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Research Article

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Characterization of microstructural changes on electroacupuncture induced rat skeletal muscle by low-field NMR and microscope image

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ABSTRACT

An electroacupuncuture method has been applied on rat skeletal muscle for determining whether this treatment can attenuate or prevent muscle degradation during the disuse. It was tested on a hind limb suspension (HS) disuse rat model, and by using a non-destructive Nuclear Magnetic Resonance (NMR) relaxation technique, and microscope image analysis technique, as well the muscle mass and density measurements to characterize the muscle microstructural changes. Our study suggests that electroacupubcuture technique is an effective method in preventing muscle degradation in a disuse rat model.

Keywords: acupuncture, NMR, Microscope image, Skeletal muscle.

INTRODUCTION

Osteopenia is a disease characterized by long term loss of bone tissue, particularly in the weight-supporting skeleton [1,2]. Meanwhile, sarcopenia, the loss of muscle mass and strength that occurs with aging, is believed to play a major role in the pathogenesis of frailty and functional impairment during aging process as well as muscle atrophy with these who experienced various injuries and disuse conditions. Therefore, finding an effective intervention for attenuating and/or preventing osteopenia and muscle atrophy become major interests in those individuals.

Loss of skeletal muscle mass has a profound effect on a patient's daily life, especially physical activity. The resulting reduction in physical activity induces further skeletal muscle atrophy, leading to a vicious circle of the atrophic process [3]. Since skeletal muscle has high plasticity, interventions such as exercise training, nutrition, and mechanical stimulation are recommended to prevent skeletal muscle atrophy [4]; it is often hard, however, for under the weightless conditions, the elderly and patients with serious diseases to continue exercise training and even to maintain daily physical activity. As an alternative to exercise training, therefore, a non-pharmacological intervention is urgently required, especially given the rapid aging of our society. Acupuncture is a branch of traditional East Asian medicine that is widely applied to various diseases [5]. The consensus of the World Health Organization is that acupuncture can be used for stroke rehabilitation, headache, menstrual cramps, osteoarthritis, low back pain, carpal tunnel syndrome, asthma, and other conditions [6,7]. In addition to these indications, electroacupuncture (EA) is used for recovery from skeletal muscle fatigue and musculoskeletal disorders [8–10]. The mechanism of

acupuncture has been extensively investigated [11]. EA increases blood flow in and oxygenation of skeletal muscles [12,13] and evokes somatosensory responses of the brain, spinal cord and muscles in humans [14], and these factors are thought to contribute to its ameliorating effect on muscle fatigue.

Hind limb suspension (HS) model has been accepted by the scientific community as the rodent model of choice for simulating weightless condition, and widely used for induction of muscle atrophy in animal model [15]. Acupuncture treatments have been used to restore muscle strength from the injury. However, little is known of effect of acupuncture treatment on the skeletal muscle function, particularly in animal model. To find an effective countermeasure on the muscle degradation, the characterization design for the muscle samples will be performed on 3 rat groups: 1) Control (CON), 2) Hind limb Suspension (HS), and 3) HS + Electroacupuncture (HS+EA/HSA).

Here we hypothesize that acupuncture in place of exercise training is an alternative non-pharmacological intervention that can help to prevent muscle atrophy. To elucidate the effects of acupuncture on skeletal muscle atrophy caused by hind limb suspension (HS), we characterized the samples with/without acupuncture performed on HS rats. In this research study it was to apply a non-destructive nuclear magnetic resonance (NMR) technique and micro-scope image to characterize skeletal muscle development associated with water distribution and cell structural changes, and these measures could subsequently be used to predict muscle quality. In this study, the NMR relaxation technique was used to assess disuse, normal, and physical treated rat skeletal muscle quality in vitro. Specifically, we tested the changes in an alteration of the NMR spin-spin (T2) relaxation time signal due to the structural changes within the different treatments. The obtained NMR signal was further processed to produce a T2 relaxation distribution spectrum [18, 20] related to water distribution and cell size changes, as well as observed by microscope image, and their derived parameters were used as descriptors of muscle microstructural changes related to the different treatments. Meanwhile, by using low-field nuclear magnetic resonance and combined with muscle soleus weighing and micro image methods to characterize the skeletal muscle changes among control, disuse (hind limb suspension (HS)), and HS plus electroacupunture (HSA) in vitro. The purpose of this study was to determinate that whether the electrocupunture treatment can attenuate or prevent muscle degradation during the disuse.

EXPERIMENTAL SECTION

In vivo animal test: disuse hind limb suspension (HS) rat model

The hind limb suspension of rats is a model of stimulated microgravity for inducing muscle atrophy (decrease in muscle mass and bone density). In HS experiment, the tail of rat was attached by a triangular metal clip, thus rat's hind limb was elevated off the ground. The animals were able to move freely with their fore limbs in the cage as shown in Figure 1.

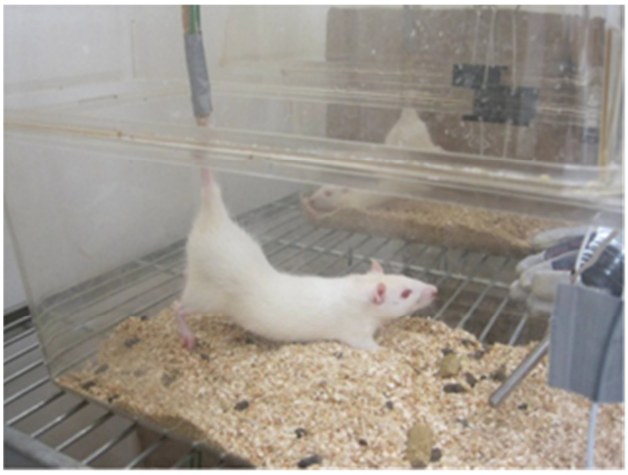

Figure1. HS consists of rat tail suspension from the cage ceiling with free access to food and water

Sample corrections

Total 25 skeletal muscle samples (n=7 in CON, n=9 in HS, and HAS, respectively) were collected and analyzed. All the specimens were stored in phosphate-buffered-saline at -20^{o}C; and prior to NMR measurements, the samples were completely thawed at room temperature.

Electroacupuncture

In electroacupuncture experiment, the sparse-wave was used, with 2-10 Hz frequency, 4-6 Voltage for 15 mins in each treatment, which is good for treating muscle atrophy. It was applied to HS rat by needing GB34 and ST36 [16] as shown in Figure 2 (a and b). The electroacupuncture treatments were 3 times per week for 3 weeks (21 days). The specific instrument for electroacupuncture treatment on rat is shown below (Figure 2c).

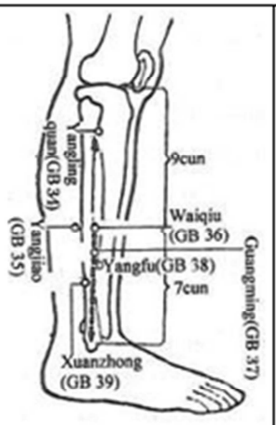

Figure 2a. shown position of needing GB34.

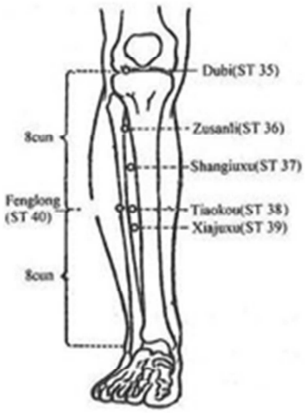

Figure 2b. shown position of needing ST36.

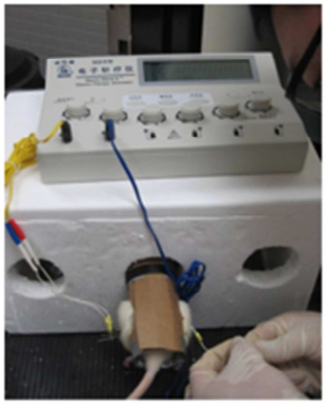

Figure 2c. specific instrument for electroacupuncture.

Relationship between NMR data and cell size

It is found that the T_2 relaxation time as measured by NMR is non-invasive and non-destructive technique for bone quality evaluation [17-21]. These techniques also can be applied to effectively determine skeletal muscle quality under various testing conditions for the animals (e.g. HS, HS+EA/HSA, and normal only). It is known that the total intensity of magnetization from the NMR free induction decay (FID) signal is due to the water (protons) present inside the muscle (mobile water), and the water (protons) that has undergone hydration with the muscle (bound water). In addition, the total amplitude of T_2 relaxation envelopes, measured by the NMR spin echo train (CPMG) [22,23], is a representation of the liquid phase inside the muscle.

In low-field NMR, at the fast diffusion limit (diffusion effect is negligible), the relaxation rate $1/T_2$ is proportional to the surface-to-volume (S/V) ratio of the pore [24]:

$$1/T_2 = \rho(S/V)_{pore} \quad (1)$$

where ρ is the surface relaxivity – a measure of the pore surface's ability to enhance the relaxation rate – which falls within a reasonably narrow band. For compact bone material, it ranges roughly from micron to tens of microns per second [20].

In the CPMG [21,22] NMR sequence (90^0 - t - 180^0 - echo - delay) for spin-spin relaxation measurement, for a fluid contained in a single pore size, the echo following the 180^0 rotation of the magnetization vector is given by

$$M(t) = M_o\exp(-t/T_2) \quad (2)$$

where M_o is the magnetization of the nuclei at equilibrium and M(t) is the observed magnetization at a variable delay time t, between the 90^o and 180^o measurement pulses. For muscle the observed nuclear magnetization (NMR signal) depends on the T_2 (i.e., pore size) of all cells' water. As shown in Equation (1), the NMR relaxation time is proportional to cell size, and it is known that muscles have distributions of cell sizes. This implies that the NMR transverse relaxation (T_2) data can be expressed as a sum of exponential function

$$M(t_i) = \sum_{j=1}^{m} f(T_{2,j})exp(-ti/T_{2,j}) \quad (3)$$

Where $f(T_{2,j})$ is proportional to the number of spins, which relax with a time constant, $T_{2,j}$. M(ti) is the NMR magnetization decay from protons (water) within muscle in different sizes of cells with the longer relaxation time corresponding to larger cell size similar as porous bone [17, 20]. Equation (3) can be inverted into a T_2 relaxation time distribution. Thus, instead of estimating a single relaxation time from a magnetization decay, it is necessary to estimate a spectrum or distribution of relaxation times $M(T_{2i})$. Since T_2 depends linearly on pore size, the T_2 distribution corresponds to pore-size distribution, with the longer relaxation times being from larger cell size [20].

NMR Experimental Studies

An BRUKER built NMR system are set up at a proton frequency of 20 MHz for these measurements. ^{1}H spin-spin (T_2) relaxation profiles can be obtained by using NMR CPMG $\{90^0 [- \tau - 180^0 - \tau (echo)]_n - TR\}$ spin echo method with a 6.2 µs wide RF-90^o pulse, τof 500 - 1000 µs, and TR (sequences repetition rate) of 15 s. The obtained data from NMR measurement, after inversion T_2 relaxation process are combined and compared with results obtained from the microscope image measurements.

Microscope image

Transverse sections (10µm) were taken from in the middle of the soleus muscle by a cryostat microtome at – 20 degree C. Myofiber of soleus muscle was visualized by Mayer's Hematoxylin& Eosin counter staining (Humanson, 1972). Then image was captured by cooled CCD camera (Carl Zeiss, Thronwood, NY) connected to NIKON microscope using 20 X, N.A. 0.16 (NIKON OPTIPHOT) and analyzed cross-sectional area using image J.

Muscle density determination

The volume of the muscle is determined by Archimedes' principle (water displaced method). The calibrated muscle is $V_B = (W_{air} - W_{water})/d_{water}$, where V_B is the volume of the muscle, W_{air} is the weight of muscle in the air, W_{wate} is the weight of muscle in the water, d_{water} is the density of water. Therefore, the density of muscle can be deter-mined by W_{air}/V_B.

RESULTS AND DISCUSSION

Our result shows our test example of the CPMG inversion T2 spin-spin relaxation spectra from HS (▲) and HSA (•). The shorter relaxation time (▲) corresponds to HS muscle (Figure 3).

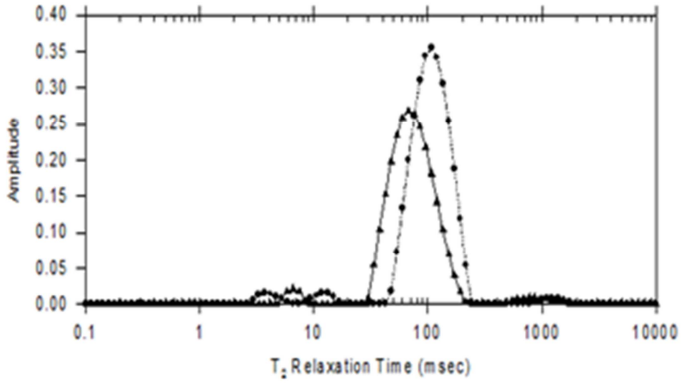

Figure 3. CPMG inversion T2 spin-spin relaxation spectra between HS (▲) and HSA (•). The shorter relaxation time (▲) corresponds to muscle degradation

The average T_2 relaxation times changes among control (CON), hind limb suspension (HS) and hind limb suspension with electroacupuncture treatment (HAS) groups were shown in Figure 4. The average of weight of Soleus mass changes are shown in Figure 5 and the example of muscle cell sizes changes is shown in Figure 6. The average muscle densities among CON, HA, and HAS samples are shown in Table1.

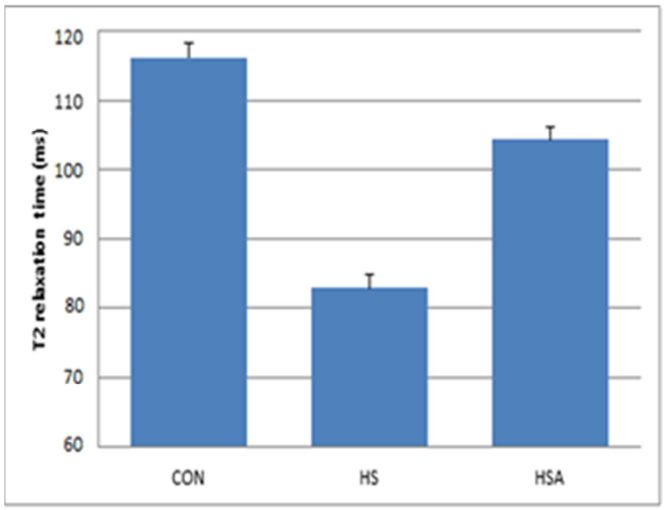

Figure 4. The average T2 relaxation times among CON, HS, and HSA groups obtained from NMR CPMG measurement

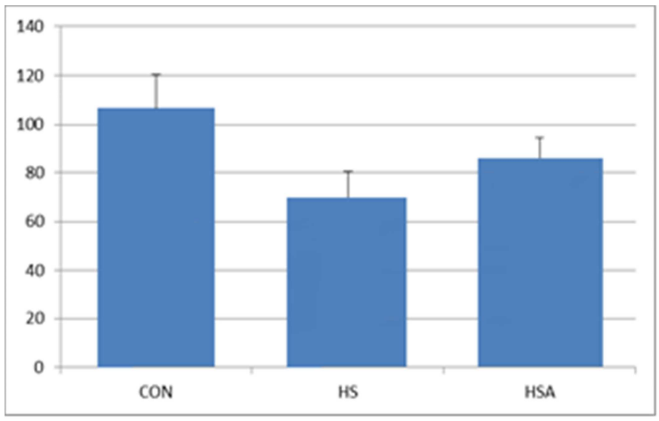

Figure 5. The average weight of Soleus mass changes among CON, HS, and HSA groups

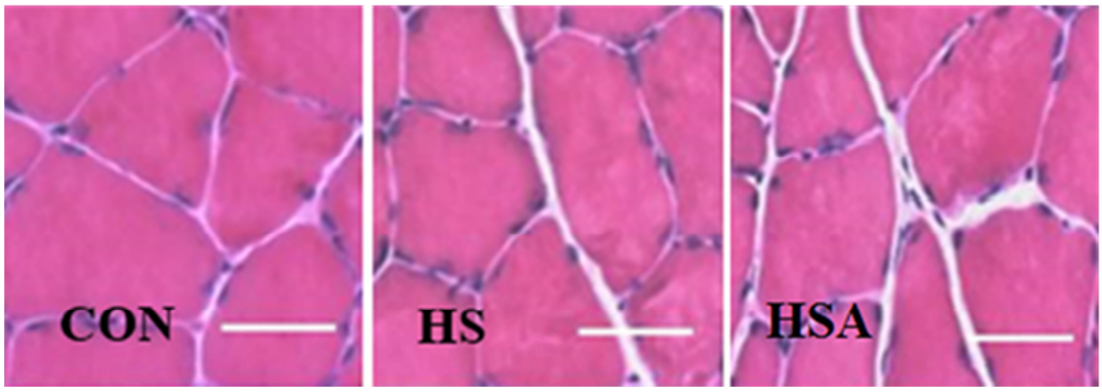

Figure 6. The example of microscope image differences (cell size) among CON, HS and HSA muscle tissues

Table 1. The average muscle densities among CON, HA, and HAS samples

sample		Mass (g)	Volume	Density
con 1		0.2719	0.2601	1.0454
con 2		0.3241	0.3156	1.0269
con 3		0.2508	0.2463	1.0183
con 4		0.3486	0.3392	1.0277
con 5		0.3885	0.3992	1.0132
con 6		0.3274	0.3205	1.0215
con 7		0.332	0.3254	1.0203
Ave				1.0248 StDev.±0.0104
HS1		0.305	0.291	1.0481
HS2		0.2943	0.2579	1.1411
HS3		0.3313	0.3089	1.0725
HS4		0.2605	0.2196	1.1862
HS5		0.2731	0.2416	1.1304
HS6		0.2948	0.2864	1.0293
HS7		0.2935	0.2865	1.0244
HS8		0.2366	0.2312	1.0223
HS9		0.2681	0.2627	1.0206
Ave				1.0751 StDev.±0.0622
HSA1		0.2816	0.268	1.0507
HSA2		0.2452	0.2398	1.0225
HSA3		0.3344	0.3275	1.0211
HSA4		0.3909	0.3882	1.007
HSA5		0.3094	0.3036	1.0191
HSA7		0.2919	0.2891	1.01
HSA8		0.2718	0.2358	1.1527
HSA9		0.3696	0.3502	1.0554
Ave				1.0423 StDev.±0.0480

The significantly difference between HS and HSA is clearly observed. The soleus muscle mass is significantly higher (11%), the skeletal muscle cell sizes are 15-20% larger, and the T_2 relaxation time is longer (20%) on HSA than on HS, respectively. Since the muscle size is decreased after HS, however, it is observed that the average muscle density is slightly increased. As shown in Table 1 the average muscle densities are HSA 1.71% higher than CON; and HS 4.91% higher than CON. It is clearly to find that the electroacupunture has the function to improve or prevent muscle degradation during the disuse.

CONCLUSION

The results from our electroacupuncture tested on the dis-use hind limb suspension (HS) rat model are positive. It is suggested this technique can be effective in attenuating muscle atrophy induced by HS rat model. In addition, the success test is quite significant, especially, this non-pharmacological intervention can be applied on human. Since NMR measurement is non-destructive and non-invasive, and it is also suggested to use MRI technique to find the recovery function by continuing to apply electroacupuncture after HAS test on rat model.

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