CHAPTER 5

Molecular Intervention with Plasma-Rich Growth Factors to Enhance Muscle Perfusion and Tissue Remodeling in Ischemic Diseases

PRGF treatment of ischemic muscle and heart

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SUMMARY

Ischemic diseases remain the first cause of morbidity and mortality worldwide. Current standard approaches based on drug-therapy or surgery have a palliative but not a repairing effect, which leads to chronification and worsening of the disease. Novel alternative approaches based on protein and stem cell therapy have been thoroughly assessed in the last two decades. Platelet-rich plasma has been shown to act as a rich depot of pro-angiogenic, pro-survival and pro-myogenic growth factors that can be locally released in the diseased tissues. This chapter focuses on the therapeutic potential of platelet-rich plasma and growth factors for protective and regenerative treatment of muscle and cardiac ischemic pathologies. We discuss the experimental and clinical results obtained when PRP/PRGF were injected in animal models of limb ischemia and myocardial infarction or angina patients, together with the mechanisms involved in such effects. Finally, the combination of platelet-derivatives with stem cells is also described as a novel protein/cellular delivery platform with improved potential for treating ischemic disease.

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1. EPIDEMIOLOGY AND PATHOLOGY OF CARDIOVASCULAR DISEASES

Ischemia defines a state in which blood flow is insufficient to meet metabolic demands. This condition occurs regularly but transiently in healthy humans, for example when they begin to exercise. On the pathological side, there are multiple mechanisms that impair the functional and structural responses of the vasculature to the metabolic demands of the muscle and cardiac tissue (among others), provoking ischemia. According to the World Health Organization, cardiovascular diseases represent the first cause of morbidity and mortality worldwide, with approximately 17 million deaths per year. Unfortunately, predictions are not favorable and it is estimated that by 2030 about 24 million people per year will die from these diseases, which represents 42% of deaths worldwide[1]. Risk factors associated with ischemia are multiple, the principal ones being hypertension, high cholesterol, obesity, diabetes, aging, family history of cardiovascular disease, gender and ethnic origin. External factors like physical inactivity, tobacco and alcohol consumption also have a great influence on the progress of these diseases.

Peripheral Artery Disease (PAD) is a common cause of great disability and morbidity. The annual incidence of this ischemic disease in Europe and the United States ranges from 500 to 1000 new cases per million people[2]. PAD is an occlusive atherosclerotic disease caused by blockage of the arteries by cholesterol plaques that cause compromised blood flow in the limbs. Following atherosclerosis and blood flow limitation, adaptation, maladaptation and injury in the distal vascular bed and skeletal muscle contribute to exacerbate the disease. Symptoms of PAD include intermittent claudication, which is defined as calf or buttock pain or cramping with walking caused by inadequate blood flow to the limb. The most severe symptomatic manifestation of PAD is Critical Limb Ischemia (CLI), defined as pain at rest due to reduced blood flow to the limb. CLI may further result in ischemic ulceration and gangrene formation. Patients with CLI have not only a high risk of amputation but also a high rate of cardiovascular death, often due to complications related to coronary artery (55%) and cerebrovascular atherosclerosis (10%)[3]. Despite the remarkable progress made in medical treatments and endovascular procedures for revascularization, patients with CLI have a very low quality of life. Nowadays, angioplasty, with or without stenting, and bypass surgery are widely used surgical interventions, and tangible results have been reported showing how they decrease mortality and limb loss while also increasing patency and improving wound healing[4].

Ischemic Heart Disease (IHD) is the leading cause of death in the developed world, representing 33% of deaths of patients aged over[3, 5]. It is estimated that each year cardiovascular diseases cause a total of about 4 million deaths in Europe alone, most due to coronary heart disease, representing 47% of all deaths. Worldwide, the annual incidence of heart failure has reached staggering numbers, culminating in 20 million cardiac-related deaths per year worldwide. Indeed, decompensated heart failure is now the primary indication for repeated hospitalization, resulting in an annual expenditure of US$120 billion worldwide and a 5-year mortality of around 50% (reviewed in[1,5]).

Myocardial ischemia generally develops when deposits of cholesterol particles accumulate on the walls of heart blood vessels and form plaques, which narrow or block the arteries that supply blood to the heart. As in ischemic limbs, the lack of blood supply leads to cell death and tissue necrosis, which activates heart tissue inflammation and remodeling responses. By these processes, necrotic cells are eliminated and replaced by a non-contractile fibrotic scar mainly composed of activated fibroblasts and extracellular matrix components[6]. These compensatory mechanisms initially prevent cardiac rupture but, with time, become a maladaptive response, leading to contractile dysfunction, arrhythmias and ultimately, heart failure.

Routine therapies driven to improve myocardial function in IHD include pharmacological treatment, percutaneous intervention and surgery.

However, these techniques are aimed at minimizing the symptoms and preventing the progression of the disease, but are not able to regenerate the tissue or to restore the heart function in a sustained manner. Therefore, the only real option for severe cases is heart transplantation with the concomitant limitations of the donor waiting lists and the need for an immunosuppressive regimen to prevent rejection. The failure of these therapies to rescue the damaged heart and the inconvenience of heart transplants have led to the emergence of alternative treatments, including protein (reviewed in[7]) and stem cell (SC)–based (reviewed in[8]) therapies. These novel strategies are directed to induce tissue revascularization as well as to intervene in the tissue remodeling processes, and are vital for treating IHC as well as PAD patients[9]. Protein-based therapies as examples of this type of treatment will be discussed in detail in the following pages.

2. TREATMENT WITH GROWTH FACTORS FOR RECOVERY OF ISCHEMIC TISSUES

Since Dr. J. Folkman first described the process of angiogenesis in 1971, much has been learned about the cells, the extracellular factors, and the signaling pathways that modulate the neovascularization process[10]. Neovascularization, the establishment of stable and functional blood vessel networks, involves multiple complex events that require several angiogenic factors to induce sprouting of pre-existing resident endothelial cells (angiogenesis), maturation and enlargement of size of pre-existing small vessels through vascular remodeling (arteriogenesis), and the recruitment of endothelial progenitor cells (vasculogenesis)[11].

Angiogenesis, arteriogenesis, and vasculogenesis are critical to the process of ischemic muscle regeneration, and they are closely related to inflammation and extracellular matrix remodeling. Although the complex interactions between these multiple cell types are still not totally understood, the major role that monocytes and macrophages play in the remodeling process is well known, as they are recruited at the injured sites and generate chemokines and growth factors that contribute to arteriogenesis and angiogenesis. These factors are important for the subsequent recruitment of leukocytes to the injured tissue as well as endothelial and smooth muscle progenitor cells, and also play a critical role in the activation of muscle-derived progenitor cells.

In recent years, many growth factors and cytokines have been identified and their function analyzed in different animal models of ischemia, including those for high limb ischemia and myocardial infarction. Among these factors, VEGF has been pointed out as a key stimulatory factor of angiogenesis[12], which strongly induces endothelial cell proliferation and migration. However, the combined action of other factors is necessary in order to induce vascular maturation and stabilization, as VEGF by itself promotes the formation of leaky and unstable capillaries[13]. Factors like PDGF-BB are essential for these processes, and the lack of this single factor leads to fragile neovasculature[14]. Morevoer, bFGF and HGF also cooperate with VEGF or PDGF-BB by acting as chemoattractans to smooth muscle cells and inducing their growth. Thus, their action favors the promotion and enlargement of collateral vessels, as it has been previously shown in several animal models of limb ischemia and myocardial infarction[15,16]. Furthermore, the anti-inflammatory and anti-fibrotic role of HGF has also been demonstrated[17]. Other factors like SDF-1 and IGF-1 have direct or indirect effects on endogenous angiogenesis. SDF-1 guides, together with VEGF, the recruitment and homing of endothelial progenitor cells to ischemic muscles, contributing to neovascularization[18], and IGF-1 also stimulates angiogenesis[19], although with a less potent effect than other angiogenic factors. Importantly, a robust anti-apoptotic and pro-myogenic role has been reported for IGF-1[20].

Positive results have been observed after treating different ischemic animal models with pro-angiogenic growth factors. For example, on this basis, therapeutic angiogenesis was proposed as a good treatment for PAD and IHD patients. However, clinical trials of single and solution forms of pro-angiogenic agents have proven to have little or no efficacy in patients with such disorders. Thus, despite Phase-I clinical trials demonstrating promising results in patients with either PAD or IHD treated with growth factors like FGF2, VEGF or HGF, multi-center, randomized, double-bind, placebo-controlled phase-II/III trials contradicted the previous results, showing no significant beneficial effects in any of the groups of ischemic patients who received single doses of recombinant proteins such as FGF2 or VEGF, among others (reviewed in[21]).

The major limitation found in these growth factors-based clinical trials is that a cocktail of growth factors and cytokines is likely to be required according to the complex intricacy of the angiogenic/arteriogenic and tissue remodeling processes; thus, administration of a single growth factor may not be sufficient to achieve a complete response[13,15]. In addition, the short half-life of the proteins due to their high instability and protease action when injected as a bolus may also counteract their beneficial effects. Also, the low local biodistribution and lack of dose control might reduce their possible benefit. To overcome these limitations, several technologies have been explored to ensure more protected, controlled and localized release of the growth factors. These new technologies, which have been thoroughly reviewed by others[22], include many biological and synthetic systems based on the preparation of functionalized scaffolds, liposomes, nano- or micro-particles, whose main function is to protect proteins from degradation and to preserve their bioactivity during their release into the damaged tissues. In that sense, the use of platelet-rich gels might be considered another release system for growth factors, and this will be described next.

3. NOVEL TREATMENTS FOR THE ISCHEMIC MUSCLE: IS PLATELET-RICH PLASMA AN ALTERNATIVE OPTION?

3.1 Platelet-rich plasma

As previously discussed, successful reperfusion of the ischemic tissue depends on stimulation of angiogenic and arteriogenesis activities[23]. Platelets are critical to hemostasis and subsequent angiogenesis in wound healing. Transfusion of platelets enhances the angiogenic recovery of blood flow in models of ischemia[24], while their depletion suppresses this process[25]. Platelets contain more than 20 growth factors that stimulate proliferation, survival, adhesion, and chemotaxis of different progenitor cells such as hematopoietic cells or endothelial cells, affecting both angiogenesis, restoration of blood flow and wound healing. Once activated, platelets release pro-angiogenic and also anti-apoptotic and pro-myogenic factors, including VEGF, SDF-1, PDGF, FGF-2, HGF, TGF and KGF (reviewed in[26]). Since platelets constitute a potential source of multiple autologous growth factors, proteins and chemokines involved in tissue regeneration, a product enriched in platelets was designed, the platelet-rich plasma (PRP) product (also termed autologous platelet)[27]. The initial rationale for PRP products was to replace the blood clot with a preparation enriched in platelets, which could, once activated, secrete a large pool of proteins and factors to the local milieu, driving the tissue regeneration mechanism. Later on, another technique was developed to obtain a type of plasma that is highly enriched with proteins and circulating growth factors and concentrated in a gelatinous form, which has been named PRGF (Plasma Rich in Growth Factor)[28]. PRGF presents the advantage over PRP that it does not need thrombin for coagulation, being produced by a simple calcium-based reaction. Finally, Platelet Rich Fibrin (PRF) has also been produced by a simplified preparation, with no manipulation of blood. A detailed description of the preparation of these three platelet-derived products and their main features and advantages for therapeutic use has been previously reviewed[29].

3.2 Application of PRP in ischemic peripheral disease

Effective restoration of blood flow by augmentation of the number of capillaries and mature vessels after PRP delivery has been demonstrated in mouse hind limb ischemia models[30,31]. Sustained release of PRP accelerates the homing of hematopoietic progenitor cells to the ischemic site, which might be explained by the presence of VEGF and SDF-1 in the PRP, inducing the arteriogenesis process. Growth factors present in PRP play bioactive roles in the proliferation and differentiation of endothelial cells and smooth muscle cells, which may contribute to the formation of functional collateral vessels and facilitate their maturation[32]. The transient fibrin scaffold, which is formed after PRP activation and polymerization, in addition to contributing to angiogenesis by sequestering and releasing different growth factors, may favor cell survival in ischemic legs through cell adhesion motifs[33]. Furthermore, this biological scaffold of fibrin contributes to wound healing of dermal tissue loss in patients affected by CLI. Platelet-derived fibrin scaffold guides the mesenchymal cells to migrate from the base and the margins of the wound, eventually maturing into a granulation tissue[34,35]. They also contain anti-bacterial proteins, which help to reduce bacterial colonization in foot ulcers[36], as well as being less invasive, painless, easier to apply, and tolerated better by the patients. In addition, in order to attain maximum therapeutic effects, the combination of platelet-rich preparations with biomaterials has made it possible to develop novel therapeutic alternatives, improving the preexisting ones and increasing their versatility. In this sense, PRP-containing-fragmin/protamine microparticles as a delivery system for proteins in PRP have been shown to induce local arteriogenesis and angiogenesis in a rabbit model of hind limb ischemia, suggesting its possible use as treatment for PAD and CLI[32].

In the full reconstruction of ischemic limb muscles, angiogenesis and myogenesis must proceed concomitantly. Vascular endothelial progenitor cells are probably essential for muscle repair, since satellite cells, the adult stem cells of the skeletal muscle, are pre-positioned near capillaries. Thus, satellite cells can easily interplay with endothelial cells to set up co-ordinated angio-myogenesis response in a functional manner[37]. It has been shown that PRGF facilitates recruitment, activation and mobilization of myogenic progenitor cells and resident macrophages, contributing to muscle repair process, in addition to the already activated endothelial cells, macrophages, and platelets in the injured area[38]. In an animal model of severe hind limb ischemia, PRGF increases blood perfusion recovery, while improving the weight and caliber of affected myofibres. PRGF, in addition to preventing accumulation of fibrous tissue, impedes the apoptotic response of endothelial and muscle progenitor cells to hypoxia, which may explain the angiogenic and myogenic activities after ischemia[30]. These results may be explained by the fact that upon activation, satellite cells accumulate and proliferate close to capillaries, receive efficient support from endothelial cells for their growth, are pro-angiogenic, and co-localize with the new vessels during their myogenic differentiation, which strongly suggests that angiogenesis and myogenesis signal reciprocally. In addition, myogenic cells undergoing differentiation secrete VEGF[39], while endothelial cells produce a series of mitogens for myogenic cells[40]. Thus, it is likely that angiogenesis and myogenesis share growth factors and cytokines contained in PRGF as co-regulatory factors. For example, VEGF effects extend to a variety of non-endothelial cell types, including myogenic cells[41]. VEGF stimulates myogenic cell growth and their migration[42], protects them from apoptosis[41,42], up-regulates myoglobin expression and promotes formation of regenerating myofibers[41]. Similarly, bFGF, IGF1, HGF, and PDGF-BB influence muscle progenitor cells growth[40]. Furthermore, SDF-1, as in endothelial cells, recruits satellite cells from their native position to damaged areas for muscle repair[43]. Thus, harmonious increase of myofiber capillarization and caliber ensues, allowing larger myofibers to benefit from an appropriately enhanced blood supply.

3.3 Application of PRP in ischemic heart disease

As well as in ischemic peripheral diseases, the therapeutic potential of PRP has also been tested in diverse experimental models of myocardial infarction (Table 1). Similarly, the angiogeneic factors present in the PRP play a key role in rescuing the ischemic heart. Other factors like SDF-1 involved in the attraction of endogenous progenitors, or IGF-1, a potent factor that favors cell survival and induces the activation and proliferation of cardiac progenitor cells[20], greatly contribute to the regeneration of heart tissue. In that sense, the application of PRP, which releases many of these factors, exerts a beneficial effect in the ischemic heart (Figure 1). Thus, in a first study performed in 2008 by the group of Dr. Li and colleagues, thrombin-activated PRP derived from rat blood was injected in a rat model of MI induced by the permanent ligation of the descendent coronary artery. A positive effect over tissue remodeling and accelerated healing was observed four weeks post-implant. In

Animal Model	Treatment	Post-implant time analysis	Analysis	Plasma-derivatives Effect	Reference
Rat AMI	Rat Allogenic-PRP	4 weeks	Histological	Angiogenesis activation Limitation of ventricular expansion Attenuation of myocardial hypertrophy	Li et al. (2008)
Mouse AMI Mouse I/R	Human-PRP (RevaTen)	3 weeks	Functional (MR) Histological	Functional improvement (higher EF) Limitation of scar formation	Mishra et al. (2011)
Rat AMI	Autologous Rat Platelet-gel	6 weeks	Functional (Echo) Histological	Preserved cardiac function Limitation of ventricular expansion Attenuation of myocardial hypertrophy Angiogenesis activation No immune-inflammatory reactions	Cheng et al. (2012)
Rabbit I/R	Nanosecond Pulsed Rabbit-PRP	2 weeks	Hemodynamics	Improved LV function Cardioprotection	Hargrave et al. (2012)
Sheep SubAcute MI	Autologous PRGF	9 weeks	Histological	No toxicity or Immune-inflammatory reactions Angiogenesis activation	Gallo et al. (2013)
Pig AMI	Autologous PRP +/- anti-oxidant & anti-inflammatory agents +/- Hidrogel	8 weeks	Functional (Echo) Hemodynamics Histological	Functional improvement (higher EF) and Limitation of ventricular expansion Higher dp/dtmax Angiogenesis activation *Greater effects with combined treatment	Vu et al. (2014)
Rat AMI	Rat Platelet Gel + CSC	3 weeks	Functional (Echo) Histological	Functional improvement (higher EF) Limitation of ventricular expansion Angiogenesis and myogenesis activation	Cheng et al. (2012)
Rat AMI	PRF + Autologous ADSC	6 weeks	Functional (Echo) Histological / WB	Preserved LV function Attenuated LV Remodeling Angiogenesis activation	Sun et al. (2014) Chen et al. (2015)
Rabbit dilated-CM (Doxorubicin-induced)	PRP + ADSC	15 days	Functional (Echo and Electro) Histological	Impaired function and no remodeling improvement	Mosbacher et al. (2016)
Angina Class III/IV Patients	TMR + Autologous PRP	6 months	Functional (Echo)	Lower/none angina episodes Functional improvement	Wehberg et al. (2009)

TABLE 1 Summary of studies performed with platelet-rich derivatives in ischemic heart disease.
AMI: Acute Myocardial Infarction; SubAMI: Subacute Myocardial Infarction; I/R: Ischemia-Reperfusion; CM: Cardiomyopathy; TMR: Transmyocardial Rvascularization; MR: Magnetic Resonance; Echo: Echocardiography; WB: Western Blot; Electro: Electrocardiography; EF: Ejection Fraction.

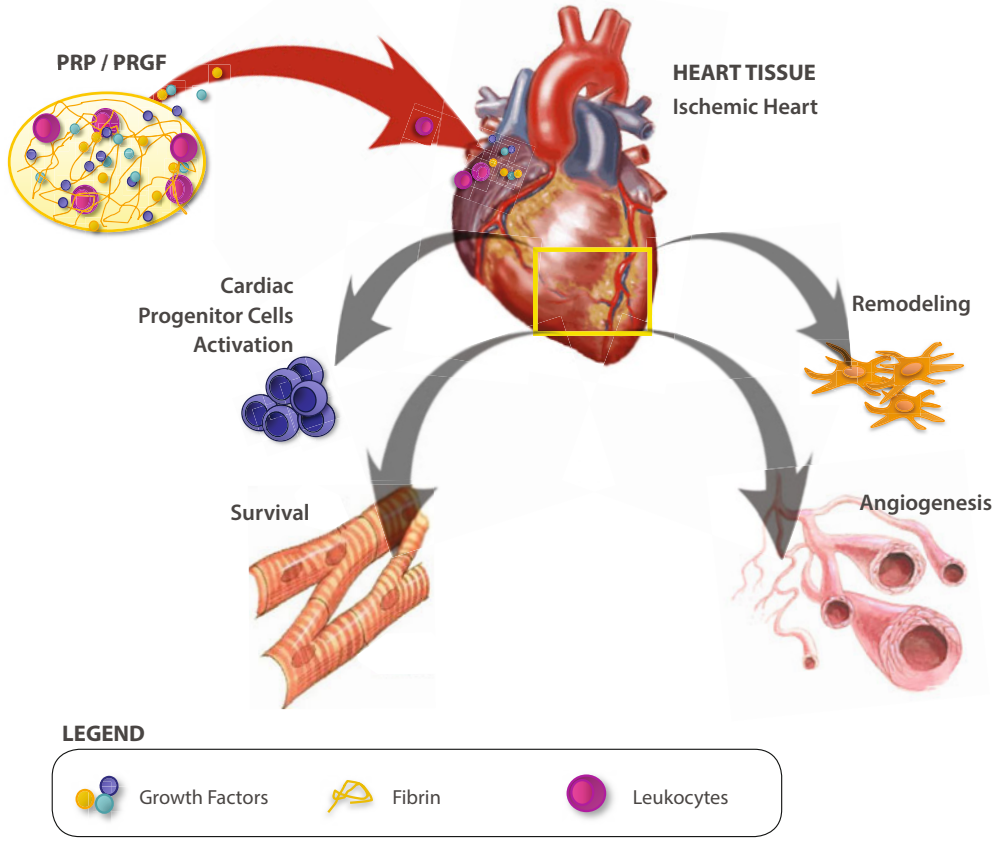

FIG. 1 Therapeutic potential of Platelet-rich plasma and growth factors in the ischemic heart.
Several mechanisms have already been described for PRP and PRGF action in ischemic diseases. The main one is by release of a cocktail of cytokines and growth factors (VEGF, PDGF, HGF or IGF among others) responsible to trigger several responses involved in anti-apoptotic signaling, cell proliferation, angiogeneic processes, fibrosis regulation or recruitment and activation of resident stem cells/progenitors able to reconstitute the damaged tissue. Also, leukocytes can immunomodulate and more robustly secrete growth factors and proteins also involved in regulating the aforementioned processes. Furthermore, fibrin content can give a robust mechanical support to the injured heart. Thus, to better know the key factors and optimal dose involved in such processes would help to improve the beneficial effect of this therapy.

more detail, the limitation of ventricular expansion, the attenuation of myocardial hypertrophy in the non-infarct region and the activation of the angiogeneic and arteriogeneic processes in the infarct area were also detected as consequence of the PRP-treatment[44]. Later on, another study was also performed in a rodent model of MI, induced either by permanent ligation or by 45 min-artery occlusion and following reperfusion[45]. Importantly, functional analyses performed by magnetic resonance were included in this study, demonstrating in both models a functional improvement 21 days after injection of human PRP (RevaTen). These functional results were later corroborated by a study performed by Chen and colleagues where an autologous platelet gel was also intra-myocardially injected in infarcted rats[46]. Deterioration in the cardiac function was evidenced in the vehicle-injected animals over the 6-week time course, while that was avoided in the platelet gel-injected animals. As in the previously described study, enhanced tissue protection together with an increment in capillary density and a lower compensatory myocyte hypertrophy were evidenced. In addition, the gel did not exacerbate inflammation in the heart whereas recruitment of endogenous cells involved in tissue regeneration was observed as a consequence of the gel injection.

Interestingly, a new way to activate the platelets based on the use of nanosecond pulsed electric fields (instead of thrombin addition) has recently been described[47,48]. This method also allows releasing growth factors stored in the platelets α-granules and has the advantages over the traditional PRP production that it can preserve the antioxidant properties of the platelets and avoid the putative adverse effects of thrombin. Moreover, an improvement in cardiac function at the mechanical and electrical level has been demonstrated in an ischemia-reperfusion model of MI in rabbit when treated with nano-pulsed PRP. The mechanisms behind these effects have been related to cardiac protection as an increase in the expression of the cardioprotective proteins Hsp27 and Hsp70, together with the stabilization of the mithocondria following diminished generation of free radicals has been demonstrated in vitro[48].

In view of the positive experimental results, the protective/regenerative potential of PRP has also been tested in two large animal models. In a first study performed in a chronic sheep model of MI, the promotion of vessel formation was histologically determined, confirming the pro-angiogenic capacity of PRP[49]. In more detail, a recent study performed in a pig preclinical infarct model, has shown the therapeutic benefit of PRP not only at the tisular but also at the functional level[50]. In this last study, infarct was induced by permanent ligation of the left circumflex coronary artery in animals randomized for intra-myocardial injection of the different treatments. Pigs received autologous PRP alone or combined with a hyaluronic acid-based hydrogel and anti-oxidant and anti-inflammatory factors, and the benefit then compared with a control group treated with saline, with the hydrogel alone or the hydrogel combined with the anti-oxidant and anti-inflammatory factors. Eight weeks after MI, a functional improvement was detected by echocardiography when treated with PRP, revealing an increase in the ejection fraction, a reduced left ventricular dilation and greater cardiac contraction. Also, an attenuated reverse remodeling and a positive impact in heart revascularization were evidenced. Interestingly, the combination of PRP with a cocktail of anti-inflammatory and anti-oxidant compounds together with a hydrogel that provided mechanical support to the heart has been shown to greatly improve heart performance. A smaller left ventricle size and better contractile function were shown with this combined treatment, demonstrating a boosting of PRP action.

Confirming all these experimental results, a clinical trial has been performed in 25 patients with refractory class III/IV angina who had no conventional revascularization options[51]. All patients underwent trans-myocardial revascularization and received PRP intra-myocardial injections or not. Interestingly, the patients that were also treated with PRP presented a lower or negligible angina score and an increase in the ejection fraction 6 months post-treatment, suggesting the clinical benefit of PRP treatment.

4. PLATELET-RICH FACTORS AND STEM CELLS FOR THE TREATMENT OF ISCHEMIC DISEASES

As well as with protein-based therapies, many efforts have been directed to develop novel therapies based on application of stem cells. As early as 1997, Asahara and colleagues isolated endothelial progenitor cells from human peripheral blood, and demonstrated that these cells retain the capacity to differentiate into mature and functional endothelial cells[52]. These findings had significant clinical implications and generated great optimism regarding offering new treatments for ischemic diseases: stem cell based approaches to promote angiogenesis and improve tissue function. However, although much progress has been made since Asahara´s findings, stem cell therapy has been slow to come to clinical use. A number of stem and progenitor cells have been examined since then for boosting angiogenesis and compensating the tissue remodeling processes within

ischemic tissues, including endothelial progenitor cells, bone marrow-derived mononuclear cells, bone or adipose mechsenchymal cells and even pluripotent stem cell-derived endothelial cells. To date, more than 50 clinical studies have been reported on the treatment of PAD[53] and more than one hundred for the treatment of IHD with stem/progenitor cells[54], but with relatively limited success.

One of the main limitations of stem cell therapy is the fact that most therapeutic donor cells do not engraft and survive in the injured host tissues. The environment of ischemic tissues may have a deleterious effect on engraftment and survival of donor cells, which in turn may influence their stem cell function. Thus, making a more favorable environment in ischemic tissues by reducing the fibrotic content and the accumulation of inflammatory cells while improving the angiogenesis response of donor cells should improve the benefit of cell therapy for ischemic tissues. Therefore, a number of groups have developed approaches to enhance donor cell survival and function, along with paracrine factor delivery. In this sense, if the paracrine effects of growth factors can improve stem cell therapy, co-administering platelet-derived products could be of great relevance in this type of approach.

Few studies have been performed combining the platelet-derivatives with several populations of stem cells in order to boost the benefit of both treatments, but those that do exist hold considerable interest (Table 1). In 2012, the group of Dr. Marban, a well known expert in the isolation and characterization of cardiac derived stem cells, in particular a population named cardiosphere-derived cells (CDC), tested their potential in a rat model of acute MI when transplanted in a platelet-gel[46]. This study clearly showed the benefit of using transplanted cells pre-seeded in a platelet gel. A functional improvement three weeks post-implant was indeed observed in the animals implanted with the cellularized gel in comparison with the platelet gel alone. Also, greater recruitment of endogenous cells and the induction of neo-angiogenesis together with a positive impact on tissue remodeling were shown after treatment, in comparison with the control and only-gel implanted animals. This in vivo effect was explained by the fact that the cellularized-gel released a greater dose of "regenerative" cytokines, such as VEGF, IGF-1 and SDF-1, than the gel alone. Furthermore, although the contribution of CSC to heart tissue had been previously shown, the mechanism of action for tissue repair was mainly attributed to their trophic effect rather than to the exogenous cells' contribution through their own differentiation towards cardiovascular cells. On the other hand, a group implanted only with CDC was not included in this study and even though the benefit of the combined therapy was clearly demonstrated, it is not possible to evaluate the real impact the gel exerts over the transplanted cells in order to elucidate whether its addition significantly increases the benefit of the cells alone. In fact, the therapeutic benefit of the CDC has been experimentally (reviewed in[55]) and clinically demonstrated, as a Phase I/II clinical trial (CADUCEUS trial) was performed in patients with myocardial infraction[46]. Even though positive results are expected, it would be interesting to be able to quantify the real impact of combining the cells with the gel.

Also, the group of Dr. Yip, in two different studies, used a rat acute model of MI to test the potential of Platelet-rich fibrin (PRF)[29] combined with autologous ADSC. In both studies, 1 million cells were embedded in the platelet gel (also derived from the rat blood) and implanted in the heart one hour after MI induction. Other groups of animals were implanted only with ADSC[56] or only with PRF[57]. Six weeks after treatment, an improvement in cardiac function together with a reduction in ventricular remodeling and an induction of angiogenesis was detected in the animals implanted with the combined treatment when compared with a non-treated group. A superior effect was also detected when compared either with the PRF alone or the ADSC alone. Thus the known therapeutic effect of ADSC[58,59] was significantly boosted by the combination with PRF, probably due to greater engraftment and retention of the cells when implanted with the fibrin patch. Indeed, the advan-

tage of transplanting cells with a matrix or scaffold has already been shown and explained as a consequence of their increased retention and the induction of adhesion-related survival pathways that increase their permanence in the tissue[60]. Moreover, the growth factors released by the PRF could exert a pro-survival effect (among other benefits) not only in the heart tissue but also in the implanted ADSC, heightening their beneficial effect even further.

Finally, a very recent manuscript has been published in which PRP combined with ADSC has been tested for the treatment of dilated cardiomyopathy. In this case, a rabbit model induced by doxorubicin was used. Surprisingly, a benefit was only observed after cell treatment alone, but not with the combination with PRP or PRP alone, when in fact, the disease was found to worsen. Functional examination was performed only 15 days post-infarct, so longer-term functional analyses to understand the impact of the treatment are missing. More detailed studies using this model would be of great interest in order to clarify the mechanisms behind these results[61].

5. CONCLUDING REMARKS AND FUTURE PERSPECTIVES

The therapeutic benefit of PRP/PRGF has already been demonstrated in several animal models of hind limb ischemia and myocardial infarction. The pro-angiogenic, pro-myogenic, immunomodulatory and anti-fibrotic effect induced by the released growth factors and the platelets/leukocyte action together with the mechanical support exerted by the fibrin matrix, justifies its therapeutic action in the ischemic tissues. Elucidation of the optimal platelet-rich formulation, dosage and delivery route for each disease will improve the potential benefits.

Moreover, safety is another advantage of this treatment. Low or negligible morbidity is associated with patient blood extraction for PRP/PRGF isolation and the autologous origin of the product avoids the viral risks of xenogeneic or allogenic products, antibody formation and risk of graft-versus-host disease[62]. Furthermore, the quick, easy protocol for preparing these products makes them a potentially cost-effective alternative compared to other novel therapies[63,64].

Finally, in view of the demonstrated benefit of PRP not only in experimental animal models but also in angina patients, and the potential of various stem cell populations like ADSC and CSC in infarcted patients, it would be of great interest to determine the therapeutic effect of PRP/PRGF in combination with stem cells and/or biomaterials, in these patients and in those suffering PAD diseases.

In conclusion, the striking functional benefits, together with the simplicity of manufacture and the autologous origin of PRP, make it an excellent candidate for the treatment of ischemic pathologies at the clinical level. Furthermore, since the actions of stem cells delivered in ischemic tissues are boosted when these are combined with PRP/PRGF, this approach opens up new therapeutic avenues for synergic treatment. In the future, the effect of such treatments is likely to be enhanced still further by fruitful combinations with bioengineering strategies.

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