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● *Original Contribution*

THE NON-THERMAL EFFECTS OF PULSED ULTRASOUND IRRADIATION ON THE DEVELOPMENT OF DISUSE MUSCLE ATROPHY IN RAT GASTROCNEMIUS MUSCLE

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Abstract—This study examined the effects of therapeutic pulsed ultrasound (US) on the development of disuse muscle atrophy in rat gastrocnemius muscle. Male Wistar rats were randomly distributed into control, immobilization (Im), sham US, and US groups. In the Im, sham US and US groups, the bilateral ankle joints of each rat were immobilized in full plantar flexion with a plaster cast for a 4-wk period. The pulsed US (frequency, 1 MHz; intensity, 1.0 W/cm^2; pulsed mode 1:4; 15 min) was irradiated to the gastrocnemius muscle in the US group over a 4-wk immobilization period. The pulsed US irradiation delivered only non-thermal effects to the muscle. In conjunction with US irradiation, 5-bromo-2'-deoxyuridine (BrdU) was injected subcutaneously to label the nuclei of proliferating satellite cells 1 h before each pulsed US irradiation. Immobilization resulted in significant decreases in the mean diameters of type I, IIA and IIB muscle fibers of the gastrocnemius muscle in the Im, sham US and US groups compared with the control group. However, the degrees of muscle fiber atrophy for all types were significantly lower in the US group compared with the Im and sham US groups. Although the number of capillaries and the concentrations of insulin-like growth factor and basic fibroblast growth factor did not change in the muscle, the number of BrdU-positive nuclei in the muscle was significantly increased by pulsed US irradiation in the US group. The results of this study suggest that pulsed US irradiation inhibits the development of disuse muscle atrophy partly *via* activation of satellite cells. (E-mail: nakano-j@nagasaki-u.ac.jp)

Key Words: **Pulsed ultrasound, Disuse muscle atrophy, Satellite cell, Growth factor, Capillary, Rat.**

INTRODUCTION

Therapeutic ultrasound (US) is a well-established deep-heating modality that converts mechanical energy into a form of sound waves. Therapeutic US, which has been widely used in physical therapy, reduces edema, relieves pain, increases the range of motion and accelerates tissue repair (van der Windt et al. 1999). It is one of several physical therapy modalities suggested for the management of pain and loss of function due to locomotive syndrome, and it can be used as part of an overall rehabilitation program (Rand et al. 2007). US may be administered in either a continuous or a pulsed mode (Rutjes et al. 2010). Pulsed US produces non-thermal effects and is used to aid in the reduction of inflammation (Johns 2002; Rutjes et al. 2010). The non-thermal effects of pulsed therapeutic US are thought to occur by mechanical stimulation of sound wave to tissues and cells.

On the other hand, mechanical stimulation leads to secretion of insulin-like growth factor (IGF)-1 and other growth factors in skeletal muscle, which play a role in muscle fiber hypertrophy. The secretion of IGF-1 in the muscle fibers increases within 1h–4 d after muscle fiber was loaded (McKoy et al. 1999; Perrone et al. 1995; Yang et al. 1997). IGF-1 activates protein translation in the ribosome, which increases the muscle fiber volume (Goldspink 1999). In addition, mechanical stimulation

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loading is known to increase vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF), which results in the development of more skeletal muscle capillaries (Folkman et al. 1988). An adequate supply of nutrition and oxygen by the increased number of capillaries contributes to muscle fiber hypertrophy and prevents muscle fiber atrophy (Deveci et al. 2002; Nakano et al. 2009; Plyley et al. 1998). Several previous reports regarding the effects of US on cells showed that the irradiation of pulsed US increased VEGF and FGF in fibroblasts and angiogenic cells in culture (Reher et al. 1999; Toyama et al. 2012). Furthermore, low-intensity pulsed US promoted the differentiation of osteoblasts and the proliferation of Schwann cells in culture (Tsuang et al. 2011; Ying et al. 2012), and pulsed US induced an increase in IGF-1 gene expression in undamaged skeletal muscle in humans (Delgado-Diaz et al. 2011). The action of US and the mechanism of hypertrophy induced by mechanical stimulation in concert led us to hypothesize that pulsed therapeutic US affects muscle fiber size *via* growth factor secretion or cell proliferation.

Satellite cells, which are un-differentiated myogenic stem cells located between the muscle fiber plasma membrane and the basement membrane, are thought to serve as the source of new muscle fiber nuclei. The importance of satellite cells has been documented during normal muscle growth, regeneration, hypertrophy and recovery after atrophy (Ambrosio et al. 2009; Gallegly et al. 2004). The application of passive stretch to muscle fibers, *i.e.,* mechanical stimulation, induces an increase in muscle fiber nuclei with enlargement of the muscle fiber size, which is explained by the incorporation of satellite cell nuclei with the adjacent muscle fiber *via* cell fusion (Carson and Alway 1996; Shenkman et al. 2010). It is not known totally whether the mechanical stimulation by US could affect satellite cells in like a passive stretch.

The effects of therapeutic pulsed US on muscle fiber hypertrophy and atrophy have not been investigated in skeletal muscle and, especially, the influences of pulsed US on satellite cells has not been clarified. If pulsed therapeutic US can induce growth factor release, angiogenesis, and satellite cell differentiation and proliferation in muscle *in vivo*, then disuse muscle atrophy would be prevented. Therefore, this study examined the effects of pulsed therapeutic US, especially the non-thermal effects, on the development of disuse muscle atrophy in the immobilized hind limbs of rats.

MATERIALS AND METHODS

Animals

All experiments and procedures were approved by the Ethics Review Committee for Animal Experimentation at Nagasaki University. We obtained 62, 8-wk-old, male Wistar rats (220 ± 10 g) from Kudo Laboratories (Tokyo, Japan). The animals were housed in cages inside a room with a 12-h dark/light cycle. The temperature and relative humidity of the room were maintained at 25°C and 50%, respectively. Food and water were available ad libitum.

The previously described animal model of disuse muscle atrophy by cast immobilization (Okita et al. 2004) was used in this study. We randomly distributed 46 rats into four groups: Control (n = 13), only cast immobilization for 4 wk (Im, n = 13), pulsed US irradiation during cast immobilization (US, n = 13) and sham US during cast immobilization (sham US, n = 13) groups. Rats in the Im, US and sham US groups were anesthetized with pentobarbital sodium (40 mg/kg) and their bilateral ankle joints were subsequently fixed in full plantar flexion with plaster casts with the gastrocnemius muscle immobilized in a shortened position. The plaster cast was positioned from above the knee joint to the distal foot. The immobilization period was set for 4 wk, which was previously shown to be adequate for induction of muscle fiber atrophy (Takekura et al. 1996). Rats in the Im group were immobilized throughout the 4 wk without treatment. For pulsed US irradiation and sham treatments, bilateral ankle casts in the sham US and US groups were removed under pentobarbital sodium anesthesia (40 mg/kg) during the immobilization period at a frequency of 6 d/wk. The bilateral ankle joints were re-immobilized after completion of the daily treatment. The number of rats was not consistent between the groups, because induction of edema by casting and failures of tissue preparation and anesthesia resulted in the exclusion of several rats. Finally, 46 rats were used for analysis of the gastrocnemius muscle (control, n = 12; Im, n = 9; US, n = 13; and sham US, n = 12).

The remaining 10 rats were used in a pilot study for the measurement of core and muscle temperatures during pulsed US irradiation.

Measurement of core and muscle temperatures during pulsed ultrasound irradiation

The time course changes of core and muscle temperatures were measured during US irradiation in a pilot study. We randomly distributed 10 rats into the US (n = 5) and sham US (n = 5) groups. After the animals were anesthetized with pentobarbital sodium (40 mg/kg), all hair on the right hind limb was subsequently removed, and a needle thermo-sensor (PTN-800, Unique Medical Inc., Tokyo, Japan) was carefully inserted in the proximal direction, horizontal to the Achilles' tendon. To target the deep tissue of the gastrocnemius muscle under the US irradiation area, the tip of the needle thermo-