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Autologous Platelet Rich Plasma (Platelet Gel): An Appropriate Intervention for Salvaging Cardiac Myocytes under Oxidative Stress after Myocardial Infarction

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Abstract

Background: The prompt restoration of blood flow (reperfusion) to the ischemic myocardium after an acute myocardial infarction is critical to the survival of non damaged heart tissue. However, reperfusion is responsible for additional myocardial damage. Our objective was to investigate the role of autologous platelet rich plasma or platelet gel prepared using nanosecond pulsed electric fields (nsPEFs) in improving left ventricular mechanical function after ischemic reperfusion.

Methods: The hearts of nine adult female New Zealand White rabbits were perfused using the Langendorff model. The hearts were exposed to global ischemia for 30 min and reperfused for 60 min. The hearts were injected with 600 µl of platelet gel supernatant prepared using nsPEF or platelet gel supernatant prepared using bovine thrombin or with bovine serum albumin (BSA) which served as our control. HUV-EC cells or H9c2 were used *in vitro* to determine the effect of platelet gel on ROS formation and mitochondrial depolarization using flow cytometry. Metalloproteinase's and their inhibitors were assessed using western blot analysis. HPLC was used to identify the presence of endogenously secreted antioxidants in the platelet gel.

Results: Platelet gel improved left ventricular mechanical function of the heart, reduced ROS formation and reduced mitochondrial depolarization. Platelet gel also decreased MMP-2 and increased TIMP-1. Catalase and superoxide dismutase were preserved at greater concentrations in the platelet gel made using nsPEFs than in the platelet gel made with bovine thrombin.

Conclusion: Platelet gel is cardioprotective to non- ischemic reperfused cardiac tissue after acute myocardial infarction and reperfusion.

Keywords: Oxidative stress; Myocardial infarction; Platelet gel

Summary

Platelet gel improves left ventricular mechanical function in the ischemic reperfused Langendorff rabbit heart, decreases ROS production and mitochondrial depolarization in HUV-EC cells (Homo sapiens, human Umbilical Vein Endothelium) *in vitro*, protects catalase and superoxide dismutase when prepared using nanosecond pulsed electric fields rather than bovine thrombin and decreases the metalloproteinase MMP-2 and increases the metalloproteinase inhibitor TIMP-1 in H9c2 cells (*Rattus norvegicus* H9c2 cardiac myoblast cells) *in vitro*.

Introduction

In patients admitted to a hospital with myocardial infarction, some of the myocardial tissue is irreversibly damaged by necrosis, but a larger part is under ischemic stress and may still be saved by appropriate intervention. It is essential to restore coronary flow to the ischemic region (reperfusion), but if no additional measures are taken, reperfusion causes the production of reactive oxygen species (ROS), which can lead to substantial tissue death (reperfusion injury) [1].

Autologous Platelet-rich plasma (PRP) or platelet gel is emerging as a biological tool to reduce cardiovascular reperfusion injury in animal experiments [2]. PRP is made from an animal's own whole blood (autologous). The platelets are concentrated and the release of growth factors from the platelets is induced, typically by combining the platelet concentrate with bovine thrombin and calcium. The resulting "activated" PRP contains a super-physiological concentration of growth factors, cytokines, and other proteins [3]. Injecting it into the myocardium after infarction and prior to reperfusion significantly improves left ventricular mechanical function during reperfusion *in vivo* [4] in rabbit hearts and promotes angiogenesis and mitogenesis in the sheep heart when injected 3 weeks after coronary ligation and evaluated 9 weeks later [5]. In addition to the growth and healing promoting proteins, PRP provides a scaffold that traps cells such as stem cells in the region of injury giving the proteins time to help the cells differentiate into cardiac myocytes.

Several problems have so far precluded the use of activated PRP in the cardiovascular system of patients: 1) injecting a PRP that contains red blood cells into the heart may lead to thrombi that can cause stroke or myocardial infarction; 2) thrombin used to prepare PRP can cause serious bleeding abnormalities in some patients who develop antibodies against certain clotting factors as well as other adverse events 3 (thrombin, itself, causes the generation of ROS and 4) lack of a mechanism explaining the mode of action of activated PRP.

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We have recently modified our production of PRP in two important ways: First, we have a new effective and safe way to activate PRP (without using thrombin), using nanosecond pulsed electric fields (nsPEFs) for the release of the stored growth factors. PRP activated by nsPEFs (ns-PRP) is similarly effective, if not more so, to thrombin-activated PRP (t-PRP). Second, we have eliminated the presence of red blood cells (RBC) in what we call our "PRP-supernatant".

In this study we investigated the effect of the ns-PRP supernatant with that of a thrombin activated PRP supernatant both *in situ* using the ischemic and reperfused Langendorff heart and *in vitro* using HUV-EC- Homo sapiens, human Umbilical Vein Endothelium (PCS-100-013 ™_ATCC; Manassas, VA, USA) cells or H9c2 (*Rattus norvegicus* H9c2 cardiac myoblast) cells. We also examined whether freezing the PRP supernatant at -20°C had any effect of its effectiveness *in vitro or in situ*.

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.

Preparation of Platelet Rich Plasma (PRP)

PRP was prepared as described by Harvest Technologies (Plymouth, MA). Sixty ml of whole blood was withdrawn from a donor rabbit using a sterile syringe containing 6 ml of ACD-A anticoagulant. 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 centrifuged for 14 min to separate the blood components from the plasma. Platelet poor plasma was used to re-suspend the concentrated platelets to a final volume of 7 ml.

Hematological analysis of rabbit whole blood used to prepare PRP was analyzed using a HESKA Diagnostic System (Loveland, Colorado). The PRP had an average platelet count of $295 \times 10^3/\mu l$. The Harvest Technology System concentrator (Harvest Technologies, Plymouth, MA) concentrated the platelets in the whole blood 4-7 times providing a platelet concentrate between $1180 \times 10^3/\mu l$ and $2065 \times 10^3/\mu l$ of platelets.

Nanosecond Pulse Electric Field (nsPEF) 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 [6,7].

Platelets were activated using a 300 ns pulse generator. The 300 ns pulse generator used for these studies was described by Zhang et al. [6] and Schoenbach et al. [7]. The electrical pulses were applied to an electroporation cuvette with an electrode gap of 0.2 cm. The aluminum cuvette plate electrode, had an area of 1 cm² for the 0.2 cm gap cuvette. The shape and amplitude of the pulse voltage, was monitored using a 500 MHz oscilloscope.

Activation of platelets using nsPEFs

The electrical pulses were applied to the PRP concentrate in the presence of 2 mM calcium in sterile electroporation cuvettes. The PRP concentrate (1 ml) was exposed to 5 pulses at an electric field of 30 kV/cm for a duration of 300 ns.

Activation of platelets using bovine thrombin

Preparation of a bovine thrombin activated platelet gel was prepared as described above in Preparation of Platelet Rich Plasma (PRP) with the exception that thrombin was used as the platelet activator. For comparison, t-PRP was activated using an APC+™ activator (Harvest Technology, Plymouth, MA) made by combining bovine thrombin with 10% calcium chloride at a ratio of 1:10. The thrombin –calcium combination was added to the platelet concentrate and 600 μl of t-PRP supernatants or BSA (control) was injected into the left ventricular heart muscle in the Langendorff model.

Method used for making Platelet Gel Supernatant

After the PRP was activated using nsPEFs or bovine thrombin as previously described it was kept at room temperature for 10 min or until the clot was formed. The activated PRP (clot) was centrifuged at 14,000 RPM for 10 min to pellet down any red blood cells and platelet fragments. The supernatant was removed and used fresh or it was frozen for 24 hr at -20°C then used in the Langendorff heart or in *in vitro* experiments. For all experiments using frozen PRP, the supernatant was thawed only once.

Ischemic Reperfusion Heart Experiments (Langendorff Model)

Nine female New Zealand White rabbits (3.5-4.0 kg, CoVance, Denver, PA) were sedated by administering acepromazine (10 mg/kg) and ketamine (25 mg/kg), intramuscular. A surgical plane of anesthesia was induced by allowing the animal to breathe isoflurane and oxygen. A midline thoracic-abdominal incision was made. Sixty milliters of whole blood was removed from the inferior vena cava for the preparation of platelet gel. The heart was then quickly removed and placed into a modified Tyrode's solution chilled to 0-4°C. The heart was mounted as previously described [8]. Briefly, after mounting, a balloon catheter attached to a pressure transducer was inserted into the left ventricle and inflated. Left ventricular systolic and diastolic pressures were recorded every 10 seconds through a polyvinyl catheter using a COBE CDX III transducer and Micro-Med 100 Blood Pressure Analyzer (Louisville, KY). The preparation was allowed to beat spontaneously and permitted to equilibrate for 15 min prior to initiation of the experimental protocol. The heart was given 10 min to re-stabilize prior to closing off flow through the aortic cannula to create global ischemia. This ischemia was maintained for 30 min with the heart encased in a heated water jacket and maintained at 37°C. At the end of the 30-min ischemic period the aortic cannula was re-opened and the heart reperfused for 60 min. All injections (ns-PRP (600 μl) or t-PRP (600 μl) or our control BSA (600 μl) were injected into the myocardium 10 min prior to reperfusing the heart. All data were normalized to a baseline of 100% and stated as the mean ± SD.

In Vitro ROS production in the presence and absence of PRP supernatant using flow cytometry

These cell culture experiments were performed using human umbilical vein endothelial cells, termed HUV-EC, over a three day period. HUV-EC are primary cells that are considered 'primary cultures ', in the truest sense; meaning that they have been preserved after only 1 to 3 passages from the initial source material (ATCC; Manassas, VA). The cells were grown in sterile T- 75 cm² 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 (FBS, Atlanta Biologicals, Inc., Lawrenceville, GA). Once the cells

reached 85-95% confluency, approximately 800,000 HUV-EC cells were transferred onto a sterile T-25 cm² tissue culture flask on day 1. On day 2, after a 24 hr incubation in DMEM the cells were treated with fresh DMEM and served as controls or with 3.5 ml of DMEM containing ns-PEF or bovine thrombin activated PRP (not the supernatant) (0.525g) or PRP supernatant fresh or frozen. The cells were then returned to the incubator for 24 hours. On day 3 ROS detection analysis was performed using Flow Cytometry (BD FACSAria, San Jose, CA). The cells were washed with fresh cell culture medium, trypsinized and suspended in cell culture media. They were then loaded with 20 μM concentration of CM-H_2DCFDA dye solution per manufacturer instructions (Invitrogen-Molecular Probes, Eugene, OR), then placed in the dark at 37°C for 1 hr. After the 1 hr loading period the cells were stimulated for 5 min with 0.15% hydrogen peroxide (H_2O_2) and analyzed for ROS production using Flow Cytometry.

Mitochondrial depolarization analysis in HUV-EC cells treated with PRP and analyzed using flow cytomerty

These experiments were also performed over a 3 day period using HUV-EC cells in culture. During the 1st two days the cells were treated as described above for ROS analysis. Using nsPEFs to activate platelets we investigated the effect of PRP (which contained red blood cells along with the growth factors) prepared fresh on the day of treatment or frozen for 24 hr then used to pretreat the cells as well as PRP supernatant made fresh on the day of treatment or frozen for 24 hr then used to treat cells. None of these experiments were performed using bovine thrombin-all PRP supernatants were prepared using nsPEFs. On day 3 of the experiment the media was removed and discarded. Cells were stimulated with either 0, 0.03 (8.8 mM), 0.07 (20.5 mM), or 0.15% (44 mM) of H_2O_2 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 re-suspended in media. The media was discarded after centrifugation at 200 g for 10 minutes. The cells were again re-suspend in 0.5 mL of 1X MitoPT™ JC-1 buffer solution (ImmunoChemistry Technologies, LLC, Bloomington, MN) and incubated at 37°C in a 5% CO_2 incubator for 15 minutes. Following this loading period, the cells were washed with and re-suspended 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 (Immuno-Chemistry Technologies, Bloomington, MN), to assess mitochondrial health/depolarization in cells pretreated with PRP supernatant or cell culture media only (control). JC-1 dye exhibits potential-dependent accumulation in mitochondria, indicated by a fluorescence emission shift from green (~529 nm) to red (~590 nm). When cells stained with MitoPT™ JC-1 were analyzed with the flow cytometer, depolarization was measured by monitoring the amount of red fluorescence. Healthy cells fluoresced red. However, as the mitochondria depolarized the amount of red fluorescence decreased.

HPLC analysis of PRP supernatant

The content of ns-PRP or t-PRP was subjected to chromatography over gel filtration Superdex 200 10/300 column (GE Healthcare Life Sciences, Piscataway, NJ) and equilibrated with Assay Buffer containing 10mM HEPES, 140 mM NaCl, 5.4 mM KCl, 2 mM $CaCl_2$, 10 mM Glucose, pH=7.3. One milliliter of the each sample was loaded onto a column and eluted in Assay Buffer at a flow rate 0.6 ml/min. The 0.6 ml fractions were collected between 6-24 ml of elution volume. Every five fractions were combined and concentrated to 0.5 ml (Amicon Ultra-4, Millipore, Billerica, MA). Fractions 11-20 and 15-29 collected between 10-20 ml of elution volume were chosen for further analysis for catalase

or superoxide dismutase enzymatic activity using assay kits described below.

Catalase (CAT) assay kit: Cayman's CAT assay kit (Ann Arbor, MI) was used to measure the concentration of CAT. This assay used the peroxidatic function of CAT for determination of enzyme activity. The method is based on the reaction of the enzyme with methanol in the presence of an optimal concentration of H_2O_2. The formaldehyde produced was measured colorimetrically with Purpald as the chromogen. The HUV-EC cells were homogenized, centrifuged, and the supernatant removed and assayed per manufacturer instructions.

Superoxide Dismutase (SOD) assay kit: Cayman's SOD assay kit was used to measure the concentration of SOD. This kit used a tetrazolium salt for detection of superoxide radicals generated by xanthine oxidase and hypoxanthine. The assay measures all three types of SOD. The HUV-EC cells were homogenized, centrifuged, and the supernatant removed and assayed per manufacturer instructions.

Western blot analysis

To determine the effect of platelet gel on the proteins MMP-2 and TIMP-1 we used H9c2 cells in culture. The protein concentration in each sample was determined using a BCA protein assay (Thermo Fisher Scientific, Rockford, IL). Rabbit monoclonal MMP-2 and rabbit polyclonal TIMP-1 primary antibodies (Abcam, Cambridge, MA) were diluted 1:2 in blocking buffer. Goat anti-rabbit secondary antibodies conjugated with an infra-red fluorophore IRDye-800CW (LI-COR Biosciences) was diluted 1:5000 in blocking buffer. The blots were treated with the primary antibodies at 4°C overnight, washed 4 times (5 min each) in PBST with 0.1% Tween-20, treated with secondary antibodies for 1 hr, and washed again. The membranes were imaged using an Odyssey 9120 Infrared Imaging System (LI-COR Biosciences) in the 700 nm channel. The images were quantified using MetaMorph software. Data was normalized to the housekeeping protein (β Actin).

Statistical Analysis

The Langendorff heart data were analyzed using an analysis of variance corrected for repeated measures and the data stated as the mean ± standard deviation. 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 at least five times and analyzed using a Student-T test. Data are stated as the mean ± SD. Data were considered significant at $p < 0.05$.

Results

Figure 1 shows work function (pumping ability) of the left ventricle in the Langendorff rabbit hearts treated with ns-PRP supernatant (n=3) made using nsPEFs, t-PRP supernatant (n=3) or bovine serum albumin (BSA) (n=3) which was our control. Work function was calculated by multiplying left ventricular pressure times heart rate. The data were then normalized to a baseline of 100%. Thirty min into reperfusion the ns-PRP treated hearts had a significantly greater left ventricular pumping function than the hearts treated with t-PRP or BSA. The pumping activity remained significantly greater in the ns-PRP treated hearts after 60 min of reperfusion. Left ventricular pressure and heart rate are shown in Tables 2 and 3, respectively.

Figure 2 shows the effect of PRP which contained RBCs (ns-PRP) made fresh on the day of treatment or frozen for 24 hr before use or ns-PRP supernatant on mitochondrial depolarization No studies were performed using bovine thrombin. The percent of healthy (non-
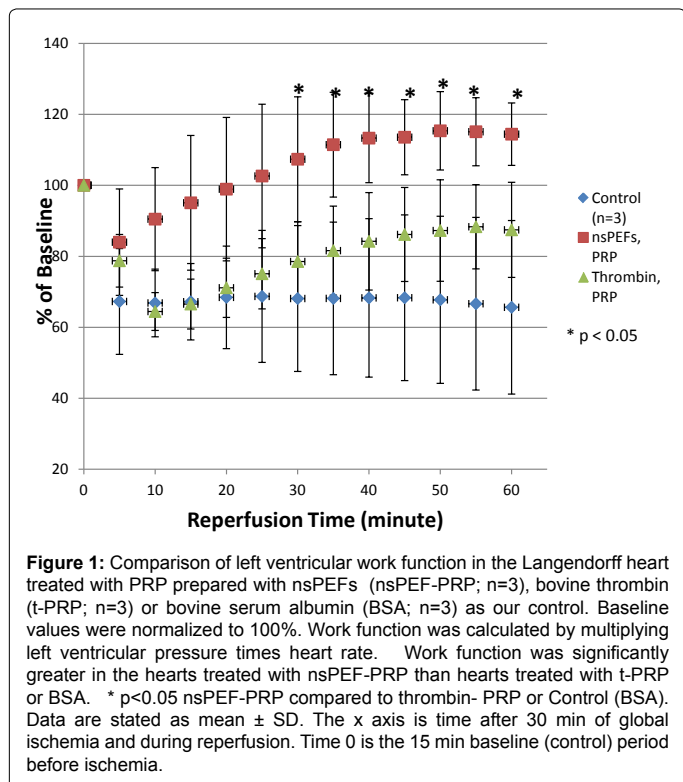
Figure 1: Comparison of left ventricular work function in the Langendorff heart treated with PRP prepared with nsPEFs (nsPEF-PRP; n=3), bovine thrombin (t-PRP; n=3) or bovine serum albumin (BSA; n=3) as our control. Baseline values were normalized to 100%. Work function was calculated by multiplying left ventricular pressure times heart rate. Work function was significantly greater in the hearts treated with nsPEF-PRP than hearts treated with t-PRP or BSA. * p<0.05 nsPEF-PRP compared to thrombin- PRP or Control (BSA). Data are stated as mean ± SD. The x axis is time after 30 min of global ischemia and during reperfusion. Time 0 is the 15 min baseline (control) period before ischemia.

	H9c2 Cells Alone	H9c2 Cells + 1% H_2O_2	H9c2 Cells + PRP	H9c2 Cells + PRP + 1% H_2O_2
TIMP-1	0.145	0.3007	2.238	0.985
MMP-2	5.06	5.83	0.099	0.097

Table 1: Quantative analysis of western blot for TIMP-1 and MMP-2 using an Odyssey Infrared Imaging System. H_2O_2=hydrogen peroxide, PRP=platelet rich plasma, TIMP-1- metalloproteinase inhibitor, MMP-2- metalloproteinase.

Time (min)	Fresh PRP (n=3)	Frozen PRP (n=3)	BSA (control) (n=3)	Thrombin PRP (n=3)
0	100 ± 0	100 ± 0	100 ± 0	100 ± 0
10	75 ± 15	78 ± 12	92 ± 26	73 ± 8
20	86 ± 17	91 ± 8	106 ± 28	93 ± 18
30	91 ± 19	94 ± 5	114 ± 25	101 ± 18
40	92 ± 20	94 ± 5	117 ± 24	105 ± 18
50	91 ± 20	93 ± 5	118 ± 23	107 ± 17
60	90 ± 20	90 ± 7	108 ± 10	111 ± 21

Table 2: Left ventricular Systolic Pressure in Langendroff hearts treated with nsPRP or Thrombin PRP supernatant or BSA (control) measured using a COBE CDX III transducer and Micro-Med 100 Blood Pressure Analyzer. Time 0 is the control period prior to ischemia.

depolarized) versus depolarized cells pretreated with the various forms of PRP in the absence of H_2O_2 was significantly greater in all cases except the frozen nsPRP. However, the cells pretreated with PRP and stimulated with 0.15% H_2O_2 had significantly less mitochondrial depolarization. These data suggest that PRP, whether freshly prepared or frozen and thawed may have a protective effect on the mitochondria of the HUV-EC cells and are consistent with our previous findings using H9c2 cells [4] suggesting that this effect is not cell specific.

Figure 3 shows a comparison of the effect of fresh on the day of treatment or frozen ns-PRP on ROS generation in HUV-EC cells stimulated with 0.15% H_2O_2. Again, cells pretreated with PRP (fresh,

frozen or supernatant) and stimulated with H_2O_2 generated significantly less ROS than cells treated with cell culture media only. These data are also consistent with our previous findings in H9c2 cells [4] and suggest that this response is not cell specific.

The degree of heart function that remains after a myocardial infarction is partly due to the degree of degradation of the extracellular matrix (ECM) of the heart by enzymes such as the metalloproteinases. In this study we asked the question whether PRP modulated the metalloprotienases (MMPs) designed to degrade the ECM and/or their inhibitors (TIMPs) which are designed to prevent or control the degree of degradation by the MMPs. We pretreated H9c2 cells with PRP, stimulated them with 1% H_2O_2 and analyzed the response using Western Blot Analysis. Thrombin prepared PRP was not used in these studies. Figures 7A and B are representative examples of changes in the metalloprotinease inhibitor TIMP-1 and the metalloprotinease MMP-2. We also analyzed changes in MMP-9 and TIMP-4 but were unable to detect a change from baseline values (data not shown). Baseline levels of MMP-2 were greater than baseline levels of the tissue inhibitor TIMP-

Time (min)	Fresh PRP (n=3)	Frozen PRP (n=3)	BSA (control) (n=3)	Thrombin PRP (n=3)
0	100 ± 0	100 ± 0	100 ± 0	100 ± 0
10	92 ± 3	116 ± 20	85 ± 5	96 ± 11
20	91 ± 12	109 ± 20	80 ± 10	90 ± 16
30	96 ± 19	114 ± 20	76 ± 12	89 ± 18
40	97 ± 24	121 ± 17	75 ± 13	90 ± 16
50	98 ± 25	125 ± 16	74 ± 14	89 ± 13
60	98 ± 27	128 ± 15	73 ± 9	88 ± 10

COBE CDX III transducer and Micro-Med 100 Blood Pressure Analyzer. Time 0 is the control period prior to ischemia.

Table 3: Left ventricular Heart Rate in Langendroff hearts treated with nsPRP or Thrombin PRP supernatant or BSA (control) measured using a COBE CDX III transducer and Micro-Med 100 Blood Pressure Analyzer. Time 0 is the control period prior to ischemia.
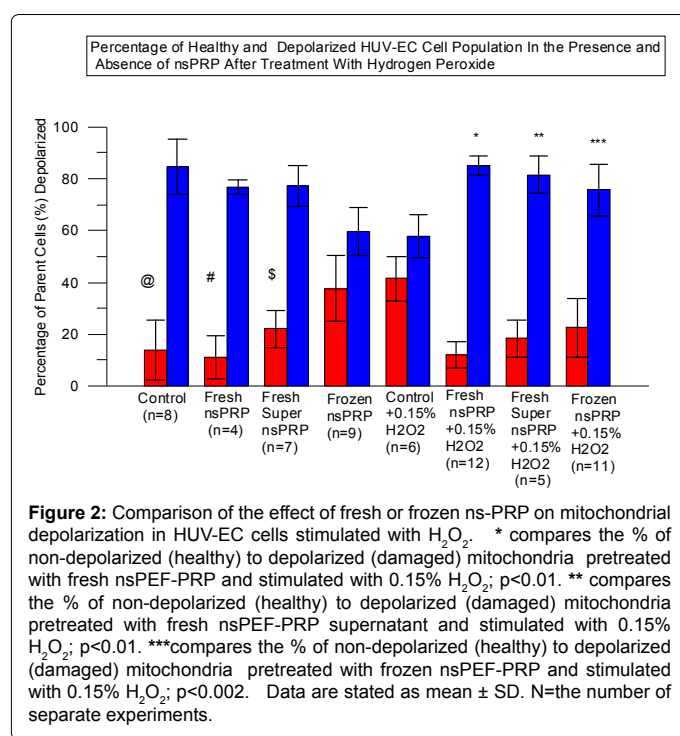

Figure 2: Comparison of the effect of fresh or frozen ns-PRP on mitochondrial depolarization in HUV-EC cells stimulated with H_2O_2. * compares the % of non-depolarized (healthy) to depolarized (damaged) mitochondria pretreated with fresh nsPEF-PRP and stimulated with 0.15% H_2O_2; p<0.01. ** compares the % of non-depolarized (healthy) to depolarized (damaged) mitochondria pretreated with fresh nsPEF-PRP supernatant and stimulated with 0.15% H_2O_2; p<0.01. ***compares the % of non-depolarized (healthy) to depolarized (damaged) mitochondria pretreated with frozen nsPEF-PRP and stimulated with 0.15% H_2O_2; p<0.002. Data are stated as mean ± SD. N=the number of separate experiments.

1. We are unable to explain why basal levels of MMP-2 were so high. However, when the cells were treated with PRP there was a drastic fall in MMP-2 and an increase in TIMP-1. Stimulation of the cells with 1% H_2O_2 was not associated with an increase MMP-2 but was associated with an increase in TIMP-1. When exposed to H_2O_2 TIMP-1 decreased slightly and may be the result of its utilization in the inhibition of MMP-2. Table 1 provides a quantitative description of the data.

Discussion

Ischemic reperfusion is a major cause of myocyte loss in the heart. Significant loss of cardiac myocytes after ischemia and reperfusion can lead to extensive dysfunction of the left ventricle and eventually loss of life. While clinicians have tools available to re-open closed or clogged coronary vessels, there is still no reliable therapeutic strategy for protecting the stressed, non-ischemic myocytes from a phenomenon called "reperfusion injury". The term reperfusion injury describes a number of events that result in myocyte damage through myocardial stunning (persistent mechanical dysfunction despite the restoration of blood flow; usually reversible), the no-flow phenomenon after opening an infarcted artery; reperfusion arrhythmia and irreversible injury of the myocardium (necrosis) [9]. The non-ischemic myocytes adjacent to the infarcted area are placed under oxidative stress and many of the undamaged cells may become apoptotic and die thereby increasing the size of the infarcted area of the heart.

We investigated the strategy of using the supernatant of platelet rich plasma or platelet gel to assess its effect on left ventricular function in the ischemic reperfused heart both *in vivo* [4] and *in situ* (Figure 1). In both cases, left ventricular function was significantly improved in the hearts treated with platelet gel, particularly the gel prepared using nsPEFs. Whether the platelets are activated with nsPEFs or bovine thrombin appears to play a role in the effectiveness of the platelet gel. We show that the work performance of the Langendorff hearts treated with platelet gel prepared using nsPEFs was significantly greater than the hearts treated with platelet gel made with bovine thrombin. We have previously reported improvement in left ventricular mechanical function in the rabbit heart *in vivo* [4]. The hearts were infarcted,
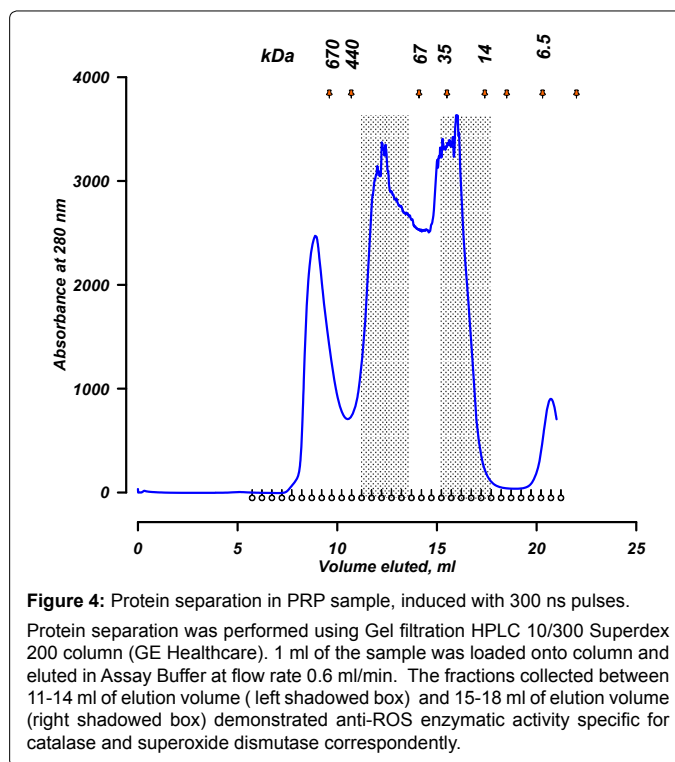

Figure 4: Protein separation in PRP sample, induced with 300 ns pulses. Protein separation was performed using Gel filtration HPLC 10/300 Superdex 200 column (GE Healthcare). 1 ml of the sample was loaded onto column and eluted in Assay Buffer at flow rate 0.6 ml/min. The fractions collected between 11-14 ml of elution volume (left shadowed box) and 15-18 ml of elution volume (right shadowed box) demonstrated anti-ROS enzymatic activity specific for catalase and superoxide dismutase correspondently.

treated with platelet gel and stressed with dobutamine 14 days post infarct. In these studies not only was positive and negative dp/dt better in the platelet gel treated hearts but infarct size was also smaller [4]. These data suggest that the hearts treated with PRP supernatants made using nsPEFs had a better work performance than either of the other two groups.

There are several possibilities that may explain why left ventricular function was enhanced to a greater extent in the hearts treated with platelet gel both *in vivo* and *in situ* in general and with platelet gel made with nsPEFs specifically. First, we demonstrate in this study using HUV-EC cells (endothelial cells) *in vitro* and previously *in vitro* using H9c2 (*Rattus norvegicus* H9c2 cardiac myoblast) cells that pretreatment with platelet gel reduced the amount of reactive oxygen species produced by the cells stimulated with H_2O_2. Microvascular dysfunction is manifestation of reperfusion injury and is characterized by endothelial cell dysfunction which causes vasoconstriction and increased ROS production [10-12]. Platelet gel reduced the amount of ROS produced in response to H_2O_2 in HUV-EC cells suggesting that one mechanism used by platelet gel to enhance left ventricular heart function may be to reduce ROS production in the microvascular in response to ischemia and reperfusion, thereby decreasing vasoconstriction of the coronary vessels. It has been known for some time that that ROS generated during reperfusion participates in myocardial stunning and that anti-oxidants reduce ROS stunning [13]. The controversy regarding whether antioxidants reduce infarct size is still a major question. There are investigators that feel antioxidants reduce infarct size [14-16] while others find no reduction in infarct size with antioxidant use [17-19]. Our *in vitro* data show that platelet gel reduces ROS formation in HUV-EC and H9c2 cells. Therefore, a reduction in ROS, would be beneficial to preserving non necrotic heart tissue and reducing infarct size. For further analysis, the content of the ns-PRP supernatant was separated with HPLC size exclusion chromatography and the entire eluate collected into 0.6 ml fractions as described in Materials and Methods.
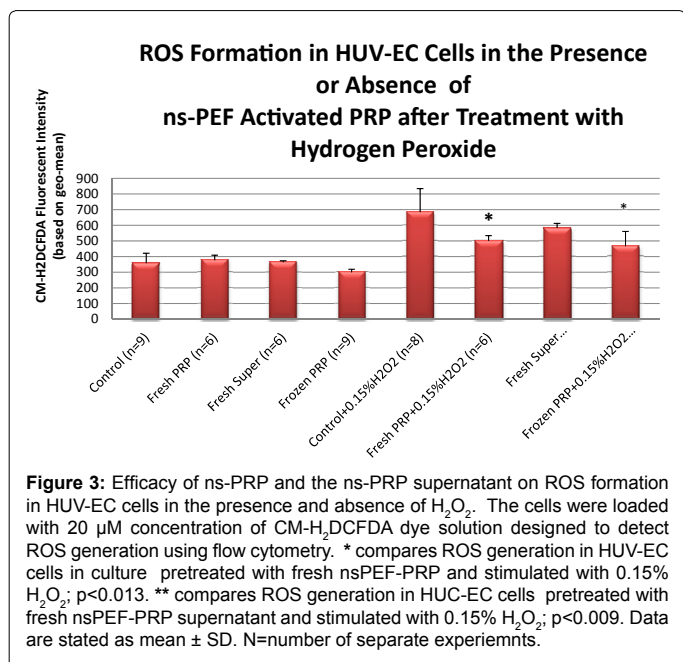
Figure 3: Efficacy of ns-PRP and the ns-PRP supernatant on ROS formation in HUV-EC cells in the presence and absence of H_2O_2. The cells were loaded with 20 μM concentration of CM-H_2DCFDA dye solution designed to detect ROS generation using flow cytometry. * compares ROS generation in HUV-EC cells in culture pretreated with fresh nsPEF-PRP and stimulated with 0.15% H_2O_2; p<0.013. ** compares ROS generation in HUC-EC cells pretreated with fresh nsPEF-PRP supernatant and stimulated with 0.15% H_2O_2; p<0.009. Data are stated as mean ± SD. N=number of separate experiemnts.

Gel filtration column elution profile is depicted in Figure 4. Inhibition of H_2O_2 -derived ROS production and mitochondrial depolarization in HUV-EC cells were found in the fractions collected between 11-14 ml of elution volume (left shadowed box) and 15-18 ml of elution volume (right shadowed box). Suspecting that eluted molecules should be in a range between 100-400 kDa and 14-40 kDa we hypothesized that catalase (CAT, 240 kDa) and superoxide dismutase (SOD, 32.5 kDa) may be responsible for anti-oxidative properties of ns-PRP supernatant. The fractions from active regions were analyzed with enzyme specific assays for catalase and superoxide dismutase as described in Materials and Methods. Enzymatic activity for catalase was found in fractions 12-16 (Figure 5A), which collected between 11-14 ml of elution volumes. Enzymatic activity for SOD was found in fractions 21-23 (Figure 5B) which collected between 15-18 ml of elution volumes. In contrast to ns-PRP, matching regions of t-PRP derived eluate retained very minor activity for both enzymes (Figures 5A and 5B). We quantified enzymatic activity in PRP samples that were not processed with HPLC. Again there was a significantly greater activity of catalase (p<0.003) (Figure 6A) and superoxide dismutase (p<0.002) (Figure 6B) in the PRP supernatant made with nsPEFs when compared to the PRP supernatant prepared using bovine thrombin. The data show that the method used to prepare PRP can influence its contents and may account for, at least in part, why some investigators report a lack of response to platelet gel prepared using bovine thrombin as the platelet activator.

Secondly, platelet gel appears to protect the mitochondria in both H9c2 [4] and HUV-EC cells *in vitro* (Figure 2). The paradox with these results is that mitochondria are both a source of ROS via the electron transport chain during electron transport at complexes I and III and a target for ROS damage [20]. The flow of electrons down the chain in the mitochondria is inhibited by ischemia and is associated with a number of other changes such as a reversal of the role of the proton-translocating enzyme FoF1ATP synthase. This enzyme which normally produces ATP now consumes ATP which is used to pump protons from the matrix into the inter-membrane space [20,21]. These changes ultimately lead to elevated cytosolic calcium and activation of proteins which can degrade enzymes such as nucleases, phospholipases and
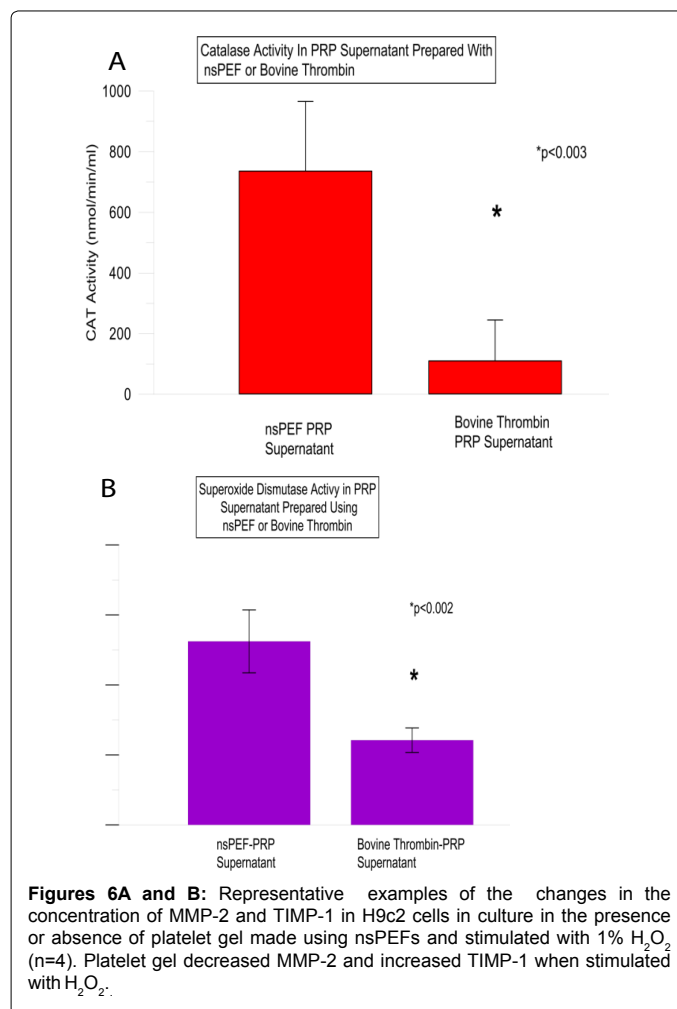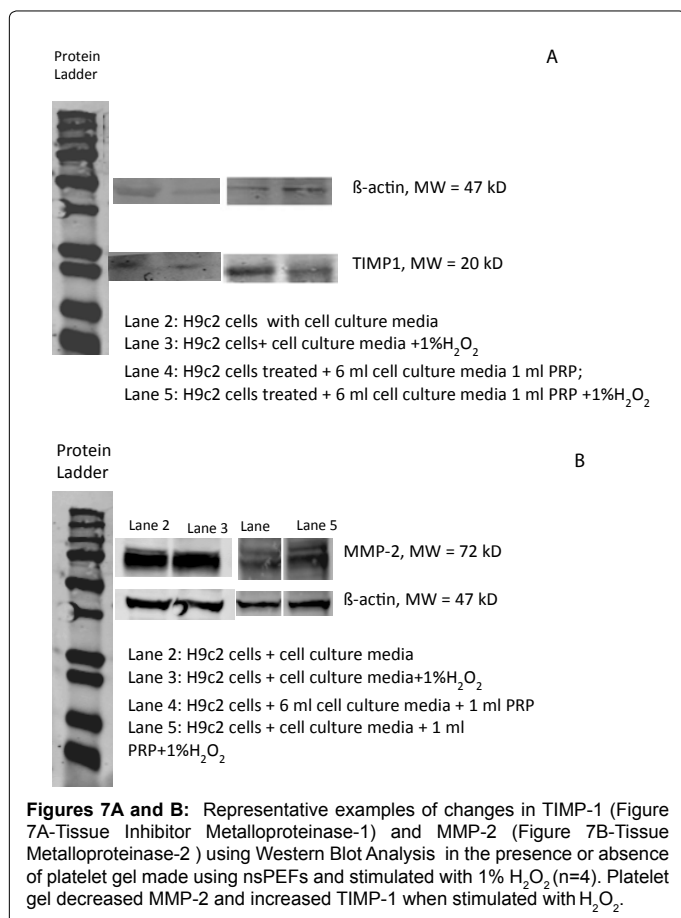
Figures 6A and B: Representative examples of the changes in the concentration of MMP-2 and TIMP-1 in H9c2 cells in culture in the presence or absence of platelet gel made using nsPEFs and stimulated with 1% H_2O_2 (n=4). Platelet gel decreased MMP-2 and increased TIMP-1 when stimulated with H_2O_2.
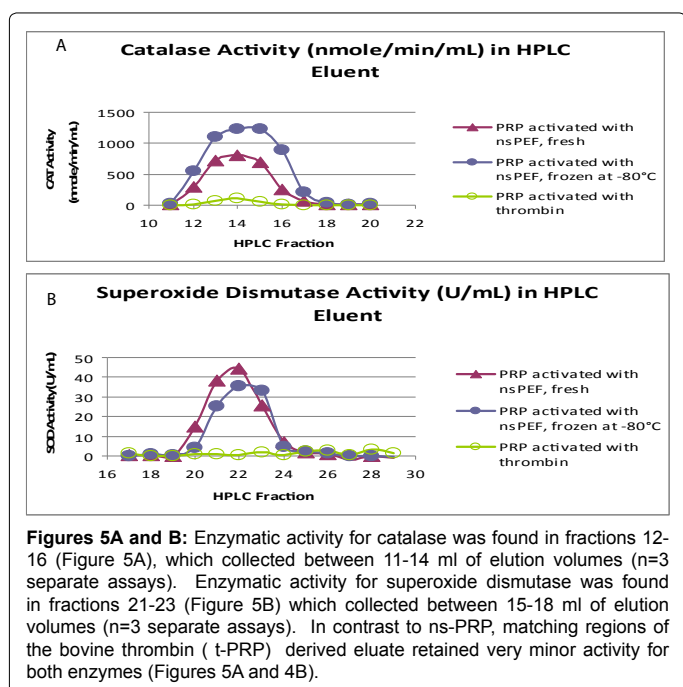
Figures 5A and B: Enzymatic activity for catalase was found in fractions 12-16 (Figure 5A), which collected between 11-14 ml of elution volumes (n=3 separate assays). Enzymatic activity for superoxide dismutase was found in fractions 21-23 (Figure 5B) which collected between 15-18 ml of elution volumes (n=3 separate assays). In contrast to ns-PRP, matching regions of the bovine thrombin (t-PRP) derived eluate retained very minor activity for both enzymes (Figures 5A and 4B).

proteases, leading to destruction of the membrane integrity [22,23]. During reperfusion, a decrease in pH, calcium overload and increased oxidative stress can cause the opening of large conductance transition pores (mPTP) which can change the membrane potential of the mitochondria. There is a positive correlation between mitochondrial membrane potential and production of ROS by electron transport [20]. Implying that the greater the change in mitochondrial membrane potential the more ROS is produced or vice versa. Nevertheless, our data suggest that platelet gel may alter this relationship. We show that pretreatment with platelet gel significantly reduces mitochondrial depolarization in HUV-EC (Figure 2) and in H9c2 cells [4], *in vitro*. This is in addition to the changes in ROS we described earlier. These data suggest that platelet gel may provide a degree of protection to mitochondria in heart and endothelial cells under oxidative stress thereby reducing ROS generation. The mechanism used by platelet gel to protect mitochondria from damage is unclear and currently under investigation in our laboratory.

Thirdly, we investigated the effect of platelet gel on metalloproteinases and their inhibitors in H9c2 cells using Western Blot analysis. In the heart, activation of MMPs triggers cardiac myocyte necrosis and left ventricle dysfunction and promotes adverse left ventricle remodeling [24]. Matrix metalloproteinase (MMP-2) is a zinc dependent endopeptidase that plays a detrimental role in ischemia and reperfusion

Lane 2: H9c2 cells with cell culture media
Lane 3: H9c2 cells+ cell culture media +1%H_2O_2
Lane 4: H9c2 cells treated + 6 ml cell culture media 1 ml PRP;
Lane 5: H9c2 cells treated + 6 ml cell culture media 1 ml PRP +1%H_2O_2

Lane 2: H9c2 cells + cell culture media
Lane 3: H9c2 cells + cell culture media+1%H_2O_2
Lane 4: H9c2 cells + 6 ml cell culture media + 1 ml PRP
Lane 5: H9c2 cells + cell culture media + 1 ml PRP+1%H_2O_2

Figures 7A and B: Representative examples of changes in TIMP-1 (Figure 7A-Tissue Inhibitor Metalloproteinase-1) and MMP-2 (Figure 7B-Tissue Metalloproteinase-2) using Western Blot Analysis in the presence or absence of platelet gel made using nsPEFs and stimulated with 1% H_2O_2 (n=4). Platelet gel decreased MMP-2 and increased TIMP-1 when stimulated with H_2O_2.

injury of the heart. In addition, MMP-2 degrades cardiac contractile proteins in response to increased oxidative stress [25,26]. The baseline level of MMP-2 was 35 fold greater than TIMP-1. When H9c2 cells were treated with platelet gel there was a 98% decrease in MMP-2 and a 600% increase in TIMP-1. When the cells were stimulated with hydrogen peroxide there was no increase in MMP-2 but TIMP-1 decreased slightly but did not return to baseline. We also measured MMP-9, and TIMP-4 but could not show a change from baseline in these proteases. MMP-2 and MMP-9 are inactivated by TIMP-1 and TIMP-2 [27]. Loss of the coordinated expression of MMPs and TIMPs is believed to generate tissue degradation under inflammatory conditions. One mechanism that may be used by platelet gel to reduce or prevent further damage to the myocytes after ischemia and reperfusion is to coordinate the ratios of MMPs and TIMPs. In support of this concept we showed that platelet gel prepared using nsPEFs had significantly greater amounts of the endogenous antioxidants catalase and superoxide dismutase than the gel prepared using thrombin. These data suggest 2 important points: 1) that the greater concentration of catalase and superoxide dismutase in the ns-PRP may account for, at least in part, why the Langendorff hearts treated with the platelet gel made using nsPEFs performed better in terms of work function than the hearts treated with platelet gel made with thrombin or with treated with BSA. 2) thrombin may interfere with the function of the platelet gel by reducing the concentration of endogenous antioxidants. 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 pro-apoptotic and pro-inflammatory [28] and may have adverse effects on cardiac myocytes, which are independent of

its pro-coagulant activity [28,29]. It has been shown that in humans, thrombin generation during reperfusion after coronary artery bypass surgery is associated with postoperative myocardial damage [30,31] and that its thrombotic activity is only partially suppressed by heparin [29]. Other investigators have shown that while PRP has the potential to increase the osteo-inductivity of demineralized bone matrix, its activation with bovine thrombin immediately prior to implantation significantly inhibits this activity [32], again, suggesting that thrombin may interfere with the efficacy of PRP. Finally, thrombin has been shown to cause the generation of ROS [31].

There are some aspects of this study that may appear as limitations such as the small sample size. We do not view this as a limitation because one goal of the investigator was to use the least number of animals possible to provide statistically significant data a fact that is expected of each investigator by the Old Dominion University IACUC. We show statistically significant data in our Langendorff hearts treated with platelet gel prepared using nsPEFs versus platelet gel made with bovine thrombin or with bovine serum albumin (control). To simply increase the number of animals for the purpose of having a large "n" is unethical and was not performed by this investigator. We view the use of animals in research as a privilege and not a right so we strive to use as few animals as possible to get statistically significant data. Another possible limitation may be that an asanguinous isolated preparation (Langendorff heart model) was used, asking the question whether similar results would be realized if blood (rather than Tyrodes media) was perfused through the heart. We answer this concern by noting that we have performed similar studies using the rabbit heart *in vivo* and show similar results i.e. that hearts treated with platelet gel perform better than hearts treated with our control [4]. Lastly, one may consider the calculation of work function a rudimentary measurement of LV function. While we admit that there are more sophisticated equipment that could be used by studies performed with large financial budgets, the results were the same regarding the improvement in left ventricular function in hearts treated with platelet gel whether we used a balloon catheter (which was used in this study) or a Millar catheter which was used previously [4].

In summary, we propose a safe, non-chemical, economically feasible and effective Intervention for salvaging cardiac myocytes under oxidative stress after myocardial infarction and after blood flow to the ischemic heart has been restored. There are several procedures available to the clinical community for the restoration or reperfusion of coronary blood flow which include coronary artery bypass grafting, thrombolysis and or percutaneous coronary intervention. However, we strongly agree with the sentiments voiced by Bolli et al. who state that the field of cardio-protection in acute MI, cardiac surgery and cardiac arrest is at a crossroads [33]. A very important point made by these investigators is that treatments designed to protect the heart from a basic research level are often inefficient[34] and expensive from a therapeutic and pharmacological standpoint. The results from our study provide evidence for a non-chemical, safe, relatively inexpensive and effective method to bridge the gap between the restoration of blood flow and salvaging non-lethal ischemic heart tissue, thereby preventing enlargement of infarct size and possibly reducing remodeling of the heart. The use of platelet gel is an autologous process that only requires the isolation and activation of platelets whose releasates contain most, if not all, factors present to 1) reduce further ROS formation by preserving and/or increasing the concentration of endogenous antioxidants, 2) reduce injury to mitochondria, thus protecting the energy source for the heart 3) reduce the concentration of proteases activated by ischemia and reperfusion designed to degrade components of the extracellular

matrix (MMP-2) of the heart and to increase the inhibitory proteases designed to prevent or control this degradation (TIMP-1).

In conclusion we provide evidence for the use of an old agent/concept (activated platelet supernatant) in a new paradigm that addresses a clinically relevant outcome (reduction in damage to the ischemic reperfused heart) in addition to some of its mechanisms action.

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