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In-vitro production and pre-validation of lyophilized canine platelet-rich plasma for therapeutic use[1]

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ABSTRACT.- Freitas N.P.P., Maior M.M.M.S., Silva B.A.P., Bezerra M.R.L., Nunes J.F., Neto S.G., Tavares K.C.S. & Martins L.T. 2021. ***In-vitro* production and pre-validation of lyophilized canine platelet-rich plasma for therapeutic use.** *Pesquisa Veterinária Brasileira 41:e06999, 2021.* Núcleo de Biologia Experimental, Universidade de Fortaleza, Av. Dr. Valmir Pontes 300, Edson Queiroz, Fortaleza, CE 60811-905, Brazil. E-mail: nataliafreitasvet@gmail.com

Platelet-rich plasma (PRP) has been considered a promising therapeutic alternative, since platelets are rich in growth factors that are used in the Regenerative Medicine field. However, fresh PRP cannot be stored for long periods. This study aimed to develop a protocol for obtaining lyophilized canine PRP capable of maintaining viability after its reconstitution. For that purpose, canine PRP extraction and lyophilization protocols were initially tested. Subsequently, assays were carried out to quantify the growth factors VEGF and TGF-β, before and after the lyophilization process, gelation test and the three-dimensional gel structure analysis of the reconstituted lyophilized PRP by electron microscopy, as well as *in vitro* cell proliferation test in lyophilized PRP gel. Additionally, the immunogenicity test was performed, using allogeneic samples of lyophilized PRP. The results showed that the lyophilized PRP had adequate therapeutic concentrations of growth factors VEGF and TGF-β (9.1pg/mL and 6161.6pg/mL, respectively). The reconstituted PRP gel after lyophilization showed an *in vitro* durability of 10 days. Its electron microscopy structure was similar to that of fresh PRP. In the cell proliferation test, an intense division process was verified in mesenchymal stem cells (MSCs) through the three-dimensional mesh structure of the lyophilized PRP gel. The immunogenicity test showed no evidence of an immune reaction. The findings were promising, suggesting the possibility of having a lyophilized canine PRP that can be marketed. New *in vivo* and *in vitro* studies must be carried out for therapeutic confirmation.

INDEX TERMS: In-vitro production, lyophilization, canine, platelet-rich plasma, regenerative medicine, healing, dogs.

RESUMO.- [Produção e validação de plasma rico em plaquetas canino liofilizado para fins de utilização terapêutica.] O plasma rico em plaquetas (PRP) é uma alternativa terapêutica promissora, pois as plaquetas são ricas em fatores de crescimento com ação na regeneração de tecidos. No entanto, o PRP fresco não pode ser armazenado por longos períodos. Esse trabalho teve como objetivo desenvolver um protocolo de obtenção de PRP liofilizado canino capaz de manter a viabilidade pós reconstituição. Portanto, foram testados diversos protocolos de extração e liofilização. Para validação do PRP canino liofilizado foi analisada a concentração dos fatores de crescimento VEGF e TGF-β antes e após o processo de liofilização, a estrutura tridimensional do PRP liofilizado reconstituído em forma

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de gel por microscopia eletrônica e seu efeito in vitro na proliferação de células-tronco mesenquimais. Os resultados demonstraram que o PRP liofilizado apresentou concentrações terapêuticas adequadas dos fatores de crescimento VEGF e TGF- β (9,1pg/ml e 6161,6pg/ml, respectivamente). O gel de PRP reconstituído após liofilização apresentou uma durabilidade *in vitro* de 10 dias, sua estrutura tridimensional mostrou-se semelhante ao PRP fresco e proporcionou intensa proliferação de células-tronco mesenquimais durante o cultivo. O teste de imunogenicidade não demonstrou evidências de reação imune. Os achados foram promissores, sugerindo a possibilidade de uso de PRP canino liofilizado para o mercado. Novos estudos *in vivo* e *in vitro* deverão ser conduzidos para comprovação terapêutica.

TERMOS DE INDEXAÇÃO: Produção *in vitro*, plasma rico em plaquetas, caninos, liofilização, medicina regenerativa, cicatrização, cães.

INTRODUCTION

The tissue regeneration capacity is part of the nature of living organisms, which is differentiated and involves more or less complex biomolecular reactions (Tambella et al. 2018). In general, it can be observed that there are two possibilities of regeneration that take place with the functional restoration of the tissue or the formation of fibrotic scar with tissue restoration, but with tissue functional loss (Ciccocioppo et al. 2019). In this context, platelets play a fundamental role as, once activated, they secrete a wide range of growth, hemostatic, angiogenic and pro-inflammatory factors, interleukins and metalloproteinases, among other bioactive molecules (Kaplan et al. 1979, Assoian et al 1983, Brunner et al. 1993, Banks et al. 1998, Coppinger et al. 2004, Blair & Flaumenhaft 2009, Garbin & Olver 2020). These molecules are contained in alpha-platelet granules and act directly and indirectly on the regenerative process, stimulating functions such as cell proliferation/mitogenesis, angiogenesis, cell differentiation, chemotaxis, extracellular matrix formation, collagen synthesis and inflammatory modulation, finally culminating in tissue repair and regeneration (Antoniades & Williams 1983, Marx 2004, Cole et al. 2010, Malhotra et al. 2013, Dhurat & Sukesh 2014, Shiga et al. 2017)

Aiming to increasingly validate effective and physiological therapies to induce and aid the regenerative process, preventing tissue function loss, several studies have reported the potentiality of using platelet-rich plasma (PRP) as a therapeutic alternative (Tambella et al. 2018, Pereira et al 2019, Acebes-Huerta et al. 2020). PRP is a blood product that has a considerably higher concentration of platelets than that found in whole blood (Lee et al. 2013, Pereira et al. 2019). Therefore, PRP constitutes a very rich fraction in growth factor classes and bioactive molecules that are physiologically active in tissue regeneration. Consequently, it can be considered that therapies based on PRP use are based on a supplementary *in situ* application of substances that are normally found in the physiological regeneration process. Several favorable results regarding the use of PRP for regeneration of different types of tissue injuries in different species have been published to date (Szponder et al. 2018, Pereira et al. 2019, Venator et al. 2020).

Despite the high therapeutic potential and wide applicability, the PRP use in the veterinary clinic still has important gaps to be overcome, such as: (a) the use of several protocols for PRP production in a relatively empirical way in the daily life of Regenerative Medicine, which results in inconsistencies regarding PRP composition and, consequently, in its therapeutic potential; (b) the fact that clinics do not normally have quality control systems that can minimally ensure the stability of variables such as composition, quality, safety and therapeutic potential of different PRP production batches; (c) the fact that therapies are based on the use of autologous PRP, produced from possibly debilitated animals, which can negatively influence the quality and therapeutic potential of the PRP, in addition to, in certain cases, completely preventing the possibility of using this therapeutic approach; and (d) PRP has a very short shelf-life, which amounts to four to six hours after collection, at room temperature (Hauschild et al. 2017) (Arthrex ACP®), making transport and storage practices markedly difficult.

In view of the current scenario, it can be inferred that the production of lyophilized PRP and its use in allogeneic treatments could generate significant advances towards the solution of several limitations currently associated to the use of PRP in the Regenerative Medicine field. Therefore, issues related to the difficulty in transporting and storing PRP, PRP production from young and proven healthy donors, problems with quality control associated with PRP composition, safety and therapeutic potential would be considerably solved.

This preliminary study aimed to assess the *in vitro* viability of using lyophilized canine PRP through assays aimed at quantifying growth factors, gelation test followed by electron microscopy of the gel structure, CTM proliferation test in the PRP gel mesh and immunogenicity test.

MATERIALS AND METHODS

Ethics statement. The experiment was conducted at "Núcleo de Biologia Experimental" (Nubex - Experimental Biology Nucleus) of the "Universidade de Fortaleza" (Unifor), Fortaleza/CE, Brazil, in accordance with the rules established by the National Council for Control of Animal Experimentation (CONCEA), and it was approved by the Ethics Committee on Animal Use (CEUA-Unifor) at the meeting held on March 27, 2020 (Protocol #8614100220).

PRP preparation and lyophilization. PRP samples were produced from a pool of canine blood collected from three young and healthy donors (Table 1), received in 500mL bags containing sodium citrate anticoagulant, purchased from a specialized company (LABOVET - Clinical Analysis Laboratory). In order to obtain a more homogeneous sample and, consequently, decrease variability and allow a decrease in the number of animals to be used, only medium-sized (between 8 and 12kg), adult (three to five years old) and clinically-healthy male dogs were used in the study. The blood processing protocol for preparing the PRP was defined after reviewing and modifying several other protocols described in the literature (Lee et al. 2013, Malhotra et al. 2013, Shiga et al. 2016, 2017) until achieving the determination

Table 1. Platelet and leukocyte count in total blood from three canine donor patients

Patient	Total blood	
	Platelets	Leukocyte
1	278.000/µL	12.700/µL
2	220.000/µL	9.900/µL
3	252.000/µL	8.300/µL

of parameters that would result in a minimally-stable platelet and leukocyte count between different production batches. All procedures related to PRP preparation were performed in a laboratory with a biosafety certificate (Biosafety Certificate Number 0294/10), using laminar flow, sterile materials and reagents. A blood processing system based on the use of two consecutive centrifugation steps (model 5810 - Eppendorf centrifuge) was used. After the centrifugation, the whole blood was initially placed in 50-mL tubes with 25mL of blood each. The first centrifugation, performed to separate the red blood cells from the blood plasma, was carried out at 720g for 10 minutes, at a temperature of 15°C. Blood plasma was removed and redistributed in 50-mL tubes with 40mL of plasma each and submitted to the second centrifugation step, which was carried out at 2170g for 20 minutes, at a temperature of 15°C. After the second stage, the upper two-thirds, called platelet-poor plasma (PPP), was removed and the lower third (final 5mL of each tube), which is considered the PRP, was submitted to the final count of the number of platelets and leukocytes.

PRP samples destined for the lyophilization process received 35mM of trehalose (Sigma-Aldrich) (Wolkers et al. 2001), were stored in glass bottles at volumes of 1 to 3mL and frozen in an ultra-freezer at -80°C. The PRP samples were dried out in a bench lyophilizer (Model L101 - Liotop). The still frozen samples were stored in the lyophilizer chamber already in operation and kept at -55°C under 34μHg pressure until completely dried. The dried products were stored in a hermetically sealed desiccator storage chamber containing silica, for 10 to 20 days, until the validation analyses were performed. Prior to the performance of all validation analyses, the lyophilized PRP was reconstituted to a liquid state. For this purpose, ultrapure water (Sigma-Aldrich) was added to an amount equal to the original volume of the fresh PRP samples before the lyophilization process.

Quantification of growth factors. Reconstituted PRP samples after lyophilization and fresh PRP control samples processed on the same day of the quantification experiment were submitted to analysis by immunoenzymatic tests using enzyme-linked immunosorbent assay (ELISA) kits. The experiment was carried out twice and the samples were analyzed in triplicates. To determine the concentration of the canine vascular endothelial growth factor (VEGF), the Canine VEGF Quantikine ELISA Kit (R&D Systems, Minneapolis/MN) was used and for the canine transforming growth factor beta (TGF-β) the Mouse/Rat/Porcine/Canine TGF-beta 1 Quantikine ELISA Kit (R&D Systems™, Minneapolis/MN) was used. The analyses were performed according to the manufacturer's protocols. The absorbance was read at 450nm wavelengths, in an ELISA reader (BioRad Laboratories™), and correlated to a standard with a range of 39.1-2,500pg/mL for VEGF and 31.2-2,000pg/mL for TGF-β. Samples that were outside this range of results were re-analyzed after further dilution. The regression curve of the analysis and the conversion of optical densities obtained in the readings were carried out with the software Prism GraphPad®.

Gelation test. The gelation capacity and durability of the reconstituted PRP gel after lyophilization was tested, in addition to the microstructure of the gel, which was analyzed by electron microscopy. As controls, fresh PRP and fibrin gel (FG) samples were used for the gelation test, whereas only FG samples were used as control for the electron microscopy analysis.

i) Gelation test: the preparation of reconstituted PRP gel after lyophilization and fresh PRP gel was carried out by adding 40mM of calcium chloride (Sigma-Aldrich) (Silver et al. 1995). For the FG production, a fibrinogen precipitate obtained by cryoprecipitation, plus 400 IU of bovine thrombin and 40mM of calcium chloride was used, as described by Silver et al. (1995), with modifications. Samples of reconstituted PRP gel after lyophilization were evaluated in comparison with samples of fresh PRP and FG. A volume of 3mL of each of the three different gel types was individually placed in glass bottles (Fig.1), kept in an incubator with saturated humidity, at 37°C and 5% CO_2 for a period of 30 days. Visual assessment of the consistency and integrity of the gel structure was performed daily. For the integrity analysis, the volume of liquid material formed from the degradation of the gel samples was also measured.

ii) Electron microscopy: after the production of the reconstituted PRP gel after lyophilization and FG, as previously described, the samples were placed on Petri dishes and fixed using a modified Karnovsky fixative solution, consisting of 2.5% glutaraldehyde and 2.5% formaldehyde in 0.05M sodium cacodylate buffer with a 7.2 pH. Subsequently, the samples were dehydrated in an alcoholic series as follows: 35% ethanol for 5 minutes; 50% ethanol for 5 minutes; 70% ethanol for 5 minutes; 80% ethanol for 10 minutes; 95% ethanol for 10 minutes; 100% ethanol for 10 minutes; and again 100% ethanol for 10 minutes. For the electron microscopy, the samples were dried by a critical point drying technique, fixed with double-sided carbon tape, mounted on aluminum stubs and submitted to silver metallization with a 20-nm cover layer. The images were obtained with the use of a secondary electron (SE) detector, with acceleration voltage ranging between 5.0 and 7.5kV and working distance (WD) between 9.3 and 10.8mm. The images were recorded at the magnifications of 1,000x, 5,000x and 10,000x.

Cell proliferation test. The cell proliferation test was performed in a culture of canine MSCs in the reconstituted PRP gel mesh after lyophilization. For this purpose, canine MSCs at passage 1 were purchased from Fortgen Technologies S/S Ltd. and subsequently grown in low glucose DMEM medium (Gibco®) plus 10% fetal bovine serum (Gibco®) and 1% antibiotic/antimycotic solution (Gibco®) until passage 3. The MSCs were centrifuged at 400g for 10 minutes, resuspended in 2mL of reconstituted PRP after lyophilization, then 40mM of calcium chloride was added to form the gel and the material was then placed on 60-mm Petri dishes and added to

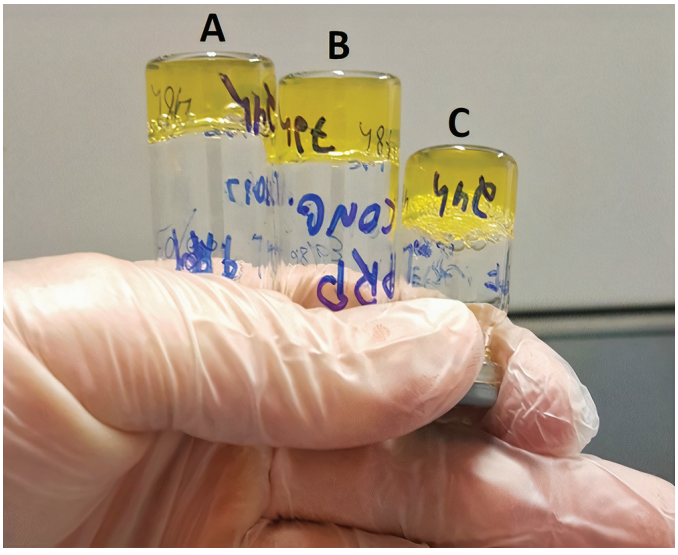

Fig.1.Platelet-rich plasma (PRP) gel integrity test. Observe the gel formation a few seconds after adding calcium chloride and maintenance of the PRP natural properties after lyophilization. Fresh PRP gel sample (A), reconstituted PRP gel after lyophilization (B) and FG in the gelation test (C).

1mL of culture medium. The samples were kept in an incubator with saturated humidity, at 37°C and with 5% CO_2 for 10 days. The culture medium was changed every 3 days. The evaluation of cell proliferation through the PRP gel mesh was carried out using an inverted optical microscope (Nikon Eclipse Ti-S).

Immunogenicity test. A crossmatch test was performed to detect the presence of preformed anti-leukocyte antigen antibodies in blood samples from recipients against leukocyte antigens present in samples of reconstituted canine PRP after lyophilization. In this case, a crossmatch test with T lymphocytes detects the presence of antibodies against antigens of the Class I Histocompatibility Complex and a crossmatch test with B lymphocytes detects the presence of preformed antibodies against antigens of the Class I and/or II Histocompatibility Complex. The crossmatch test was performed as recommended by Salvalaggio et al. (2009) with minor modifications. The evaluation of the cytotoxicity reaction was performed based on the percentage of cells that underwent cell lysis (positive reaction), represented by the following scores:

Ø- indeterminate due to the presence of bubbles, few or no cells
Score 1: up to 10% of cell lysis (negative)
Score 2: 10 to 30% of cell lysis (doubtful negative)
Score 3: 30 to 50% of cell lysis (doubtful positive)
Score 4: 50 to 80% of cell lysis (positive)
Score 5: 80 to 100% cell lysis (positive)

As a positive control, a crossmatch test was performed between B and T lymphocytes isolated from samples of reconstituted canine PRP after lyophilization and human blood serum from a sample bank maintained by the "Centro de Hematologia e Hemoterapia do Ceara" (HEMOCE - Hematology and Hemotherapy Center of the State of Ceará), Brazil.

RESULTS

PRP production and lyophilization

It was not possible to reproduce results reported in previous studies regarding the repeatability of important PRP characteristics, such as clarity, platelet and leukocyte counts. Thus, after several adjustments in the time and velocity variables, in addition to an adjustment related to the size of the centrifuge tubes and the volume of blood allocated in these containers, an effective and repeatable protocol was achieved regarding the PRP quality based on its clarity, platelet and leukocyte count. Even the model of the centrifuge used in the study can influence the results, since the repetition of all variables and parameters resulted in different final characteristics of the PRP obtained from the centrifugation in different brands of equipment. As recommended by previous studies (Lee et al. 2013, Malhotra et al. 2013, Shiga et al. 2016, 2017), the protocol established in this study provided a consistent platelet count result that was 3 to 5-fold higher and a leukocyte count that was 5 to 7-fold lower than the donors' whole blood (Table 2).

The total drying of the PRP samples submitted to the lyophilization process occurred between 36 and 48 hours. The effectiveness of the lyophilization protocol, including the use of 35mM trehalose as a cryoprotectant, was demonstrated by assessing how easily the lyophilized product was reconstituted by adding ultrapure water and through the other validation tests performed in the present study. The lyophilized products were reconstituted quickly and completely by shaking the vials in circular movements for approximately 30 seconds. The results of the other validation tests are described below.

Quantification of growth factors

As shown in Table 1, the concentrations of VEGF and TGF-β growth factors in the reconstituted PRP samples after lyophilization were higher than in fresh PRP. Thus, it was observed that PRP samples submitted to the lyophilization process showed concentrations of the VEGF and TGF-β factors of 63.9% and 159.4%, respectively, compared to fresh PRP samples.

Gelation test

Gel formation after the addition of calcium chloride occurred within a few seconds, very similarly between samples of reconstituted PRP after lyophilization and fresh PRP. The analysis of durability showed total maintenance of gel integrity (100% gel and 0% liquid) for 10 days for samples of lyophilized and fresh PRP gel, and for 5 days for FG (Table 3). From then on, it was possible to observe the beginning of the disintegration process through the formation of liquid material near the edges of the gel. At the end of 30 days of evaluation, the percentage of gel/liquid was 52/48, 53/47 and 30/70 for samples of reconstituted PRP gel after lyophilization, fresh PRP gel and FG, respectively.

In addition to the gelation test that confirmed the maintenance of the PRP natural properties after the lyophilization process, the microstructure of the reconstituted PRP gel after lyophilization was analyzed by electron microscopy in comparison to the FG. As shown in Figure 2-7, there was a marked similarity between the PRP gel and the FG samples in terms of porosity, pore size and general microstructure of the three-dimensional mesh, especially under a magnification of 1000x. Under magnifications of 5,000x and 10,000x, it was possible to observe that the pore size was slightly larger in the PRP gel samples when compared to that of the FG.

Cell proliferation test

The evaluation of cell proliferation and, consequently, of biocompatibility between the reconstituted PRP after lyophilization and MSCs showed promising results, as intense cell proliferation was observed in the three-dimensional mesh of the gel. Figure 8-10 shows that cell multiplication occurred actively within the PRP gel mesh over the 6-day culture period. On day 6, however, the MSCs showed a morphology that was

Table 2. Platelet and leukocyte count from a pool of canine fresh and lyophilized PRP

Cell type	Pool from PRP	
	Fresh	Lyophilized
Platelets	750.000/µL	788.000/µL
Leukocyte	250/µL	0/µL

PRP = platelet-rich plasma.

Table 3. Test of integrity and durability of the reconstituted canine PRP gel after lyophilization

Samples	Durability
Fresh PRP	10 days
Lyophilized PRP	10 days
Fibrin gel	5 days

Samples of fresh PRP and fibrin gel were used as controls; PRP = platelet-rich plasma.

slightly different from the fibroblastoid profile normally observed in MSC cultures. The cells had a slightly thinner shape than normal, in addition to a broader and more complex intercellular contact network than commonly found in MSC cultures using a standard culture medium.

Immunogenicity test

The reconstituted canine PRP after lyophilization resulted in a negative reaction (cell lysis rate <10%) when submitted to a crossmatch test with blood serum from the recipient dog. In this case, it can be inferred that the antibody activity present in the blood serum of the recipient dog against B and T lymphocytes extracted from lyophilized canine PRP samples was nonexistent or negligible. However, when lyophilized canine PRP samples were submitted to crossmatch test with human blood serum samples, used as a positive control, the reaction was positive and classified with a score of 5 (80 to 100% cell lysis).

DISCUSSION

The regenerative potential of PRP has resulted in both the generation of several studies aimed at analyzing its composition and therapeutic effect when used in the treatment of several clinical conditions (Tambella et al. 2018, Pereira et al. 2019, Acebes-Huerta et al. 2020) and its use in the clinical and surgical routine of Veterinary and Human Medicine. The great interest in PRP use is based on the very rich composition of growth factors and other bioactive molecules secreted by platelets when applied *in situ*, for the treatment of different types of lesions, in the form of PRP. Among the main factors and molecules involved in this process, hemostatic factors (i.e., factor V and fibrinogen), angiogenic factors (i.e., angiogenin

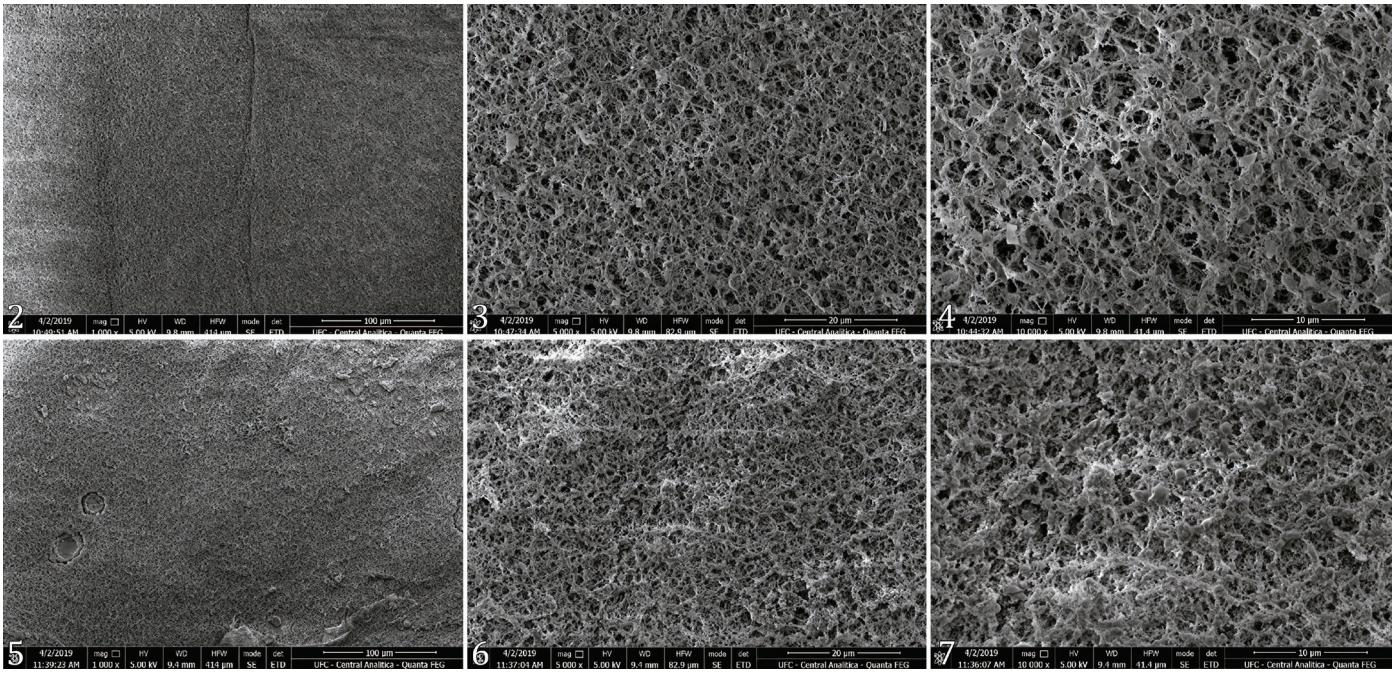

Fig.2-7.Electron microscopy assay carried out in samples of reconstituted platelet-rich plasma (PRP) gel after lyophilization and fibrin gel (FG). Observe a marked similarity between PRP and FG gel samples regarding porosity, pore size and general microstructure of the three-dimensional mesh, under 1000x magnification and a subtle increase in the pore size of the PRP gel samples in relation to FG, at the magnifications of 5000 and 10000x. (**2**-**4**) PRP gel samples at magnifications of 1000, 5000 and 10000x, respectively. (**5**-**7**) FG samples at magnifications of 1000, 5000 and 10000x, respectively.

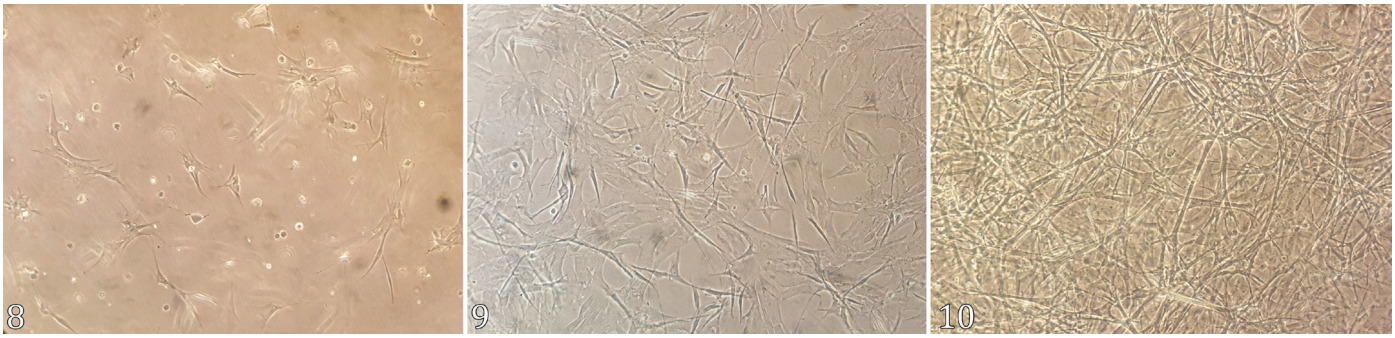

Fig.8-10.Cell proliferation test of canine mesenchymal stem cells (MSCs). The figure shows that cell multiplication occurred actively within the three-dimensional mesh of platelet-rich plasma (PRP) gel over the 6-day culture period after lyophilization.

and vascular endothelial growth factor - VEGF, fibroblastic growth factor - FGF, transforming growth factor beta - TGF-β), platelet-derived growth factor - PDGF, epidermal growth factor - EGF), proteases (i.e., metalloproteinase 9 - MMP-9) and pro-inflammatory factors (i.e., tumor necrosis factor-α - TNF-α) and interleukin-1 beta (IL-1β), among others (Kaplan et al. 1979, Assoian et al. 1983, Brunner et al. 1993, Banks et al. 1998, Coppinger et al. 2004, Blair & Flaumenhaft 2009).

Despite several studies and use in clinical and surgical routines, issues related to the repeatability of protocols and quality control related to the production of PRP are important gaps yet to be solved. When carrying out the present study, different protocols described in the literature were attempted for the production of canine PRP. However, there was a great difficulty to reproduce the results, considering variables such as plasma clarity, platelet and leukocyte count, even when using protocols apparently identical to what has been described in different publications (Lee et al. 2013, Malhotra et al. 2013, Shiga et al. 2016, 2017). This finding corroborates the importance of standardization and, possibly, of an industrialized PRP production, following broadly controlled production and validation conditions. It is known that clinicians produce PRP under poorly controlled and repeatable conditions, which may be responsible for insufficient and variable results. The protocol used when carrying out this study was defined based on changes in the variables time, velocity and temperature of the first and second centrifugation until clear plasma was repeatedly obtained, as well as adequate platelet and leukocyte count in the PRP, which, in turn, has a direct association with PRP therapeutic potential and immunogenicity, respectively (Slichter & Harker 1976).

A highly desirable feature for any biomaterial consists of prolonged stability and shelf-life, which can greatly facilitate issues related to the dissemination of the technique use and a larger-scale production while in compliance with quality control standards. The PRP shows exactly opposite characteristics, and it is recommended that its application should not exceed the period of four to six hours from the moment it is extracted from whole blood, until its application (Sobczyńska-rak et al. 2014, Hauschild et al. 2017) (Arthrex ACP®). Lyophilization is recognized as a process capable of maintaining the chemical properties and the therapeutic effect of bioactive molecules and drugs for long periods (Rocha et al. 2017). In the case of PRP, despite platelet sensitivity and instability, there are subsidies in the literature that support the viability of their lyophilization (Rocha et al. 2017). In the present study, a non-toxic cryoprotectant, trehalose (Crowe et al. 2001, Wang et al. 2015) was used, aiming to stabilize the PRP samples for lyophilization, in addition to allowing the production of a harmless compound that can be applied directly to patients after reconstitution without the risk of side effects. The established lyophilization protocol showed to be effective, allowing rapid and total reconstitution of dry products through the addition of ultrapure water, conservation of growth factors and maintenance of the gel formation property.

The quantification of growth factors constitutes one of the most representative tests used to analyze the effectiveness of PRP extraction and lyophilization processes. In this study, the quantification of VEGF and TGF-β was higher in lyophilized canine PRP samples than in fresh samples (Table 4). The most likely explanation for this finding lies in the phenomenon of platelet activation, which is well described in the literature and must have occurred more significantly throughout the PRP lyophilization and reconstitution processes (Spicer & Mikos 2010). From the perspective of the therapeutic potential of the lyophilized PRP samples and, consequently, of the viability of the lyophilization process used in the study, the obtained results can be viewed optimistically, since the main obstacle to be overcome in the first instance was to avoid reducing the concentration of growth factors after the lyophilization. It can be inferred that lyophilized samples may exert their regenerative function through the presence of growth factors in their free form in reconstituted PRP, as well as through the *in situ* activation of the remaining intact platelets (Sánchez et al. 2007, Sundman et al. 2011). Complementary studies should be carried out to quantify the number of platelets in PRP samples before and after the lyophilization process, in addition to measuring the concentration of other growth factors present in the PRP. Regarding the quantification of VEGF and TGF-β, growth factors involved in regenerative activities such as neovascularization, mitogenesis and tissue repair (Tang & Pikal 2004), the concentrations obtained in this study corroborate the values reported in the literature (Clifford et al. 2001, Shiga et al. 2016, 2017).

Gelation capacity is a PRP property that is normally activated in the presence of calcium ions (Tangsupati & Murdiastuti 2018). Thus, the potential for gel formation can be used as an assay to analyze the preservation of PRP natural properties after different processing methods, including lyophilization. Moreover, the use of PRP in the gel state can represent a therapeutic strategy for delivery of growth factors, other bioactive molecules and even drugs, in addition to being a framework for cell therapy through the application of stem cells enmeshed in the three-dimensional gel mesh (Clifford et al. 2001).

In this study, samples of reconstituted PRP after lyophilization, samples of fresh PRP and FG samples were submitted to the gelation test. The durability of the gel prepared from lyophilized samples was similar to that of fresh PRP gel and superior to the FG. This result confirms the effectiveness of the PRP lyophilization process used in this study. Moreover, the maintenance of 100% of the gel state for 10 days and 52% of the gel state after 30 days of the experiment confirms the potential of using reconstituted PRP gel after lyophilization as a matrix for the delivery of several molecules or the framework for MSCs in regenerative clinical treatments (Nail & Gatlin 2016). In this context, it is noteworthy the fact that a wide variety of substances, as well as MSCs, have been found to be substantially labile when applied to the patients' bodies. Growth factors, for example, are rapidly metabolized, while MSCs applied systemically or locally, can be inactivated by the

Table 4. Quantification of growth factors VEGF and TGF-β from fresh and lyophilized canine PRP samples

Growth factor	Type of PRP	Concentration (pg/mL)
VEGF	Lyophilized	9.10
	Fresh	5.55
TGF- β	Lyophilized	8228.57
	Fresh	3171.70

PRP = platelet-rich plasma, VEGF = vascular endothelial growth factor, TGF- β = transforming growth factor beta.

host's immune system and/or lost through the bloodstream (Valeri 1974, Tidball 2005, Uebel 2006, Vajta et al. 2007).

The three-dimensional structure of the reconstituted PRP gel mesh after lyophilization was analyzed in parallel to the FG by electron microscopy (Fig.2-7). Both structures showed to be similar, confirming the potential use of PRP gel as a therapeutic strategy, in addition to raising the possibility of replacing FG by PRP gel, considering the significant simplicity associated with the preparation of PRP gel in relation to the FG. In the present study, the gelation test and the analysis by electron microscopy were performed in comparison to the FG because this biomaterial has been studied for decades in the field of human regenerative medicine and is considered a good framework for the delivery of various substances and MSCs (Janmey et al. 2009, Spicer & Mikos 2010, Reddy et al. 2018), constituting a valuable control for the generation of comparison parameters.

To verify the biocompatibility and safety of the lyophilized PRP, an MSC proliferation assay was performed on the three-dimensional PRP gel mesh (Fig.8-10). It is worth mentioning that, during the regenerative process, several factors and molecules secreted by platelets constitute important signaling peptides for the tissue repair process, promoting cell differentiation, chemotaxis and cell migration induction, especially of MSCs (Anitua et al. 2004, Everts et al 2006). Moreover, there is evidence that the PRP provides a favorable microenvironment for MSC proliferation and differentiation, in addition to attracting endogenous MSC populations, accelerating the tissue regeneration process *in vivo* (De la Fuente et al. 2010, Yamada 2011).

In this study, the cell proliferation test showed that MSCs effectively multiplied through the three-dimensional mesh of the reconstituted PRP gel after lyophilization, resembling, although this comparison was not performed in the present study, the cell multiplication profile normally seen in cultivation of MSCs in standard culture media in our laboratory. This result is in agreement with the literature findings regarding the cultivation of MSCs in FG mesh (Wergin & Kaser-Hotz 2004, Wahl et al. 2015). Thus, the lyophilized PRP, in the presence of trehalose, showed to be biocompatible with MSCs and did not disclose signs of cytotoxicity, additionally confirming the potential for its use as a framework for therapy with MSCs. However, a point that deserves to be better studied is the change in the cell morphology and communication network of MSCs (Fig.8-10) throughout the culture period. Morphological changes may be associated with cell differentiation, which is often not welcome in the field of stem-cell therapy, as it may be associated with changes and/or loss of cell functions (Wiens et al. 2007). However, it is worth considering that the bioactive molecules physiologically secreted by platelets act by modulating gene expression, secretome and the differentiation of cells such as fibroblasts, osteoblasts, chondrocytes and MSCs, stimulating functions such as cell proliferation/mitogenesis, angiogenesis, differentiation, chemotaxis, extracellular matrix formation, collagen synthesis and inflammatory modulation, culminating, in the end, in tissue repair and regeneration (Barbaree & Sanchez 1982, Barbaree et al. 1985, Ayala García et al. 2012, Sobczyńska-rak et al. 2014, Bain 2016, Rocha et al. 2017). In this context, the morphological change identified in this study may be a consequence of a normal and possibly beneficial effect caused by factors present in the PRP. Moreover, the changes identified may be only a consequence of an adaptation of the MSCs to the cultivation in the PRP gel three-dimensional mesh.

The results discussed so far provide a preliminary endorsement of the use of lyophilized PRP in the field of regenerative therapies, showing advantages such as the potential for large-scale production and industrialization, which could raise the level of quality control and, consequently, therapeutic effectiveness and repeatability, not only experimentally, but also in clinical and surgical routines. Thus, limitations associated to the lack of consistency of protocols and procedures, lack of specialized labor, lack of standardization regarding the composition and therapeutic effect of PRP could be minimized. Still, a major obstacle currently found in the field of Regenerative Medicine is in the fact that autologous PRP is mainly used. In this case, the donor's age and/or health status is frequently incompatible with the production of therapeutically viable PRP batches. To overcome this scenario, the use of allogeneic PRP obtained from young and healthy donors is a potential alternative. Thus, a crossmatch test was carried out to analyze the compatibility of leukocyte antigens and, consequently, the immunological compatibility between individuals, aiming at assessing the immunogenicity of the allogeneic lyophilized canine PRP when in contact with the blood of another animal. It is also noteworthy that this test assesses the compatibility related to the antigens of the Class I and II Major Histocompatibility Complex, one of the main histocompatibility determinants in different types of transplants and grafts (Wilmink & Van Weeren 2004). Fortunately, the preliminary result of the crossmatch test was negative, indicating that the allogeneic lyophilized canine PRP was not immunogenic. This finding confirms results reported in previous studies, where no signs of immunogenicity were found with the use of allogeneic and even heterologous PRP (Wu et al. 2012, Zhang et al. 2013, Zhou et al. 2016). More specific and broader studies still need to be performed to certify the biosafety concerning the use of allogeneic PRP.

CONCLUSIONS

The present study demonstrated, *in vitro*, the preliminary and very promising results related to the production and use of lyophilized canine platelet-rich plasma (PRP) as an alternative in Regenerative Medicine. It was demonstrated that the reconstituted canine PRP after lyophilization did not suffer any loss in relation to the concentrations of vascular endothelial growth factor (VEGF) and transforming growth factor beta (TGF-β), maintaining the gel-forming capacity of which microstructure was similar to that observed in the fibrin gel (FG), as well as provided support for mesenchymal stem cell (MSC) proliferation in the three-dimensional gel mesh and did not result in any evidence of immunogenicity when allogeneic PRP was used.

There was also a preliminary demonstration that the lyophilized PRP can be used therapeutically in the gel state, enhancing clinical and surgical approaches that aim to achieve a slow release of growth factors and other bioactive molecules or that require a framework for cell therapy. New studies with a larger number of animals, donors and patients, and a higher number of repetitions should be carried out to generate more conclusive data regarding the quantification of growth factors, biocompatibility and immunogenicity. Moreover, studies aimed

at the analysis of stability and shelf life, in addition to *in vivo* tests aiming to attest safety and efficacy, will be necessary for the final validation of lyophilized canine PRP.

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