PESQUISA VETERINÁRIA BRASILEIRA
Brazilian Journal of Veterinary Research
ISSN 0100-736X (Print)
ISSN 1678-5150 (Online)

Pesq. Vet. Bras. 40(6):474-478, June 2020
DOI: 10.1590/1678-5150-PVB-6512

Original Article
Small Animal Diseases

Evaluation of platelet-rich plasma gel as an angiogenesis-inducing agent in canine advancement skin flaps[1]

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ABSTRACT.- Aleixo G.A.S., Coelho M.C.O.C., Almeida T.L.A., Pereira M.F., Teixeira M.N., Andrade L.S.S., Bessa A.L.N.G. & Evêncio-Neto J. 2020. **Evaluation of platelet-rich plasma gel as an angiogenesis-inducing agent in canine advancement skin flaps.** *Pesquisa Veterinária Brasileira 40(6):474-478*. Departmento de Medicina Veterinária, Universidade Federal Rural de Pernambuco, Rua Dom Manuel de Medeiros s/n, Dois Irmãos, Recife, PE 52171-900, Brazil. E-mail: grazielle@yahoo.com

This work aimed to evaluate the effect of platelet-rich plasma (PRP) on advancement skin flaps in dogs regarding improvement of vascularization, with focus on increasing its viable area, since there are reports that it is a potential angiogenesis stimulator. The experimental group was composed of eight adult bitches, in which two advancement skin flaps were made in the ventral abdominal region. No product was applied in the control flap (CF), while PRP was used in the contralateral flap, called treated flap (TF). The areas were clinically evaluated every two days until the 7th postoperative day regarding skin color and presence of necrosis. At 10 days, both flaps were removed and submitted to histological examination and blood vessel morphometry. The vessels counted in each group were statistically analyzed by the F-test at 1% probability. Results showed no significant difference in macroscopic changes in the wound, or CF and TF vascularization, thus suggesting that PRP gel did not improve advancement skin flap angiogenesis in bitches under the experimental conditions in which this research was developed.

INDEX TERMS: Platelet-rich plasma gel, angiogenesis, canine, skin flap, platelet gel, growth factors, dogs.

RESUMO.- [**Avaliação do gel de plasma rico em plaquetas como agente indutor da angiogênese em flapes cutâneos de avanço em cães.**] Objetivou-se com o presente artigo avaliar a ação angiogênica do gel de plasma rico em plaquetas (PRP) em flapes cutâneos de avanço em animais da espécie canina, visando aumentar a viabilidade da pele após o procedimento, uma vez que existem relatos de que o produto é um potente estimulador da angiogênese. O grupo experimental foi composto por oito cadelas adultas, onde foram confeccionados dois flapes de avanço (de padrão subdérmico) na região abdominal ventral. Em um dos flapes, considerado controle (FC) não foi aplicado nenhum produto, enquanto que no flape contralateral, denominado tratado (FT), foi usado o gel de PRP. As áreas foram macroscopicamente avaliadas a cada dois dias até o 7º dia de pós-operatório em relação à coloração da pele e presença de área de necrose, e com 10 dias ambos os flapes foram coletados por biópsia e submetidos ao exame histológico e morfometria dos vasos sanguíneos. Os vasos contados em cada grupo foram estatisticamente analisados pelo teste de F ao nível de 1% de probabilidades. Os resultados demonstraram que não houve diferença significativa nas alterações macroscópicas das feridas e na morfometria vascular dos FC e FT, sugerindo dessa maneira que dentro das condições experimentais nas quais a pesquisa foi executada, que o gel de PRP não incrementou a angiogênese de flapes de avanço em cadelas.

TERMOS DE INDEXAÇÃO: Plasma rico em plaquetas, indução, angiogênese, flapes cutâneos, cães, retalhos cutâneos, gel de plaquetas, fatores de crescimento, neoangiogênese, caninos.

[1] Received on February 6, 2020.
Accepted for publication February 24, 2020.

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INTRODUCTION

Flaps consist of a skin segment transferred from a donor site to a recipient site to cover flaws while keeping a connection to the former through a pedicle that preserves its blood irrigation (MacPhail 2014). Since it has its own vascularization and does not depend on the vascularization of the recipient area, the blood from its pedicle is essential for its survival (Estevão et al. 2013).

Skin flaps have the advantage of providing an immediate cover for a region, which decreases the healing period (MacPhail 2014, Ober et al. 2019) and treatment expenses, also providing excellent aesthetic and functional results (Biondo-Simões et al. 2000).

Flap viability is highly dependent on its vascularization (Nardi et al. 2016); therefore, the use of substances aiming to improve its irrigation is a relatively common practice (Estevão et al. 2009).

Platelet-rich plasma (PRP) derived from autologous blood is defined as a plasma volume with platelet concentration higher than physiological levels, obtained via centrifugation (Marx 2004, Silva et al. 2007, Garcez et al. 2016). The therapeutic use of PRP has been described since 1990 (Floryan & Berghoff 2004) as contributing in soft and hard tissue healing (Arora et al. 2009).

The product is applied on the wound as a gel or solution (Martinez-Zapata et al. 2012) and at that moment, it deposits growth factors (GFs), which are important for tissue recovery, since they promote angiogenesis, mitogenesis, chemotaxis (Maia & Souza 2009), and cellular differentiation (Vendruscolo et al. 2012). Approximately seven GFs have already been identified (Marx 2004); however, the only ones proven to participate in vascular formation are: platelet-derived growth factor (PDGF), transforming growth factor beta (TGFß), epithelial growth factor (EGF), fibroblast growth factor (FGF), and vascular endothelial growth factor (VEGF) (Vendramin et al. 2010).

Vascular endothelial growth factor also known as vascular permeability factor (VPF), is one of the most important factors for stimulation of "*in vivo*" angiogenesis through a complex process which involves endothelial cells mitosis. It also has the ability to increase the permeability of microvessels in the skin, peritoneal wall, mesentery, and diaphragm (Bao et al. 2009). Platelet-derived growth factor acts by stimulating mitosis in the vascular endothelium cells (Moreira et al. 2008) and the smooth muscle cells of blood vessels, since angiogenesis is one of its main functions. The EGF also demonstrates mitogenic potential in endothelial cells, while FGF stimulates endothelial and smooth muscle cells (Litwack 2018).

Despite many papers describing that PRP application on wounds has the potential to increase tissue recovery, it is still not widely used and there are many controversies in the literature regarding the benefits of its use (Arora et al. 2009, Vendruscolo et al. 2012), especially because there are numerous protocols available (Vendruscolo et al. 2012).

Based on such information, the present experiment aimed to evaluate the use of platelet-rich plasma gel for stimulation of angiogenesis to consequently improve the integration of experimentally produced advancement skin flaps in dogs.

MATERIALS AND METHODS

Prior to start of the research, the project was submitted to and approved by the Ethics Commission on Animal Use (CEUA) of the "Universidade Federal Rural de Pernambuco" (UFRPE) (Protocol No. 23082.005174).

In order to obtain a more homogeneous sample and, consequently, decrease variability and allow a lower number of animals to be used, only medium-sized (between 10 and 15kg), adult (two to five years old) and clinically healthy females were used.

Three weeks before the date chosen for the surgical procedure, the eight selected bitches were placed in the kennel of the "Departmento de Medicina Veterinária" (DMV), where they remained in individual stalls for environmental and handling adaptation. Their diet consisted of commercial dog food given twice daily and water *ad libitum*.

After a 12-hour food fasting and 4-hour water fasting period, and approximately three hours before the surgical procedure, blood samples were collected for producing the PRP gel which would be used in the skin flap. Blood was collected from the cephalic or saphenous veins using a vacuum collection system. Four 4.5mL glass tubes containing sodium citrate as an anti-coagulant (for PRP production) and one 2mL plastic tube with Ethylenediaminetetraacetic acid (EDTA) (for the complete blood count - CBC, and platelet count from the total blood) were filled.

The four tubes of blood used to produce PRP were centrifuged at 104.83g for 10 minutes in an ordinary laboratory centrifuge (Baby I Model 206, Fanem). After the first centrifugation, all plasma and buffy coat were pipetted from each tube using an automated precision pipette and sterile tips, which were transferred to four other sterile tubes for another centrifugation at 186.37g for 10 minutes. Later, 80% of the supernatant plasma was discarded and the rest was homogenized to resuspend the remaining platelets (platelet button), obtaining the PRP. One of the tubes was separated to perform a quantitative evaluation of the PRP. This was done by direct count and morphological evaluation of the platelets via microscopic examination of a blood smear.

From the blood collected in the tube with EDTA, complete blood count and platelet count were obtained to evaluate the patient's health for surgery and to determine the platelet count, which would serve as reference for the value found in the PRP.

The anesthetic protocol used was composed by 0.1mg/kg of acepromazine and 1mg/kg of tramadol hydrochloride administered intramuscularly (IM) as pre-anesthetic medication. Later, 0.1mg/kg of meloxicam and 5mg/kg of enrofloxacin were administered subcutaneously (SC). After moving the patient to the operating room, the anesthetic induction was carried out with 4mg/kg of propofol, intravenously (IV), followed by endotracheal intubation for anesthesia maintenance with isoflurane via a semi-closed circuit for inhalation anesthesia. The patient received sodium lactate ringer, IV, throughout the surgical procedure.

Two flaps with 4cm in length and 1cm in width were made in the ventral abdominal region: one cranial (treated flap) and one caudal. With a pair of surgical scissors, 1cm of tissue was removed from the distal extremity of each flap to create an advancement flap. Afterwards, simple interrupted stitches were done with n. 3-0 monofilament nylon thread leaving only an opening big enough to place the PRP gel between the treated flap (TF) and its receptive field.

Before placing the PRP on the surgical bed, the three remaining tubes containing the product were homogenized and deposited in sterile stainless steel bowls where thromboplastin was added in a 2:1 proportion (1mL of PRP per 0.5mL of thromboplastin) in order to activate the platelets. No product was applied between the caudal flap and its receptive field and, consequently, this flap was considered the control (CF).

Throughout the post-operative period, the dogs were prescribed 5mg/kg of enrofloxacin antibiotic once a day orally (P.O.) and 0.1mg/kg of meloxicam (anti-inflammatory) every 24 hours P.O. for seven and four days, respectively. An Elizabethan collar and surgical garment were placed on the dogs throughout the post-operative stay in order to avoid self-inflicted injuries.

On the first day after surgery (D_1), the first clinical evaluation of CF and TF was made and, afterwards, the bandage was changed and flaps were clinically examined every two days until the seventh day (D_3, D_5, and D_7).

At each time (D_1, D_3, D_5, and D_7), both flaps were photographed with a digital camera for macroscopic recording of the region and further calculation of the necrotic area using Imagelab software. The pictures were taken with the camera always at a 5cm focal distance between the lens and the region to be photographed.

On day 10 (D_{10}), biopsy of both flaps (CF and TF) were done, and they were submitted to histological analysis. Tissue characteristics, such as the presence of necrosis and angiogenesis, were observed by examination of hematoxylin-eosin (HE) stained slides. Morphometry of blood vessels was performed by adapting a 100-squares grid reticle to the microscope lens. In the histological cut, three fields were selected and, in each area, the vessels found within 10 squares of the central column of the grid reticle were counted.

The experiment was arranged in a completely randomized design as a function of the homogeneity between the animals, and the statistical analyses for the vessel count in CF and TF were carried out by the F test at 1% probability using the Systat 10 software (demo version).

RESULTS

Macroscopically, the necrotic area was evaluated by skin color, where the areas that were initially pale from the third post-operative day, were continuously evolving into a purple color and, on the seventh day, to a black color. Necrosis was identified in three patients (3/8) in both groups; however, the area was restricted to the distal extremity of the flap. The necrotic regions were calculated by the Imagelab 2000 software (Table 1).

The necrotic area identified clinically and by the Imagelab 2000 software on the distal extremity of three flaps from each group was confirmed by histology, and the absence of cell nucleus were observed, compromising the epidermis and papillary dermis.

Microscopically, the presence and intensity of new vessel formation were similar in both groups. The analysis of variance for morphometric counting, performed via F test, presented lower results than the tabulated ones, thus showing that there are no statistically significant differences between the control and treated groups (Table 2).

Table 1. Necrotic areas of the control and treated flaps, calculated using Imagelab® 2000 software

Patient	Necrotic area	
	Control flap	Treated flap
1	0.00%	0.00%
2	0.00%	0.00%
3	14.00%	17.77%
4	0.00%	0.00%
5	0.00%	0.00%
6	10.00%	14.51%
7	10.48%	11.67%
8	0.00%	0.00%

DISCUSSION

Experimental research in dogs aiming to evaluate integration of skin flaps are rare. Since most researchers used rats or other laboratory animals as an experimental model (Biondo-Simões et al. 2000, Almeida et al. 2004, Estevão et al. 2009, Pazzini et al. 2016, Kemper et al. 2018), finding scientific papers to conduct a comparative analysis with the results obtained herein was a limiting factor.

Determining the total area that would be necessary for maintaining flap irrigation, focusing on improving tissue viability, is still a great challenge for surgeons (Almeida et al. 2004). In this study, it was decided to exceed the limits recommended by most authors using a 4:1 proportion between length and height by removing 1cm from the distal margin of the skin flap, in order to evaluate the tissue viability and vascularization under the least favorable tension conditions and venous return, as described by Estevão et al. (2009).

Studies have been developed with the aim of increasing the survival rate of skin flaps by enhancing its vascularization. Since several authors (Moreira et al. 2008, De Rossi et al. 2009, Bosch et al. 2011, Pazzini et al. 2016) have already reported the angiogenesis potential of the GFs present in PRP, it was chosen to test this product's efficacy on advancement skin flaps in dogs.

After production of PRP, it was observed that platelets were well preserved, since they did not present morphological alteration upon microscopic examination, and the material acted quickly upon stimulation of the agonist, creating a consistent clot only in a few seconds, just as observed by Pazzini et al. (2016). According to Yung et al. (2017), a good platelet function is retained during the PRP processing if there is a satisfactory response from platelets after adding the clotting factor, with resulting clot formation.

Based on the platelet count obtained on total blood and PRP (Table 3), it was observed that it was possible to provide a four to five times increase on the count, obtaining the concentration

Table 2. Mean number of vessels per mm² identified in the control and treated flaps

Treatments	Means
Control flap (CF)	21.125 per mm²
Treated flap (TF)	28.125 per mm²

Table 3. Platelet count in total blood and in PRP of all patients

Patient	Platelet count	
	Total blood	PRP
1	218.000/µL	1.197.000/µL
2	304.000/µL	1.517.000/µL
3	204.000/µL	1.078.000/µL
4	241.000/µL	1.114.000/µL
5	246.000/µL	1.395.000/µL
6	237.000/µL	1.129.000/µL
7	287.000/µL	1.345.000/µL
8	217.000/µL	1.293.000/µL

PRP = platelet-rich plasma. The calculation of sample sufficiency demonstrated that the number of patients used was adequate for performing the experiment.

considered as therapeutic by many researchers, such as Marx (2004), Whitlow et al. (2008), and Pazzini et al. (2016).

Macroscopic observation of the flap for the presence of necrosis was performed continuously every two days until the 7th day from the first 24-hours evaluation, because, according to Pavletic (2007), periodical observations are necessary to determine survival of a flap on the days following transplant. Tobias (2017) describes that flap necrosis may not be evident until six days after surgery, making it necessary to wait for a week to identify viable and non-viable areas.

Factors such as infection, compression and tension may contribute to development of necrosis in a flap, though tissue irrigation is the main cause, once vessels are responsible for local nutrition (Nardi et al. 2016). The cause of necrosis in all flaps was attributed to improper vascularization, since no signs of infection were observed and, if excessive tension had been the cause, some sutures would have probably broken and areas of dehiscence would have been identified. The flaps were also not submitted to excessive compression during the post-operative period, since the wound was protected via a bandage composed by a single layer of hypoallergenic tape and non-compressive post-surgical garment. Moreover, according Estevão et al. (2013), the setting-in of ischemia and subsequent necrosis depends on the blood supply provided by the blood vessels in the pedicle.

In the control and treated groups, in all evaluations, no odor or dehiscence was observed, even on necrotic regions as previously mentioned, though dehiscence is one of the main complications related to the use of skin flaps (Nardi et al. 2016). This probably happened because the necrosis was more superficial, compromising only the epidermis and the papillary dermis. It is believed that the force exerted by the reticular dermis was enough to keep the flap and the edges of the surgical wound closed by the stitches since this layer presents thick collagen and elastic fiber bundles (Kierszenbaum & Tres 2016).

Aside from the macroscopic evaluations, which are considered more subjective because they are determined according to the evaluator's observation, microscopic analysis was conducted in order to substantiate or complement the information from clinical examinations. In the work carried out by Suaid et al. (2007), evaluating PRP action in grafts for covering gingival retraction in dogs, the evaluations were based only on clinical observations, nevertheless, the same authors mentioned that it is not enough to analyze the action of the platelet gel, requiring histological examination. In another study conducted by Kemper et al. (2018), where histopathological analyses were performed to evaluate the use of PRP in rabbit skin grafts, the results showed that there were no statistically significant differences between the treated and control groups considering the average numbers of vessels, corroborating our findings, despite the fact they evaluated skin grafts and not flaps.

In spite of the promising results cited by some researches using PRP activated by thromboplastin, no differences were found in the use of the product when compared with CF, which agrees with results found by Silva et al. (2007), Gurgel et al. (2007), Suaid et al. (2007), Martinez-Zapata et al. (2012), and Kemper et al. (2018).

An important factor to be considered regarding papers that presented favorable effects of PRP on tissue recovery, more specifically, on angiogenesis, is that most of them base their satisfactory results only on clinical analyses, especially with experiments carried out in humans. However, it is recommended that histology should be an essential part of evaluating the effectiveness of PRP, since many alterations, such as intensity of neovascularization, can only be identified via microscopic analysis.

CONCLUSION

Within the conditions in which the experiment was conducted, the results obtained made it possible to conclude that platelet-rich plasma (PRP) gel did not improve angiogenesis or, consequently, the viability of advancement skin flaps in dogs.

Conflict of interest statement.- The authors report no conflict of interest.

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