doi: 10.17221/24/2016-VETMED

Concentrations of growth factors in platelet-rich plasma and platelet-rich fibrin in a rabbit model

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ABSTRACT: Platelet-rich plasma (PRP) and platelet-rich fibrin (PRF) are platelet concentrates which have been used to improve healing and regeneration of damaged tissues. The concentration of growth factors greatly influences the final outcome of the treatment. For studying the effects of PRP and PRF, an animal model is required. The New Zealand White rabbit appears to be a good candidate for studying the features of these blood derivatives. This article presents for the first time the various preparation methods of blood derivatives with regard to concentration of growth factors – insulin-like growth factor, transforming growth factor and vascular endothelial growth factor. There was no statistically significant difference among the methods in the case of the first two factors. In the case of vascular endothelial growth factor, the lowest amount was detected in PRF. This study, even though performed on a limited number of animals, reports novel characterisation of the rabbit as an animal model for *in vivo* studies of the action of these blood derivatives.

Keywords: blood derivate; thrombocyte; PRP; PRF; healing; animal model

Platelet-rich plasma (PRP) and platelet-rich fibrin (PRF) are autologous blood derivatives containing concentrated thrombocytes. Alpha-granules of thrombocytes contain growth factors (GFs) associated with tissue healing, such as transforming growth factors (TGF) β1 and β2, insulin-like growth factor (IGF), and vascular endothelial growth factor (VEGF) (Lubkowska et al. 2012). Therefore, they have been used for various purposes including orthopaedic, maxillofacial, periodontal, plastic surgeries, and sport medicine (Lubkowska et al. 2012; Knezevic et al. 2016; Richards et al. 2016). They have been shown to have antimicrobial and healing properties leading to reduced postoperative swelling, lower consumption of analgesics and improved surgery outcomes (Cohn and Lockhart 2015; Knezevic et al. 2016). On the other hand, critics of PRP have pointed out a large number of variables used in the studies on the basis of which positive effects of PRP are assumed (Kuffler 2015). There are two types of separation for PRP. The first type of PRP collection uses haematology blood separators, the second type utilises various modifications of a double centrifugation method. Haematology blood separators are found mainly in transfusion banks but because of high running costs they are seldom used except in university centres. A much cheaper although not as effective way of PRP preparation is the double centrifugation method. Peripheral blood is drawn into a syringe containing anticoagulant solution that inhibits platelet degranulation and activation. The first soft-spin centrifugation separates the blood into three fractions: red blood cells (RBC) at the bottom, platelet-poor plasma (PPP) on the top and

Supported by the Ministry of Health of the Czech Republic (Project No. NT 14321), the Ministry of Education, Youth and Sports of the Czech Republic (Project No. LO 1218), and the Ministry of Agriculture of the Czech Republic (Project No. RO 0516).

doi: 10.17221/24/2016-VETMED

buffy coat (BC) in the middle. PPP, BC and some uppermost RBC are then transferred into another tube without anticoagulant for the second hard-spin centrifugation. Just before application PRP is activated with thrombin and calcium in order to release GFs from the α-granules (Marx et al. 1998).

Preparation of the second generation of blood plasma derivatives – PRF, is simpler and does not require anticoagulant or thrombin as an activator (De Pascale et al. 2015). Peripheral blood is immediately centrifuged in 10 ml tubes. Fibrinogen starts to transform into fibrin during the centrifugation when in contact with circulating thrombin. Fibrin clot (FC) is formed between the uppermost PPP and the RBC at the bottom of the tube. Platelets are activated by contact with the tube walls and release the content of the granules.

One of the most frequent applications of these derivatives is periodontal surgery. For this purpose, animal models have been used in studies of the effectiveness of autologous platelet concentrates in pulp and dentin regeneration. The results of these studies are summarised in Del Fabbro et al. (2016). The authors stated that further studies are needed to assess a contribution of PRP in endodontic regenerative therapy. In our study, the rabbit was selected as a sufficiently large animal model, much more suitable than the mouse or pig. This is advantageous when re-operation is needed to collect samples for subsequent laboratory analyses. Since both of these blood plasma derivatives – PRP and PRF, are prepared in different ways and GFs are the most important components of both, the aim of the study was to compare their concentrations in these two blood derivatives.

MATERIAL AND METHODS

Animals and sample processing. Four New Zealand White adult rabbits were used in the experiment. Peripheral blood was taken from auricular vein: 8 ml of into a tube with 50 IU heparin to block the blood coagulation and 5 ml into a tube without any anticoagulants. The animals were used in this study with the agreement of the Branch Commission for Animal Welfare of the Ministry of Agriculture of the Czech Republic.

Five samples were derived in this study. Sample 1 – platelet-rich plasma, was prepared as follows: heparinised blood was centrifuged for 10 min (400 × *g*) without brake at 21 °C. Two ml of superficial platelet-poor plasma were drained and 2 ml of buffy coat together with a small portion of red blood cells were transferred into a new tube. The second centrifugation lasted also 10 min at 500 × *g* at 21 °C without brake. Again, the superficial layer amounting to 0.7 ml was drained and the rest (1.3 ml) was used as PRP. After every centrifugation, total counts of leukocytes, erythrocytes and thrombocytes were determined using a haematology analyser (BC-2800Vet, Shenzhen Mindray Bio-Medical Electronics, Shenzhen, People's Republic of China). Samples 2 and 3 – platelet-rich fibrin, were prepared as follows: blood without any anticoagulants was centrifuged immediately in a PC02 centrifuge (1500 spins/min, Complete A-PRF® system, Rawex ApS, Denmark) for 14 min according to an already described protocol (Choukroun et al. 2000). This centrifuge was developed especially for PRF preparation and thus provides standardised and reproducible results. Between superficial PPP (Sample 2) and RBC that were at the bottom of the tube a PRF clot was formed. The PRF clot was gently removed with sterile tweezers and stored in a PRF box for later use. For detection of GFs the PRF clot was placed into a sterile Petri dish for 10 min after which the supernatant was collected for ELISA (Sample 3). Samples 4 and 5 – samples from whole blood, were prepared as follows: for non-activated samples, heparinised blood was immediately centrifuged at 1000 × *g* for 15 min. Supernatants were used as Sample 4. For activation, blood was left to settle for 30 min and then centrifuged for 15 min at 1000 × *g*. Supernatants were used as Sample 5.

Quantification of growth factors. The amounts of VEGF, IGF, and TGF were measured in all samples using a commercially available enzyme-linked immunosorbent assay kit (Cusabio Biotech Co., Ltd., Wuhan, China) according to the manufacturer's recommendations. Absorbances were read at 450 nm using the multichannel spectrometer, SynergyTM 2 (BioTek, USA).

Statistical analysis. Due to small number of experimental animals, the statistical significance of changes was tested with the nonparametric ANOVA test for repeated measures (Friedman test). Differences at the level of $P < 0.05$ were considered statistically significant. All calculations were performed with Prism® (Graph Pad Software, Inc., USA) software.

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Table 1. Counts of thrombocytes, leukocytes and erythrocytes during the process of platelet-rich plasma preparation

	Original amount		After 1st centrifugation		After 2nd centrifugation	
	mean ± SD	median	mean ± SD	median	mean ± SD	median
Thrombocytes (× 10^9/l)	189.1 ± 65.5	198	349.2 ± 202.7	316	536.1 ± 323.8	486
Leukocytes (× 10^9/l)	10.7 ± 3.4	10.4	18.3 ± 8.5	17.3	25.3 ± 11.7	23.3
Erythrocytes (× 10^{12}/l)	5.9 ± 0.5	5.9	4.2 ± 1.1	4.1	6.1 ± 1.3	5.8

RESULTS

Regarding the counts of thrombocytes, their median rose from 198 × 10^9/l in blood to 316 × 10^9/l after the first soft centrifugation to 486 × 10^9/l in the final PRP ($P < 0.001$). The number of leukocytes also increased from 10.5 × 10^9/l to 17.4 × 10^9/l to 23.3 × 10^9/l ($P < 0.001$). The numbers of erythrocytes remained the same and ranged between 4–6 × 10^{12}/l. All these results are summarised in Table 1.

The amounts of GFs released into samples are shown in Figure 1. Statistical analyses showed that only in the case of VEGF were there significant differences among the groups. The amount of VEGF was the lowest in Samples 2 and 3 – i.e. platelet-rich fibrin prepared form blood collected without any anticoagulants.

DISCUSSION

The concentrations of GFs reported in publications vary due to many factors. One of the most important factors is the method of activation. For example, Kim et al. (2010) compared four methods of thrombocyte activation and concluded that different activators led to differential release of particular GFs. Moreover, Weibrich et al. (2002) did not find any correlation between GF amount and thrombocyte counts in samples. Information regarding the concentration of GFs in rabbit PRP or PRF is very limited mainly due to a lack of immunoreagents able to detect them. One of the exceptions is the publication by Yin et al. (2016), in which the authors measured TGF-β levels. Their results were higher when compared with ours but they used a commercially available kit from a different producer. We found that amount of VEGF was the lowest in PRF. VEGF is a growth factor responsible for the induction of angiogenesis and neocapillarisation is an important part of the action

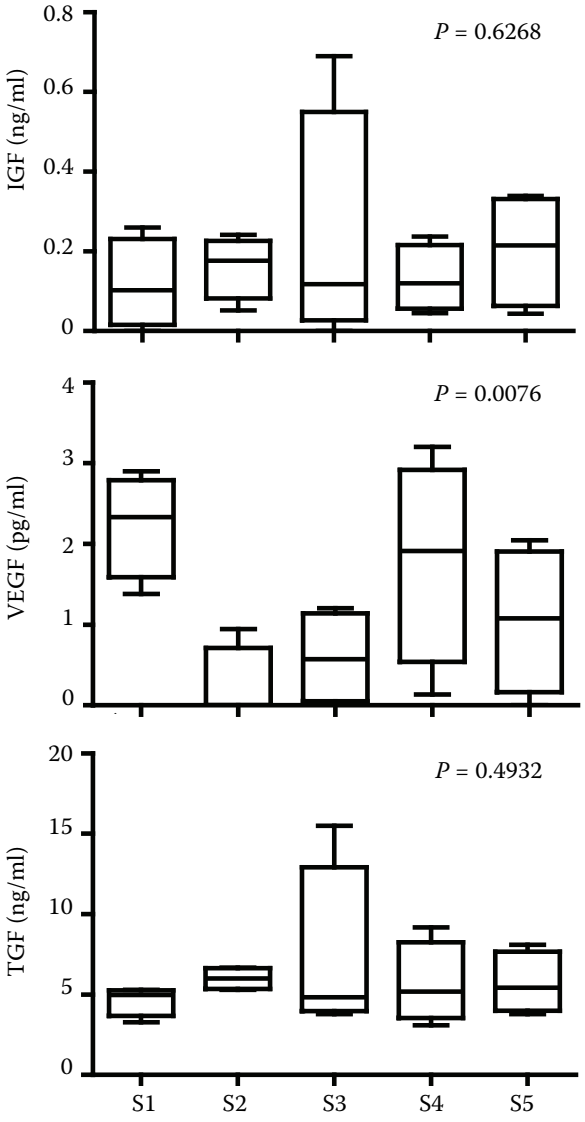

Figure 1. Amount of insulin-like growth factor (IGF), vascular endothelial growth factor (VEGF), and transforming growth factors (TGF) in platelet-rich plasma (S1), platelet-poor plasma (S2), platelet-rich fibrin (S3), non-activated sample of heparinised blood (S4), and activated sample of heparinised blood (S5). Data are shown as median (min.–max. and 25–75%, $n = 4$). P = results of non-parametric ANOVA test for repeated measures (Friedman test)

doi: 10.17221/24/2016-VETMED

of blood derivatives (Wang et al. 2016). Therefore, PRF seems to be less useful as a source of healing factors.

In this work, the concentration of GFs was measured for the first time in rabbit PRP and PRF preparations. We thus obtained new information regarding the rabbit as an animal model for *in vivo* studies of the action of these blood derivatives.

Acknowledgement

The authors greatly appreciate the technical assistance of Lenka Leva and Hana Kudlackova from the Department of Immunology of the Veterinary Research Institute.

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Received: 2016–02–05
Accepted after corrections: 2016–07–11

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