THE EFFECTS OF PLATELET RICH PLASMA AND COLD ATMOSPHERIC PLASMA ON RAT DORSAL SKIN FLAPS – A COMPARATIVE STUDY

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Abstract

The aim of the study was to compare the effects of platelet rich plasma (PRP) and cold atmospheric plasma (CAP) on two different rat dorsal skin flap model (ischemia free and induced ischemia). PRP has been used on rat dorsal skin flaps and showed vasodilatory effects and lowered the skin necrosis rate. To the best of our knowledge, there is no record of using CAP on this flap models or a comparative study of the two types of plasma (autologous and synthetic). Our study comprised 3 groups of 6 animals each (control, PRP and CAP) on which a modified McFarlane flap was performed (free ischemia) and 3 groups with the same design, but supplementary the left skin pedicle was surgically cauterised (induced ischemia). Autologous and synthetic plasma solutions were administered subcutaneously and the results evaluation was performed in the 5th and 7th postoperative day. The data interpretation follows the correlation between oxidative stress and haematological parameters, PRP and CAP administration in the case of induced or without induced ischemia. CAP had a better effect on cutaneous vascularization of the McFarlane dorsal flap, with lower necrosis rates compared with PRP.

Rezumat

Scopul studiului a fost de a compara efectele plasmatelor îmbogățite în trombocite (PRP) și plasmă rece la presiune atmosferică (CAP) asupra a două tipuri de lambou cutanat dorsal la șobolan. PRP a fost utilizată pe lambouri fasciocutanate dorsale la șobolan și a dovedit efecte vasodilatatoare, scăzând rata de necroză cutanată. Nu există însă nici o dovadă a utilizării soluției de CAP pe acest tip de lambou și nici un studiu comparativ al efectelor celor două tipuri de plasmă, autologă și sintetică. Studiul a cuprins 3 grupuri a câte 6 animale (martor, PRP și CAP) pe care s-a efectuat un lambou McFarlane modificat și 3 grupuri cu același design, la care pediculul stâng a fost cauzatizat chirurgical, pentru a induce ischemia lamboului. Soluțiile de plasmă au fost administrate subcutanat, iar evaluarea rezultatelor a fost efectuată în a 5-a și a 7-a zi postoperator. Interpretarea datelor a avut în vedere correlația ale markerilor stresului oxidativ și ale unor parametri biochimici după administrarea PRP și CAP la lorurile fără ischemie și la cele cu ischemie indusă. Soluția CAP a avut un efect mai bun asupra vascularizației cutanate a lamboului dorsal de șobolan McFarlane, cu rate de necroză mai mici, comparativ cu PRP.

Keywords: platelet rich plasma, cold atmospheric plasma, rat dorsal skin flaps, oxidative stress

Introduction

Chronic or acute wounds, with the exposure of vital structures such as nerves, bones or joints, need a robust coverage with a fascio-cutaneous flap or musculo-cutaneous flap. Unfortunately, depending on the local conditions, medical history of the patient or complexity of the procedure, complications can occur during or after the surgery: the most serious one is the total or partial necrosis of the flap, with or without abnormal scarring [21].

The rat dorsal skin has been used as an experimental model for flaps in order to test the effects of various vasoactive substances [10]. The most common design of a flap is the McFarlane type, which can be modified in several ways. It usually has a caudal base, and the perforator iliac skin pedicles can be cut in order to transform it in a purely random vascular type, with
a predictable area of necrosis [15]. This flap is composed of skin, subcutaneous tissue and superficial fascia with a very thin layer of muscle [18, 20]. There is a huge variety of substances already tested on this flap, in order to find which one has a strong vasodilatory effect, in order to lower the rate of the skin necrosis. PRP (platelet rich plasma) is one of them, and the results are encouraging, with proven proangiogenetic and anti-inflammatory effects [10]. Plasma is defined as an ionised gas produced by disintegration of polyatomic gas molecules or the removal of electrons from monoatomic gas shells [1]. It can be divided into high-temperature, thermal and non-thermal groups [5]. Plasma is the fourth state of matter, and occurs on earth as lightning in temperatures peaking over 24,000 K. The plasma generated at room temperature (239 K) is known as cold atmospheric plasma (CAP) or non-thermal plasma. There are many methods to create CAP such as a dielectric barrier discharge, when two electrodes create an electric discharge that energises the air to create plasma [11]. Low pressure cold plasma was first used for sterilisation and decontamination purposes, with high capacity of reducing bacterial load on a wound [11]. There are several devices for clinical or experimental use, which use CAP. They have similar characteristics: to accelerate acute or chronic wound healing by stimulating re-epithelisation, angiogenesis and collagen synthesis, concomitant with the antibacterial actions [4]. Different imaging techniques have been described to analyse the effects of the administrated substances on the rat dorsal skin flap such as the Doppler ultrasound, infrared thermography or percutaneous endoscopy [2, 7, 13, 17]. Blood samples provide researchers with information regarding coagulation status, inflammatory process, blood loss quantity after surgery and the level of oxidative stress [3].

To the best of our knowledge, no studies were published before regarding the comparative effects of PRP and CAP on McFarlane rat flap. The purpose of the study was to evaluate clinical and paraclinical differences in the effects of two vasoactive plasma solutions (PRP and CAP) on different rat dorsal skin flap models, without ischemia and with induced ischemia.

Materials and Methods

The study included 36 adults male Wistar rats, individually accommodated in special cages, with constant temperature, and a day/night cycle from 7 a.m. to 7 p.m., with access to water and food ad libitum. The groups comprised 6 animals each, and were divided in two experimental studies. The first experimental study was represented by 3 groups: control group, PRP group and CAP group. All of them were operated using a McFarlane surgical technique. The control group didn’t have any solution administrated, after the elevation of the flap and before suturing back a sterile synthetic material was interposed in order to prevent revascularisation from underlying tissue. The animals from the PRP group received 5 subcutaneous injections, 20 μL each, starting from a 2 cm distance from the pedicles and moving cranially. The CAP solution was administrated in the same manner. The second experimental study was similar to the first one, but after the dissection of the flap, the left perforator iliac pedicle was cut for induced ischemia. The PRP and CAP solutions were administrated in the same way as described above.

The experimental study was approved by the Ethics Committee of “Grigore T. Popa” University of Medicine and Pharmacy, Iași, Romania and was performed at the Advanced Research and Development Centre for Experimental Medicine (CEMEX).

The viability of the flaps was assessed on the 5th postoperative day, by clinical examination which included the integrity of the flap, colour, suture state, suppuration, turgor, temperature, fixation of the adjacent tissue and smell. The photographic documentation (Nikon D3200 device – Nikon Inc. NY, U.S.A.) was part of the protocol too. The captured images were analysed using the Fiji software (Eliceiri/LOCI group – University of Wisconsin-Madison, USA, Jug and Tomančak labs – MPI-CBG, Dresden, Germany): the selection of normal and pathological areas of the flaps being acquired with polygon selection tool, following the contour of the normal and pathological areas, first being acquired by the entire area of the flap and there after the pathological regions. To determine the area of necrosis, the ratio between area of necrosis and entire flap, multiplied by 100, was calculated.

PRP solution preparation: 6 - 8 mL of fresh blood were collected from 4 donor rats into special heparinized tubes through cardiac puncture. After the collection of the blood, the tubes were centrifugated at 3100 rpm for 10 minutes. The supernatant was transferred into new tubes and centrifuged at 3600 rpm for 15 minutes. The PRP solution (the supernatant of the second centrifugation) was transferred into new tubes. After the blood collecting procedure completed, the animals were euthanized through anaesthetic overdose.

CAP solution preparation [9]: an air dielectric barrier discharge (DBD) plasma source was designed to produce the pulsed plasma directly into 24 well plates, for a quick and safe manipulation of activated liquids. One column of the well plate can be exposed one at a time, using an array of 4 stainless steel electrodes, inserted into each well, leaving an open space between them and the walls of the wells. A gap of 3 mm is imposed between the electrode array and well plate bottom. The plate itself acts as a dielectric layer and then the circuit can contain supplementary dielectric layers to prevent the breakdown of well plates. A planar back electrode, made
Haematological assessment: on the 7th postoperative day, the animals were euthanized, after blood samples and histopathological fragments were collected. About 6 - 8 mL of blood were collected, from abdominal veins, in test tubes with ethylenediaminetetraacetic acid (EDTA) solution. After maximum 15 minutes the samples were transferred in the lab for further analysis. Malondialdehyde (MDA) quantification: for the evaluation of oxidative stress, was performed by a spectrophotometric method, using a Lambda 25 spectrophotometer, Perkin Elmer, USA. Glutathione (GSH) levels were determined by a chromatographic method, using an Altima HILIC column, 150 x 4.6 mm, 5 μm. Multi-level calibration curves were used for the quantification and good linearity (r > 0.999) was achieved for the tested intervals that included the whole concentration range found in the samples. The limit of quantification (LOQ) was calculated as 3x standard deviation of the analysed values for the procedural blanks, LOQ was calculated for a signal-to-noise ratio equal to 10 based on the signal obtained for the standard [19]. Clinical evaluation of haematological parameters: complete blood count is very commonly used as a simple screening test in various pathologies including anaemia and evaluation of an inflammatory process. Complete blood count consists in determining the following parameters: leucocyte count (WBC), erythrocyte count (RBC), haemoglobin (Hb), erythrocyte indices, mean erythrocyte (corpuscular) volume (MCV), mean erythrocyte (corpuscular) haemoglobin (MCH), concentration of mean erythrocyte (corpuscular) haemoglobin (MCHC), erythrocyte distribution width (RDW), platelet count (PLT), leucocyte formula (neutrophils, lymphocytes, monocytes, eosinophils, basophils).

Statistical analysis: the statistical study follows the analysis of oxidative stress as well as the paraclinical parameters variation. The extensive statistical analysis is performed to highlight the ability of PRP and CAP to influence oxidative stress in the cellular system, since data show some mixed effects of these compounds. Variable analysis was applied in order to explain a certain phenomenon. All statistical data analysis was performed using Microsoft Excel program and STATISTICA 10 software package. Multiple variables analysing techniques, by definition are performed by processing several variables that may have different scaling factors of the measurements. The results and interpretation of an analysis in which multiple variables are operated, critically depend on these factors.

The data normalisation process is necessary, especially if the values for a series of measurements of a variable have an asymmetric distribution, the series of values being characterized by the presence of several extremely large values. The Shapiro-Wilk’s normality test was performed, in order to observe if the study data are normally distributed. The test results show that the data analysed are not normally distributed (p > 0.05). The Spearman’s correlation coefficient was used to measure the strength of the correlations between haematological parameters and oxidative stress parameters. The value of p < 0.05 was considered statistically significant.

Results and Discussion

Quantification and clinical evaluation of oxidative stress parameters

Oxidative stress is an imbalance between oxidants and antioxidants produced by reactive oxygen species (ROS) called free radicals. Free radicals are substances derived from incompletely oxidized compounds, which have undergone partial combustion, having in their structure oxygen groups able to initiate aggressive oxidation reactions on the surface of cellular membranes or even inside cells. The antioxidant capacity of newly synthesized or used compounds is a permanent interest for researchers, many studies using different methods to evaluate the antioxidant capacity or activity also [6, 8]. Depending on the intensity, oxidative stress can occur intra- or extracellularly. Intracellular oxidative stress can cause cell necrosis or, more or less marked, cell disorganization, with catastrophic effects if a cell cannot replicate. Extracellular oxidative stress is also cytotoxic. Malondialdehyde (MDA) can be determined in many biological samples (serum, plasma and urine) and has become one of the most important biomarkers that can assess oxidative stress on lipids [19, 20]. Glutathione (GSH) is a localized intracellular tripeptide with many physiological functions: essential for cell proliferation, regulation of apoptosis, protection against free radicals.
The results obtained and the statistical analysis show a normal distribution of the oxidative stress parameters, respectively MDA and GSH. Therefore, Figure 1 shows the distribution of data by Quantile-Quantile plot (Q-Q plot) diagrams for oxidative stress parameters, as well as for the percentage of flap necrosis (% Necrosis), for all groups.

Figure 1. 
Q-Q plot for a) MDA (nmol/L), b) GSH (µg/g) and c) % Necrosis

The results of the quantification assessment of oxidative stress markers in plasma, are presented in Table I. Thus, in the case of the group of rats without induced ischemia, lower concentrations of MDA for the PRP group (mean = 0.61 nmol/L) and the CAP group (mean = 0.81 nmol/L) are observed compared to the control group (mean = 0.74 nmol/L). The profile of MDA concentrations shows mean concentration values of 0.61 nmol/L in the case of the PRP group, 0.81 nmol/L in the case of the CAP group and 0.74 nmol/L for the control group. Literature studies have shown that PRP is a widely used technique to stimulate the healing of human tissues, with a major role in improving endogenous antioxidant protective systems [12]. Moreover, PRP is considered to be an abundant reserve of various growth factors and activated platelets play an important role in endothelial damage during lesion production, especially inhibiting the production of ROS [16].

Table I
Oxidative stress parameters

<table>
<thead>
<tr>
<th>Studied parameter</th>
<th>Mean</th>
<th>SD</th>
<th>Median</th>
<th>Mean</th>
<th>SD</th>
<th>Median</th>
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<tbody>
<tr>
<td></td>
<td>Control group</td>
<td>PRP group</td>
<td>CAP group</td>
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<tr>
<td>GSH (µg/g)</td>
<td>135.57</td>
<td>73.12</td>
<td>139.10</td>
<td>117.29</td>
<td>19.41</td>
<td>118.34</td>
</tr>
<tr>
<td>MDA (nmol/L)</td>
<td>0.74</td>
<td>0.39</td>
<td>0.72</td>
<td>0.61</td>
<td>0.08</td>
<td>0.62</td>
</tr>
<tr>
<td></td>
<td>0.62</td>
<td>0.81</td>
<td>0.53</td>
<td>105.67</td>
<td>44.27</td>
<td>104.41</td>
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|                   | Control group | PRP group | CAP group |
| GSH (µg/g)        | 209.90 | 78.62 | 191.31 | 218.36 | 58.01 | 215.96 |
| MDA (nmol/L)      | 0.39   | 0.34  | 0.36   | 0.51   | 0.19  | 0.5   |
|                   | 0.35   | 0.32  | 0.33   | 165.33 | 51.93 | 150.15 |

The profile of plasma GSH concentrations is characterised by low values in the case of the CAP group without induced ischemia, values of the mean concentration of 105 µg/g, lower than the values of the mean concentration of the PRP group (117.29 µg/g). It has been shown that GSH has a protective role against cells apoptosis through a multifactorial mechanism involving detoxification and modulation of the redox state, being an important antioxidant in xenobiotic detoxification [14]. At the same time, serum GSH plays an important role in oxidative defence in animals, protecting against the negative effects of free radicals [22].

The study design follows the correlation between the effects of PRP and CAP administration and the oxidative stress, in the case of McFarlane flaps with or without induced ischemia. In Figure 2 lower concentrations of plasmatic MDA can be easily observed for PRP-ischemia group versus PRP-without induced ischemia group as well as lower concentrations of the same parameter for CAP-ischemia group versus CAP-free ischemia group. As for the control group the MDA value for the ischemic groups is lower than for the control group (0.39 nmol/L and 0.74 nmol/L). Both of the control groups had similar skin necrosis rates (around 50%), meaning that the impact of sacrificing one skin pedicle is not so strongly reflected upon the tissue damage. Also, sacrificing the artery right after flap elevation and immediate suture of the flap back into its original position enhanced small and medium skin vessels anastomosis in response to ischemia [16].

Similar results to assess ischemia exposure in the PRP technique have been described in other studies [16], which have argued that ischemia has a beneficial effect of stimulating angiogenesis and PRP activates the antioxidant response element.
An increased number of studies are evaluating oxidative stress because ROS produced during PRP infusion could activate apoptosis cascades. In this study, PRP decreased the concentrations of ROS by quantifying lower concentrations of MDA. In the case of CAP-induced ischemia group we found lower concentration of MDA (0.35 nmol/L) while in the CAP group, level of MDA was 0.81 nmol/L. In fact, we observed here that CAP acts as a protective factor on the lesion apparently by reducing the level of concentrations of ROS, in comparison with the control and PRP group.

One of the highlights of this study was to clinically evaluate the effects of PRP and CAP administration on the percentage of necrosis. Table II presents the results of the percentage of necrosis on the flap surface for the investigated groups.

Table II

<table>
<thead>
<tr>
<th>% of necrosis in the flap surface</th>
<th>Free ischemia groups</th>
<th>Induced ischemia groups</th>
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<tr>
<td></td>
<td>Mean</td>
<td>SD</td>
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* p < 0.005 vs. lot control-induced ischemia group; ** p < 0.005 vs. PRP-induced ischemia group; *** p < 0.001 vs. PRP-ischemia group; **** p < 0.05 vs. CAP-ischemia group; ***** p < 0.001 vs. PRP-ischemia group; **** p < 0.05 vs. PRP-ischemia group.

Table III and Table IV. Therefore, different values of mean platelet concentrations are observed in all analysed groups, as well as for the PDW (platelet distribution width) parameters. In the case of platelets, significantly higher values were determined in the induced ischemia-CAP group (with an average value of 1275 • 10^3/µL), compared to the induced ischemia-PRP group (with a mean concentration value of 954 • 10^3/µL).

Blood platelets are also involved in inducing the inflammatory process and play a role in the body's antimicrobial defence and tissue repair processes. Platelets mediate the adhesion of leucocytes to each other, as well as their adhesion to endothelial cells [3]. The characteristics of the two groups analysed were compared using the Man-Whitney U test. In the case of non-ischemic groups, statistically significant differences were determined between the control group and the CAP group for the following parameters: platelets (p < 0.05), HB (p = 0.004) and HCT (p = 0.008). Statistically significant differences were also determined between the control-ischemia group and the CAP-ischemia group for both platelets (p = 0.004) and MCV (p = 0.024), HB (p = 0.006), HCT (p = 0.006), RBC (p = 0.004), segmented neutrophils (p = 0.03).

In the case of the control group and the PRP-ischemia group, statistically significant differences p < 0.05, were determined for the MPV and PDW indices. Leukocytes and especially neutrophils are the first line of cells defence against infection. In the early stages, one of the systemic effects of inflammation is leukocytosis with a high level of neutrophils. The results for neutrophils in the investigated groups show values between 1.76 • 10^3/µL and 3.10 • 10^3/µL for the group with induced ischemia (Table III), as well as values of 3.25 • 4.45 • 10^3/µL for the group without induced ischemia. The clinical aspect of the ischemic and non-ischemic flaps did not show any signs of infection, but the number of postoperative complications (wound dehiscence, superficial seroma or exudate) was higher for the ischemic flaps, especially on the side where the artery was cut.
In order to highlight the oxidative stress in the case of the group with induced ischemia, the variation of the haematological parameters as well as those of the evaluation of the oxidative stress was analysed, applying the correlation matrix between the plasma solutions. The results of the Spearman correlations between the investigated clinical parameters in the case of the group with induced ischemia were analysed. Applying the correlation matrix in haematological parameters and oxidative stress evaluation parameters, very good correlations were obtained with values of r = 0.41 - 0.98; p < 0.05.

Significant correlations were obtained between neutrophils and WBC (r = 0.994, p = 0.000), RBC (r = 0.719, p = 0.000), PLT (r = 0.83, p = 0.000), but also plasma GSH concentrations and PDW (r = 0.42, p = 0.038). Plasma platelets may vary depending on size and volume PDW (platelet distribution width) aiming to show the heterogeneity of these cells in a given volume and is evaluated together with MPV.

The behaviour of the investigated clinical parameters for the group with ischemia exposure was performed using factor analysis. It is therefore observed that the factor analysis can be explained by the contribution of 3 factors. The first factor represents 51% of the total variation of the data set and it is represented by the plasma platelet concentration and the concentration of segmented neutrophils. The second factor represents 23% of the total variation of the data set and is represented by MPV, PDW platelet indices. The third factor represents 16% of the total variation of the data set and it is represented by the percentage of necrosis in the flap surface and the concentration of the evaluation parameters of the respective oxidative stress, GSH and MDA. Figure 3 shows the share of the three factors resulting from the factor analysis in the case of evaluating the PRP and CAP administration, for the group of rats with induced ischemia.
as clinical parameters MDA and GSH, important parameters in assessing oxidative stress for the percentage of necrosis evaluation in the case of PRP and CAP administration.

Conclusions
Cold atmospheric plasma solution (CAP) had a better effect on cutaneous vascularization of the McFarlane at dorsal flap, with lower necrosis rates compared with platelet rich plasma (PRP), both for rats without induced ischemia and for the group with induced ischemia.

The profile of plasma glutathione concentrations (GSH) is characterised by low values for the CAP group without induced ischemia, lower than the values of the PRP group. GSH plays an important role in oxidative defence in animals, protecting against the negative effects of ROS.

The clinical aspect of the flaps is correlated with the values of the oxidative stress and those of the haematological parameters. The results of the skin viability percentage in the group with PRP administered do not differ significantly from those in the literature.

CAP solution may be a good candidate for lowering skin flap necrosis in human flaps as well.

Conflict of interest
The authors declare no conflict of interest.

References