Pulsed acoustic cellular expression as a protective therapy against I/R injury in a cremaster muscle flap model

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\textbf{Abstract}

Background: Tissue ischemia and reperfusion (I/R) affects blood flow restoration and oxygen delivery to the damaged tissues contributing to tissue morbidity and microcirculatory compromise. Pulsed acoustic cellular expression (PACE) technology is known to support tissue neovascularization. The aim of this study was to test PACE conditioning mechanism of action on microcirculatory hemodynamics in ischemia–reperfusion injury model.

Methods: 34 rat cremaster muscle flaps were monitored under intravital microscopy system in 4 experimental groups: 1) non-ischemic controls (n=10), 2) 5 h ischemia without conditioning (n=8), 3) pre-ischemic (5 h) PACE conditioning (n=8), 4) post-ischemic (5 h) PACE conditioning (n=8). Standard microcirculatory hemodynamics of RBC velocity, vessel diameters and functional capillary perfusion were recorded for 2 h after I/R. Immunohistochemistry assessed expression of proangiogenic factors: VEGF and vWF, whereas real-time PCR assessed proangiogenic (VEGF, eNOS) and proinflammatory factors (iNOS; chemokines: CCL2, CXCL5 and chemokine receptor CCR2).

Results: Pre-ischemic PACE conditioning (group 3) resulted in increased RBC velocity of second (A-2) and third order arterioles (A-3) and venule (V-1) by 40%, 15% and 24% respectively comparing to ischemic group without conditioning (p<0.05). Post-ischemic PACE conditioning (group 4) revealed: 1) increase in RBC velocity in second (A-2) and third order arterioles (A-3) by 65% and 31% respectively comparing to ischemia without conditioning (group 2), 2) 33% increase in first order arterioles diameter (A-1) (p<0.05) compared to ischemic controls, 3) 21% increase in number of functional capillaries compared to ischemia without conditioning (group 2) (P<0.05). Immunostaining assays showed that PACE postconditioning up-regulated proangiogenic factors vWF and VEGF, whereas real-time PCR assessed proangiogenic (VEGF, eNOS) and proinflammatory factors (iNOS; chemokines: CCL2, CXCL5 and chemokine receptor CCR2).

Conclusions: As expected 5 h ischemia resulted in deterioration of microcirculatory hemodynamics confirmed by decreased vessels diameters and RBC velocities. This was alleviated by pre- and post-ischemic PACE conditioning which improved functional capillary density and stimulated angiogenesis as confirmed by up-regulated VEGF expression. Furthermore, post-ischemic PACE conditioning correlated with decreased expression of early proinflammatory factors (iNOS, CCL2, CXCL5). Both types of PACE conditioning ameliorated deleterious effect of ischemia–reperfusion injury on microcirculatory hemodynamics of muscle flaps.
Experimental studies have demonstrated that accumulation of inflammatory cells (including neutrophils, tissue mast cells, monocytes, and platelets) during reperfusion plays a crucial role in I/R injury due to release of oxygen-derived free radicals, proteases and leukotrienes. Thus, modulation of cytokine inflammatory responses may have a direct impact on I/R injury and free flap survival (Zhang et al., 2005).

Nitric Oxide (NO) is a potent biological messenger acting in a variety of tissues, with a wide range of physiological functions such as vasodilatation, inhibition of platelet aggregation, regulation of neurotransmission and natural defense of the immune system (Garcia and Stein, 2006; Lanas, 2008; Wallace and Miller, 2000). Cellular production of NO requires the presence of one or more of the three isoforms of NO synthase (NOS). Two of them [endothelial NOS (eNOS) or Type III NOS and neuronal NOS (nNOS) or Type I NOS] are constitutively expressed in the mature skeletal muscle fibers and on the vascular endothelium of the skeletal muscle (eNOS), and the presence of intra-cellular calcium and calmodulin is required for their function. The other isoform [inducible NOS (iNOS) or Type II NOS] is calcium- and calmodulin-independent and it is usually induced during an inflammatory process in the presence of certain inflammatory cytokines and/or bacterial products. Each gene of NOS has a selective effect and origin of expression. Constitutively expressed NOS isoforms (eNOS and nNOS) may be protective in I/R injury of skeletal muscle, as the intravenous infusion of a low dose of NO has been shown to attenuate I/R injury in a rat skeletal muscle (Phillips et al., 2009; Raat et al., 2009; Schulz et al., 2004). In contrast, iNOS expressed in normal skeletal muscle fibers is generally up-regulated during acute and chronic pathological states (asthma, chronic heart failure) and produces a high level of NO, leading to a deleterious effect on tissues (Baeuerle and Baichwal, 1997).

Proinflammatory chemokines are expressed in the tissues submitted to ischemia and, in response, regulate monocyte and lymphocyte recruitment and activation at the ischemic sites. The role of CCL2 and CXCL5 in the ischemic myocardium has been well described in the literature (Formigli et al., 2001; Kajihara et al., 2003; Kumar et al., 1997; Matsumori et al., 1997). These studies confirmed that decreased expression of proinflammatory chemokines attenuates monocyte activation and protects cardiac tissue against I/R injury.

There is an interest in clinical practice to find therapies which will alleviate the effect of tissue ischemia and reperfusion injury. Pulsed acoustic cellular expression (PACE) is an interesting technology using acoustic waves (Extracorporeal Shock Waves — ESW). By focusing these acoustic waves with a semi-ellipsoid reflector, they can be transmitted to a specific tissue site at which a cellular response will be elicited. EWSs have been clinically used since 1980 for lithotripsy and are currently widely used in medicine (Lingeman et al., 1986; Ogden et al., 2001; Thiel, 2001).

In orthopedics, shock wave therapy has been used to treat different musculoskeletal disorders (i.e.: non-union of long bone fractures, calcifying tendonitis, tennis elbow, fasciitis plantaris–heel spur etc.) (Rompe et al., 1996; Wang et al., 2001, 2007).

In cardiology, shock wave therapy has improved left ventricular remodeling after acute myocardial infarction in experimental models (Fukumoto et al., 2006; Nishida et al., 2004; Uwatoku et al., 2007; Zimpher et al., 2009).

The role of shock wave treatment in reduction of skin flap necrosis has been confirmed as demonstrated by improvement of blood supply to the ischemic tissues (Arno et al., 2010; Davis et al., 2009; Huemer et al., 2005; Meurer et al., 2005a, 2005b, 2007).

We have recently reported the beneficial effect of PACE therapy on muscle angiogenesis following PACE application as a short-acting treatment in a rat experimental model. PACE application resulted in up-regulation of proangiogenic chemokine gene expression in skeletal muscle and in up-regulation of proangiogenic factors such as VEGF and vWF expressed on the endothelium of the small vessels (Krokowicz et al., 2010).

Based on our recent study and literature reports, it is evident that PACE conditioning may induce angiogenesis and improve blood supply to the ischemic tissues. PACE may also modulate inflammatory responses via a direct effect on cytokine release and nitric oxide synthase activity. Despite many reports on shock wave therapy application in a clinical scenario, the exact mechanism of its action is still not well known. Thus, our interest was to define the pre- and post-conditioning effect of PACE on microcirculatory hemodynamics, cytokine expression and tissue neovascularization during I/R injury.

Our well established rat cremaster muscle model for direct in vivo recordings of microcirculatory hemodynamics was applied to test the effect of PACE on modulating I/R injury in skeletal muscle flap.

**Material and methods**

This study was approved by the Institutional Animal Care and Use Committee of Cleveland Clinic. All animals used in this study received humane care in compliance with the Guide for the Care and Use of Laboratory Animals published by the National Institute of Health. The Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) certifies the Cleveland Clinic animal care facility for accreditation of laboratory animal care. Animals were caged at room temperature on a 12-h light/dark cycle. Standard laboratory food and water were provided to the animals freely.

For the purpose of PACE application and surgical intervention, the rats were anesthetized with pentobarbital (50 mg/kg) intraperitoneally and given supplements (20% of initial dose) as needed. Body temperature was maintained between 35 and 37 °C with a heat lamp during the operations and the observation period. After the microcirculatory recordings were completed, animals were euthanized with an intravenous injection of pentobarbital.

**Experimental groups**

A total number of 34 male Lewis rats weighing 130–160 g were used in this study. Rats were randomly divided into 4 experimental groups:

- **Group 1 (n = 10):** non-ischemic controls. Animals did not receive any treatment before cremaster muscle dissection.
- **Group 2 (n = 8):** 5 h Ischemia without conditioning. After cremaster muscle dissection, the femoral and iliac vessels were clamped for 5 h to induce ischemia. After ischemia and 15 min of reperfusion, microcirculatory recordings were taken for 2 h.
- **Group 3 (n = 8):** pre-ischemic (5 h) PACE conditioning. Before cremaster muscle dissection, 500 impulses of PACE (0.10 mJ/mm² energy flux density; recently revised to 0.23 mJ/mm² by the manufacturer) were applied to the scrotum. Next, the cremaster muscle was dissected and placed upon a tissue bath. Once microcirculatory hemodynamics were stabilized, the iliac and femoral vessels were clamped for 5 h to induce ischemia. After ischemia and 15 min of reperfusion, standard microcirculatory recordings were taken for 2 h.
- **Group 4 (n = 8):** post-ischemic (5 h) PACE conditioning. Following dissection and before PACE application, the femoral and iliac vessels were dissected and clamped for 5 h to induce ischemia. After 5 h of ischemia the clamps were released and, after 15 min of reperfusion, 500 impulses of PACE (0.10 mJ/mm² energy flux density; recently revised to 0.23 mJ/mm² by the manufacturer) were applied to the cremaster muscle, which was then placed upon a tissue bath in preparation for microcirculatory recordings, which were taken for 2 h.

**Induction of ischemia in the cremaster muscle flap**

To induce ischemia, microvascular clamps were applied, for 5 h, to the iliac and femoral vessels above and below the origin of the cremaster muscle pedicle (Fig. 1A). In group 2, vessels were clamped after muscle dissection; in group 3, the cremaster muscle received...
500 impulses of PACE, which was followed by muscle dissection and application of clamps, for 5 h of ischemia; in group 4, vessels were clamped before PACE application and, after 5 h of ischemia, PACE was administered to the scrotum, followed by dissection of the cremaster muscle. In each group microcirculatory recordings were first taken after 15 min of reperfusion and were then recorded for an additional 2 h at three time points: 0, 1 h and 2 h.

**PACE application**

Anaesthetized rats were placed in a dorsal position. Ultrasound transmission gel was used as a contact medium between the PACE apparatus (dermaPACE, SANUWAVE, Alpharetta, GA/USA) and scrotum. Next, 500 impulses of an energy flux density of 0.23 mJ/mm² were applied to the scrotum region. The PACE apparatus uses a focused energy system. The applicator was placed on the right testicle and then randomly moved to provide a full application of PACE to the cremaster muscle. The rationale for the dosage used in this experimental design was based on our experience (Krokowicz et al., 2010) The cremaster muscle flap dissection was performed as previously described (Anderson et al., 1988; Siemionow and Moreira-Gonzalez, 2005). Briefly, skin was incised through a right ventral inguinal incision. After the testes and spermatic cord were extracted, the cremaster muscle, along with its pudic-epigastric pedicle, was isolated up to the pedicle’s origin at the iliac vessels, under an operating microscope (Carl Zeiss OPMI 6-SD, Carl Zeiss, Goettingen/Germany). The cremaster muscle, along with its pudic-epigastric pedicle, was isolated up to the pedicle’s origin at the iliac vessels, under an operating microscope (Carl Zeiss OPMI 6-SD, Carl Zeiss, Goettingen/Germany). The cremaster muscle was then opened along its anterior wall and a round island flap with an axial pattern of main feeding vessels was created; C — Preparation of cremaster muscle flap for microcirculatory recordings by spreading it out over the tissue plexiglas bath and fixing with 7/0 silk sutures.

**In vivo microcirculatory recordings**

After being secured on the tissue bath, the rats were placed on the stage of an intravital microscope (Nikon Optiphot-2/Japan) equipped with an optical Doppler flow velocimeter (custom made, Texas A&M, College Station, TX/USA), a 19-in. monitor (Sony Trinitron, Japan), and a color digital camera (Carl Zeiss AxioVision Rel.4.6, Carl Zeiss, Goettingen/Germany). The microcirculation image was displayed on a computer’s monitor and saved on a hard drive (Hewlett Packard HPL1940T and HP xw8400 Workstation, Hewlett Packard, Palo Alto, CA/USA). Final magnification on the computer’s monitor was × 1800.

In experimental groups measurements were taken at different time points for 2 h (3 time points — 0, 1 h and 2 h) after the cremaster muscle flap underwent ischemia and received PACE treatment.

**Vessel diameters**

Using a digital image measurement device (Carl Zeiss Axiocam MR and Carl Zeiss Axiovision Rel.4.6, Carl Zeiss, Goettingen/Germany), vessel diameters of the first order venule (V-1), first order arteriole (A-1), second order arterioles (A-2), and third order arterioles (A-3), were recorded.

**Red blood cell velocity**

Using an optical Doppler velocimeter (Texas A&M, College Station, TX/USA), RBC velocity was recorded for the first order A-1 and second order A-2 arterioles, as well as for V-1 venules.

**Capillary density**

Three regions with clear visualization were chosen for measurements of capillary perfusion in the proximal, middle and distal area of the flap, for the evaluation of functional capillary density (FCD). The number of flowing capillaries was recorded in 9 high power fields (HPF) in the area of 3 pre-selected postcapillary venules (total of 27 fields per cremaster muscle).
Leukocyte–endothelial interactions

Leukocyte behavior in the selected postcapillary venules of the proximal, middle and distal regions of the cremaster muscle flap, was recorded.

The number of rolling, sticking (stationary for more than 20 s) and transmigrating leukocytes was counted in each region of the flap, for a 2-min period, using a manual cell counter.

Tissue harvesting for real-time PCR evaluation and immunostaining

Cremaster muscle samples for real-time PCR evaluation and immunostaining were harvested at the end of the 8-h experimental procedure (1 h muscle dissection, 5 h ischemia and 2 h of recordings during reperfusion).

RNA isolation from cremaster muscle tissue

At the end of the experimental procedure, the cremaster muscle tissues were harvested and preserved in RNALater® Solution and kept overnight in 4 °C. Total RNA was isolated the next day using TRI-ZOL® Reagent (Invitrogen). Tissue samples (100–120 mg) were homogenized in TRIZOL® Reagent by PowerGen 125 homogenizer (Fisher Scientific).

The isolation procedure was carried out according to the manufacturer’s (Invitrogen™) instructions. Total RNA (10 μg) was additionally treated with TURBO DNA-free™ to remove DNA contamination from the RNA preparation. The concentration and quality of extracted total RNA were measured spectrophotometrically with NanoDrop® ND-1000 (Thermo Scientific). The ratio of sample absorbance A260/A280 in the range of 1.8–2.2 was considered to be an acceptable measure of RNA purity. The RNA integrity was estimated by visual examination of two distinct rRNA bands (28S and 18S) on denaturing 1% agarose gel stained with ethidium bromide. Only RNA samples with clear and sharp 28S and 18S bands were used for further experiments.

cDNA synthesis

1 μg of total RNA was reverse-transcribed to cDNA in a total volume of 20 μl, using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystem), in accordance with the manufacturer’s instructions. The amount of cDNA synthesized in a single reaction was sufficient to amplify all of the genes via PCR.

Relative quantification real-time PCR

Two groups of genes: proangiogenic factors (VEGF, and endothelial nitric oxide synthase eNOS) and proinflammatory factors (iNOS; chemokines: CCL2, CXCL5 and chemokine receptor CCR2) were amplified using real-time PCR technique in the 7300 Real-Time PCR detection system with 7300 System SDS software (Applied Biosystem). Amplification was carried out in a total volume of 25 μl containing TaqMan Universal PCR Master Mix (2×), Gene Expression Assay Mix (20×) and 1 μl cDNA (undiluted reverse-transcription product derived from 1 μg of total RNA) under standard conditions (initial setup 2 min–50 °C; 10 min–95 °C; denaturation 15 s–95 °C; annealing 1 minute–60 °C) and 40 cycles. All PCR runs were performed in triplicate to achieve reproducibility. Non-template controls were run with every assay and no indication of PCR contamination was observed. Reported changes in expression for all examined genes were normalized to endogenous GAPDH, in each sample, and were relative to the expression of the gene in untreated cremaster muscle tissue.

Immunostaining

At the end of each experiment, sections of cremaster muscle flap specimens were snap frozen in liquid nitrogen and kept at −80 °C until immunohistochemical staining. Immunohistochemical staining was performed to identify the presence of proangiogenic factors VEGF and vWF. Prior to immunostaining, frozen tissues were cut into 4 μm sections and fixed for 10 min in acetone. Next, sections were blocked for endogenous peroxidase in 3% H2O2. Monoclonal antibodies for VEGF (C-1) (Santa Cruz Biotechnology, Inc) and for vWF Ab-2 (clone F8/86) mAb (LabVision, Corp) were applied for 30 min. The binding of primary antibodies was detected using a DAKO LSAB®2 System, Peroxidase (AEC) (DAKO, Carpinteria, California/USA), in accordance with the manufacturer’s instructions. Slides were counterstained in hematoxylin and mounted in Paramount.

Vessel density for VEGF and vWF was assessed in the tissue sample 8 h after cremaster flap dissection and was scored for staining intensity. The intensity of staining was scored subjectively, as follows: no staining (0), weak (+), moderate (++), and strong (+++).

Statistical analysis

To compare the number of functional capillaries, RBC velocities and vessel diameter changes, repeated measures of ANOVA models were fit to each outcome and location. An overall model included all treatment groups and 3 time points. In each model, the mean level of response for an outcome was modeled as a function of the treatment group, time, and an interaction between time and group. When the p-value for the interaction among all groups was statistically significant, comparisons of each treatment group with the control group were performed. An overall significance level of 0.05 was assumed, and individual group comparisons with the control group were performed using the Bonferroni corrected significance level of 0.0125. However, given the small sample size per group, all results smaller than 0.05, even in the paired comparisons, are considered potentially interesting and described in the text and figures. All statistical analyses were performed using SAS (version 9; Cary, NC, USA).

Results

The following data summarize the hemodynamic responses and leukocyte-endothelial activation following PACE treatment in a 5-h ischemia–reperfusion model.

Vessel diameter changes following ischemia/reperfusion injury

Non-ischemic controls were used to establish baseline values of arteriole and venule diameter affected only by microsurgical trauma. The diameter values for arterioles were as follows: A-1 (92 μm), A-2 (70.5 μm) and A-3 (55 μm). The diameter of the venule (V-1) was 265.5 μm. After 5 h of ischemia, a decrease in venule (V-1; 201 μm) and arteriole (A-1, 73 μm; A-3 52 μm) diameters was observed (P<0.05).

PACE preconditioning resulted in a 24% increase of mean venule diameter compared to the ischemia without conditioning group (201 μm) (P<0.05) (Figs. 2A, B; Table 1).

In response to PACE postconditioning, a 33% increase in the mean diameter of the first order arteriole (A-1) (97 μm) was observed when compared to the ischemia without conditioning group (73 μm) (P<0.05) (Fig. 2A; Table 1).

No significant changes were observed in the diameters of the second and third order arterioles during microcirculatory recordings (Figs. 2C, D; Table 1).

Red blood cell velocity response following ischemia/reperfusion injury

Baseline average values of RBC velocity in a non ischemic group were represented by venules V-1 (8 mm/s), arterioles A-1 (12 mm/s),
A-2 (10 mm/s), and A-3 (8.92 mm/s). Ischemia/reperfusion conditioning decreased the RBC velocity of A-1, A-2 and A-3 arterioles, however it did not affect RBC velocity of V-1 venules.

In group 3 (pre-ischemic (5 h) PACE conditioning), the RBC velocity of the main venule (V-1), as well as RBC velocity of all arterioles (A-1, A-2, A-3), was significantly decreased compared to the non-ischemic control group (P < 0.05). In group 4 (post-ischemic conditioning), RBC velocity of the venule (V-1) and first and second (A-1, A-2) order arterioles was similar to the control group. In both group 3 and group 4, the RBC velocity of second and third order arterioles (A-2, A-3) significantly increased compared to group 2 of ischemia without conditioning (P < 0.05). In group 3, a 40% increase was observed in A-2 arterioles (7.71 mm/s) and a 15% increase in A-3 arterioles (6.43 mm/s). In group 4, a 65% increase was observed in A-2 arterioles (9.11 mm/s) and a 31% increase in A-3 arterioles (7.32 mm/s), compared to group 2 of ischemia without conditioning, in which A-2 and A-3 arterioles revealed RBC velocity of 5.51 mm/s and 5.56 mm/s, respectively (P < 0.05) (Figs. 3A–D; Table 1).

### Functional capillary density following ischemia/reperfusion injury

The mean number of functional capillaries in the groups submitted to 5 h of ischemia followed by 2 h of reperfusion (groups 2, 3, 4) was significantly decreased compared to the baseline controls (group 1) (8.15, 8.0, 9.85 vs. 12.1, respectively; p < 0.05). When post-ischemic...
PACE conditioning was applied, the number of functional capillaries increased by 21% per HPF (9.85) compared to group 2 (ischemia without conditioning) (8.15) (P < 0.05) (Fig. 4A; Table 1). Pre-ischemic PACE conditioning did not affect functional capillary density and the number of capillaries was comparable to the number observed under ischemic control.

Leukocyte–endothelial interactions

Changes of the number of rolling leukocytes following pre- and post-ischemic PACE conditioning

The mean number of rolling leukocytes in groups submitted to 5 h of ischemia (group 2, 3, 4) was significantly decreased compared to
Pre- and post-ischemic PACE conditioning does not affect sticking leukocytes. There were no significant changes observed in the mean number of transmigrating leukocytes during microcirculatory recordings in all experimental groups (Fig. 4D; Table 1).

Pre- and post-ischemic PACE conditioning does not affect transmigrating leukocytes. There were no significant changes observed in the mean number of transmigrating leukocytes in all groups submitted to 5 h ischemia (groups 2, 3, 4) was significantly increased compared to the baseline control (group 1) (4.22, 4.07, 3.78 vs. 2.77, respectively), (P<0.05) (Fig. 4C Table 1).

PACE conditioning did not have an effect on sticking leukocytes, and there were no significant changes in the number of sticking leukocytes observed in group 3 (pre-ischemic PACE conditioning) and group 4 (post-ischemic PACE conditioning) when compared with group 2. There was an increase in the number of sticking leukocytes after PACE pre-ischemic and post-ischemic conditioning (groups 3 and 4) when compared with group 1 (control without ischemia) (P>0.05) (Fig. 4B; Table 1).

The mean number of sticking leukocytes in all groups submitted to 5 h of ischemia/reperfusion did not affect the gene expression level of the proangiogenic factor eNOS, but did increase expression of VEGF. After applying pre- and post-ischemic PACE conditioning, VEGF gene expression levels were up-regulated up to 140% and 180%, respectively. We did not observe any changes in gene expression levels after PACE therapy (Fig. 5).

Real-time PCR evaluation of gene expression of proinflammatory and proangiogenic factors after pre- and post-ischemic PACE treatment. Ischemia/reperfusion did not affect the gene expression level of the proangiogenic factor eNOS, but did increase expression of VEGF. The application of pre-ischemia PACE conditioning resulted in decreased gene expression for chemokine receptor Ccr2 in all experimental groups: non-ischemic controls (group 1), ischemic controls (group 2), 500 impulses of PACE treatment applied before (pre-ischemic PACE conditioning (group 3)) or after (post-ischemic PACE conditioning (group 4)) 5 h of ischemia. In the group after post-ischemic PACE conditioning a significant down-regulation of gene expression level for iNOS and proinflammatory chemokines: CCL2 and CXCL5 were observed (P<0.05). The application of pre-ischemia PACE conditioning resulted in increased gene expression for chemokine receptor Ccr2 (Fig. 6).

Evaluation of vessel density by von Willebrand factor and VEGF expression. Expression of proangiogenic factors on the vessel endothelium of the ischemic cremaster flaps is summarized in Table 2 and illustrated in Fig. 7. Our experiment revealed a weak expression of VWF and VEGF on the vessels from the non-ischemic control group, the 5-h ischemia group (without conditioning), and the pre-ischemic (5 h) PACE conditioning group (groups 1, 2, and 3). The post-ischemic (5 h) PACE conditioning group (group 4) showed a moderate increase in the expression of both proangiogenic factors.

Discussion

PACE conditioning has many medical applications due to its confirmed ability to improve blood supply to the tissues and its potential to stimulate angiogenesis and neovascularization. Many experimental studies have been performed using PACE therapy. However, to the best of our knowledge, the present study is the first to assess the effects of PACE conditioning on I/R injury during direct in vivo monitoring of the microcirculatory hemodynamics. In this study, we used a well-established cremaster muscle flap model introduced in 1988 by Anderson et al. (1988). This model has multiple applications that mimic clinical situations due to its structural and functional similarity to other skeletal muscles. Access to the main neurovascular pedicle of the muscle flap allows for the study of I/R injury and the effects of...
tissue innervation and denervation during direct in vivo recordings of peripheral microcirculation (Adanali et al., 2001; Anderson et al., 1988; Ozer et al., 2002; Siemionow et al., 1994; Siemionow and Moreira-Gonzalez, 2005; Unsal et al., 2002). For experienced microsurgeons, our cremaster muscle I/R model is easy to prepare, and yields a high animal survival rate as well as the opportunity to monitor peripheral microcirculatory responses to applied treatment modalities. The advantages of this model include direct in vivo observation of multiple microcirculatory parameter changes, in real time. The major disadvantage is the fact that observations are performed on surgical model requiring muscle isolation, thus exposing it to surgical trauma. However, since each experiment requires surgical isolation, the effect of various test conditions can always be compared with the standard control muscle.

Based on previous experience with PACE application to healthy tissue, we know that PACE conditioning does not have a negative impact on the treated tissues. To the contrary, 500 impulses of PACE conditioning enhance angiogenesis and decrease inflammatory response following surgical intervention (Krokowicz et al., 2010).

In the present study, as expected the negative impact of I/R injury on microcirculatory hemodynamics was observed as confirmed by decreased red blood cell velocity and decreased diameters of the main feeding vessels. Conditioning with PACE therapy applied either before or after I/R reversed the negative effects of I/R injury and stimulated recovery of microcirculatory hemodynamics to the pre-ischemic values.

The disruptive effects of I/R injury on the microcirculatory hemodynamics of the cremaster muscle flap were clearly visible at the capillary level, where a significant reduction of functional capillary perfusion was observed. However, conditioning with PACE after I/R (group 4) resulted in a significant increase in capillary perfusion, which correlated with increased RBC velocity in the second and

### Table 2
Expression of von Willebrand factor and VEGF in cremaster muscle.

<table>
<thead>
<tr>
<th>Experimental group</th>
<th>von Willebrand factor</th>
<th>VEGF</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group 1 (Control)</td>
<td>0/+</td>
<td>0/+</td>
</tr>
<tr>
<td>Group 2 (Ischemia)</td>
<td>0/+</td>
<td>0/+</td>
</tr>
<tr>
<td>Group 3 (Pre-ischemic PACE treatment)</td>
<td>0/+</td>
<td>0/+</td>
</tr>
<tr>
<td>Group 4 (Post-ischemic PACE treatment)</td>
<td>+</td>
<td>+++</td>
</tr>
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Intensity of staining was scored subjectively as follows: no staining (0), weak (+), moderate (++), and strong (+++).

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**Fig. 7.** Expression of proangiogenic factors VEGF (vascular endothelial growth factor) and vWF (von Willebrand factor) on the vessel endothelium in all experimental groups: Non-ischemic controls (group 1), ischemic controls (group 2), 500 impulses of PACE treatment applied before (pre-ischemic PACE conditioning) or after (post-ischemic PACE conditioning) 5 h of ischemia. Application of post-ischemic PACE conditioning resulted in a moderate increase in the expression of VEGF and vWF proangiogenic factors.
third order arterioles (A-2 and A-3). Under physiological conditions, 20–25% of capillaries in the vessel bed are dormant. The mechanical stimulation of the cremaster muscle created by PACE conditioning helps to activate these dormant capillaries that are needed to provide better oxygenation and nutrient distribution to the tissue, thus improving tissue recovery after I/R injury. The number of sticking and transmigrating leukocytes after pre-and post-ischemic PACE conditioning was comparable to the number of leukocytes observed in the ischemic controls. However, both pre- and post-ischemic PACE conditioning decreased the number of rolling leukocytes in comparison to ischemic controls. This observation was associated with down-regulation of proinflammatory chemokines CCL2 and CXCL5, in the post-ischemic PACE conditioning group. Although PACE conditioning decreases expression of the proinflammatory chemokines responsible for attracting leukocytes to the site of inflammation, it does not affect the expression of selectins responsible for adhesion and transmigration of leukocytes.

It is hypothesized that PACE conditioning, similar to ESW, affects treated tissues by cavitation which generates shear stress to the cells’ membranes. This results in infiltration of bio-active molecules into the cells which activates/inhibits various pathways of intracellular signaling (Gambihler et al., 1994). Shock waves may also modify microcirculatory blood flow by enhancing the production of nitric oxide by the vascular endothelium. Under normal conditions, NO has a protective effect on cells and tissues and prevents the formation and activation of free radicals. During I/R, the balance between NO and free radicals is disrupted due to low NO secretion and increased production of the peroxide ($O_2^-$) and hydrogen peroxide ($H_2O_2$) by xantine oxidase. This leads to development of tissue inflammation correlating with the activation of cell adhesion molecules and secretion of platelet activating factor (PAF) (Carden and Granger, 2000).

In our experimental design, I/R resulted in increased expression of the iNOS gene responsible for NO production. When PACE conditioning was applied before I/R, the level of iNOS expression did not change and was elevated. However, when PACE conditioning was applied after I/R, iNOS expression was comparable to that in the non-ischemic control group. Similar results of decreased expression of iNOS after shock wave conditioning were observed by Ciampa et al., (2005).

Chemokines (CCL2, CCL3, CCL4 and CXCL8) influence the migration and activation of various types of leukocytes. Increased chemokine expression has been reported in patients with chronic inflammatory diseases (Koch et al., 1992), in patients after acute myocardial infarction (Matsumori et al., 1997), and in an ischemia/reperfusion study of the liver (Kurrelmeyer et al., 2000). It has also been shown that proinflammatory chemokine inhibition can attenuate tissue damage caused by I/R and can improve tissue function (Kajihara et al., 2003). In our I/R model without PACE conditioning, the expression of CCL2 and CXCL5 was highly elevated. However, PACE conditioning especially after ischemia, lowered the values of proinflammatory chemokines, which correlated with the levels recorded before ischemic insult. These results were similar to findings reported in the literature which showed that PACE therapy ameliorated inflammatory responses, stimulated angiogenesis, and decreased tissue necrosis (Davis et al, 2009; Ito et al., 2009; Krokowicz et al., 2010; Stojadinovic et al., 2008; Wang etal.).

It is well known that von Willebrand factor (vWF) and Vascular Endothelial Growth Factor (VEGF) are crucial components of vasculo-genesis and are necessary for its initiation at early stages. VEGF is the most important mitogen during the process of angiogenesis and is synthesized in macrophages and tumor cells in response to low oxygen levels in tissues and induction of hypoxia inducible factor (HIF). Elevated levels of VEGF are detected during I/R, angiogenesis and tumor growth (Broxmeyer et al., 1995; Folkman and Shing, 1992). In this study, we observed weak expression of VEGF in the groups without PACE conditioning and in the group submitted to pre-ischemic PACE conditioning (group 3). In contrast, PACE application after I/R (group 4) resulted in increased VEGF expression on the vascular endothelium as confirmed by immunostaining. Furthermore, the increased expression of VEGF protein on the vascular endothelium correlated with up-regulation of VEGF gene expression in the ischemic cremaster flap, when PACE conditioning was applied after I/R injury. This concomitant up-regulation of proangiogenic factors in response to the ischemic insult could be interpreted as leading to the stimulation of natural defense mechanisms which, via the induction of local angiogenesis, could support delivery of oxygen and nutrients to the ischemic tissues. Our observation that post-ischemic PACE treatment is more effective in stimulating VEGF expression and subsequently new blood vessel formation, is consistent with studies that used a cerebral ischemic rat model in which post-ischemic VEGF administration enhanced angiogenesis within the ischemic boundary and improved functional recovery (Yang et al., 2010).

Up-regulation of VEGF gene expression was also observed in the pre-ischemic PACE conditioning group. However, in this case, there was no correlation with the expression of VEGF protein, which was found to be low, on the vessel endothelium. These results indicate that post-ischemic PACE conditioning affects VEGF gene and protein expression and is associated with increased eNOS gene expression in endothelial cells, which serves a protective role and has a confirmed a proangiogenic capacity (Phillips et al., 2009).

The results of this study are supported by observations previously reported in the literature. Application of shock waves in the skin flaps compromised by ischemia increased expression of both VEGF and eNOS (Meier et al., 2007a; Kuo et al., 2007, 2009).

We have also confirmed in the present study that increased VEGF expression on the vascular endothelium, in the post-ischemic PACE treatment group, is associated with the expression of VWF. The presence of stronger VEGF expression correlating with low expression of vWF on the vascular endothelium, confirms proangiogenic activity on the I/R damaged tissues. Distribution of VWF on the vascular endothelium is heterogeneous and VWF is expressed strongly on the venules but not on the arterioles of the microcirculation. Heightened expression of VWF is preferably seen in larger vessels. (Table) (Flamme et al., 1997; Folkman and Shing, 1992; Risau, 1997, 1998; Zanetta et al., 2000)

Conclusions

PACE conditioning applied after I/R injury (group 4) restored microcirculatory hemodynamics, which correlated with an increase of main vessel diameter and RBC velocity, as well as activation of capillary perfusion. PACE conditioning also had a proangiogenic and anti-inflammatory effect on the post-ischemic tissues, which was confirmed by elevated expression of proangiogenic VEGF and VWF factors and down regulation of iNOS and proinflammatory chemokines. PACE induced the expression of anti-inflammatory and proangiogenic factors correlating with improvement in microcirculatory hemodynamics, in the tissues submitted to ischemic insult. This makes PACE therapy a promising tool with potential application in clinical cases where tissue I/R injury is expected or diagnosed.

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References

