Received: June 25, 2019 Revised: December 13, 2019 Accepted: January 2, 2020

(onlinelibrary.wiley.com) DOI: 10.1111/ner.13105

Pulsed Radiofrequency Neuromodulation Contributes to Activation of Platelet-Rich Plasma in *In Vitro* Conditions

Anna Michno, PhD*; Zbigniew Kirkor, PhD†; Ewelina Gojtowska, MSc*; Marek Suchorzewski, PhD‡; Irmina Śmietańska, PhD‡; Bartosz Baścik, PhD§

Objectives: Recent years have brought new developments in interventional chronic pain management, namely regenerative orthopedics utilizing platelet-rich plasma (PRP) as well as further evolution of pulsed radiofrequency neuromodulation (PRF). Both methods have been used separately. Here, we investigated whether PRF may potentiate the activation of platelets in PRP samples when both these techniques are combined together in *in vitro* conditions.

Materials and Methods: Studies were performed on concentrated PRP samples (PRPs) obtained from acid citrate dextrose-treated blood taken from 11 healthy volunteers. PRPs were divided into four groups: 1) nonactivated PRP; 2) thrombin-activated PRP as a positive control for maximal platelets activation; 3) PRF-treated PRP exposed for 20 min to PRF energy generated by neurotherm radio frequency generator at 500 kHz, with a voltage of 40 V and maximal temperature of 42°C; and 4) a combination of groups 2 and 3.

Results: PRF-induced platelet activation measured by platelet factor 4 (PF4) and ATP release from PRPs was significantly higher compared to nonactivated PRPs, and similar to PF4 and ATP release from thrombin-activated PRPs. Thrombin activation did not potentiate PF4 release in PRF samples and even reduced ATP level. Additionally, PRF neither induced any platelet membrane damage measured by lactic dehydrogenase release from PRP nor modified any platelets viability or metabolism measured by MTT.

Conclusions: We confirmed that PRF may activate PRP without additional platelet activators. So, a combination of both methods PRF and PRP application may provide a more effective opportunity for tissue regeneration in dentistry, surgery, dermatology, or in orthopedics.

Keywords: Chronic pain, platelet activation, platelet-rich plasma, pulsed radiofrequency neuromodulation, regenerative treatment

Conflict of Interest: None of the authors listed have any conflicts of interest to disclose.

INTRODUCTION

Recently, much attention has been focused on new developments in several fields of regenerative medicine including dermatology, dentistry, surgery, or pain management in orthopedics utilizing stem cells and platelet-rich plasma (PRP) injections and further modifications of pulsed radiofrequency neuromodulation (PRF) (1–30).

An attractive alternative for tissue regeneration is to harness the potential of PRP for direct use in tissue healing. There are several protein growth and angiogenic factors secreted by activated platelets ((3,4)), which regulate growth and differentiation of chondrocytes, osteoblasts, epithelial, endothelial or mesenchymal cells, stimulate chemtaxins and mitogenesis in fibroblasts, glial or smooth muscle cells, regulate collagen synthesis all to initiate wound healing process, and promote tissue regeneration (3). There is a growing evidence from preclinical and clinical studies on the benefits of PRP injections in treating and accelerating natural healing process including dental surgery, osteoarthritis, melisma, alopecia, peripheral nerve regeneration, chronic tendinopathies, acute ligament injuries, muscle and ligament tears, ankle sprains, cartilage damage or degenerative disk disease (10). On the other hand, other studies reported no additional benefits of PRP therapy compared to standard treatment for treating sport injuries (9,11–13). The observed differences in effectiveness of PRP may be due to a wide variation in PRP preparation and a lack of standardization in blood processing (10,13).

Moreover, PRP, which is obtained from citrated-anticoagulated blood samples, requires over 20 mM $CaCl_2$ to reverse the citrate chelating effect and initiate the coagulation process leading to

Address correspondence to: Anna Michno, PhD, Department of Laboratory Medicine, Medical University of Gdańsk, Dębinki 7, 80-211 Gdańsk, Poland. Email: mianna@gumed.edu.pl

* Department of Laboratory Medicine, Medical University of Gdańsk, Poland;
† Algocells, Regenexx, UK;
‡ Anaesthesiology and Intensive Care Department, Medical University of Gdańsk, Poland; and
§ Radiology Department, Medical University of Gdańsk, Poland

For more information on author guidelines, an explanation of our peer review process, and conflict of interest informed consent policies, please go to http://www.wiley.com/WileyCDA/Section/id-301854.html

Source(s) of financial support: Dr. Anna Michno provided funding for the manuscript from Department of Laboratory Medicine (St-57) at Medical University of Gdańsk.

thrombin generation for subsequent platelet activation sufficient enough for the release of the granules containing various growth factors (3,4,14). In *in vitro* experimental conditions, exogenous activators such as thrombin, collagen, $CaCl_2$, or a combination of $CaCl_2$ with thrombin are used (3,4,14). On the other hand, there are a lot of discrepancies on whether to activate PRP before injection in clinical applications. Although anticoagulated PRP injected into the skin or the enclosed joint cavity is exposed to the extracellular fluid and the mechanical trauma, $CaCl_2$ concentration in the fluid is still not high enough to reverse the effect of chelate action of citrate (3,4). Even though injected PRPs are exposed to collagen in the tissue, blood platelets are activated partially and slowly in such conditions (14). The key issue is whether the tissue collagen activates platelets enough to support the inflammatory effects and obtain the optimal tissue regeneration (5–7). On the contrary, the activation of platelets in PRP by thrombin induces their degranulation up to 90% within just 30 min (4). So it seems that either the addition of strong platelet activators such as thrombin or recalcification of citrated PRP may potentiate the utility of PRP in clinical applications (14,15). PRF has been used in pain management for more than 30 years (1–16,21).

PRF technique utilizes a needle-based, non-necrotic and minimally invasive treatment provided by controlled pulsed electric current generating electromagnetic field at the tip of the electrode placed close to the targeted tissue. Radiofrequency current is induced in short, high-voltage bursts, with the silent phases to prevent heat over 42°C around the treated area (22–24). A series of animal and clinical studies showed the effectiveness of PRF in the treatment of chronic radicular or articular pain without the permanent damage to the tissues (22,23,25–28).

The mechanisms of PRF action on tissues have not been fully explained. One of the possible mechanisms of PRF action is a reduction of small nerve endings growth into the joint structures or the disks in the area of inflammation. Others propose that PRF may restore homeostatic mechanisms between pain-causing inflammatory mediators' production including tumor necrosis factor α and pain relieving inhibitory factors in the treated tissues, which may also accelerate their healing like nerve growth factor and brain-derived neurotrophic factor (22).

Clinical findings revealed benefits of either PRF or PRP treatment separately as nonsurgical and nonpharmacological techniques of short-term pain relief used in orthopedics or PRP application in regenerative treatment in dentistry, surgery, and dermatology (1–32). To our knowledge, there are no data on a combined application of both techniques into one treatment in order to achieve higher efficacy compared with PRF or PRP alone.

Here, we investigated whether PRF may potentiate the activation of platelets in PRP samples when both these techniques are combined together in *in vitro* conditions.

We employed PRF standard parameters used in radicular pain management (exposition for 20 min to PRF energy at 500 kHz, at a pulse rate of 5 ms/5 Hz, for a period of 120 sec, with an adjusted voltage of 40 V, to permit maximum temperature of 42°C) to treat samples containing PRP in order to investigate whether and to what extent PRF could modify blood platelet activation *in vitro* conditions. Additionally, we analyzed the impact of PRF on metabolism and functional integrity of blood platelets and leucocytes present in PRPs to verify the safety of PRF on cellular components of PRP. Thereby our preliminary *in vitro* research provided a rationale for a future clinical application of the combination of both PRF and PRP in several fields of regenerative medicine including orthopedics. Data presented here suggest that it may be the case.

MATERIALS AND METHODS

Reagents and Materials

Reagents for lactic dehydrogenase (LDH), thiazolyl blue tetrazolium bromide (MTT), ATP, acid citrate dextrose (ACD), thrombin were supplied by Sigma Chemicals Co. (Poznan, Poland); Commassie Brillant Blue G-250 was from Bio-Rad (Munchen, Germany); RGDS (Arg-Gly-Asp-Ser) was from Tocris Bioscience (Abingdon, UK); Imunoclone Platelet Factor 4 ELISA (PF4) was from Sekisui Diagnostics (Stamford, UK). Radiofrequency Thermocouple Curved Electrodes (Gauge 22 ga) were delivered by NeuroTherm (Croydon, UK).

Study Group and Sample Collection

The study was performed on PRP obtained from 30 mL of blood collected on ACD from 10 healthy volunteers (aged 25-65). The study protocol was approved by the Bioethical Commission at the Medical University of Gdańsk (NKBBN/122/2017). None of the volunteers took any antiplatelet or anti-inflammatory medicines for at least two weeks before blood collection. Only one volunteer was a regular smoker, the rest of them were nonsmokers.

Washed platelets (PLTs) were obtained from buffy coats provided as by-products of whole blood fractionations by Regional Blood Centre in Gdansk.

PRP Preparation

PRP was obtained from whole blood taken on ACD. Blood was centrifuged at room temperature at 150*g* for 25 min in a centrifuge (Jouan CT 1000; Thermo Scientific, Gliwice, Poland). All plasma including one-third of the buffy coat was collected and transferred to fresh tubes and the content was centrifuged at 900*g* for 15 min. The upper layer of platelet poor plasma was discarded and only 3 mL of the bottom layer was left and suspended to obtain PRP 3–4 condensed. Platelet and leucocyte count was assessed with hemocytometer Superior (Marienfeld, Germany). The characteristics of the PRP is shown in Table 1.

Washed Platelets Isolation

Washed platelets (PLTs) were obtained from platelet rich buffy coats provided as by-products of whole blood fractionations by Regional Blood Centre in Gdansk.

Whole blood was collected from healthy volunteers in standard ACD solutions. RBCs and plasma were fractionated according to standards for blood banks. The remaining parts of buffy coats were diluted in PBS and anticoagulated with sodium citrate to the final concentration of 3.2% and centrifuged at 100*g* at room temperature for 25 min. The upper fractions containing platelets and leucocytes were collected into fresh tubes and centrifuged to

Table 1. Characteristics of PRP From Study Groups.

Data	PRP characteristics
No. of subjects	11
Men/women	5/6
Age (year)	43 ± 12
Platelet count in whole blood (giga/L)	257 ± 70
Platelets count in PRP (giga/L)	997 ± 163
Leucocytes count (giga/L)	8.8 ± 2.0

Data are the means ± SD from 11 observations.

obtain platelet supernatant and leucocyte pellet at 100*g* for 15 min. After that the platelet supernatant was centrifuged in fresh tubes to obtain platelet pellet at 800*g* for 15 min. Platelets were finally reconstituted Tyrode's buffer containing 5 mM glucose to a final concentration 3×10^8/ml.

Neuromodulation Studies on PRP

For neuromodulation studies, freshly prepared PRPs including nonactivated and thrombin-activated controls with addition of 10 mM $CaCl_2$ to reverse the anticoagulation with ACD and were placed into fresh polystyrene round plastic tubes. In our *in vitro* studies on PRPs which did not interact with any other cells or extracellular fluid such a sub-optimal recalcification was to obtain at least approximate physiologic conditions. For studies on thrombin-activated positive controls PRPs, 0.5 U of thrombin was added after PRP incubation with 0.6 µmol/L RGDS peptide to prevent PLT aggregation after their maximal activation by thrombin PRP samples (PRPs) were divided into groups:

1. nonactivated PRP as a negative control;
2. thrombin-activated PRP as a positive control for maximal platelet activation;
3. nonactivated PRP exposed for 20 min to PRF energy at 500 kHz, at a pulse rate of 5 ms/5 Hz, for a period of 120 sec, with an adjusted voltage of 40 V, to permit maximum temperature of 42°C; and
4. thrombin-activated PRP as a positive control and immediately exposed for 20 min to PRF to obtain the combined conditions as in groups 2 and 3;

To start studies 22-gauge, 5-mm active tip needles were placed perpendicularly to the tubes containing PRP samples (group 3 or 4) to allow energy exposure generated by Neurotherm Radio Frequency Lesion Generator Model NT2000 (Croydon, UK).

Once neuromodulation was terminated PRP samples including all controls were transferred to Eppendorf tubes placed on an ice bath, followed by centrifugation for 3 min at 10,000 *g*. Collected supernatants and blood cell pellets were used for LDH and MTT assay or stored at −80°C for up to four weeks before used for PF4 and ATP assays.

Cell Viability Assay

The analysis of cell viability was performed by incorporation with MTT assay (33). PRP samples were diluted in PBS and plated at a density of 2×10^7 cells in duplicate in 96-well plates (Corning Incorporated) then 0.5 mg/mL MTT was added. After four hours of incubation in dark at 37°C, DMF/SDS solution (20%/3%, pH 4.8) was added to dissolve the reduced formazan product. Finally, the plates were read in a microplate VICTOR 1420 Multilabel Counter (PerkinElmer, Warsaw, Poland) at 570/690 nm. The results were assessed as % compared to controls, which were 100% of cell viability.

LDH Assays

For LDH assay, collected supernatants were homogenized and solubilized in 0.2% v/v Triton X-100. The activity of LDH was assayed by spectrophotometric methods described elsewhere (34). Immediately before the assays, platelet membranes were solubilized by the addition of Triton X-100 at final concentration 0.2% by volume. Assays were performed at 37°C in Ultrospec 3 spectrophotometer (Amersham-LKB, Cambridge UK). The positive control that showed the maximal LDH release was blood cells pellets obtained by PRP samples centrifugation ad homogenized in Triton X-100.

ATP Release

In our *in vitro* studies, ATP release was analyzed as a marker of platelet dense granules and the platelet activation. Collected supernatants stored at −80°C were thawed and assessed by a luminometric method using luciferin/luciferase method on Berthold Junior LB 9509 luminometer (Berthold Technology, Bad-Wildbad, Germany) (35).

Platelet Factor 4 Release

Platelet factor 4 (PF4) release was assessed as a specific and a unique marker of platelet alpha granules and the platelet activation. Samples stored at −80°C were thawed and supernatants were assayed on the Imunoclone Platelet Factor 4 ELISA kit (Sekisui Diagnostics, Stamford, UK) as directed by the manufacturer. Briefly, the kit consisted of a 96-well plate precoated with an affinity purified rabbit polyclonal antibody specific for human PF4. The antibody captured the PF4 protein during an incubation for one hour at room temperature. Following a wash step, an affinity purified rabbit polyclonal antibody specific for human PF4 coupled to horseradish peroxidase (HRP) was added to the microwells for one hour at room temperature. Following another wash step, the peroxidase substrate 3,3′,5,5′ – tetramethylbenzidine (TMB), in the presence of hydrogen peroxide (H_2O_2), was added to the microwell and the subsequent enzymatic reaction yielded a blue colored solution. Last, the addition of sulfuric acid stopped the reaction and turned the solution color to yellow. The absorbance of the solution was measured at 450 nm on microplate VICTOR 1420 Multilabel Counter (PerkinElmer).

Platelet Aggregation

Isolated platelets were suspended in 0.3 mL of medium containing Tyrode's buffer (pH 7.4) and 5 mmol/L glucose to obtain density of 3×10^8/mL and preincubated for 5 min at 37°C in APACT aggregometer (Labor, Ahrensburg, Germany). Platelets were activated by the addition of thrombin (0.1 U/mL) and continued for 10 min (35,36).

Statistical Analysis

PRP data were presented as the means ± SD from 11 observations. Data for isolated platelets were means ± SD from three to six donors. The data distribution was tested by Kolmogorov–Smirnov normality test. Values of $p < 0.05$ were considered statistically significant. Differences between nonactivated negative control, thrombin-activated positive control, PRF treatment or thrombin-activated and PRF treatment groups were tested by Student's *t*-test. All statistical analyses were performed using the Graph Pad Prism 4.0 statistical package (Graph Pad Software, San Diego, CA, USA).

RESULTS

Characteristics of PRP From Study Groups

PRP preparation resulted in an approximately fourfold increase of platelet count compared with whole blood (Table 1). Leucocytes count in PRP was less than 1% and the mean PLT: WBC ratio was 113 (Table 1). There were only single erythrocytes present in PRP samples.

Effect of Temperature on Blood Platelet Aggregation

PRF energy gains maximal temperature of 42°C at the site of a cannula. Therefore, the effect of increased temperature on platelet aggregation was assessed. There was no difference in thrombin-induced platelet aggregation in samples exposed to 42°C for 20 min or in samples treated by PRF for 20 min compared with physiologic 37°C in both early phase and late phase of aggregation (Fig. 1).

Effect of Neuromodulation on Blood Platelets Granule Release

PF4 release as a specific and a unique marker of platelet alpha granules in PRP in resting conditions was 85.5 ng/mL PRP (Fig. 2a). Thrombin activation caused 2.5-fold increase of PF4 release in PRP (Fig. 2a). Treatment of PRP by PRF for 20 min induced threefold PF4 release from blood platelets (Fig. 2a).

Interestingly, there was no combined effect of PF4 release in thrombin-activated PRP samples treated by PRF (Fig. 2a).

The ratio of ATP-release in thrombin-activated or PRF-treated PRP was comparable with PF4 release in the same conditions (Fig. 2a,b). So ATP release as a marker of dense granules activation in PRP in resting conditions was 24.0 nmoL/mL PRP (Fig. 2b). Thrombin caused twofold increase of ATP release in PRP (Fig. 2b). The treatment of PRP with PRF for 20 min caused a similar release of ATP compared with thrombin activation (Fig. 2a,b). Unlike for PF4 release, the combined PRP activation with thrombin and PRF treatment caused a significant reduction of ATP release to the level comparable with resting conditions (Fig. 2a,b).

PRF treatment caused a significant release of both PF4 and ADP from PRP (Fig. 2a,b). In PRP treated with PRF, we found a significant correlation between PRF4 and ATP release ($r = 0.795$, $p < 0.05$) (Fig. 3). It confirmed a strong relationship between ATP release and PF4 release in PRP samples treated with PRF.

Effect of PRF on Platelet Viability and Survival

To assess the influence of PRF on metabolism, viability and survival of blood platelets and leucocytes in PRP, we used MTT test and LDH activity in PRP supernatants (Fig. 4). PRF technique did not have any influence on MTT in PRP both in resting conditions and in thrombin-induced PRF-treated PRPs (Fig. 4a). Simultaneously, there was no difference in LDH release in PRPs between resting PRF or thrombin-activated PRPs (Fig. 4b). The PRF technique altered neither viability nor survival of platelets in analyzed PRP samples (Fig. 4a,b).

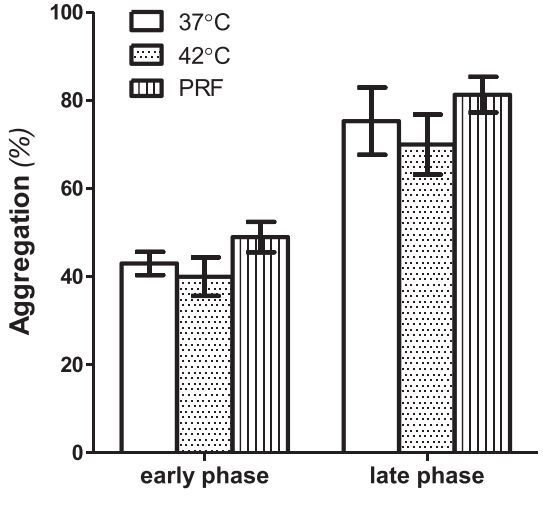

Figure 1. Effect of temperature and PRF on blood platelet aggregation. Data are means ± SD from three to six donors. Isolated platelets (200 giga/L resuspended in Tyrod's buffer) were incubated for 20 min at 37°C or 42°C or exposed to PRF for 20 min and then activated by thrombin (0.1 U). Early phase of aggregation was after 1 min and late phase after 10 min following thrombin addition.

DISCUSSION

The fourfold increase of platelet count in PRP compared to the mean platelet count in whole blood of donors indicates adequate technique of PRP preparation (Table 1) (13,37). Moreover, obtaining PRP close to 10^9/ml is required for bone and soft tissue healing enhancement and our PRP samples filled these criteria (Table 1) (13). Additionally, the minimal contamination of PRP by erythrocytes shows the lack of RBCs interference in the presented studies. However, the fact that PLT:WBC ratio was 113 shows that we obtained leukocyte-rich PRP (L-PRP) for our studies (Table 1) (13).

Active secretion of growth factors by blood platelets starts within 10 min upon activation and lasts up to one hour so it confirms that our 20-min exposure of PRP to PRF or thrombin was sufficient to observe PF4 and ATP release from α- and δ- granules, respectively (Fig. 2a,b) (37).

Our observation that incubation of platelets at temperature up to 42°C, which might be locally induced by PRF neuromodulation, did not affect thrombin-induced early platelet activation and late aggregation, viability and survival of PRP samples suggests that local hyperthermia associated with PRF did not have any impact on platelet metabolism and function (Figs. 1 and 4). Our results are contradictory to some reports, which linked hyperthermia to bleeding complications observed in patients with fever (38–40). Moreover, other studies showed that hyperthermia (40–42°C) inhibited platelet function due to selective down-regulation of the release of their alpha-granule proteins, ATP release, reduced platelet aggregation or accelerated their apoptosis (38–41). However, in the majority of those studies, the antiplatelet effect of hyperthermia was observed after at least one hour exposure of platelets to 42°C (39–41). This may suggest that hyperthermia may have an impact on platelet function but only after longer exposition, while in our studies, we exposed PRPs only for a short time, that is, 20 min (Fig. 1) (39–41). In our studies, such a short-time exposition did not affect platelet function and viability (Figs. 1–4). Therefore, these data confirm the safety of the neuromodulation technique with the parameters used in our studies for blood cells components (Figs. 1 and 4).

Presented data showing over twofold PF4 and ATP release from PRP upon PRF treatment revealed that neuromodulation technique, when applied to resting platelets (negative control), may induce their activation and granule release (Figs. 2a,b and 3). Moreover, the fact that the observed PRF-induced PF4 and ATP release was similar to thrombin-induced (positive control) granules release suggests that PRF has pro-platelet activating properties comparable with strong agonists of platelet activation such as thrombin (Fig. 2Aa,b) (35,42). Therefore, our data indicate that PRF technique may be useful to activate platelets in PRP samples injected to treated tissues or joints without any need to use additional activators (Fig. 2a,b). Since, PRF-applied parameters used in our experiments did not influence platelet viability or necrosis, it may suggest the safety of such a treatment on platelet concentrates; however, more data are required to confirm the safety of the technique in any clinical applications (Fig. 4).

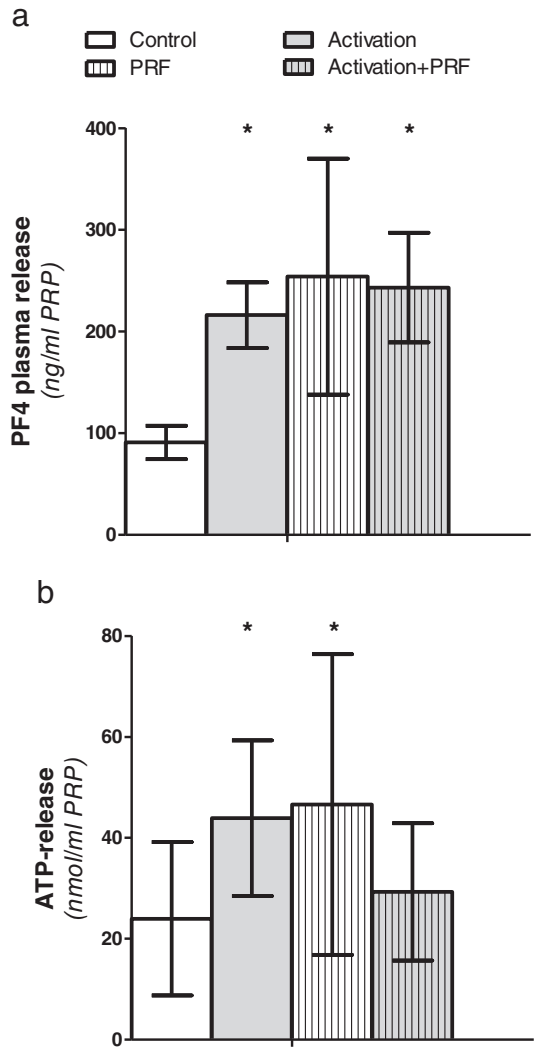

Figure 2. Effect of PRF on platelet granules release. (a) Effect of PRF on PF4 release from blood platelets in PRP. (b) Effect of PRF on ATP release from blood platelets in PRP. Data are means ± SD from 11 studies performed in duplicate. Significantly different from: $^{*}p < 0.05$, nonactivated conditions (paired Student's *t*-test), respective thrombin-activated conditions $^{+}p < 0.05$ (paired Student's *t*-test). PRF, pulsed radiofrequency neuromodulation; PRP, platelet-rich plasma; PF4, platelet factor 4.

The observation that PRPs activated by thrombin and simultaneously treated with PRF released similar level of PF4 compared with only PRF treatment or isolated thrombin-activation suggests no additional benefits of activating PRP samples when PRF is applied (Fig. 2). Moreover, combining both thrombin activation and PRF reduced ATP release to the level observed for resting PRP samples (Fig. 2b). This observation may be linked to increased dephosphorylation of the released ATP to ADP, AMP, and adenosine by leukocyte ecto-nucleotidases, which were present in PRP samples, due to over-activation of platelets (Table 1, Fig. 2b) (35,42). Such an observation also confirms no benefits of platelet or PRP over-activation by combining several activators (Fig. 2a,b). So PRF itself may be sufficient to activate blood platelets in PRPs.

The fact, that we observed a strong correlation between PF4 and ATP release from PRPs upon PRF treatment indicates that both α- and δ-granules release may be regulated by neuromodulation

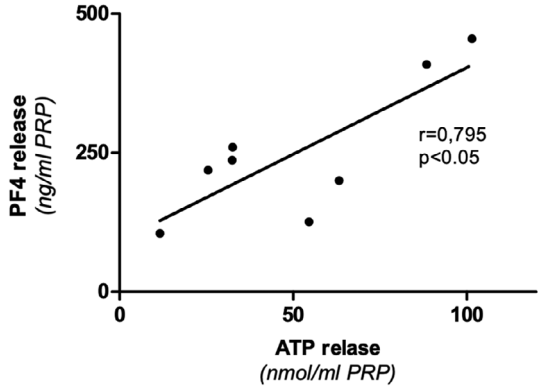

Figure 3. Correlation between ATP and PF4 induced by PRF in PRP. PRF, pulsed radiofrequency neuromodulation; PRP, platelet-rich plasma; PF4, platelet factor 4.

(Fig. 3). Additionally, the increased release of δ-granules by PRF confirms the ability of neuromodulation not only to activate platelets but also to potentiate their function (Figs. 2a,b and 3) (35).

Our data show that PRF applied to PRP samples activates blood platelets as strong as thrombin; however, the mechanisms of PRF action on platelets are unclear and require further investigation (Figs. 2a,b and 3).

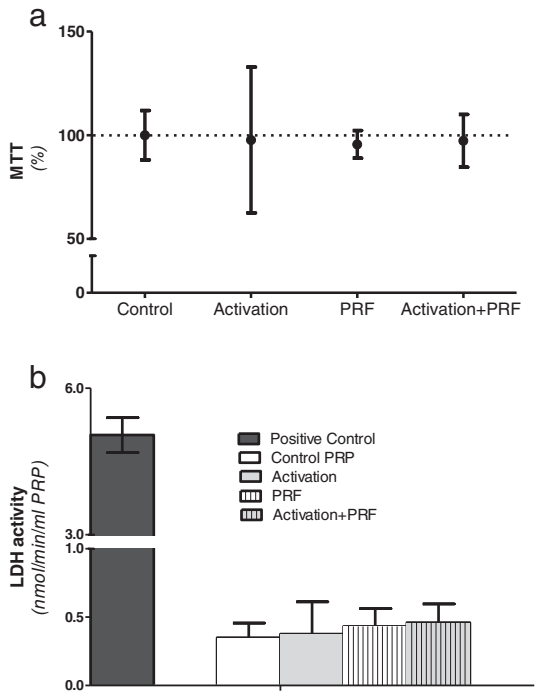

Figure 4. Effect of PRF on blood platelet viability and PRP cell components survival. (a) Effect of PRF on blood platelet viability. Data are means ± SD from three studies performed in duplicate. Isolated platelets (200 giga/L resuspended in Tyrode's buffer) were prepared as described in Materials and methods section and MTT assay was performed. (b) Effect of PRF on PRP cellular components. Data are means ± SD from 11 studies performed in duplicate. PRP was treated as described in the Materials and Methods section and LDH was assayed. The positive controls were pellets containing platelets and leucocytes as described in Table 1. PRF, pulsedradiofrequency neuromodulation; PRP, platelet-rich plasma; PF4, platelet factor 4.

One of possible explanations may be linked to previous observations that PRF induces permeabilization of cell membranes, which lead to elevation of intracellular Ca^{2+} (30,43). So, a rise in Ca^{2+} accompanies platelet granule secretion induced by most physiological agonist including thrombin (44). Therefore, we conclude that PRF may activate platelet granule secretion in a Ca^{2+}-dependent manner. That may also be supported by the observation that PRF induced granule secretion in a thrombin-activation pathway dependent mechanism (Fig. 2a,b). However, the precise mechanisms how PRF activates platelets requires further investigations.

These findings support the hypothesis that neuromodulation applied as PRF potentiated the activation of resting platelets measured by their alpha granules secretion in PRP samples when both methods were combined together in *in vitro* conditions. Additionally, PRF settings used in our experiments had no impact on functional integrity of blood platelets and leucocytes present in PRPs which confirms the safety of PRF but only on cellular components of PRP in *in vitro* conditions. Thereby, our preliminary research provides a rationale for the future clinical application of the combination of both PRF and PRP, currently used separately, to accelerate natural healing process in several fields of regenerative medicine including orthopedics, dentistry, surgery, or dermatology.

However, some limitations should be noted. First, we used *in vitro* model to define the PRF role in PRP activation. So our studies should be regarded as preliminary studies and further investigation on cell cultured enthothelial cells, fibrablasts or animal model needs to be carried for the final confirmation of our observation.

Second, we fully activated platelets with the use of exogenous activators such as thrombin to show maximal platelet activation and granule release to prove how potent neuromodulation is in PRP activation. Many clinical studies and practice are based on nonactivated PRPs. However, in cases where activated PRP is more preferable to redirect the inflammatory and regulatory effects or to use PRP as gel at surgical sites, we propose alternative options such as PRP application combined with neuromodulation instead of exogenous platelet activators. Finally, it is also worth assessing PRP activation and granule releases by different techniques in the future. Hence, our future work will be orientated toward more complex analysis on combination of PRP with neuromodulation on cell lines and tissues.

CONCLUSIONS

Activation of blood platelets in PRP by PRF potentiated their alpha and dense granules secretion in *in vitro* studies. PRF had no impact on platelet integrity, metabolism, and function. PRF induced granule secretion in a thrombin-dependent mechanism. These data indicate that combination of both PRF and PRP application may be useful in controlling pain and accelerating healing in regenerative medicine but further clinical studies are required to support our preliminary findings.

Acknowledgements

The work was funded by ST-57 project grant (Medical University of Gdańsk, Poland). The authors thank The Student Research Circle at the Department of Laboratory Medicine, Medical University of Gdańsk, Poland.

Authorship Statement

Dr. Anna Michno, Dr. Zbigniew Kirkor, Miss Ewelina Gojtowska, Dr. Marek Suchorzeski, Dr. Irmina Śmietańska and Dr. Bartosz Baścik designed and conducted the study, including patient recruitment, data collection and statistical analysis. Dr. Anna Michno prepared the manuscript with the intellectual input from Dr. Kirkor, Dr. Suchorzeski, Dr. Śmietańska, Baścik, and Dr. Miss Gojtowska. All the authors approved the final manuscript.

How to Cite this Article:

Michno A., Kirkor Z., Gojtowska E., Suchorzewski M., Śmietańska I., Baścik B. 2020. Pulsed Radiofrequency Neuromodulation Contributes to Activation of Platelet-Rich Plasma in *In Vitro* Conditions.
Neuromodulation 2020; E-pub ahead of print. DOI:10.1111/ner.13105

REFERENCES

1. Contreras Lopez WO, Navarro PA, Vargas MD, Alape E, Camacho Lopez PA. Pulsed radiofrequency versus continuous radiofrequency for facet joint low Back pain: a systematic review. *World Neurosurg* 2019;122:390–396.
2. Coppinger JA, Cagney G, Toomey S et al. Characterization of the proteins released from activated platelets leads to localization of novel platelet proteins in human atherosclerotic lesions. *Blood* 2004;103:2096–2104.
3. Flaumenhaft R. Platelet secretion. In: Michelson AD, editor. *Platelets*. Third ed. Amsterdam: Elsevier, 2013; p. 343–366.
4. Kark LR, Karp JM, Davies JE. Platelet releasate increases the proliferation and migration of bone marrow-derived cells cultured under osteogenic conditions. *Clin Oral Implants Res* 2006;17:321–327.
5. Anitua E, Andía I, Sanchez M et al. Autologous preparations rich in growth factors promote proliferation and induce VEGF and HGF production by human tendon cells in culture. *J Orthop Res* 2005;23:281–286.
6. Nagai M, Sato S, Kamoi H, Kamoi K. Effects of application of platelet releasate in periodontal regeneration therapy. *Nagai Int J Periodontics Restorative Dent* 2005; 25:571–583.
7. Moulin V, Lawny F, Barritault D, Caruelle JP. Platelet releasate treatment improves skin healing in diabetic rats through endogenous growth factor secretion. *Cell Mol Biol* 1998;44:961–971.
8. Yuan T, Zhang C-Q, Wang J. Augmenting tendon and ligament repair with platelet-rich plasma (PRP). *Muscles Ligaments Tendons J* 2013;3:139–149.
9. Wang JH, Nirmala X. Application of tendon stem/progenitor cells and platelet-rich plasma to treat tendon injuries. *Oper Tech Orthop* 2016;26:68–72.
10. Koji A, Junichi Y, Erikka TL, Akihiro S, Koichi M. Platelet-rich plasma in the management of chronic low back pain: a critical review. *J Pain Res* 2019;12:753–767.
11. Moraes VY, Lenza M, Tamaoki MJ, Faloppa F, Belloti JC. Platelet-rich therapies for musculoskeletal soft tissue injuries. *Cochrane Database Syst Rev* 2013;12: CD010071.
12. Reurink G. Managing acute hamstring injuries in athletes. *Br J Sports Med* 2017; 51:614–615.
13. Dhurat R, Sukesh MS. Principles and methods of preparation of platelet-rich plasma: A review and Author's perspective. *J Cutan Aesthet Surg* 2014;7:189–197.
14. Cavallo C, Roffi A, Grigolo B et al. Platelet-rich plasma: the choice of activation method affects the release of bioactive molecules. *BioMed Res Int* 2016;2016: 6591717.
15. Cares Huber S, Rosenberis Cunha J Júnior, Montalvão S, Queiroz da Silva L, Urban Paffaro A, Ramos da Silva FA. *In vitro* study of the role of thrombin in platelet rich plasma (PRP) preparation: utility for gel formation and impact in growth factors release. *J Stem Cells Regen Med* 2016;12:2–9.
16. Koh W, Choi SS, Karm MH et al. Treatment of chronic lumbosacral radicular pain using adjuvant pulsed radiofrequency: a randomized controlled study. *Pain Med* 2015;16:432–441.
17. Lee DG, Cho YW, Ahn SH, Chang MC. The effect of bipolar pulsed radiofrequency treatment on chronic lumbosacral radicular painrefractory to monopolar pulsed radiofrequency treatment. *Pain Physician* 2018;21:97–103.
18. Chang MC, Cho YW, Ahn SH. Comparison between bipolar pulsed radiofrequency and monopolar pulsed radiofrequency in chronic lumbosacral radicular pain: a randomized controlled trial. *Medicine (Baltimore)* 2017;96:e6236.

19. Brossi PM, Moreira JJ, Machado TSL, Baccarin RYA. Platelet-rich plasma in orthopedic therapy: a comparative systematic review of clinical and studyal data in equine and human musculoskeletal lesions. *BMC Vet Res* 2015;11:98.
20. Schmitt A, Griensven M, Imhoff AB, Buchmann S. Application of stem cells in orthopedics. *Stem Cells Int* 2012;2012:394962.
21. Vuka I, Vučić K, Repić T, Ferhatović Hamzić L, Sapunar D, Puljak L. Electrical stimulation of dorsal root ganglion in the context of pain: A systematic review of in vitro and in vivo animal model studies. *Neuromodulation* 2018;21:213–224.
22. Van Boxem K, Huntoon M, Van Zundert J, Patijn J, van Kleef M, Joosten EA. Pulsed radiofrequency: a review of the basic science as applied to the pathophysiology of radicular pain: a call for clinical translation. *Reg Anesth Pain Med* 2014;39:149–159.
23. Schianchi PM, Sluijter ME, Balogh SE. The treatment of joint pain with intra-articular pulsed radiofrequency. *Anesth Pain Med* 2013;3:250–255.
24. Cahana A, Vutskits L, Muller D. Acute differential modulation of synaptic transmission and cell survival during exposure to pulsed and continuous radiofrequency energy. *J Pain* 2003;4:197–202.
25. Van Boxem K, de Meij N, Kessels A, Van Kleef M, Van Zundert J. Pulsed radiofrequency for chronic intractable lumbosacral radicular pain: a six-month cohort study. *Pain Med* 2015;16:1155–1162.
26. Koh W, Choi SS, Karm MH et al. Treatment of chronic lumbosacral radicular pain using adjuvant pulsed radiofrequency: a randomized controlled study. *Shin J Pain Med* 2015;16:432–441.
27. Choi GS, Ahn SH, Cho YW, Lee DG. Long-term effect of pulsed radiofrequency on chronic cervical radicular pain refractory to repeated transforaminal epidural steroid injections. *Pain Med* 2012;13:368–375.
28. Chang MC. Effect of bipolar pulsed radiofrequency on refractory chronic cervical radicular pain: A report of two cases. *Medicine (Baltimore)* 2017;96:e6604.
29. Erdine S, Yucel A, Cimen A, Aydin S, Sav A, Bilir A. Effects of pulsed versus conventional radiofrequency current on rabbit dorsal root ganglion morphology. *Eur J Pain* 2005;9:251–256.
30. Protasoni M, Reguzzoni M, Sangiorgi S et al. Pulsed radiofrequency effects on the lumbar ganglion of the rat dorsal root: a morphological light and transmission electron microscopy study at acute stage. *Eur Spine J* 2009;18:473–478.
31. Hagiwara S, Iwasaka H, Takeshima N, Noguchi T. Mechanisms of analgesic action of pulsed radiofrequency on adjuvant-induced pain in the rat: Roles of descending adrenergic and serotonergic systems. *Eur J Pain* 2009;13:249–252.
32. Laver L, Marom N, Dnyanesh L, Mei-Dan O, Espregueira-Mendes J, Gobbi A. PRP for degenerative cartilage disease: a systematic review of clinical studies. *Cartilage* 2017;8:341–364.
33. Wang P, Henning SM, Heber D. Limitations of MTT and MTS-based assays for measurement of antiproliferative activity of green tea polyphenols. *PLoS One* 2010;5:e10202.
34. Koh JY, Choi DW. Quantitative determination of glutamate mediated cortical neuronal injury in cell culture by lactate dehydrogenase efflux assay. *J Neurosci Methods* 1987;20:83–90.
35. Michno A, Bielarczyk H, Pawełczyk T, Jankowska-Kulawy A, Klimaszewska J, Szutowicz A. Alterations of adenine nucleotide metabolism and function of blood platelets in patients with diabetes. *Diabetes* 2007;56:462–467.
36. Panse M, Block HU, Förster W, Mest HJ. An improved malondialdehyde assay for estimation of thromboxane synthase activity in washed human blood platelets. *Prostaglandins* 1985;30:1031–1040.
37. Marx RE. Platelet-rich plasma (PRP): what is PRP and what is not PRP? *Implant Dent* 2001;10:225–228.
38. Etulain J, Lapponi MJ, Patrucchi SJ et al. Hyperthermia inhibits platelet hemostatic functions and selectively regulates the release of alpha-granule proteins. *Thromb Haemost* 2011;9:1562–1571.
39. Aliasgharzadeh A, Gharehbaghian A, Taherian AA, Ghasemzadeh M, Salimian M. Modulation of hyperthermia-induced platelet aggregation inhibition in the presence of urea. *Int J Hyperthermia* 2013;29:256–258.
40. Wang Z, Shi Q, Li S, Du J, Liu J, Dai K. Hyperthermia induces platelet apoptosis and glycoprotein Ibα ectodomain shedding. *Platelets* 2010;21:229–237.
41. Shi Q, Wang Z, Yan R et al. The effects of hyperthermia on platelet physiology. *Blood* 2009;114:2422.
42. Heptinstall S, Johnson A, Glenn JR, White AE. Adenine nucleotide metabolism in human blood—important roles for leukocytes and erythrocytes. *J Thromb Haemost* 2005;3:2331–2339.
43. Rols MP, Dahhou F, Mishra KP, Teissie J. Control of electric field induced cell membrane permeabilization by membrane order. *Biochemistry* 1990;29:2960–2966.
44. Varga-Szabo D, Braun A, Nieswandt B. Calcium signaling in platelets. *Thromb Haemost* 2009;7:1057–1066.

COMMENT

The advent of platelet-rich plasma therapies in the field of pain medicine is noteworthy and deserving of further elucidation. As is often the case, patients receive treatment with several concomitant modalities, both pharmacologic and interventional. Awareness of the influence of dual therapies on each other is critical and can be detrimental, inconsequential, or even advantageous. Owing to the lack of knowledge regarding the efficacy of PRP, investigations such as this study into the potential role and mechanism of combination therapies are novel, important, and may lead to significant insights that in turn guide future developments.

Mark Jones, MD
Boston, MA, USA

