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Multiple benefits of platelet-rich plasma for regenerative medicine therapies

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Abstract

Platelet rich plasma is rich in growth factors that exert a beneficial effect on cellular processes, favor tissue regeneration, stimulate fibroblast activation and actively participate in wound healing process. Growth factor concentration in PRP directly correlated to the efficiency of tissue regeneration, thus it is critical to determine it quantitatively in order to obtain a positive and visible effect in patients that undergo wound healing procedures or skin rejuvenation treatments. This study highlights some of the applications of PRP, both in vitro and in vivo, and confirms that therapy with PRP can be a useful tool in aesthetic and regenerative medicine.

Keywords: platelet rich plasma, growth factors concentration, tissue regeneration, regenerative medicine.

1. Introduction

Platelet-rich plasma (PRP) is defined as a platelet concentrate derived from whole blood, that gained a special interest due to its capability to improve the healing process in many medical fields such as plastic, reconstructive and cosmetic surgery, cartilage and tendon repair, orthopedic surgery, oral-maxillofacial surgery (QIAN & al. [1]; TAMBELLA & al. [2]). PRP is rich in growth factors such as epidermal growth factor (EGF), insulin-like growth factor (IGF-1), platelet-derived growth factor (PDGF), transforming growth factor β1 (TGF- β1), vascular-endothelial growth factor (VEGF), fibroblastic growth factor (FGF), interleukin (IL), keratinocyte growth factor, connective tissue growth factor, fibronectin growth factor, platelet factor (PF). These factors are thought to promote cell migration, proliferation and differentiation and subsequently exert a positive effect on wound healing and tissue regeneration processes (IQBAL & al. [3]; ZHENG & al. [4]; SINGH & SINGH [5]; ZBUCHEA & al. [6])). In recent years it has gained a lot of interest especially in the cosmetic and dermatological field due to PRP's positive influence on dermal fibroblast proliferation and type I collagen synthesis. In this area, PRP is used for facial rejuvenation, hair growth and skin quality of scars (ALVES & GRIMALT [7]; ZHANG & al. [8]). Another use of PRP is along with fat grafts in soft tissue defects. Many studies have reported better cell proliferation in vitro and in vivo, improving stem cells regenerative potential and the graft survival (LIAO & al. [9]; VIRZI & al. [10]). Despite its broad use, there is no standard approach in the protocol used to treat different affection and different group age of people (CHAHLA & al. [11]). This inconsistency was noted by Laver & al. in a review on PRP's use to treat degenerative osteoarthritis. They observed the wide variance between studies concerning the type of isolation

protocol of PRP used, PRP concentration, number and interval of injections administered (LAVER & al. [12]). Concerning PRP's use in bone tissue engineering (BTE), it is widely believed that PRP positively influences bone regeneration, being present in the process of wound healing and thus being able to resemble the conditions for bone healing (IQBAL & al. [3]). Various studies indicate that PRP has the tendency to improve and to stimulate the bone formation process, upregulating osteogenesis-specific marker genes, such as OPN, RUNX2 and OSX (WEI & al. [13]). Results have shown that, in vitro, PRP has the ability to enhance the proliferation of bone marrow stem cells (BMSC) and to promote the osteogenic differentiation (ZOU & al. [14]), and along with the capability of PRP to accelerate the healing process (ROUBELAKIS & al. [15]), this proves PRP to be highly effective for BTE. BTE combines the knowledge of biology, engineering and material science to design architectural supports for cells (SHI & al. [16]). Three main components are the key for bone tissue engineering: a scaffold that closely mimics bone's extracellular matrix, a cell source which can convert into bone cells and inductive growth factors (ROSE & al. [17]). In order to have a scaffold with good properties, it must have a few indispensable characteristics, the most important one being biocompatibility, so that cells can adhere, function normally and proliferate. Also, high porosity and a high mechanical resistance are required (NGA & al. [18]; SUCIU & al. [19]). Tissue-engineered scaffolds should provide mechanical properties that can support tissue growth and also have the ability to degrade as new tissue is synthesized (DE LA LASTRA & al. [20]). Thus, another important feature that scaffolds must have is biodegradability (BRIEN [21]). Over the years, experiments with different types of biomaterials have been carried out, finally leading to the development and improvement of BTE (LEE & al. [22]). Cellulose and its derivatives, such as cellulose acetate (CA), are among the most preferred biomaterials for BTE. CA is often chosen because it possesses electrospinning skills and exhibits good mechanical properties, although it presents a low degree of crystallization. More of its features include: nanotoxicity and transparency. Other materials such as polystyrene require the use of a solvent (like chloroform) which is not miscible with water and therefore it can remain in the material, damaging in this way the biocompatibility of the material (KIM & al. [23]). On top of that, CA is preferred for BTE, for in vitro assays, but especially for in vivo experiments because it can easily pass through the kidneys after its degradation, because this type compound presents a smaller molecular weight than 50 kDa. As a further matter, CA is able to encourage osteoblast growth in vitro and bone maturation in vivo (ATILA & al. [24]). Carbon nanotubes (CNTs) are one-dimensional macromolecules with unique properties that have been abundantly used in research since their discovery in 1991. More specifically, cylindrical molecules consisting of carbon atoms found in sp2 conformation and they possess thermal, chemical and mechanical properties (CANCIAN & al. [25]). Their density is similar to that of graphite and much smaller than metal-based materials used for bone tissue engineering. However, CNTs are the most rigid biomaterials and the implantation of these in the bone can improve the mechanical properties of the injured bone. From this point of view, CNTs can on one hand be used to stimulate bone repair, and on the other hand to provide constant mechanical support for bone. On the top of that, this type of scaffold own flexibility and elasticity. Having a one-dimensional structure, they are ideal for the production of composite biomaterials (ZANELLO & al. [26]). They play an important role in the osteoblast differentiation process. Due to the Van der Waals interactions, these materials can form bubbles in an aquatic environment, which can lead to slippage between nanotubes or to cracks due to applied forces (CANCIAN & al. [25]). The aim of this study was (1) to determine growth factors concentration in PRP and (2) to investigate the effect of PRP on fibroblast proliferation, *in vitro* bone differentiation and *in vivo* skin rejuvenation, as primary applications and medical use of PRP.

2. Materials and methods

2.1. Optimization of PRP preparation and characterization in growth factors

Whole blood was collected from adults after obtaining the informed consent and centrifuged twice according to Glofinn technology in order to purify the platelet-rich plasma fraction. Once obtained, the PRP was activated using a calcium chloride solution in order to release the growth factors from α-granules. The concentration of six growth factors found in PRP- EGF, bFGF, VEGF, PDGF, IGF1 and TGF-β1- was quantitatively assessed using Milliplex MAP kits (Millipore) and magnetic beads technology.

2.2. Cell proliferation studies

For proliferation study, a culture of human fibroblasts was used. Briefly, cells were seeded in a low density and cultured in complete Dulbecco Modified Eagle's media (DMEM), supplemented with 10% fetal bovine serum (FBS). After 24 h, treatment with 1 and 10% PRP was added to the media and cultures were monitored for 96 hours to determine the proliferation. After 48 and 96 h in the presence or absence of PRP, cells were stained in green with calcein and assessed by fluorescence microscopy (Olympus).

2.3. Applications- PRP effect on *in vitro* osteogenic differentiation

Next, we wanted to test the effect of PRP during *in vitro* osteogenic differentiation. For this, human adipose-derived stem cells (hASCs) were cultured on the surface of a biomaterial based on calcium acetate (CA) and carbon nanotubes (CNT) that was previously proven to be biocompatible (IONITA & al. [27]). Then, the 3D culture obtained was exposed to osteogenic differentiation media (ThemoFisher) and to treatment with PRP, to determine whether PRP enhances osteogenesis or not. The effect was evaluated both at gene level by qPCR and protein level by immunostaining and fluorescence microscopy. Alkaline phosphatase (Alp) was used as a marker for the osteogenic process and its expression was analyzed both at gene and protein levels.

2.4. Applications- PRP effect on skin rejuvenation

PRP was used to treat patients undergoing skin rejuvenation procedures after obtaining their informed consent. Improvement was monitored during 3-6 months and pictures were taken before and after PRP administration.

All experiments were performed in triplicate. Statistical analysis was performed using GraphPad Prism software, One-way ANOVA algorithm, followed by Bonferroni correction. Statistically significant data was considered for $p<0.05$.

3. Results and discussion

3.1. Concentration of main growth factors found in PRP

PRP's effect relies strongly on its composition in growth factors and proportion between them in order to create a synergic positive effect of tissue regeneration. Here, we adapted a procedure for PRP isolation and purification using a commercial technology (Glofinn) in order to enrich the platelet fraction and consequently the growth factors concentration. Data obtained was analyzed and the resulting PRP fraction consisted of higher concentrations of IGF1, TGF-β1 and PDGF (in the range of 20000-75000 pg/mL), while VEGF, bFGF and EGF were found in the range of 75-100 pg/mL (fig. 1). PDGF, TGF-β and IGF-1 are found in high concentrations in PRP and they modulate fibroblasts chemotaxis and proliferative activity. PDGF and TGF-β are also involved in collagen synthesis, while PDGF and IGF-1 stimulate bone formation. EGF and VEGF are found in lower concentration in PRP and they regulate cell proliferation, angiogenesis and differentiation of epithelial cells (ALSOUSOU & al. [27]).

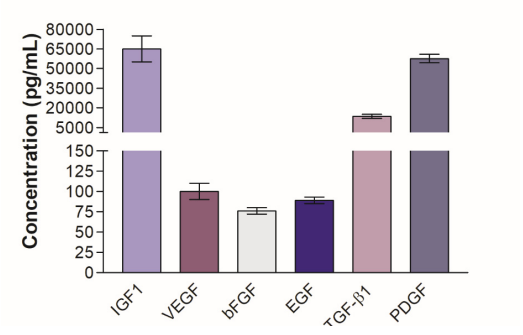

Figure 1. The concentration (pg/mL) of growth factors found
in a fraction of PRP obtained from an adult blood sample.

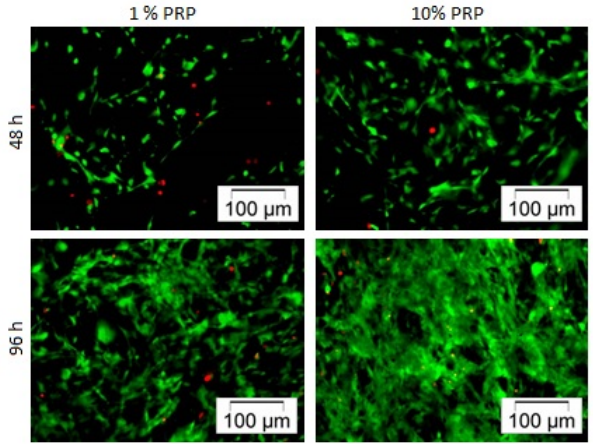

Figure 2. The effect of 1 and 10 % PRP on fibroblast proliferation
evaluated after 48 and 96 hours of culture.

3.2. PRP effect on fibroblast proliferation

The general effect of PRP on cell proliferation was tested on *in vitro* culture of fibroblasts (fig.2). Normally, when a tissue is damaged and regeneration process starts, fibroblasts are recruited from dormant state and determined to proliferate. Here, we wanted to evaluate the effect of PRP *in vitro* on fibroblast proliferation and to test if the quantity of PRP influences the proliferation process. An important cell proliferation rate was observed by fluorescent microscopy in the case of adding 10 % PRP to cell culture media after 4 days of culture, significantly higher than when adding only 1 % PRP. This effect is probably due to the higher growth factors concentration in the case of 10% PRP in the media, which act as activators of the proliferation process. Interestingly, the proportion of dead cells (marked in red) in the culture diminished during proliferation, showing also a non-toxic effect of PRP on fibroblast culture. Cell shape remained constant during treatment with PRP, suggesting that PRP does not affect cell phenotype. Other studies also reported positive effect of PRP on cell proliferation (ZHENG & al. [4]; SINGH & SINGH [5]) and even the use of PRP instead of fetal bovine serum (FBS) as supplement in cell culture media (GONZALES & al. [28]).

3.3. Preliminary study on the effect of PRP on *in vitro* bone regeneration- a potential application for the use of therapeutic properties of PRP

One of the applications of PRP in clinics is the positive contribution to joint and bone defects repair. It has been shown that PRP improves and stimulates the bone formation process, upregulating osteogenesis-specific markers (WEI & al. [13]). Therefore, we investigated the evolution of a stem cell differentiation process to bone lineage *in vitro* in the presence and absence of PRP in the culture media. A material based on CA and CNT which was previously characterized and proven to be biocompatible (IONITA & al. [29]) was used as a support for osteogenic differentiation, considering the possibility to obtain a synergic effect of PRP and CNT on the differentiation process. Fig.3 illustrates (a) gene expression levels for one of the early osteogenic markers- alkaline phosphatase (*alp*) and (b) Alp protein expression as determined by immunostaining and fluorescence microscopy in a culture system obtained on a CA/CNT material designed for BTE.

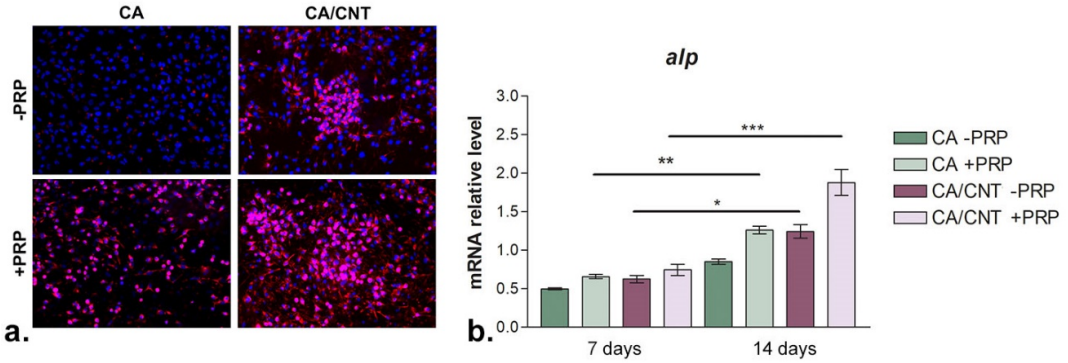

Figure 3. *In vitro* osteogenic differentiation on the surface of CA/CNT biomaterial, in the presence or absence of PRP; (a) Alp protein expression observed by fluorescence microscopy after 14 days of differentiation; (b) profile of *alp* gene expression after 7 and 14 days of differentiation.

Gene expression results showed that the addition of PRP to the differentiation conditions favored the activation of *alp* gene. After 14 days of differentiation, there was a statistically significant increase in *alp* expression in the presence of PRP in cells cultivated on CA biomaterial ($p<0.01$), as well as on CA improved with CNT ($p<0.001$), On the other hand, significant results ($p<0.05$) were also obtained during bone differentiation on the CA/CNT material after 14 days, in the absence of PRP treatment, thus showing the positive effect of CNT on bone formation. Similarly, bone-specific cell groups, as well as Alp expression were found on CA/CNT and to a higher extent on CA/CNT in the presence of PRP, as shown by fluorescence microscopy. Overall, the highest Alp expression was registered in the case of cells cultivated on CA/CNT material and treated with PRP, suggesting that there was an increased osteogenic differentiation due to CNT and PRP synergic effect. Similar positive synergy was described in other studies (El Backly & al. [30]), confirming that PRP enhances osteogenic differentiation process.

3.4. PRP therapy in patients for skin rejuvenation

Correlated with the analysis of PRP composition in growth factors, patients involved in aesthetic restauration procedures received treatment with PRP. After 3-6 months (fig. 4), results showed amelioration of skin defects, better skin elasticity, as well as improved skin wrinkles, texture, and skin tone. In all cases, accelerated cell renewal was observed and it was concluded that PRP could be used as a 'natural filler' to help restore tissue by using skin's own rejuvenation potential.

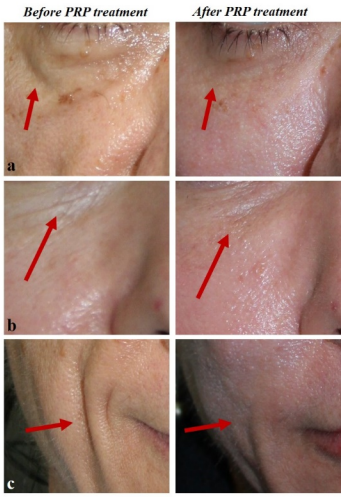

Figure 4. *Skin rejuvenation in patients exposed to treatment with PRP.*

4. Conclusions

PRP is rich in growth factors that positively influence tissue regeneration and cellular processes such as proliferation. Our study confirmed that 10% PRP addition to culture media enhanced cell proliferation. We showed that PRP could be used in BTE because it upregulates the gene expression of osteogenesis markers, such as alkaline phosphatase. This study proved that a synergetic effect can be created by using PRP in cell culture media together with CNT in the biomaterial composition. This confirms that PRP is able to improve wound healing process and represents a powerful tool for aesthetic restauration and regenerative medicine therapies.

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