Ultrasound in Med. & Biol., Vol. ■, No. ■, pp. 1–12, 2016

0301-5629/$ - see front matter

http://dx.doi.org/10.1016/j.ultrasmedbio.2016.08.004

● *Original Contribution*

THERAPEUTIC PULSED ULTRASOUND PROMOTES REVASCULARIZATION AND FUNCTIONAL RECOVERY OF RAT SKELETAL MUSCLE AFTER CONTUSION INJURY

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(*Received* 27 *August* 2015; *revised* 26 *July* 2016; *in final form* 2 *August* 2016)

Abstract—The mechanism by which therapeutic pulsed ultrasound (TPU) promotes the repair of damaged gastrocnemius muscle was investigated. Male Wistar rats were divided into uninjured, sham-treated injured and TPU-treated injured (TPU) groups. Injury was induced by mass-drop technique. TPU was applied to the injured muscle for 5 min, daily, started at day 1 post-injury and continuing for 3, 7 and 14 d. For 3 d post-injury, a significant reduction in muscle force was observed in both the sham-treated injured and TPU groups. TPU treatment significantly increased recovery force of the injured muscle after day 7 post-injury. This effect of TPU is associated with increased centronucleated fibers and cross-sectional area, mRNA expression of the vascular endothelial growth factor and capillary density of the regenerated fibers, but not with mRNA expression of nitric oxide synthase. We conclude that TPU hastens muscle recovery, at least in part, by upregulating angiogenesis. (E-mail: tossaporn.yim@mahidol.ac.th) © **2016 World Federation for Ultrasound in Medicine & Biology.**

Key Words: **Therapeutic pulsed ultrasound, Muscle regeneration, Injury, Angiogenesis, Vascular endothelial growth factor.**

INTRODUCTION

Muscle contusion injuries are common during sporting activities and pose a major challenge in sports medicine. Even though skeletal muscles are able to repair themselves after an injury, their regenerative capacity is limited. For instance, skeletal muscle function may not fully recover after the injury, resulting in the risk for recurrent injuries (Huard et al. 2002; Jarvinen et al. 2005). After an injury, inflammatory cells infiltrate the lesion area to remove necrotic muscle fibers and cellular debris. Subsequently, the damaged muscle fibers differentiate and undergo myogenesis (Karalaki et al. 2009; Wagers and Conboy 2005). This process depends exclusively on the function of satellite cells lying between the basal lamina and the sarcolemma of mature myofibers (Zammit et al. 2006). Activated satellite cells proliferate and migrate to the injured area, differentiate into myoblasts and, subsequently, fuse the remaining fibers to replace the damaged muscle fibers (Jarvinen et al. 2005). Although the activation of satellite cells is known to be a crucial step for the growth, maintenance and healing of skeletal muscle, revascularization of the damaged muscle also plays an important role during healing and could influence the success of muscle regeneration (Frey et al. 2012; Jarvinen et al. 2005; Karalaki et al. 2009). During the healing process, new capillaries sprout toward the center of the damaged area, allowing newly formed blood vessels to provide a necessary blood supply, which optimizes the aerobic metabolism of the regenerated myofibers (Jarvinen et al. 2005).

As maintained in Clapp et al. (2009), angiogenesis is essential for embryogenesis, organogenesis and fetal development, but the physiologic role of angiogenesis in adult tissues is highly restricted and occurs only in the early phase of muscle and fracture healing. Among several pro-angiogenic factors, vascular endothelial growth factor (VEGF) is the most potent growth factor promoting angiogenesis (Clapp et al. 2009). The physiologic significance of VEGF in muscle regeneration and healing has been well documented. Previous reports suggested that VEGF not only promotes growth of regenerated myofibers, but also protects myogenic cells from

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apoptosis (Arsic et al. 2004). Apart from facilitating the early regeneration of damaged neuromuscular junctions, VEGF helps to restore contractile function of the muscle and minimize fibrosis (Arsic et al. 2004; Borselli et al. 2010; Frey et al. 2012). In particular, reports indicate that mRNA expression of VEGF is upregulated shortly after muscle injury and peaks within 5 to 7 d. This elevated VEGF expression is accompanied by increasing angiogenesis of the injured area (Ota et al. 2011; Wagatsuma 2007).

Therapeutic pulsed ultrasound (TPU) has been used as a therapeutic intervention to promote bone healing (Claes and Willie 2007; Pounder and Harrison 2008). TPU not only accelerates bone healing in animals (Suzuki et al. 2009; Tang et al. 2006) and humans (Handolin et al. 2005), but also increases production of VEGF, basic fibroblast growth factor (bFGF) and interleukin-8 (IL-8), all of which are angiogenic factors (Reher et al. 1999). In addition, nitric oxide (NO) is known to play a central role in vasodilation, in stimulation of proliferation and migration of endothelial cells and in revascularization of ischemic heart and limb muscles (Jozkowicz et al. 2001). It has also been reported that TPU increases NO production (Reher et al. 2002; Tang et al. 2007) and VEGF-A expression (Reher et al. 1999; Wang et al. 2004) in human osteoblasts.

Several lines of evidence suggest that TPU treatment may benefit the healing process of non-mineralized tissues including ligament (Sparrow et al. 2005; Warden et al. 2006) and skeletal muscle (Montalti et al. 2013; Piedade et al. 2008). TPU also enhances muscle regeneration and improves physiologic performance recovery after laceration (Chan et al. 2010). So far, the cellular mechanism(s) by which TPU enhances muscle healing is, however, not well understood. In the study described here, we investigated the effect of TPU on mRNA expression of VEGF and angiogenesis in rat skeletal muscle. We hypothesized that TPU promotes healing of the muscle by increasing expression of VEGF and angiogenesis through a mechanism that may involve NO.

METHODS

Animals

Eight-week-old male Wistar rats (weight 300–320 g) were obtained from the National Animal Center, Salaya, Mahidol University, Nakhon Pathom, Thailand. All procedures were approved by the Animal Care and Use Committee, Faculty of Science, Mahidol University (Protocol No. MUSC 55-013-259) and were performed in accordance with the Ethical Principles and Guidelines of the National Research Council of Thailand. After 1 wk of acclimation, rats were randomly assigned to three groups: a control non-injured group (Control) (n = 7/group), a sham-operated injured group (ShU) (n = 21/group) and a TPU-treated injured group (TPU) (n = 21/group). The ShU and TPU groups were further divided into three subgroups each (n = 7/group), according to specified post-injury time points (*i.e.,* 3, 7 or 14 d). All rats were housed in a temperature-controlled room (21–22°C), maintained on a 12-h light–dark cycle at the Faculty of Science, Mahidol University, and fed standard rat chow and water *ad libitum*.

Induction of muscle injury

Rats were anesthetized with an intra-peritoneal injection of a cocktail of zoletil (25 mg/kg weight) and xylazine (8 mg/kg weight). Under deep anesthesia, the right hind limbs of rats in the ShU and TPU groups were shaved, and the mid-belly gastrocnemius muscle was injured using a contusion technique. Briefly, the hind limb was fixed in a hip fully lateral rotation, knee extended, and ankle dorsiflexion at angle 90° position. A stainless-steel mass (650 g) was dropped from a height of 30 cm from a contusion apparatus onto the junction of the proximal and middle thirds of the right gastrocnemius muscle belly, as previously described by Kami and Senba (2002) with minor modifications. The rats were returned to their cages after fully recovering normal consciousness.

Ultrasound treatment

Therapeutic pulsed ultrasound (Intelect Mobile, Chattanooga, Vista, CA, USA) was applied at frequency of 1 MHz and intensity of 0.3 W/cm^2 SATP (spatial average temporal peak), using a 5-cm^2-diameter head with an effective radiating area (ERA) of 4 cm^2. The protocol used a pulse duration of 20% duty cycle (2 ms on, 8 ms off), which corresponded to 0.06 W/cm^2 SATA (spatial average temporal average) and a total energy of 72 J. TPU had been calibrated by the manufacturer before the experiment. The transducer was calibrated by emitting ultrasound from the probe surface onto a submerged cone in the water, which was the calibration target. The power output measured using an ultrasound power meter (UPM-DT-10-AV, Ohmic, St. Charles, MO, USA) was within 20% of the intensity indicated on the ultrasound machine. TPU was applied for 5 min at every 24 h, beginning 24 h post-injury and continuing daily for 3, 7 or 14 d until sacrifice. A commercially available ultrasound gel was used as a coupling agent. During ultrasound, rats were stabilized using a restraint device, and their hind limbs were manually fixed (Fig. 1). The ShU group was treated similarly to the TPU group, except that the power was not turned on. The experimental protocol is depicted in Figure 2.

Evaluation of contractile function

Isometric contractile properties of gastrocnemius muscles were determined according to the method