

Nanosecond Pulse Electric Field Activation of Platelet-Rich Plasma Reduces Myocardial Infarct Size and Improves Left Ventricular Mechanical Function in the Rabbit Heart

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Abstract: In the current study, we used the novel, nonchemical method of nanosecond pulsed electric fields (nsPEF) to investigate the efficiency of a protocol involving the in vivo treatment of the ischemic and reperfused heart and heart cells in culture with platelet-rich plasma (PRP). Associated with the restoration of blood flow to the ischemic tissue is a phenomenon referred to as “ischemic reperfusion injury.” Clinically a type of reperfusion injury occurs during coronary bypass surgery once blood perfusion to the heart is restarted. Although the restoration of oxygen to ischemic myocardial cells is critical for tissue survival, reperfusion causes myocardial oxidative stress, attributable in part to the increased production of reactive oxygen species (ROS). Enhanced ROS production is associated with mitochondrial damage. Adult female New Zealand white rabbits were anesthetized and a left thoracotomy performed to expose the heart. The distal segment of the left anterior descending coronary artery was occluded for 15 minutes and then released so reperfusion of the

tissue could occur. PRP (.21 mg/heart) or saline was injected into the ischemic area of the myocardium. Mechanical function of the left ventricle was analyzed using a Millar catheter attached to a Micro-Med Analysis System. H9c2 cells in culture were treated with 1 mL of nsPEF activated PRP (1.05 mg/flask) for 24 hours before analysis for ROS production or mitochondrial depolarization damage). The left ventricle contracted and relaxed faster and infarct size was reduced in hearts treated with PRP compared with saline. ROS production and mitochondrial depolarization were reduced in H9c2 cells treated with PRP and stimulated with hydrogen peroxide. These results provide evidence that nsPEFs can successfully be used to prepare PRP and that the PRP is functional in heart protection possibly by reducing ROS generation and stabilizing the mitochondria of the ischemic/reperfused heart. **Keywords:** platelet rich plasma, ischemic reperfusion injury, reactive oxygen species, mitochondria JECT. 2012;44:198–204

Platelet-rich plasma (PRP) or platelet gel is a therapeutic strategy used to enhance healing of surgical and nonsurgical wounds. The efficacy of this approach lies in the release of a wide range of proteins from platelets that are involved in hemostasis, renewed growth, angiogenesis, immune control, trapping circulating stem cells, and tissue remodeling (1–3). The rationale for using PRP is to enhance and support the body’s natural wound healing process by maximizing the availability of “healing factors” (proteins) at the site of the wound.

Myocardial infarction occurs when blood flow through coronary vessels is blocked or drastically reduced. However, associated with the restoration of blood flow to the ischemic

tissue is a phenomenon referred to as “ischemic reperfusion injury.” Although the restoration of oxygen to ischemic myocardial cells is critical for tissue survival, reperfusion causes myocardial oxidative stress, attributable in part to the increased production of reactive oxygen species (ROS) (4–11). ROS are major contributors to oxidative stress and include free radicals such as superoxide anions, hydroxyl radicals, lipid radicals, and hydrogen peroxide (5). Enhanced ROS production is associated with mitochondrial damage (6,7).

Currently, in most PRP preparations, the platelets are activated to release proteins that are stored within their alpha granules by exposing them to thrombin. Although thrombin is considered to be the “gold standard” for platelet activation, many of the principal actions of thrombin may be harmful to the heart. Thrombin is a multifunctional protease, which is proapoptotic and proinflammatory (12), and may have adverse effects on cardiac myocytes, which are independent of its procoagulant activity (12–15). It has been shown that in humans, thrombin generation during reperfusion after coronary artery bypass surgery is

Received for publication June 27, 2012; accepted December 2, 2012.

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The senior author has stated that the authors have reported no material, financial, or other relationship with any healthcare-related business or other entity whose products or services are discussed in this paper.

associated with postoperative myocardial damage (15,16) and that its thrombotic activity is only partially suppressed by heparin (13). Recently, Han et al. reported that PRP has the potential to increase the osteoinductivity of demineralized bone matrix but that its activation with bovine thrombin immediately before implantation significantly inhibits this activity (17) suggesting that thrombin may interfere with and/or antagonize the efficacy of PRP. Of importance to the heart is the fact that thrombin has been shown to cause the generation of ROS (13) and to induce apoptosis (16).

The novel aspect of this work was that we used nanosecond pulse electric fields (nsPEFs) to activate platelets. Nanosecond pulse electric fields are ultrashort, high-voltage pulsed electric fields. The pulses are of short duration and they affect intracellular as well as extracellular structures (membranes) and functions (cell signaling). Nanopulses convey intense, high-power (megawatts) but low-energy (joule) electric fields to platelets. nsPEFs charge cellular membranes of platelets and create a pore without permanent damage to the cell membranes (18,19). The 300-ns pulse generator used for these studies has been previously described (18,19).

The three objectives of the present study were: 1) to demonstrate that PRP prepared using nsPEFs improves function of the ischemic left ventricle; 2) to show that a nsPEF; prepared PRP reduced the size of the infarct; and 3) to investigate the effects of PRP prepared using nsPEFs on ROS production and mitochondrial depolarization.

MATERIALS AND METHODS

The protocols used in all studies involving animals were approved by the Animal Care and use Committee at Old Dominion University and in accordance with the Guide for the Care and Use of Laboratory Animals, 8th edition, 2011 National Research Council.

Nanosecond Pulse Electric Field and the Pulse Generator

Nanosecond pulse electric fields are ultrashort, high-voltage pulsed electric fields. The pulses are of short duration and they affect intracellular as well as extracellular structures (membranes) and functions (cell signaling). Nanopulses convey intense, high-power (megawatts) but low-energy (joule) electric fields to platelets. nsPEFs charge cellular membranes of platelets and create a pore without permanent damage to the cell membranes (18,19).

Preparation of Platelet-Rich Plasma

PRP was prepared as described by Harvest Technologies (Plymouth, MA). Sixty milliliters of whole blood was withdrawn from a donor rabbit using a sterile syringe containing 6 mL of ACD-A anticoagulant. Removal of this amount of

blood is incompatible with life so after the blood was removed, the blood donor animal was euthanized with FatalPlus (2 mL/kg) given intravenously. A SmartPREP[®]-2 Platelet Concentrate System and a sterile processing disposable pack (Terumo Cardiovascular Systems, Ann Arbor, MI) were used to prepare PRP and platelet-poor plasma. The processing disposable was placed into the centrifuge and centrifuged for 14 minutes to separate the blood components from the plasma. Platelet-poor plasma was used to resuspend the concentrated platelets to a final volume of 7 mL. The electrical pulses were applied to PRP in the presence of 1 mM calcium in sterile electroporation cuvettes with an electrode gap of .4 cm. The aluminum cuvette plate electrodes had an area of 1 cm² for the .4-cm gap cuvette. The shape and amplitude of the pulse voltage were monitored using a 500-MHz oscilloscope. After five pulses at an electric field of 30 kV/cm for a duration of 300 ns, the platelets were quickly removed from the cuvette and used to treat the experimental animals or used in *in vitro* studies.

Hematological analysis of rabbit whole blood used to prepare PRP was analyzed using a HESKA Diagnostic System (Loveland, CO). The PRP had an average platelet count of $295 \times 10^3/\mu\text{L}$. The Harvest Technology System concentrator (Harvest Technologies, Plymouth, MA) concentrated the platelets in the whole blood four to seven times providing a platelet concentrate between $1180 \times 10^3/\mu\text{L}$ and $2065 \times 10^3/\mu\text{L}$ of platelets.

Ischemic Reperfusion In Vivo in the Rabbit Heart Model

Eight New Zealand white rabbits (Harlan, Inc., Frederick, MD) were sedated with a combination of acepromazine (2 mg/kg) and ketamine (25 mg/kg) and intubated using a pediatric endotracheal tube size 3. Once the endotracheal tube was in place, general anesthesia was induced using isoflurane in oxygen (1.5–2% for the duration of the procedure). The endotracheal tube was attached to a Harvard respirator and the animal ventilated at a tidal volume of approximately 50 mL of O₂/min. The left thoracic area was shaved and cleansed with 70% ethanol and Chlorhex-Q scrub. Once a surgical plane of anesthesia was established, the animal was surgically prepared for the creation of ischemia and reperfusion by performing a left thoracotomy and pericardiotomy. To make the heart ischemic, the distal branch of the left anterior descending artery was transiently occluded by placing a 5-0 proline suture around the distal segment of the vessel. The coronary vessel was occluded for 30 minutes by placing tension on the suture. Ten minutes before release of the occlusion (reperfusion), 200 μL (.21 mg/heart) of PRP activated with nsPEFs or 200 μL saline was injected directly into the myocardium of the left ventricle at the level of the infarcted tissue using a sterile 25-G 1/2-inch needle. Preliminary studies in our laboratory using the rabbit Langendorff heart demonstrated that 200 μL of platelet gel

was the minimum amount of gel that could be injected and improvement of left ventricular function observed. The injection was given 10 minutes before reperfusion to allow enough time for the components of the platelet gel to diffuse within the infarcted area. We therefore used this amount of platelet gel in the current *in vivo* studies. Care was taken not to place the PRP or saline into the ventricular chamber. After the injection of activated PRP or saline into the muscle of the left ventricle, the thoracic cavity was closed and care taken to remove excess air from the cavity to prevent the development of a pneumothorax. An Angiocath (16 gauge) was used as a chest tube to remove air from the chest cavity. The chest tube was then removed. Once the chest was closed and the incision sutured, the isoflurane was stopped and the animal was allowed to breathe room air. The endotracheal tube was removed when there were signs that the animal was breathing comfortably without the ventilator. All animals were returned to their housing facility for 14 days.

Measurement of Left Ventricle Mechanical Function In Vivo during Dobutamine Stress

Fourteen days after coronary occlusion and reperfusion, general anesthesia was again induced with isoflurane and oxygen as previously described. A midline incision was made over the trachea and the left carotid artery exposed. A Millar Probe Catheter-Model SPR 524 (Millar Instruments, Houston, TX) was gently passed into the left ventricle through the carotid artery for the measurement of left ventricular pressures, heart rate, and positive and negative dp/dt. A 25-G catheter was placed into the marginal ear vein for the administration of dobutamine (5, 10, and 20 $\mu\text{g}/\text{kg}/\text{min}$), which was used as a stressor. The dobutamine (Sigma-Aldrich, St. Louis, MO) dose was increased every 3 minutes until the maximum dose was achieved. Data (left ventricular maximal pressure, heart rate, positive and negative dp/dt) were collected for 60 minutes after the maximal dobutamine dose was given using a Micro-Med Analyzer (Louisville, KY).

Infarct Staining and Measurement of Infarct Size

With the animal under a surgical plane of anesthesia, the heart was removed after completion of the dobutamine stress test and a cannula was placed in the aorta. The heart was perfused with saline (.9%) followed by perfusion of a red dye (2,3,5-triphenyltetra-dodium chloride; Sigma-Aldrich) and then with a blue dye (Blue Heubach-I Pigment Dispersion dye/stain; Sigma-Aldrich), which served as our counterstain at a ratio of 1:4. A tight suture was placed around the vessel that was originally ligated at the level of the ligation to prevent the counterstain, which is the blue stain from entering this region.

That air suture made when the heart was infarcted marked this area. The heart was placed on a flat surface and all tissue above the left ventricle removed and dis-

carded. The left ventricle was weighed and separated into the parts above and below the ligation of the coronary vessel. The part of the left ventricle below the ligation was cut into slices, weighed, and placed in 10% formalin for 24 hours. A digital picture of each slice was taken and analyzed using a Metamorph Imaging program. Infarct quantification was performed as described by Bohl et al. on digital photographs by manually contouring the differentially colored left ventricle subsets (20). The relative areas of the subsets were obtained using a pixel count tool. The infarcted area stained white. The area was then corrected for slice weight.

H9c2 Cell Culture

In Vitro Reactive Oxygen Species Production in the Presence and Absence of Platelet-Rich Plasma: The cell culture experiments were performed over a 3-day period. *Rattus norvegicus* H9c2 cardiac myoblast cells (CRL-1446; ATCC, Manassas, VA) were grown in sterile T-75 cm^2 tissue culture flasks in an incubator at 37°C and 5% atmospheric CO_2 . Cell culture media consisted of 15 mL Dulbecco's Modified Eagle's Medium™ (DMEM) (Mediatech, Inc., Manassas, VA) containing 10% fetal bovine serum (Atlanta Biologicals, Inc., Lawrenceville, GA). Once the cells reached 85–95% confluency, approximately 800,000 H9c2 cells were transferred onto a sterile T-25 cm^2 tissue culture flask on Day 1. On Day 2, after a 24-hour incubation in DMEM, the cells were treated with fresh DMEM as previously described or 3.5 mL of DMEM containing nsPEF-activated PRP (.525 g). The cells were then returned to the incubator for 24 hours. On Day 3, ROS detection was performed in H9c2 cells in culture using flow cytometry. The cells were washed with fresh cell culture medium, trypsinized, and suspended in cell culture media. The cells were then loaded with 20 μM concentration of CM-H₂DCFDA solution per manufacturer instructions (Invitrogen-Molecular Probes, Eugene, OR), placed in the dark at 37°C for 1 hour, then stimulated for 5 minutes with H₂O₂. ROS formation was measured using flow cytometry (BD FACSAria, San Jose, CA).

Mitochondrial Depolarization Analysis Using Flow Cytometry

These experiments were also performed over a 3-day period. During the first 2 days, the cells were treated as described for ROS analysis. On Day 3, the media was removed and discarded. Cells were stimulated with 0, .03 (8.8 mM), .07 (20.5 mM), or .15% (44 mM) of hydrogen peroxide in DMEM growth medium at 37°C in a 5% CO_2 incubator for 15 minutes. The cells were then washed in fresh media, trypsinized, and suspended in media. The supernatant was discarded after centrifugation at 200 g for 10 minutes. The cells were then resuspended in .5 mL of 1 × MitoPT™ JC-1 solution (ImmunoChemistry Technologies,

LLC, Bloomington, MN) and incubated at 37°C in a 5% CO₂ incubator for 15 minutes. After this loading period, the cells were washed with and resuspended in assay buffer. The changes in mitochondrial membrane potential were evaluated using a flow cytometer. A membrane-permeant JC-1 dye was used per manufacturer instructions (ImmunoChemistry Technologies, LLC) to assess mitochondrial health in the presence or absence of PRP. JC-1 dye exhibits potential-dependent accumulation in mitochondria, indicated by a fluorescence emission shift from green (approximately 529 nm) to red (approximately 590 nm). When cells stained with MitoPT™ JC-1 were run through the flow cytometer, the instrument measured depolarization by monitoring the amount of red fluorescence in each region. Healthy cells fluoresced red. However, as the mitochondria depolarized, the amount of red fluorescence dropped.

Statistical Analysis

The in vivo heart data were analyzed using an analysis of variance corrected for repeated measures and stated as the mean ± standard deviation (SD). The Tukey or Student-Newman-Keuls post hoc tests were used to determine which groups were statistically different from each other. ROS or mitochondrial depolarization experiments were repeated five times and analyzed using a Student's *t* test. Data are stated as the mean ± SD. Data were considered significant at $p < .05$. A power analysis was performed by a statistician (Dr. D. Niak in the Department of Statistics at Old Dominion University) and was required for protocol approval by the Internal Animal Care and Use Committee. The sample size calculations were done in SAS for comparison of two means using paired *t* test with $\alpha = .05$ and minimum power of 80%.

RESULTS

Ischemia and Reperfusion Rabbit In Vivo Heart Experiments

Left ventricular positive (Figure 1A) and negative dp/dt (Figure 1B) were analyzed to determine the effects of activated PRP on left ventricular function in vivo 14 days after ischemia and reperfusion and with the heart under a dobutamine stress. Under stress, the left ventricle of the activated PRP-treated hearts contracted (positive dp/dt) and relaxed (negative dp/dt) faster than the hearts treated with saline.

Infarct Size in the Presence and Absence of Platelet-Rich Plasma

We measured the size of the infarcted area of the heart in eight rabbits 14 days after the heart was made ischemic and reperused and immediately after the heart was stressed with dobutamine. The size of the infarcted area

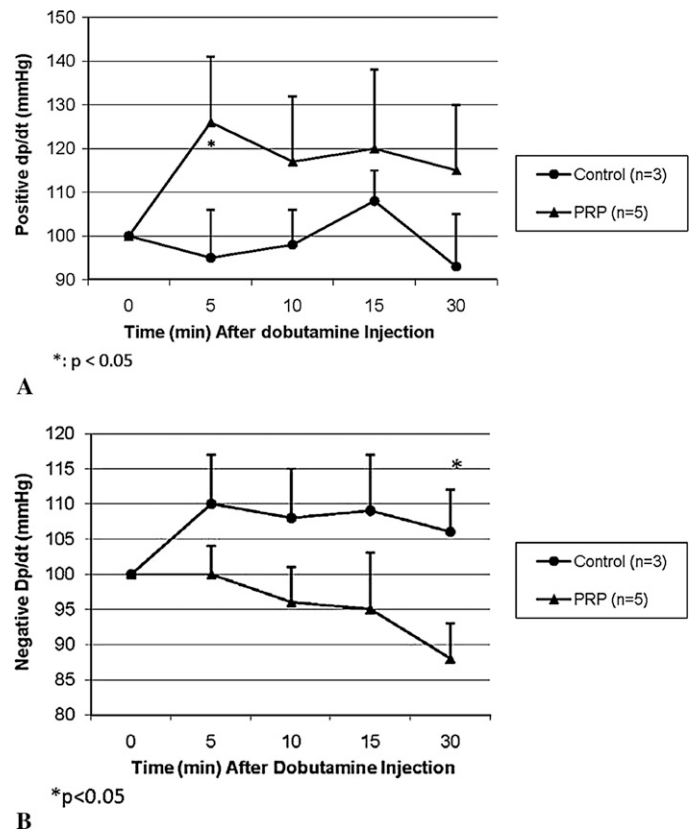


Figure 1. In vivo left ventricular positive and negative dp/dt, respectively, in the rabbit heart stressed with dobutamine 14 days after 30 min of occlusion of the distal segment of the left anterior descending coronary artery and reperfusion. Work function was significantly higher ($p < .05$) in the PRP than in the saline treated hearts five minutes after the highest dose of dobutamine was given intravenously. The left ventricle of the PRP treated hearts was significantly more relaxed ($p < .05$) than the left ventricle of the saline treated hearts 30 min after the highest dose of dobutamine was given intravenously. Data were normalized to a baseline value of 100% and stated as the mean ± SD.

in the hearts treated with activated PRP was significantly ($p < .05$) smaller than that observed in animals treated with saline (Table 1).

In Vitro Reactive Oxygen Species Production in the Presence and Absence of Platelet-Rich Plasma

To determine if activated PRP reduced ROS production in cells stimulated with H₂O₂, we loaded H9c2 cells with the free radical detection agent CM-H₂DCFDA by placing the dye in the cell culture media. ROS production was not significantly different in cells placed in cell culture media only or in media containing PRP (n = five separate experiments) in the absence of H₂O₂. In separate experiments, cells were 1) exposed to PRP placed in cell culture media; or 2) cell culture media only in the presence of 8.8 mM, 20.5 mM, or 44 mM H₂O₂ (n = five separate experiments for each concentration of H₂O₂). Activated PRP significantly reduced the ROS response in H9c2 cells stimulated with

Table 1. Size of left ventricle, risk region, and infarct.

Treatment Group	Heart (g)	LV (g)	Area at Risk (g)	Infarct (g)	Area at Risk (% of LV)	Infarct Size (% of LV)	Infarct Size (% of area at risk)
PRP	14.61 ± 3.89	8.77 ± 2.33	.50 ± .12	.05 ± .01	6.42 ± 3.63	.69 ± .34	11.02 ± 1.42
Saline control	11.60 ± 4.42	6.96 ± 2.65	.56 ± .19	.12 ± .07	9.07 ± 5.51	1.96 ± 1.75	19.51 ± 6.23
<i>p</i>							<.05

LV, left ventricle; PRP, platelet-rich plasma.

H₂O₂ at final concentrations of 44 mM ($p < .05$) and 8.8 mM ($p < .05$) (Figure 2).

In Vitro Mitochondrial Depolarization in the Presence and Absence of Platelet-Rich Plasma

To determine if activated PRP had an effect on mitochondrial depolarization, we stimulated H9c2 cells pretreated with PRP (placed in cell media, 1:7 dilution) or with cell culture media only (control) with H₂O₂ at concentrations previously described and measured changes in mitochondrial membrane potential. Cells treated with PRP and stimulated with 20.5 mM ($p < .05$) and 44 mM ($p < .05$) H₂O₂ had significantly less mitochondrial depolarization than cells treated with media only (Figure 3). Five separate experiments were performed at each concentration of H₂O₂.

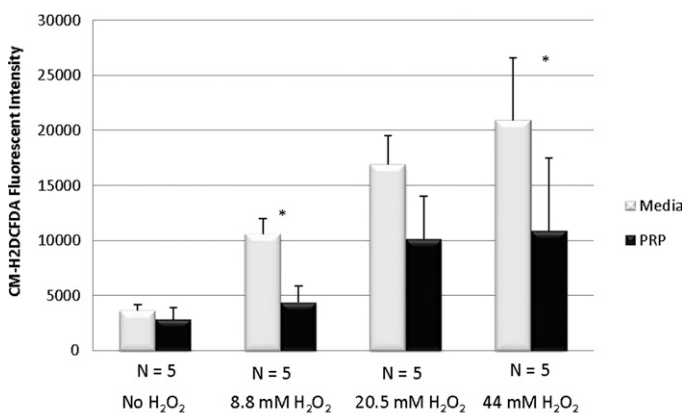
DISCUSSION

Ischemic heart disease secondary to acute myocardial infarction (AMI) is a major cause of morbidity and mortality in the United States and in the world. On rare occasions a clinically induced form of ischemic reperfusion injury may occur in patients undergoing coronary artery

bypass graft (CABG) surgery and can lead to myocardial stunning and increased cardiopulmonary bypass time. Myocardial protection after AMI and during and/or after CABG would greatly reduce the effect of myocardial stunning and the pathological restructuring of the heart that so often occurs after AMI, which can lead to heart failure.

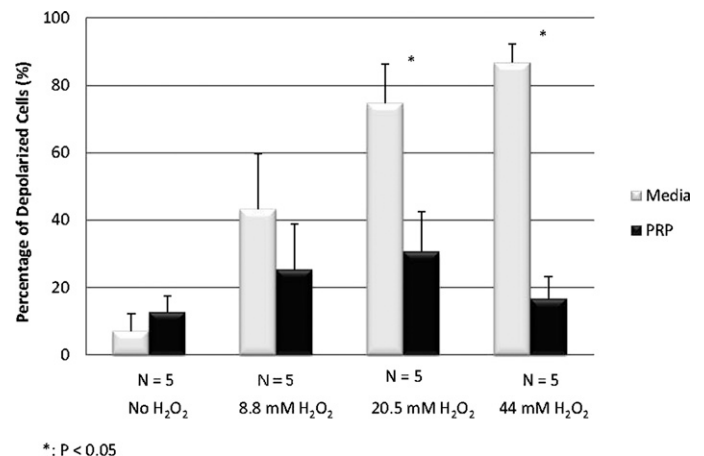
Clinically, the timely restoration of myocardial blood flow has reduced the number of deaths associated with AMI. However, the ability to limit the effects of reperfusion injury remains an area for study. Certain high-risk cardiac patients might especially benefit from a greater level of protection. Although PRP prepared with thrombin has been shown to improve heart function after ischemia, the thrombin used in the preparation of PRP may be counterproductive to the desired effects of the treatment. We have available to the clinical community a safe, nonchemical platelet activator (nsPEFs) that is easy to use in the preparation of PRP for treatment of many different types of wounds. The pulse generator never comes in contact with the patient and allows for the production of a sterile platelet gel for application to the wound.

The PRP prepared using nsPEFs has been just as effective in functional assays as the PRP prepared using thrombin (bovine or human). More importantly, repeated exposure of



*: $P < 0.05$

Figure 2. In vitro effect of PRP on ROS production as analyzed by flow cytometry. ROS production was significantly less in the PRP treated cells when stimulated with H₂O₂ at concentrations of 8.8 mM ($p < .05$) and 44 mM ($p < .05$). Data are stated as mean ± SD. Five separate experiments were performed at each concentration of H₂O₂.



*: $P < 0.05$

Figure 3. Effect of PRP on mitochondrial depolarization as analyzed by flow cytometry. Mitochondrial depolarization was significantly less in the PRP treated cells when stimulated with H₂O₂ at concentrations of 20.5 mM ($p < .05$) and 44 mM ($p < .05$). Data are stated as mean ± SD. Five separate experiments were performed at each concentration of H₂O₂.

autologous PRP to patients should not generate antibodies against certain clotting factors observed with thrombin.

In Vivo Ischemia and Reperfusion

Our experiments show that PRP injected into the ischemic myocardium before reperfusion is associated with faster contraction and relaxation of the ischemic reperfused left ventricle. These data are consistent with data published by other investigators (21–23). In addition, the infarct size was smaller in PRP-treated hearts than in hearts treated with vehicle suggesting that PRP may reduce the stress to noninjured myocytes and therefore prevent them from becoming apoptotic.

Mechanistic Considerations of Platelet-Rich Plasma in the Heart

The mechanism associated with the improved mechanical function of the left ventricle in the PRP-treated hearts is unclear and may involve the interaction of several parameters. First, our results show that PRP reduces the production of ROS in H9c2 cells stimulated with H₂O₂ in vitro and therefore may possibly reduce ROS in the ischemic heart during reperfusion. ROS are highly reactive chemical entities that can exert harmful effects on heart tissue when produced in concentrations that overwhelm the body's inherent antioxidant system. ROS have direct electrophysiological effects that contribute to arrhythmias (11) and are implicated in the pathogenesis of postischemic myocardial stunning (contractile dysfunction that is reversible). Myocardial cell death after ischemia and reperfusion results from necrosis and apoptosis, which can be activated by ROS (16) generated during ischemia and reperfusion, effectively increasing the size of the infarct. In addition to a reduction in ROS production, we observed a significantly smaller infarct area in the hearts treated with PRP. The fact that ROS production is reduced in the presence of PRP is both exciting and intriguing.

Another mechanism that PRP may use to protect the heart from further ischemic/reperfusion damage is suggested by our data showing a reduction in mitochondrial depolarization in H9c2 cells treated with PRP. H9c2 cells pretreated with PRP and stimulated with H₂O₂ showed fewer depolarized mitochondria than cells treated with cell culture media only. In the heart, mitochondria make up approximately 35% of cardiomyocyte volume. Although mitochondria are a source of ROS, mitochondria also provide ROS defense mechanisms, including enzymatic antioxidants. When there is an imbalance between ROS generation and ROS scavenging, as is the case with ischemic-reperfusion injury, accumulated ROS can alter the function of proteins, lipids, and DNA through structural modifications (5). A major source of mitochondrial injury is oxidative stress produced by ROS, which are byproducts of energy production in mitochondria.

Methodological Considerations

One methodological aspect that may have impacted the results of the present study is that we used .9% saline as our vehicle of injection for hearts serving as our controls. Platelet-poor plasma was considered for use as a vehicle in the control hearts because its viscosity was somewhat closer to that of PRP. However, the residual platelets in platelet-poor plasma (although few in number) may have altered the response of the left ventricle to ischemia and reperfusion.

CONCLUSION

We provide evidence that PRP supports left ventricular function in the ischemic reperfused heart, reduces the size of the ischemic area, reduces ROS production, and reduces mitochondrial depolarization. We present here preliminary results that demonstrate the feasibility of our approach. Although we are aware of the fact that the fundamental physiologic role of platelets is to facilitate clot formation, we are currently investigating ways to obviate the clot-forming aspect of platelet gel and to identify and isolate those factors within the platelet, which seem to demonstrate the cardioprotective effect(s) observed in this study.

ACKNOWLEDGMENT

We gratefully acknowledge the financial support for these studies by Dr. Mahamad Karim of the Office of Research at Old Dominion University and the statistical assistance by Dr. Niak, statistician in the Department of Math and Statistics. We also thank Dr. Len Murray, Rashae Cook, Anne Nix, Danelle Tajirian, and Ben Cuffee for the expert care of the animals.

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