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Impact of incubation method on the release of growth factors in non-Ca^{2+}-activated PRP, Ca^{2+}-activated PRP, PRF and A-PRF☆

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ABSTRACT

The aim of this study was to investigate the influence of different incubation methods on the growth factor content of lysates of platelet-rich fibrin (PRF), advanced-platelet-rich fibrin (A-PRF) and platelet-rich plasma (PRP) products. A comparison of related studies suggests that the method of sample preparation has a significant influence on growth factor content. There are few reports on the comparison of non-Ca^{2+}-activated PRP, Ca^{2+}-activated PRP, A-PRF, and PRF, along with a lack of information on the release of PDGF-BB, TGF-β1, and VEGF among the different incubation methods.

The lysate preparation was made of non-Ca^{2+}-activated PRP, Ca^{2+}-activated PRP, PRF, and A-PRF, using a room-temperature, 37 °C, or freeze–thaw–freeze incubation method. Afterwards the VEGF, PDGF-BB, and TGF-β1 content was investigated by running ELISA tests.

Growth factor levels were significantly increased in the non-Ca^{2+}-activated PRP with freeze–thaw–freeze incubation, and in the PRF preparation there was a significant disadvantage to using room temperature incubation for releasing growth factors.

In conclusion, the freeze–thaw–freeze method is sufficient for releasing growth factors, and calcium activation is not necessary. Finally, the study demonstrates the possibility of preparing PRP products from platelet concentrates, so that preoperative blood sampling might not be required.

1. Introduction

Bone regeneration and remodeling are coordinated by interaction among different cells, insoluble and soluble modulators, and their antagonists (Gerstenfeld et al., 2003), for example interaction of osteoblasts and osteoclasts. In tissue damage, such as fractures, necrosis, or infections, platelets are activated and growth factors are excreted from the internal α particles. There are different types of PDGF (PDGF-AA, PDGF-AB, and PDGF-BB) and two types of TGF-β as well as IGF and EGF. In particular, the growth factors PDGF and TGF-β play a decisive role in bone regeneration and healing (Frechette et al., 2005). Two distinct PDGF receptors, alpha and beta, mediate the effects of the PDGFs on target cells. PDGF-BB is the only isoform, which induces all combinations of alpha- and beta-receptor dimers (Fang et al., 2004; Colciago et al., 2009). Fiedler et al. evaluated the role of PDGF in the chemoattraction of human bone-derived osteoblasts at various stages of differentiation, showing that rhPDGF-BB produced a powerful chemotactic stimulus (Fiedler et al., 2004). TGF-β1, a member of the TGF-β family, is a regulatory protein involved in bone remodeling and fracture healing (Joyce et al., 1990). Increased levels of TGF-β1 in hematomas and serum after bone fractures show its importance for fracture healing (Sarahrudi et al., 2011). VEGF has a supporting effect as part of a cascade, especially in bone healing, by promoting vascular structures and acting on osteoblasts (Street et al., 2002; Herford et al., 2017).

Various studies have shown that platelet-rich plasma (PRP), platelet-rich fibrin (PRF) and advanced-PRF (A-PRF) may promote colonization, adhesion, proliferation, and differentiation of cells

☆ The aim of this study was to clarify if Freeze-Thaw-Freeze incubation, incubation at room temperature or incubation at 37 °C for 1 h with following 16 h at 4 °C, would increase release of TGF-β1, PDGF-BB and VEGF and to assess the differences among these methods.

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due to high concentrations of GF such as PDGF-BB, TGF-β1, and VEGF (Anitua et al., 2013; De Pascale et al., 2015; Masoudi et al., 2016). There are many different methods, which differ in speed, time, and number of centrifugations, blood volume used, anti-coagulative supplementation, and activation/incubation methods (Zimmermann et al., 2003; Mazzucco et al., 2009).

Platelet concentrates prepared from whole blood without addition of anticoagulants, called PRF, lead to the induction of a fibrin clot. Standard PRF is centrifuged at 3000 RPM for 10 min, whereas the procedure for A-PRF involves centrifugation at 1500 RPM for 14 min (Dohan et al., 2006; Ghanaati et al., 2014). Activation of GF release from platelet concentrates prior to clinical application has frequently been described, especially for PRP production. Methods for GF release from platelet derivatives can be divided into different categories: repeated freeze–thaw cycles (freeze–thaw–freeze, FTF); incubation at 37 °C for 1 h followed by 4 °C retraction for 16 h; and direct activation by adding a $CaCl_2$ solution or human/recombinant thrombin (Burnouf et al., 2016). Although many commercialized devices for clinical preparation of PRP are currently available, there is a lack of standardized manufacturing protocol. Recent studies have shown that the activation of PRP with calcium, thrombin, repeated freeze–thaw cycles, or 37 °C incubation leads to an increase in the release of GF. However, many questions regarding the optimum release of the GF from the different platelet derivatives remain unanswered.

The aim of this study was to clarify whether FTF cycles, incubation at room temperature, or incubation at 37 °C for 1 h with a further 16 h at 4 °C, would increase release of TGF-β1, PDGF-BB, and VEGF, and also to assess the differences among these methods.

2. Materials and methods

All procedures were approved by the institutional ethical committee of the University of Luebeck, Germany, and were conducted according to the ethical standards of the Declaration of Helsinki and its later amendments. All blood samples were obtained from volunteers with informed signed consent.

2.1. Preparation of PRP

PRP was prepared using the 'buffy-coat' method routinely performed in blood banks to produce pooled platelet concentrates containing at least 2×10^{11} platelets/300 mL (Bundesärztekammer, 2017). Whole blood collected from patients was first centrifuged for 20 min at 22 °C and 3500 RPM to obtain the buffy coat (Fig. 1). Afterwards, four buffy-coat units and one plasma unit (32% plasma and 68% storage solution for platelets) from four donors with identical blood groups were pooled. After a second centrifugation for 17 min at 22 °C and at a speed of 920 RPM the platelet concentrate was put into a storage bag via a leukocyte depletion filter. Initially, half of the platelet concentrate was activated with 5.5% $CaCl_2$ at a ratio of 1:10.

2.2. Preparation of PRF and A-PRF

Blood samples were taken from three healthy volunteers aged between 20 and 40 to produce the PRF. Each volunteer donated 10 ml for the six different preparation methods. Half of the blood samples were centrifuged at 1500 RPM for 14 min at 18 °C to prepare A-PRF (Fig. 1). Centrifugation of the remaining 50% was carried out at 3000 RPM for 10 min at 18 °C. Afterwards the plasma and the buffy coat/fibrin clot were removed from the tubes and the rest was discarded.

2.3. Study design

Calcium-activated PRP (PRP + Ca), non-calcium activated PRP (PRP), PRF, and A-PRF were further processed using three different incubation methods so that finally 12 separate groups were established: groups 1–4 (PRP + Ca-RT, PRP-RT, PRF-RT, A-PRF-RT) were incubated at room temperature for 1 h (Parrish et al., 2016; Glovinski et al., 2017). Groups 5–8 (PRP + Ca-Inc; PRP-Inc; PRF-Inc; A-PRF-Inc) were incubated in a 37 °C water bath for 1 h and then cooled down within 16 h at 4 °C in the refrigerator (Landesberg et al., 2000; Amable et al., 2013). Groups 9–12 (PRP + Ca-FTF, PRP-FTF, PRF-FTF, A-PRF-FTF) were prepared by direct freezing at −80 °C for 24 h and then thawing at 37 °C for 1 h in a water bath. In the latter procedure groups 9–12 were then frozen again for 24 h at −80 °C (freeze–thaw–freeze, FTF) (Diaz-Gomez et al., 2014; Kobayashi et al., 2016b; Glovinski et al., 2017). After incubation, centrifugation was performed at 3000 RPM for 20 min at 18 °C for each group. The supernatants were then removed and preserved in aliquots at −80 °C until they were used for subsequent experiments. Each experiment was performed in triplicate.

2.4. GF quantification by ELISA

The different types of PRP and PRF were tested for release of three GF: VEGF (DVE00; range 15.6–1.000 pg/ml), human TGF-β1 (DB100B; range 31.2–2.000 pg/mL), and human PDGF-BB (DBB00; range 31.2–2.000 pg/ml). The concentrations of GF were assessed using enzyme-linked immunosorbent assay tests (ELISA; RND Systems, Minneapolis, MN, USA) and conducted according to the product instruction manual.

2.5. Statistical evaluation

Statistical evaluation was carried out with the statistical package IBM SPSS Statistics Version 24. Data were expressed as mean ± SD. Values were evaluated by one-way analysis of variance (ANOVA), corrected by a Bonferroni post hoc test. SPSS adjusted the single values (results) according to the Bonferroni correction for related tests so that a p-value of <0.05 was still considered statistically significant.

3. Results

3.1. VEGF release from non-Ca^{2+}-activated PRP, Ca^{2+}-activated PRP, PRF, and A-PRF

We found a significantly higher VEGF release using the FTF method in the non-Ca^{2+} activated PRP (PRP-FTF; 753.8 ± 39.7 pg/ml; $p < 0.01$) and Ca^{+2}-activated PRP (PRP + Ca-FTF; 630.3 ± 44.0 pg/ml; $p < 0.01$) compared with other preparation methods (PRP + Ca-Inc = 412.3 ± 75.7 pg/ml, PRP-Inc = 101.6 ± 16.6 pg/ml, PRP + Ca-RT = 119.0 ± 13.0 pg/ml, PRP-RT = 109.8 ± 19.4 pg/ml, A-PRF-Inc = 163.1 ± 59.7 pg/ml, A-PRF-FTF = 135.2 ± 42.1 pg/ml, A-PRF-RT = 132.1 ± 39.1 pg/ml, PRF-Inc = 165.3 ± 68.3 pg/ml, PRF-FTF = 67.9 ± 28.1 pg/ml, and PRF-RT = 33.3 ± 20.0 pg/ml). For the Ca^{2+}-activated PRP, PRP + Ca-Inc induced a significantly higher VEGF release (PRP + Ca-Inc; 412.3 ± 75.7 pg/ml; $p < 0.01$) than in the remaining groups, except for the PRP-FTF and PRP-Ca-FTF groups. The lowest VEGF release was observed in the PRF-RT group (33.3 ± 20.0 pg/ml). Detailed values of VEGF release are shown in Table 1, Fig. 2, and Fig. 5.

3.2. PDGF-BB release from non-Ca^{2+}-activated PRP, Ca^{2+}-activated PRP, PRF, and A-PRF

Under the influence of the FTF cycle, PRP-FTF showed the highest PDGF-BB release (PRP-FTF; 14208.9 ± 1313,1 pg/ml; $p < 0.01$) compared with the other groups, except for the PRP + Ca-Inc group (12533.3 ± 869.2 pg/ml). Further assessment produced the following values: PRP-Inc = 1717.7 ± 30.1 pg/ml; PRP + Ca-RT = 1913.4 ± 353.8 pg/ml; PRP-RT = 1302.2 ± 427.2 pg/ml; A-PRF-Inc = 4571.1 ± 1290.2 pg/ml; A-PRF-FTF = 1677.8 ± 636.8 pg/ml; A-PRF-RT = 4568.9 ± 1720.0 pg/ml; PRF-Inc = 4568.9 ± 1530.2 pg/ml; PRF-FTF = 1397.8 ± 599.2 pg/ml; PRF-RT = 628.9 ± 292.6 pg/ml. PRP + Ca-FTF induced release of PDGF-BB (8631.1 ± 2390.9 pg/ml; $p < 0.02$), which was significantly higher than in other groups, except for the PRP-FTF and PRP-Ca-Inc groups. The lowest concentrations were found for PRF-RT (628.9 ± 292.6 pg/ml; $p = 0.03$) and showed significant differences from A-PRF-Inc, A-PRF-RT, and PRF-Inc. Further details are shown in Table 1, Figs. 3 and 5.

3.3. TGF-β-1 release from non-Ca^{2+}-activated PRP, Ca^{2+}-activated PRP, PRF, and A-PRF

TGF-β1 release showed the highest values in the PRP-FTF group (32603.7 ± 8765.7 pg/ml). A significant difference could be

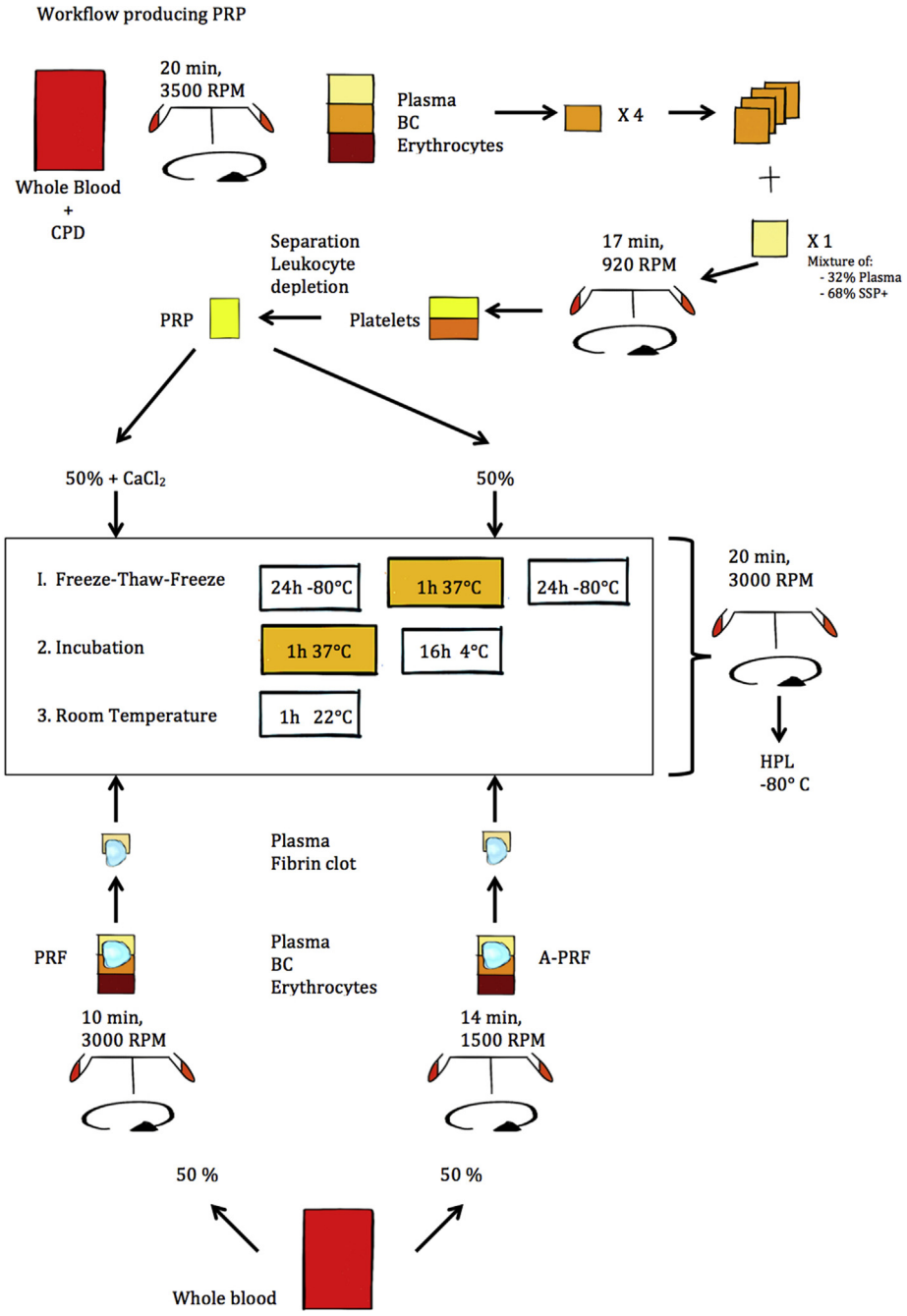

Fig. 1. Different modalities of platelet preparation. PRP is prepared using the buffy-coat method, which combines four buffy coats and a plasma (32%)/storage solution for platelets (SSP+; 68%) blending unit. Subsequently, part of the PRP is activated with calcium solution and both groups are processed using one of three different methods before a lysate is produced. PRF and A-PRF are produced from whole blood without pooling, according to Choukroun et al (Choukroun et al., 2006; Dohan et al., 2006). For lysate production, incubation is also performed using one of the three methods. CPD — citrate phosphate dextrose solution; RPM — revolutions per minute; HPL — human platelet lysate.

Table 1
Different approaches, mean concentration of VEGF expressed in pg/ml and the p-value of cross-comparison evaluated by one-way analysis of variance (ANOVA) corrected by a Bonferroni post hoc test.

VEGF	mean (pg/ml)	SD	PRP+Ca-Inc	PRP-Inc	PRP+Ca-FTF	PRP-FTF	PRP+Ca-RT	PRP-RT	A-PRF-Inc	A-PRF-FTF	A-PRF-RT	PRF-Inc	PRF-FTF	PRF-RT
PRP+Ca-Inc	412.30	75.70		<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
PRP-Inc	101.56	16.64	<0.01		<0.01	<0.01	1	1	1	1	1	1	1	1
PRP+Ca-FTF	630.27	43.96	<0.01	<0.01		0.13	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
PRP-FTF	753.85	39.70	<0.01	<0.01	0.13		<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
PRP+Ca-RT	118.96	13.01	<0.01	1	<0.01	<0.01		1	1	1	1	1	1	1
PRP-RT	109.75	19.37	<0.01	1	<0.01	<0.01	1		1	1	1	1	1	1
A-PRF-Inc	163.13	59.75	<0.01	1	<0.01	<0.01	1	1		1	1	1	0.88	0.08
A-PRF-FTF	135.15	42.10	<0.01	1	<0.01	<0.01	1	1	1		1	1	1	0.57
A-PRF-RT	132.06	39.15	<0.01	1	<0.01	<0.01	1	1	1	1		1	1	0.69
PRF-Inc	165.27	68.35	<0.01	1	<0.01	<0.01	1	1	1	1	1		0.76	0.07
PRF-FTF	67.89	28.06	<0.01	1	<0.01	<0.01	1	1	0.87	1	1	0.76		1
PRF-RT	33.25	20.02	<0.01	1	<0.01	<0.01	1	1	0.08	0.57	0.69	0.07	1	

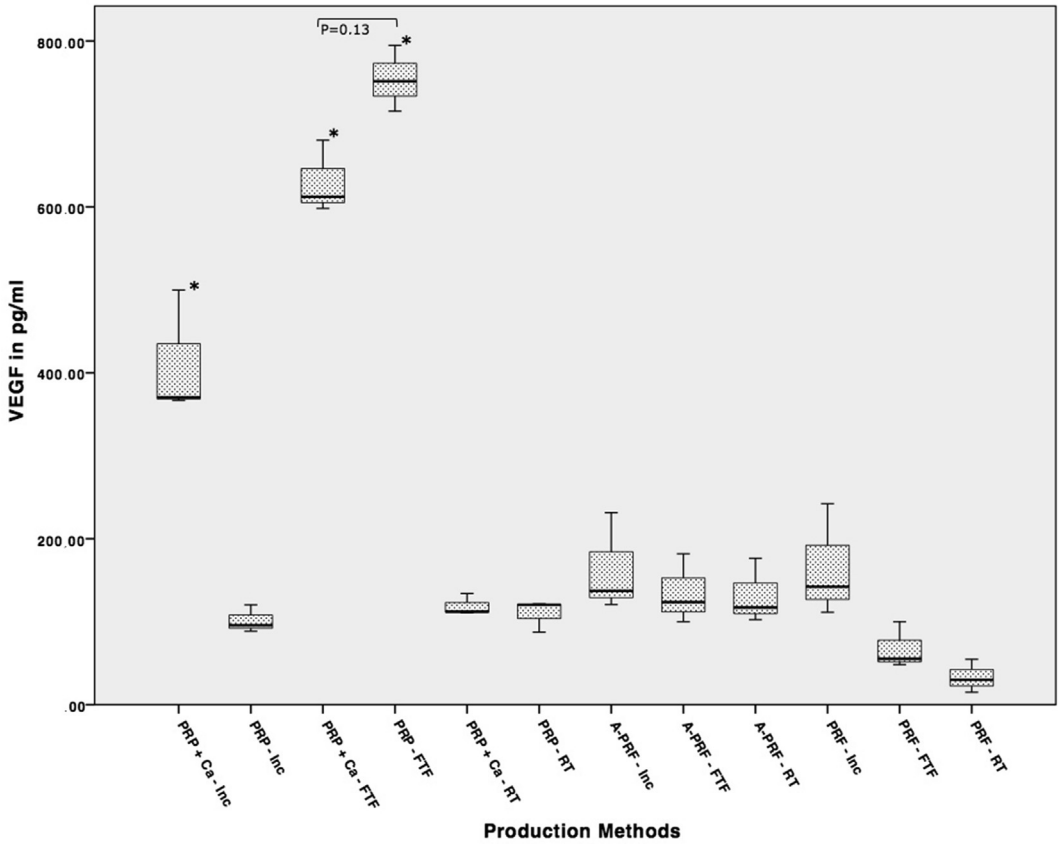

Fig. 2. Levels of VEGF released from calcium-activated PRP, non-calcium-activated PRP, PRF, and A-PRF under the different incubation methods. Inc. — incubation for 1 h at 37 °C and for 16 h at 4 °C; FTF (freeze-thaw-freeze) — 24 h at −80 °C, 1 h at 37 °C, and 24 h at −80 °C; RT (room temperature) — 1 h at 22 °C). *differ significantly from all approaches, except where indicated.

observed compared with the PRP-Inc (11670.4 ± 3087.0 pg/ml), PRP + Ca-RT (7574.1 ± 2928.3 pg/ml), PRP-RT (11259.3 ± 619.3 pg/ml), PRF-FTF (16625.9 ± 4988.8 pg/ml), and PRF-RT (4574.1 ± 2542.7 pg/ml) groups. However, there were no differences compared with PRP + Ca-Inc (23229.6 ± 5277.4 pg/ml), PRP + Ca-FTF (26629.7 ± 5843.1 pg/ml), A-PRF-Inc (24900.1 ± 3669.2 pg/ml), A-PRF-FTF (20662.9 ± 5326.4 pg/ml), A-PRF-RT (19163.0 ± 3963.2 pg/ml), or PRF-Inc (20055.6 ± 4374.3 pg/ml). The incubation method for PRP-Inc induced a significantly lower release of TGF-β1 than the Ca^{2+}-activated PRP-FTF (11670.4 ± 3087.0 pg/ml, $p < 0.05$). Similarly, there were no advantages for PRP production with incubation at

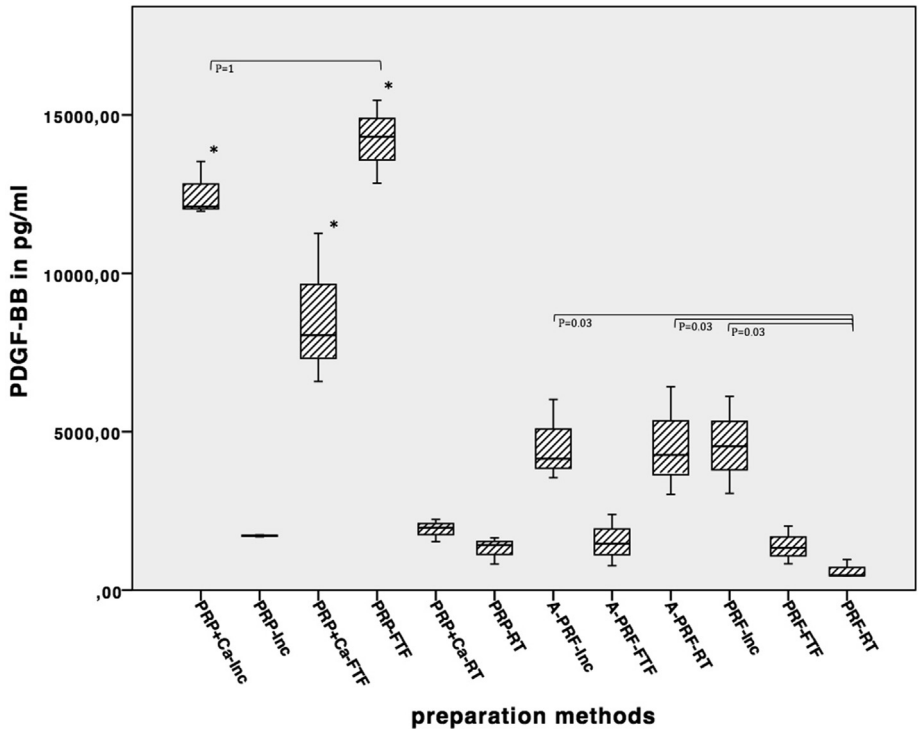

Fig. 3. Levels of PDGF-BB released from calcium-activated PRP, non-calcium-activated PRP, PRF, and A-PRF under the different incubation methods. *differ significantly from all approaches, except where indicated.

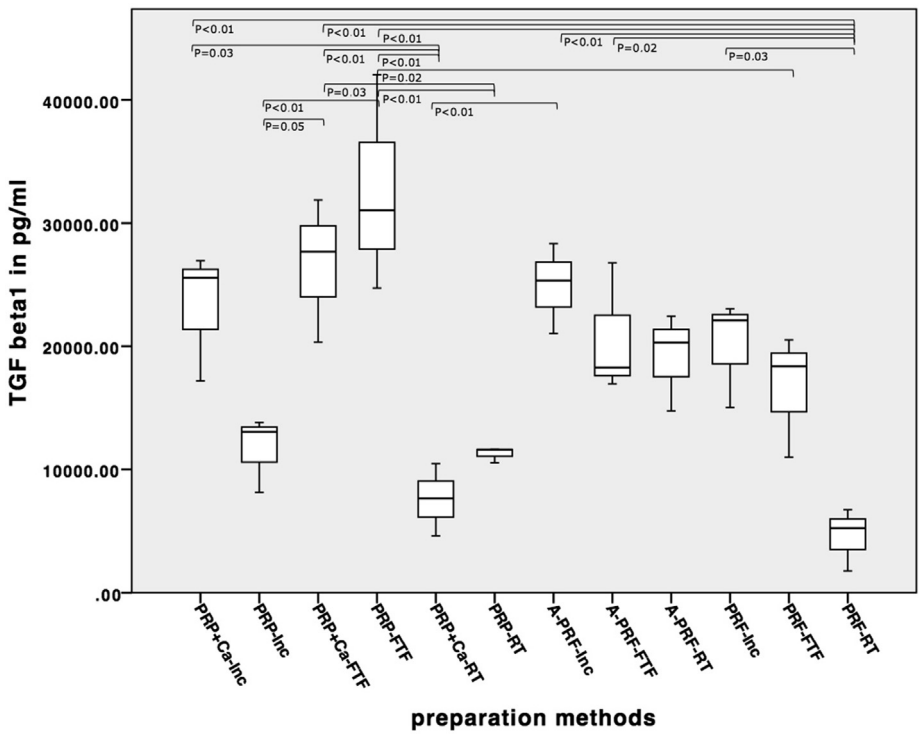

Fig. 4. Levels of TGF-β1 released from calcium-activated PRP, non-calcium-activated PRP, PRF, and A-PRF under the different incubation methods.

room temperature (22 °C). PRP + Ca-RT and PRP-RT even showed significantly less protein release compared with A-PRF-Inc, PRP + Ca-Inc, PRP + Ca-FTF, and PRP-FTF (detailed values are given in Table 1). The various A-PRF production processes did not show any different release values. The PRF-RT method induced the lowest concentration of TGF-β1 compared with PRP + Ca-Inc, PRP + Ca-FTF, PRP-FTF, A-PRF-Inc, A-PRF-FTF, and PRF-Inc (Figs. 4 and 5).

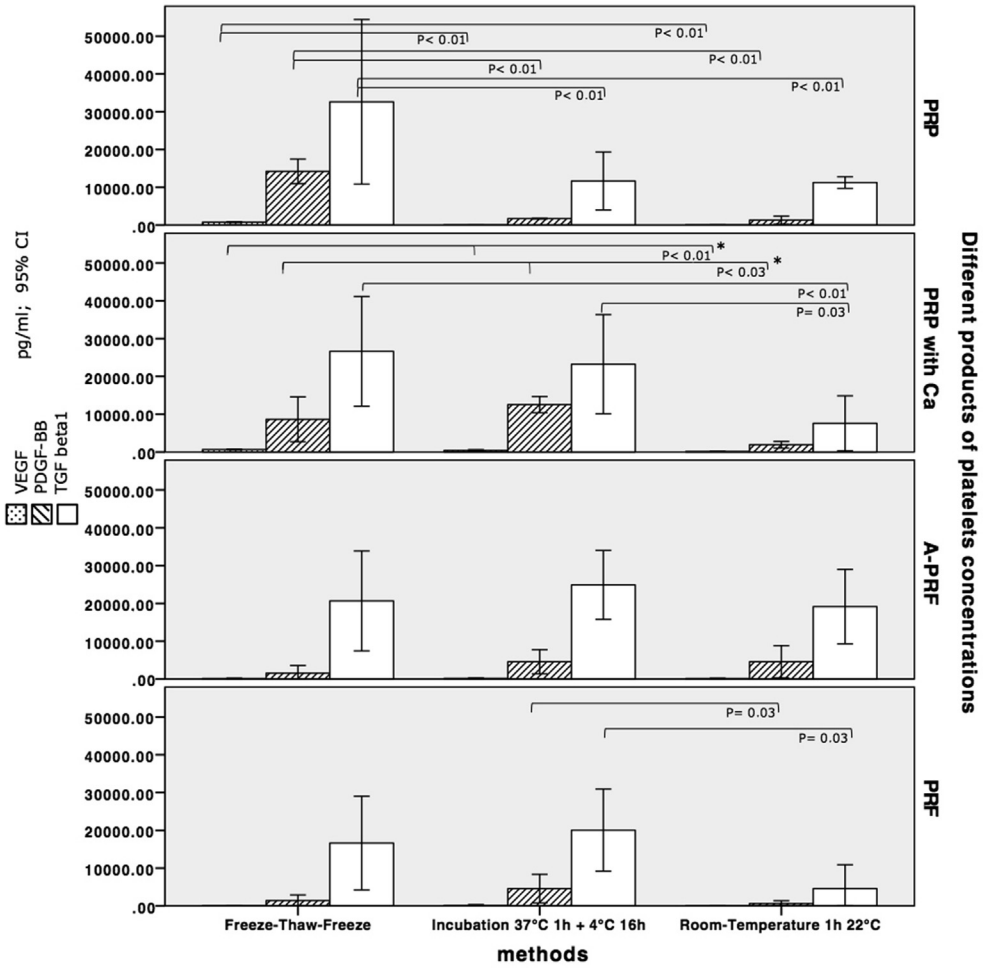

Fig. 5. Total growth factor release sorted by calcium-activated PRP, non-calcium-activated PRP, PRF, A-PRF, and the three different incubation/activation methods. *differ significantly from all approaches.

4. Discussion

The aim of this study was to investigate the effect of different processing methods on the release of PDGF-BB, VEGF, and TGF-beta1 from different platelet concentrate types. The study investigated non-Ca^{2+}-activated PRP, Ca^{2+}-activated PRP, PRF, and A-PRF. In the course of preparation, lysates were obtained from these products using three incubation methods: freeze–thaw–freeze; incubation at 37 °C for 1 h followed by a 4 °C retraction for 16 h; and 1 h storage at room temperature (22 °C). There are several activation and incubation methods described for the release of PDGF, TGF-β, and VEGF from PRP (Weibrich et al., 2003; Burnouf et al., 2016). The incubation methods investigated in this study do not require substrate additions, with the exception of calcium. The calcium method is often used before topical PRP application in order to form a clot (Landesberg et al., 2000). A comparison of related studies showed a lack of information on the release of PDGF-BB, TGF-β1, and VEGF by the different incubation methods, and so the method of sample preparation could have a significant influence on the concentration of PDGF-BB, VEGF, and TGF-β1 content in platelets derivatives (Zimmermann et al., 2003).

4.1. Release of PDGF-BB, VEGF, and TGF-β1 in PRP

The advantages of different centrifugation forces and times, anticoagulants, and activations in the preparation of PRP have frequently been discussed (Araki et al., 2012; Amable et al., 2013; do Amaral et al., 2016; Yin et al., 2017). In order to measure the release of PDGF-BB, VEGF, and TGF-β1 from PRP, this study investigated a PRP preparation method using platelet concentrates available from the local blood bank. Since platelet concentrates (2×10^{11} platelets/unit (unit = suspension of 200–450 ml)) have to contain a defined number of platelets, a product with a relatively constant quality is routinely available (Bundesärztekammer, 2017).

This study observed a higher release of TGF-β1, PDGF-BB, and VEGF in PRP than in PRF and A-PRF. This is in accordance with Kobayashi et al., who recently showed a similarly higher release in PRP than in PRF and A-PRF in the first 8 h following PRP preparation (Kobayashi et al., 2016a). There are two possible explanations for these results. First, platelet count plays a crucial role in the release of PDGF, TGF, and VEGF, and therefore pooled standardized platelet concentrates routinely containing 6×10^5 platelets/μl released higher GF, although leukocytes as a component of A-PRF — but not in the PRP prepared in this study — may also enhance GF release (Weibrich et al., 2002, 2003; Martineau et al., 2004). Second, the specific platelet aggregation inhibition performed in PRP — but not in PRF — might be an important factor in GF yield.

Platelet aggregation and activation cause a reduction in platelet content in PRP and result in the release of GF during the preparation process. Araki et al. suggested that it is crucial to minimize platelet aggregation in the preparation process, and revealed a correlation between PDGF-BB release and the number of activated

platelets (Araki et al., 2012). In our study, it could be shown that FTF and calcium activation lead to a higher GF release than when using the PRP-Inc and PRP-RT methods (Table 1 and Fig. 5). Avoiding $CaCl_2$ and thrombin addition simplifies handling, particularly in view of the tightening regulations for transfusion of blood products (Lacoste et al., 2003). Huber et al. showed that thrombin activation is not required because the release of GF from platelets with and without thrombin remains similar (Huber et al., 2016).

Among the GFs investigated in our study, TGF-β1 showed the highest concentration in all PRP preparations, followed by PDGF-BB and VEGF. Our results are in line with those of Weibrich and Kobayashi et al., who found the highest concentrations for TGF-β1 among the GFs evaluated in PRP (Dugrillon et al., 2002; Weibrich et al., 2003; Kobayashi et al., 2016b). They also showed that the yield of TGF-β1 seems to correlate with the total platelet count in the PRP. The levels of PDGF-BB and VEGF assessed in our study (ranges 0.5–14 ng/ml and 0.03–0.7 ng/ml, respectively) are comparable with those encountered by previous studies (Banks et al., 1998; Weibrich et al., 2003; Martineau et al., 2004; Kobayashi et al., 2016b).

4.2. Release of PDGF-BB, VEGF, and TGF-β1 in PRF

In this study, PRF and A-PRF were prepared according to Choukroun et al. (Choukroun et al., 2006; Choukroun, 2014) and measured the concentration of PDGF-BB, VEGF, and TGF-β1 in PRF and A-PRF lysates following the three different preparation methods. Compared with PRF, A-PRF showed a slightly higher, albeit not significantly different, release of the GFs mentioned above in all approaches (Tables 1–3). A comparable A-PRF protocol was recently modified by lowering centrifugation speed to 1300 RPM for 8 min, due to better release of GFs (Fujioka-Kobayashi et al., 2017). Further components in such preparations (e.g. plasma and leukocytes) can also augment this release (Weibrich et al., 2003; Kobayashi et al., 2016b).

Among the A-PRF preparation approaches themselves, VEGF, PDGF-BB, and TGF-β1 showed comparable values without any significant differences. For PRF, incubation at room temperature showed a significantly lower release of the three GFs compared with the other methods (Tables 1–3).

Table 2
List of different approaches, mean concentration of TGF-beta 1 expressed in pg/ml and the p-value of cross-comparison evaluated by one-way analysis of variance (ANOVA) corrected by a Bonferroni post hoc test.

TGF-beta 1	mean (pg/ml)	SD	PRP+Ca-Inc	PRP-Inc	PRP+Ca-FTF	PRP-FTF	PRP+Ca-RT	PRP-RT	A-PRF-Inc	A-PRF-FTF	A-PRF-RT	PRF-Inc	PRF-FTF	PRF-RT
PRP+Ca-Inc	23229.60	5277.37		0.4	1	1	0.03	0.31	1	1	1	1	1	<0.01
PRP-Inc	11670.40	3087.02	0.4		0.05	<0.01	1	1	0.14	1	1	1	1	1
PRP+Ca-FTF	26629.67	5843.14	1	0.05		1	<0.01	0.03	1	1	1	1	1	<0.01
PRP-FTF	32603.67	8765.74	1	<0.01	1		<0.01	<0.01	1	0.32	0.12	0.22	0.02	<0.01
PRP+Ca-RT	7574.07	2928.33	0.03	1	<0.01	<0.01		1	<0.01	0.15	0.39	0.22	1	1
PRP-RT	11259.27	619.32	0.31	1	0.03	<0.01	1		0.11	1	1	1	1	1
A-PRF-Inc	24900.07	3669.24	1	0.14	1	1	<0.01	0.11		1	1	1	1	<0.01
A-PRF-FTF	20662.93	5326.42	1	1	1	0.32	0.15	1	1		1	1	1	0.02
A-PRF-RT	19163.00	3963.17	1	1	1	0.12	0.39	1	1	1		1	1	0.06
PRF-Inc	20055.60	4374.32	1	1	1	0.22	0.22	1	1	1	1		1	0.03
PRF-FTF	16625.93	4988.80	1	1	1	0.02	1	1	1	1	1	1		0.29
PRF-RT	4574.07	2542.74	<0.01	1	<0.01	<0.01	1	1	<0.01	0.02	0.06	0.03	0.29	

Table 3
List of different approaches, mean concentration of PDGF-BB expressed in pg/ml and the p-value of cross-comparison evaluated by one-way analysis of variance (ANOVA) corrected by a Bonferroni post hoc test.

PDGF-BB	mean (pg/ml)	SD	PRP+Ca-Inc	PRP-Inc	PRP+Ca-FTF	PRP-FTF	PRP+Ca-RT	PRP-RT	A-PRF-Inc	A-PRF-FTF	A-PRF-RT	PRF-Inc	PRF-FTF	PRF-RT
PRP+Ca-Inc	12533.33	869.18		<0.01	0.03	1	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
PRP-Inc	1717.73	30.06	<0.01		<0.01	<0.01	1	1	0.44	1	0.44	0.44	1	1
PRP+Ca-FTF	8631.07	2390.89	0.03	<0.01		<0.01	<0.01	<0.01	0.02	<0.01	0.02	0.02	<0.01	<0.01
PRP-FTF	14208.87	1313.12	1	<0.01	<0.01		<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
PRP+Ca-RT	1913.40	353.84	<0.01	1	<0.01	<0.01		1	0.7	1	0.71	0.71	1	1
PRP-RT	1302.20	427.21	<0.01	1	<0.01	<0.01	1		0.15	1	0.15	0.15	1	1
A-PRF-Inc	4571.07	1290.18	<0.01	0.44	0.02	<0.01	0.7	0.15		0.28	1	1	0.2	0.03
A-PRF-FTF	1677.80	636.83	<0.01	1	<0.01	<0.01	1	1	0.28		0.29	0.29	1	1
A-PRF-RT	4568.87	1720.04	<0.01	0.44	0.02	<0.01	0.71	0.15	1	0.29		1	0.2	0.03
PRF-Inc	4568.93	1530.21	<0.01	0.44	0.02	<0.01	0.71	0.15	1	0.29	1		0.2	0.03
PRF-FTF	1397.80	599.20	<0.01	1	<0.01	<0.01	1	1	0.2	1	0.2	0.2		1
PRF-RT	628.87	292.56	<0.01	1	<0.01	<0.01	1	1	0.03	1	0.03	0.03	1	

It can be assumed that none of the three different preparation methods investigated had an influence on PDGF-BB, VEGF, and TGF-β1 release from PRF, or that the platelet activation applied was insufficient to release GFs from the fibrin clot, which is known to act as an adhesive carrier of GFs, thus maintaining their bioactivity over a longer period of time (Wolberg, 2007; Janmey et al., 2009; Kobayashi et al., 2012).

5. Conclusion

In summary, the study presented might suggest an influence of different incubation methods on the release of GFs, especially for PRP derivatives. FTF activation is an adequate method in this regard, such that additives, including thrombin and calcium, can be avoided. A-PRF seems to release higher concentrations of PDGF-BB, VEGF, and TGF-β1 compared with the conventional PRF preparation method.

Conflicts of interest

The authors declare that they have no competing interests.

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