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

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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 protein expression. This correlated with increased gene expression of VEGF (up to 180%). In contrast, gene expression of proinflammatory factors (iNOS, CCL2, CXCL5) decreased compared to ischemic controls. Pre-ischemic PACE conditioning decreased gene expression of proinflammatory chemokines (CCL2 and CXCL5), compared to ischemic controls without conditioning.

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.

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Introduction

Ischemia/reperfusion (I/R) injury is a destructive process caused by the return of blood supply to tissues submitted to variable periods of ischemia. Depletion of oxygen and energy as well as release of free radicals contributes to tissue damage in many clinical situations, including free tissue transfers, replantation, organ transplantation, myocardial infarction, and stroke (Qi et al., 2004).

Abbreviations: ESW, Extracorporeal shock waves; ESWT, Extracorporeal shock wave technology; I/R, Ischemia/reperfusion; PACE, Pulsed acoustic cellular expression; RBC, Red blood cells; VEGF, Vascular Endothelial Growth Factor; vWF, von Willebrand factor; iNOS, inducible Nitric Oxide Synthase; eNOS, endothelial Nitric Oxide Synthase; CCL2, Chemokine (C-C motif) Ligand 2; CCR2, Chemokine (C-C motif) Receptor 2; CXCL5, Chemokine (C-X-C motif) Ligand 5.

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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 intracellular 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. ESWs 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; Zimpfer 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; Meirer et al., 2005a, 2005b, 2007b).

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 illiac 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/mm^2 energy flux density; recently revised to 0.23 mJ/mm^2 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 illiac 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/mm^2 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

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