 6 ml of blood were obtained through medial laparotomy and cava vein puncture. PBMC isolation was performed through Histopaque (Sigma-Aldrich, USA) gradient and separation by centrifuge at 400 RCF for 30 min. Then, mononuclear cell layer was recovered and washed three times in phosphate-buffered saline. PBMCs were resuspended in 50-μl phosphate-buffered saline, and a final concentration of 1.5 × 10^6 cells was administered. This corresponds to 4.1 ± 0.14% of CD34+ MPC, according to previous studies (Padilla et al., 2009).

2.2.3 | Thrombin

Two IU of thrombin tissuecol (Baxter, USA) was adjusted to final concentration of 1 ml with PRP and immediately administrated.
2.3 | Design and groups

All animals were submitted to ischemic lower limb model; however, specific treatments were assigned to groups of rats (n = 4), after the second surgical procedure. All treatments were intramuscularly injected (gracilis muscle) as a single dose. Group I (control saline) received 1 ml of 0.9% saline solution; Group II (PRP control) received 1 ml of PRP; Group III (PBMC treatment) received PBMCs 1.5 x 10⁶ adjusted to 1 ml of 0.9% saline solution; Group IV (PBMCs + PRP treatment) received PBMCs 1.5 x 10⁶ adjusted to 1 ml with PRP; and Group V (PBMCs + PRP + thrombin treatment) received same components as Group III plus thrombin.

2.4 | Assessment of angiogenic mediators

Plates were prepared according to manufacturer’s instructions (Milliplex® MAP Rat Vascular Injury Panel 1—Configurable 9 Plex. RV1MAG-26 K. EMD Millipore, Merck KGaA, Darmstadt, Germany). Antibody-coated beads were dispensed into each well and washed twice. A standard curve was generated by reconstituting each cytokine standard, with further serial dilutions. A known concentration for each mediator (VEGF, TNF-α, PAI-1, and CXCL1 [GRO-α]) was included to create a standard curve, and quality control was also included. Then, samples and standards were incubated with the mixed beads overnight at 4°C under shaking conditions. The beads were washed and then incubated with a detection antibody at room temperature for 1 hr and developed with streptavidin. The beads were washed twice, resuspended in drive fluid provided by the kit, and the plate was subsequently analyzed on the Luminex MagPix® plate reader. The mean fluorescence intensity was then compared with the standard curve, as previously described, to calculate the cytokine concentration. Each standard curve was adjusted as required to achieve linearity (R² ≥ .9).

2.5 | Angiography and vascularization assessment

One month after treatment, a lower limb angiography was performed. Medial laparotomy, aortic dissection and ligation of mesenteric, sacral arteries, and aortic ligation were implemented. Then, a 0.60-mm catheter was placed inside the aorta by arterotomy, with further fixation of the catheter to lower aorta. Catheter lavages were performed with a 21G syringe, using 20 ml of 1% heparin solution (saline 20 ml and 0.2 ml of heparin [1,000 UI/ml]) each time. During the second lavage, the cava is sectioned, so the venous blood may have external flow. Lavages were performed until there was no evidence of circulating blood, evaluated visually through the cava blood flow drainage (Padilla et al., 2012). Then, corporectomy was executed and conventional angiography with ethiodized oil contrast media (Lipiodol Ultra-fluide, Guerbet, France) was performed. For vascularization assessment, vascular pattern observed at the angiography was manually traced over a transparent acetate sheet. Then, a 10 x 10 mm grid was applied and the number of vascular intersections was quantified (angiographic index) and evaluated as vascular intersection percentage (Figure 2).

3 | STATISTICAL ANALYSIS

Quantitative data were expressed as median (P50) and interquartile range (P25, P75) or mean ± standard deviation as appropriate after normality assessment by Shapiro–Wilks test. Angiogenic mediators were analysed by independent two-tailed, t test. The angiographic index of vascular intersections was compared independently by two-tailed, Mann–Whitney U test and Kruskal–Wallis test. Statistical significance was assumed when p < .05. Analysis were performed in SPSS v.21.

4 | RESULTS

4.1 | Effect on proangiogenic mediators

We measured the effect of the different treatments (PBMCs, PRP, and thrombin) over well-known proangiogenic mediators, during the experimental revascularization of the lower limb ischemia. We found that PRP alone stimulated TNFα. PBMCs seemed to stimulate PAI-1 and VEGF-A, and when PBMCs were combined with PRP, a higher induction of PAI-1 was observed, as well as an attenuation effect in the group with thrombin. Likewise, the combination containing thrombin also increased VEGF-1. GRO-α showed a sensitive induction to all proangiogenic mediators (Figure 3).

4.2 | Effect on revascularization

Concomitantly, the effect of the different treatments on revascularization was determined as the number of vessel intersections in the lower limb submitted to experimental limb ischemia (Figure 4). A comparison of a comprehensive measure of vessel intersections indicating revascularization is provided in Figure 5. Although all treatments significantly stimulated revascularization, a more significant effect was observed with the combination of PBMC + PRP treatment, which was stronger than PBMC or PRP alone.

5 | DISCUSSION

The main finding of the present study was that PBMC autologous transplant, either combined with PRP or thrombin, stimulates proangiogenic mediators and differentially affect fibrinolytic mediators. Concomitantly, they stimulated revascularization during experimental critical ischemia of the lower limb.

The modification of PBMC number and activities, induced by diseases that cause vascular damage like diabetes mellitus and atherosclerosis, is highly relevant to recognize PBMC’s clinical implications. For example, PBMC’s production of proinflammatory mediators is...
higher in patients with diabetes mellitus, whereas islet’s beta cell proteins may stimulate PBMCs and even hypoglycemia may modulate leukocyte mobilization and proinflammatory changes. It has been suggested that metabolic environment’s influence on immune cells occurs by either the expression of pattern of cell surface recognition receptors or the expression of specific downstream effectors (Brooks-Worrell, Starkebaum, Greenbaum, & Palmer, 1996; Kim et al., 2017; Ratter et al., 2017). Whether the number of PBMCs is modified by diabetes mellitus is less clear (Di Mario et al., 1987, Kopeć, 1992, Roura-Mir et al., 1993). In atherosclerotic scenarios, a lower number of EPCs along decreased functional activities such as proliferative, migratory, adhesive cytokine production, and vasculogenesis capacity have been observed (Chen et al., 2004; Moonen et al., 2007; Wang, Chen, Tao, Zhu, & Shang, 2004).

In the present study, the angiogenic role of PBMCs was evaluated, because they contain MPCs with significant angiogenic effects and due to the potential applications within tissue engineering and regenerative medicine. Likewise, we explored the role of thrombin because several studies had suggested that thrombin preconditioning enhances therapeutic efficacy of transplanted progenitor cells, probably through modulation of secretion of growth factors and cytokines like VEGF and HGF (Kim et al., 2019; Mühleder et al., 2018; Tarzami, Wang, Li, Green, & Singh, 2006).
PBMCs increased the production of PAI-1, an effect that was prevented by the addition of thrombin. In addition, PBMCs and MPCs may regulate angiogenesis and thrombolysis by their interactions with the fibrin clot. MPCs may synthesize plasminogen activators, upon PAI-1 activation, resulting in fibrin network degradation, but MPCs may also inhibit fibrinolysis and fibrin stabilization by the production of PAI-1. In our model, PBMC treatment increased PAI-1, suggesting a fibrin stabilization effect, whereas the addition of thrombin counterbalanced the expression of PAI-1 and probably induced local plasminogen activators in MPCs, which are known to be thrombin responsive (Smadja et al., 2008).

On the other hand, PBMCs increased VEGF-1, either alone or when combined with thrombin. This effect may enhance angiogenesis by inducing endothelial cell migration, proliferation, and apoptosis inhibition, as well as the formation of capillary tubes and integrin expression that modulate endothelial functions (Katsanos et al., 2009). The final proangiogenic effect of thrombin observed in this study may support potential applications of thrombin in therapeutic angiogenesis. (Tsopanoglou & Maragoudakis, 1999).

According to our results, the combination of PBMCs and PRP most significantly stimulated proangiogenic potential, at higher level than PBMC or PRP alone. The interaction between circulating cells with angiogenic potential and PRP has been previously reported (Nami et al., 2016; Raz, Lev, Battler, & Lev, 2014) and described to modulate proangiogenic mediators like VEGF, PDGF, and proinflammatory factors promoting wound healing. It is known that PRP may stimulate angiogenesis by different mechanisms. PRP contains a high level of platelets and growth factors produced by platelets owning potential angiogenic effects and facilitates cell adhesion, migration, angiogenesis, or promotion of tissue repair and/or regeneration (Rodríguez Flores, Palomar Gallego, & Torres García-Denche, 2012). In the present study, adjuvant proangiogenic effect of PRP may be explained by either facilitating the

**FIGURE 4** Effect of PBMCs, PRP and thrombin treatments on revascularization. We compared the revascularization pattern in the lower limb observed after 4 weeks from vascular ligation. Group I: saline solution, Group II: PRP, Group III: PBMCs, Group IV: PBMCs +PRP, and Group V: PBMCs+PRP + thrombin. The % intersections is expressed as the mean of four independent experiments per group. PBMC, peripheral blood mononuclear cell; PRP, platelet-rich plasma; Tb, thrombin

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differentiation of PBMCs or by increasing its survival, due to the VEGF content in PRP (Jiménez-Cuenca, 2003; Li, Hou, Wu, Chen, & Luo, 2014). In the present study, PRP increased plasma levels of TNF-α, which might induce angiogenesis because of its mutual interaction with platelet-activating factor present in PRP (Montrucchio et al., 1994), or due to the induction of mediators like LRG1 that promotes angiogenesis and mesenchymal stem cell migration (Wang et al., 2017). In addition, PRP also increased GRO-α. Consistently, production of GRO-α has been described in activated PRP supernatants in vitro (Park, Yang, & Chung, 2011) as well as in PRP-stimulated cultured tenocytes (Andia & Rubio-Azpeltia, 2014). PRP-mediated increase of TNF-α was prevented by thrombin. Conversely, other studies have described a higher induction of GRO-α in supernatants during PRP activation in presence of thrombin, which may be explained by differences regarding the in vivo model used here and the induction of experimental limb ischemia. However, we think that the in vivo model reflects a more realistic scenario of proangiogenic mediators within human disease (Park et al., 2011).

A limitation of the present study includes the PBMC treatment consisted in the administration of mononuclear cells preparation, where only 4% corresponded to CD34+ MPCs, but the remaining mononuclear cells were not characterized. Such portion of MPCs has been previously described as enough to significantly stimulate angiogenesis in the same experimental model (Padilla et al., 2003); however, the effects of additional factors present in the cell preparation were not assessed in the present study.

In conclusion, PBMCs + PRP autologous transplant stimulates revascularization during experimental critical ischemia of the lower limb, whereas particular effects over angiogenic and fibrinolytic mediators may be attributed to PBMCs and its combination with thrombin.

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CONFLICT OF INTEREST
The authors declare that there is no conflict of interest regarding the publication of this paper.

AUTHOR CONTRIBUTIONS
Conception and design: Luis Padilla. Acquisition, analysis, and interpretation of data: Juan Miguel Rodríguez-Trejo, Pilar Hazel Carranza-Castro, Javier López-Gutiérrez, Alejandro Hernández-Patricio Biol, Eduardo Vera-Gómez Biol, Alan De Jesús Gómez-Calderón Biol, and Mario Antonio Téllez-González. Drafting the manuscript and critical review: Rubén Argüero-Sánchez, Juan Antonio Suárez-Cuenca, Jaime Polaco-Castillo, Mauricio DI Silvio-López, Horacio Olguín-Juárez, and Paul Mondragón-Terán.

DATA SHARING AND ACCESSIBILITY
Data supporting the findings of this study are available from the corresponding author (LP) upon request.

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