Freeze-dried versus fresh platelet-rich plasma in acute wound healing of an animal model

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Aim: To compare freeze-dried and fresh platelet-rich plasma (PRP) preparations, in a pre-clinical study. **Materials & methods:** 30 Wistar male rats were used to compare and characterize human PRP which was applied at the perilesional area in an acute wound model, evaluated by macroscopical and histological analysis. **Results:** Despite the increased growth factor concentration after the freeze-drying process, no change in the healing kinetics was observed *in vivo*. Nevertheless, a significant increased number of myofibroblasts was demonstrated in comparison with the fresh PRP group. We also demonstrated a significant increased percentage of blood vessels in comparison with controls in both the superficial and deep epidermis. **Conclusion:** These results encourage randomized clinical trials to evaluate the effectiveness of freeze-dried PRP for skin ulcer treatment.

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With the increase in prevalence of obesity and diabetes, there is currently a simultaneous increase in chronic wounds and tissue repair defects. Chronic wounds have a negative impact upon the quality of life of patients and result in high costs for the health system [1,2]. In the EU 2% of the annual health budget is used for treatment of chronic wounds. 4 months of outpatient treatment costs between 200 and 2000 pounds, and annually 40 million are used to treat ulcers [3,4].

Healing is a complex process which includes hemostasis, inflammation and remodeling, with the aim of restoring a functional barrier. This entire process is regulated by several cell types and amounts of cytokines and/or growth factors. After injury, the growth factors present in macrophages and platelets are released and resulted in an inflammatory phase that influences all aspects of the wound healing process directly or indirectly [5–7]. More than 30 cytokines which play a central role in wound healing, have been detected in platelets, such as: VEGF, EGF, PDGF, TGF- β and IGF [8,9]. Due to the potential of improving wound healing, through molecular and cell induction responses, the use of platelet concentrates has been gaining attention [10,11]. Platelet-rich plasma (PRP) is defined as platelets concentrated over the basal number (four- to nine-fold) in a small plasma volume [12]. Until now, a number of different techniques to prepare PRP, including commercial systems and *in house* preparations, have been described. There is no consensus regarding time and speed of centrifugation, use or not of activators and leukocytes in the PRP preparations which lead to a heterogeneity of results in PRP studies. An attempt in order to standardize heterologous PRP products was made by Muraglia *et al.* focused on culture medium supplement, improving the proliferation, with genetic stability of cells [13,14]. Furthermore, Spanó *et al.* used similar methodology for *in vitro* and *in vivo* tests, in biological membranes with positive results for wound healing [11].

Some studies using PRP to treat ulcers showed no effectiveness [15,16] however others have demonstrated improvement in vascularization, decrease in wound area and rapid epithelization, contributing to an improvement in healing [17,18]. These studies used different protocols, based on *in house* techniques, and only determined platelet counts. However, the concentration of growth factors is also of great importance in the PRP context and a protocol capable of promoting a high number of platelets and growth factor concentration becomes valuable. Thus, in terms of medium culture supplement, Muraglia *et al.* published a standardized protocol showing functional action for culture of mesenchymal stem cells derived from bone marrow, from adipocyte and umbilical cord. They

evaluated the number of platelets, concentration of growth factors, including freeze-dried PRP (FD-PRP) and time of storage [13,14]. Our group recently published a study showing the maintenance of concentration and functional parameters of growth factors of PRP after freeze-dried process in cell culture experiments [19].

The use of FD-PRP can be interesting in clinical practice due to the facility of being ready to use after hydration. Pietramaggiore and colleagues compared treatment of chronic wounds in rats with human fresh frozen or FD-PRP and controls. PRP preparations increased granulation tissue in comparison to controls, spurring new ideas in the regenerative medicine field [20]. The possible use of growth factors derived from platelets, and the potential to prolong the shelf-life of platelet concentrates by freeze-drying becomes a particularly suitable method for improving wound healing treatment, as demonstrated the maintenance of function by *in vitro* tests [13,14,19].

This study aims to advance in the knowledge of healing, offering a biological mean of accelerating the healing process using PRP. As a preclinical study we compared two forms of PRP preparations, fresh and FD-PRP.

With this study we hope to assist to improve the therapeutic approach, offering an aid to treatment based on evidence to be used in clinical practice, in the healing of nonhealing ulcers, contributing to greater quality of life of patients and a decrease in public health costs.

Methodology

Fresh PRP

Two platelet concentrate bags (60 ml each) from a single donor were obtained from the blood bank (Hemocentro of the University of Campinas – Unicamp). The platelet concentrate was transferred to falcon sterile tubes and centrifuged at 700 x g for 17 min, as previously described [21]. 80% of the plasma volume was discarded and 20% was resuspended as PRP. Aliquots were used to quantify platelet number in a hematological counter (CellDyn) and for growth factors quantification. 1 ml was used for treatment as fresh PRP and the remaining volume was used for FD-PRP.

FD-PRP

In sterile vials for lyophilization, 440 µl of fresh PRP was mixed with 20 µl of each solution in a final concentration of Tris 0.05 M, glycine 0.06 M and sucrose solution 0.06 M, totaling 60 µl and homogenized. The vials were placed on the shelf of the lyophilizer, which was stored at -80°C for 2 h to freeze the samples. After 2 h, the shelf was placed in the lyophilizer, reaching the temperature and vacuum programmed, and the lyophilization process occurred for 19 h. After the process the vials were stored at -20°C. This protocol was based on a previous standardization lyophilization study of our group published by da Silva *et al.* [19]. For the experiments the FD-PRP was reconstituted with sterile water at the same volume used before the lyophilization (500 µl) and injected according to the experimental method.

Quantification of growth factors in PRP

Growth factors were quantified in FD-PRP and fresh PRP by multiplex technology (Luminex – Millipore). The growth factors analyzed were: EGF, PDGF-AA, TGF-β1 and VEGF. The protocol was conducted according to the manufacture's procedure.

Experimental animals

30 Wistar male rats were obtained from the Multidisciplinary Center for Biological Research in the Area of Science in Laboratory Animals (CEMIB), Unicamp, SP, Brazil. During the experiments, there were two animals per cage, separated by a steel partition, avoiding, thus, the contact between the rats. The animals were maintained under standard conditions of lighting (cycle of 12 h light/dark) and temperature of 22 ± 2°C. The experiments were performed with rats with 6–8 weeks of age, and all the animals received diet and water *ad libitum*. The procedures were performed in conformity with the Brazilian laws of animal experimentation approved by the Ethical Commission for Animal Use (CEUA-Unicamp).

After intramuscular anesthesia with ketamine (75 mg/kg; Ketalar, Parke-Davis, Brazil) and xilazine (10 mg/kg; União Química Farmacêutica Nacional S/A, Brazil) the animals were placed in prone position and, after shaving the dorsal region, a wound of 1 cm² was made in the midline with the aid of a scissor. The animals were divided into three groups of ten according to treatment: Control – saline, fresh PRP – PRP, and FD-PRP – FD-PRP. We use different animals for each treatment as described by other authors [22]. The use of the same animal with multiple

Table 1. Levels of growth factors in fresh and freeze-dried platelet-rich plasma.

Growth factor (pg/ml)	Freeze-dried PRP	Fresh PRP	Fold dried /fresh
PDGF-AA	192571.0	51783.2	3.72
VEGF	2428.2	1477.9	1.64
EGF	3595.5	1316.9	2.73
TGF	4138.8	1609.1	2.57

PRP: Platelet-rich plasma.

lesions is questionable as each lesion could be exposed to different stimuli, according to the site in the body. In order to minimize this variability, the same animal was used and the number of animals in each group was increased.

The method of application consisted of perilesional injections of 500 μ l of PRP (fresh or FD) or saline using a syringe and needle of 24 G in the day of the wound induction (D0). No cover was used.

Wound closure monitoring

To evaluate wound contraction, a transparent plastic film was placed over the lesion and wound margins were traced at specific time-points: days 0, 3, 7 and 10, as previously standardized [22,23]. After digitalization, the wound area was measured using Image J software (MD, USA). Wound area measurements and wound photographs were obtained on these specific days, until euthanasia, without scab removal. Wound sizes were expressed as percentage of the initial wound area; in this way, the value in cm^2 in the day 0 was considered 100% and the other days were measured in relation to day 0, as follows:

$$\text{Day 0 (cm}^2\text{)} - 100\%$$

$$\text{Day 3 (cm}^2\text{)} - X$$

Histological & immunohistochemistry evaluation of wound healing

Euthanasia was performed ten days after wounding using deep anesthesia. The ulcerated area and adjacent normal skin were excised, formalin-fixed and paraffin-embedded. 5 μ m sections were stained with hematoxylin-eosin and Masson's trichrome for the evaluation of the characteristics of the epithelia and collagen density.

Epithelial thickness was evaluated in Masson's trichrome, using ten pictures obtained with a microscope x10 objective. Each picture was measured in ten points of the epithelia, totaling 100 measures for slide using Image J software [24].

Collagen density was calculated in Masson's trichrome with the evaluation of three pictures for each slide (x10 objective). Each picture was measured using the software Image J, option color deconvolution in an area of 10,000 μm^2 of dermis. Results were expressed as density of collagen/ μm^2 [25].

Immunohistochemistry was performed by heat antigenic recovery, with a monoclonal antibody against α -smooth muscle actin (α -SMA, clone 1A4, 1:300, DBS, CA, USA), to identify functional blood vessels (arterioles) and myofibroblasts. Three animals per group were randomly chosen. The quantification of blood vessels and myofibroblasts were performed through the evaluation of five random fields per animal on the superficial region, and five random fields in the deep region of the granulation tissue using a x20 objective lens in a video microscopic system (Nikon microscope, Infinity 1 video camera) and expressed as the percentage of area. Blood vessels were determined as number of vessels/field.

Statistics

Comparative analyses were performed by Kruskal-Wallis test with Dunns post-test to compare all pairs of columns. The significance level adopted for the statistical tests was 5% ($p \leq 0.05$). Analyses were performed with using GraphPad, version 5.

Results

PRP characterization

Fresh and FD-PRP were characterized according to platelet number and growth factor concentration. The platelet number of fresh PRP was 5714×10^3 cells/ μ l, and the concentrations of growth factors are shown in Table 1.

Figure 1. Kinetics of wound closure in percentage of initial area of the different treatments during days 3 (D3), 7 (D7) and 10 (D10) of experiments. FD-PRP: Freeze-dried PRP; PRP: Platelet-rich plasma.

Figure 2. Macroscopic photographic of the wounds on day 10. (A) Control (saline), (B) PRP, (C) FD-PRP. FD-PRP: Freeze-dried PRP; PRP: Platelet-rich plasma.

The FD-PRP presented higher concentration of all growth factors when compared with fresh PRP from 1.64 to 3.72-fold. Platelet number after freeze-drying process was 268×10^3 cells/ μL , which represents a loss of 95.3% of platelet number.

Wound closure analysis

Figure 1 represents the mean and standard deviation of initial wound area expressed as percentage, measured 3 (D3), 7 (D7) and 10 (D10) days after wounding.

The mean and standard deviation (SD) of area on D3 was $112.9 \pm 16.6\%$, 107.9 ± 21.3 and 100.8 ± 30.2 for control, PRP and FD-PRP, respectively. On D7 the areas were 54.3 ± 28.9 , 63.9 ± 22.8 and 61.6 ± 20.6 for control, PRP and FD-PRP, respectively. On D10 the results were 9.9 ± 6.1 , 13.9 ± 11.2 , 7.7 ± 6.0 for control (saline), PRP and FD-PRP, respectively. Figure 2 demonstrates macroscopic photographic of the wounds at D10. We observed, though not statistically significant, a trend to smaller wound areas with FD-PRP.

Histological analysis

Epithelial thickness

Figure 3 represents epithelial thickness in different groups at D10. The mean and SD of epithelial thickness was $25.37 \pm 7.63 \mu\text{m}$ for control, $24.56 \pm 7.21 \mu\text{m}$ for PRP and $25.52 \pm 5.84 \mu\text{m}$ for FD-PRP (not statistically significant, $p = 0.08876$).

Collagen density

The mean and SD of collagen density at day 10 (D10) was 183.6 ± 32.59 pixels/ μm^2 for control, 174.6 ± 38.65 pixels/ μm^2 for PRP and 193.8 ± 25.36 pixels/ μm^2 for FD-PRP, as shown in Figure 4 (not statistically significant, $p = 0.5679$).

Figure 3. Epithelial thickness of the different treated groups measured by Masson's trichrome on day 10.
FD-PRP: Freeze-dried PRP; PRP: Platelet-rich plasma.

Figure 4. Collagen density by Masson's trichrome of the treated groups on day 10.
FD-PRP: Freeze-dried PRP; PRP: Platelet-rich plasma.

Figure 5. Image of imunohistochemistry marked with anti- α actin for blood vessels and myofibroblasts in the skin. (A) Control (saline), (B) platelet-rich plasma, (C) freeze-dried platelet-rich plasma.

Myofibroblasts through imunohistochemistry

Figure 5 shows the quality of imunohistochemistry image for all the groups.

Quantification of myofibroblasts by area in imunohistochemistry using Image J software showed that the percentage was higher with FD-PRP treatment when compared with the other groups, in deep and superficial epidermis. This increase of myofibroblasts was statistically different when comparing FD-PRP and PRP treatments ($p = 0.0095$) in deep region of epidermis at D10 (Figure 6).

Blood vessel density

Our results obtained by imunohistochemistry showed an increased percentage of blood vessels per area in the group treated with FD-PRP when compared with controls, both in deep and superficial epidermis at D10, as demonstrated in Figure 7.

Figure 6. Quantification of myofibroblasts using immunohistochemistry with smooth α -actin antibody and treated by Image J software, evidencing the mean and standard deviation of treated groups. (A) Superficial epidermis, (B) deep epidermis.

FD-PRP: Freeze-dried PRP; PRP: Platelet-rich plasma.

Figure 7. Quantification of blood vessels by immunohistochemistry using smooth α -actin antibody and treated by Image J software, evidencing the mean and standard deviation and coefficient of variation of the treated groups. (A) Superficial epidermis, (B) deep epidermis.

FD-PRP: Freeze-dried PRP; PRP: Platelet-rich plasma.

Discussion

The reason for the use of the term FD-PRP is that this terminology is used in the majority of the previously published articles [10,19]. We understand that the lyophilization process damages approximately 40–90% of the platelets, however not all of them. Moreover, the action of PRP is mainly derived from the growth factors and not from the platelets. In this way, it was observed an increase in the growth factors concentration, which was an expected result due to the platelet damage caused by lyophilization, with platelets lysis and consequent release of growth factors, increasing the concentration of these proteins.

In our study the application of PRP or FD-PRP did not significantly induce changes in wound healing kinetics measured as contraction when compared with controls during all evaluated days [3,7,10]. Though not significantly different, FD-PRP presented the smallest area in almost all days except for D7. Despite epithelial thickness and collagen density not differing among the groups, blood vessels density in percentage of area was significantly higher in those treated with FD-PRP in comparison to the controls, both in superficial and deep regions of epidermis. One relevant result of this study was the quantification of growth factors in PRP, and we believe that this finding is a result of the high concentration of VEGF present in FD-PRP and delivered to the wound area. VEGF plays a

central role in promoting angiogenesis during wound repair and previous studies demonstrated accelerated wound closure with collagen-binding VEGF topically [26].

The use of PRP is widely used for wound treatment; however, this use remains controversial, with preclinical experiments showing different results and no consensus regarding the use of PRP until now. Mehrjerdi *et al.* (2008) evaluated the topical effect of autologous PRP without leukocytes versus saline in the healing of dog wounds [15]. Macroscopic and histological evaluations demonstrated no differences in the lesion area between both treatments. Indirect collagen (hydroxyproline) and histological evaluation, demonstrated a trend, though not statistically significant, of increase of the re-epithelization with PRP treatment. These results suggested that PRP did not accelerate or improve the quality of tissue repair with topical use in the treatment of animal acute wounds [11]. Osivar *et al.* evaluated the use of PRP without leukocytes in chronic wounds of rabbits compared with saline, with a cover of polyurethane film daily. Through macroscopic and histological evaluation they showed that the healing time of the group treated with PRP was shorter, associated to an increase in the mean of vascular density, demonstrating positive effects in wound healing [18]. Spanò *et al.* evaluated two bio membranes of PRP, with different concentrations of platelets, for chronic wound regeneration. In an *in vitro* model the proliferation of keratinocytes, and mesenchymal stem cells derived from bone marrow was evaluated by measuring the level and kinetics of growth factors. Although both bio membranes induced wound healing, a higher platelet concentration resulted in a slowdown of the membrane resorption that interfered with the skin healing [11].

The use of lyophilized or frozen platelets has been studied due to the limitation of short half-life of platelet concentrate. In this way, the search for methods to improve platelet stabilization using biochemicals to preserve their biological activities has acquired great value [13,14,27,28], however the interference of lyophilization in platelet hemostatic function remains questionable [29].

PRP is currently being investigated for a wide range of therapeutic indications, focusing on the growth factors and cytokines present in PRP granules. For this means FD-PRP could be an interesting approach, based on the ability to maintain a stable preparation, with a single blood collection for multiple samples of PRP that can allow multiple applications. Indeed, we produced an autologous product with high concentration of growth factors. This is of great value in clinical practice, however there is little basic and preclinical research regarding this area.

Pietramaggiore and colleagues contributed to the field of freeze-dried and wound healing. The better effectiveness of lyophilized PRP with and without stabilizing agents in the treatment of wounds of diabetic mice compared with control was evidenced by an increased area of granulation tissue in the former. There was no difference in the concentration of growth factors (PDGF-AB, TGF- β 1, VEGF and EGF) among the different preparations, nevertheless the *in vitro* analysis showed an increase in cellular proliferation with lyophilized PRP [13,14,19]. Another experiment comparing single or multiple injection applications in the wound area of diabetic mice with FD-PRP containing trehalose, fresh PRP, multiple doses of recombinant VEGF or no treatment demonstrated multiple doses of VEGF and the use of FD-PRP promoted a faster wound closure when compared with the nontreated group [30]. Promising results with higher proliferation rate, revascularization and better re-epithelization for diabetic wound healing have been described with the use of micronized dermis in association with FD PRP [31].

Regarding the induction of differentiation into myofibroblasts with the use of PRP, the results in literature remain controversial. TGF- β is a well-known key molecule inducer for myofibroblastic differentiation. However, PRP is a complex mixture including other molecules that can counteract this myofibroblast inductive-effect. The induction was verified by authors that use PRP rich in leukocytes (L-PRP) [32,33]. Anitua *et al.* (2013, 2012) did not show this differentiation with P-PRP (called PRGF) *in vitro*, concluding that the differences found regarding myofibroblasts could be due to the different PRP composition in terms of leukocyte content [34,35]. In our study, we showed an increase in the concentration of myofibroblasts with the use of FD-PRP, and we believe that this effect was achieved due to the high concentration of growth factors induced by lyophilization process.

Some studies have suggested a positive influence of the concentrate platelets on angiogenesis. Notodihardjo *et al.* used the PRP releasate (PRPr) impregnated in a biodegradable hydrogel gelatin for wound healing in mice. After 14 days of wounding, an increase in the number of capillary formation was verified [9]. Cheng *et al.* also reported an increase in the number of new blood vessels in the group treated with PRP for hair growth [36]. The increase in blood vessels showed in our study corroborate previous articles, suggesting that the significant increase of blood vessels in the group treated with FD-PRP is due to the high concentration of growth factors induced by lyophilization.

One limitation of this study was the acute ulcer model, and we could not rule out the possibility of a better impact in treatment of chronic wounds.

Conclusion

In conclusion, results obtained with the treatment of acute ulcer in rats with PRP or FD-PRP point towards lyophilization as a good option in clinical practice. This is very interesting especially for those patients who require multiple applications.

Increased VEGF concentration in FD-PRP may be related to higher vascularization demonstrated with this treatment, which is of great value for wound healing.

Translational perspective

Since the use of PRP for the treatment of wounds has been showing promise results, the possibility of increasing the shelf-life of PRP products with lyophilization is of great interest, especially for patients who require multiple applications. The use FD-PRP is practical, safe and of great interest for clinical practice. The next step is the evaluation in clinical trials for wound healing of different etiologies.

Summary points

- The use of platelet-rich plasma (PRP) is suitable for wounds due to the presence of multiple cytokines with important roles in regeneration/ healing.
- The freeze-drying process is a possibility to use in clinical practice to enhance the shelf-life of PRP products.
- Characterization of PRP products is realized through platelet number and growth factors concentration.
- *In vivo* evaluation of PRP function is conducted in fresh, and after the freeze-drying process.
- Macroscopic evaluation of wound healing is done through kinetics of wound closure according to time.
- Histologic evaluation of PRP products is performed by the epithelial thickness, collagen density and immunohistochemistry for myofibroblasts and blood vessels in deep and superficial epidermis – to evaluate the healing process of the different PRP products.
- A significant increase in blood vessels and myofibroblasts number when the animals were treated with freeze-dried PRP demonstrated promising results.
- Encouraging for clinical trials due to the increase of vascularization, the freeze-drying process represents an option for patients who require multiple applications; maintaining the properties of fresh PRP and showing positive results.

Author contributions

SC Huber was the principal investigator, responsible of the project and animal manipulation. SAL Montalvão was part of the study design, performed statistical analysis and reviewed and correct the manuscript. JL Cunha, SC Huber and LQ Silva were presented in the technical performance, such as the growth factors quantification and buffers standardization. JM Annichino-Bizzacchi guided this project, drafting, revising and finalizing the manuscript.

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Ethical conduct of research

The procedures were performed in conformity with the Brazilian laws of animal experimentation. Ethical Commission for Animal Use (CEUA-Unicamp) approved of the experimental procedures (CPEA:2290-1).

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Availability of data & material

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

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