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Strengthening of C2C12 mouse myoblasts against compression damage by mild cyclic compressive stimulation

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ABSTRACT

Deep tissue injury (DTI) is a severe kind of pressure ulcers formed by sustained deformation of muscle tissues over bony prominences. As a major clinical issue, DTI affects people with physical disabilities, and is obviously related to the load-bearing capacity of muscle cells in various in-vivo conditions. It is important to provide a preventive approach to help muscle cells from being damaged by compressive stress. In this study, we hypothesized that cyclic compressive stimulation could strengthen muscle cells against compressive damage and enhance the cell plasma membrane resealing capability. Monolayer of myoblasts was cultured in the cell culture dish covered by a cylinder 0.5% agarose gel. The platen indenter was applied with 20% strain on the agarose gel in the Mach-1 micromechanical system. The vibration was 1 Hz sinusoidal function with amplitude 0.2% strain based on 20% gel strain. Cyclic compressive stimulation for 2 h could enhance the compressive stress damage threshold of muscle cells, the muscle cell plasma membrane resealing ratio and viability of muscle cell under static loading as preventive approach. This approach might help to reduce the risk of DTI in clinic.

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1. Introduction

Pressure ulcer is severe tissue damage under prolonged mechanical loading. People with spinal cord injury and immobile elderly people confined to seats or beds are vulnerable to suffer from pressure ulcers (Bader et al., 2005; Bogie et al., 1995; Byrne and Salzberg, 1996). Generally, superficial ulcers and deep tissue injury (DTI) (Bader et al., 2005; Mak et al., 2011) are two forms of pressure ulcers. DTI is difficult to be detected and to be curbed in the early stage resulting in extensive ulceration developed fast in the deep tissue under sustained loading (Bliss, 1993). Damages due to excessive internal mechanical stress could be developed in the muscle tissues around skeletal prominences (Oomens et al., 2010; Solis et al., 2012). The loading duration that muscle cells can tolerate depends on the stress level (Yao et al., 2015). Mechanical insults beyond certain thresholds can directly cause necrosis of muscle cells leading to DTI (Bouten et al., 2001; Breuls et al., 2003; Gawlitta et al., 2007; Linder-Ganz and Gefen, 2004). Cell plasma membranes could be disrupted and permeabilized by the physical insults and could repair themselves within their resealing capability (Jagannathan and Tucker-Kellogg, 2015; Kilinc et al., 2008; McNeil and Steinhardt, 2003; Reddy et al., 2001; Duan et al., 2015). The resealing capability of cell plasma membrane could reflect the ability of the cell against the physical insults.

It is important to prevent DTI. Vibration therapy apparently could accelerate the healing of pressure ulcers in older adult patients (Arashi et al., 2010). Vibration could also inhibit the development of DTI (Sari et al., 2015). On the cellular level, cyclic mechanical stimulation could induce cells to produce mechano-growth factor (MGF) and insulin growth factor-1 (IGF-1), elevating cell proliferation and inhibiting oxidative stress induced apoptosis (Dugan et al., 2014; Li et al., 2009, 2012). It was also found that nitric oxide could regulate myoblast proliferation induced by cyclic stretch (Soltow et al., 2010). Cyclic mechanical stimulation in-vitro could change actin expression and affect the actin cytoskeletal network (Wang et al., 2010; Xie, et al., 2012). Integrin expression and activation of FAK and RhoA could also be effected (Zhang et al., 2007). Cyclic strain with similar mean strain but different cyclic amplitudes resulted in significant differences in cell proliferation and apoptosis (Colombo et al., 2013). The elasticity of the myoblasts could be regulated by cyclic mechanical stimulation (Takemoto et al., 2012). Cell viability, number and differentiation could also be regulated by vibration loading (Tirkkonen et al., 2011). Cyclic mechanical stimulation could regulate cellular mechanobiology. If properly applied, such cyclic stimulation could enhance the capability of cells against compressive damage.

We hypothesized that cyclic compressive stimulation could strengthen muscle cells against compressive damage and enhance

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the cell plasma membrane resealing capability. This hypothesis is relevant to the design of preventive measures against DTI.

2. Materials and methods

2.1. Cell culture

We cultured C2C12 mouse skeletal myoblasts (ATCC, Manassas, USA) in 35mm cell culture dishes (SPL Lifesciences, Korea) with a growth medium comprising high-glucose Dulbecco's Modified Eagle Medium (DMEM, Gibco, Life Technologies, USA), 10% fetal bovine serum (FBS, Gibco, Life Techologies, USA) and 1% Penicillin-Streptomycin (Gibco, Life Technologies, USA). For the cell passage process, the myoblasts were trypsinized by 0.25% Trypsin-EDTA (1 ×, Gibco, Life Technologies) from passage 16–19 every other day to maintain the continuous passage. Myoblasts were incubated in a 37°C, 5% CO_2 humidified incubator for two days. The monolayer of the myoblasts was about 90% confluent prepared for all the experiments.

2.2. Static compression

Agarose gels were prepared by dissolving 0.5% (w/v) agarose (Biosharp, Hefei, China) powder in Dulbecco's phosphate-buffered saline (PBS, 1 ×, Gibco, Life Technologies) at 100 °C. Liquid agarose solution was transferred to a 35 mm culture dish using a pipette (Fisher Scientific) and then naturally cooled in room condition to form a 3 mm thick gel layer. A cylindrical gel of thickness 3 mm and diameter 14.8 mm was cut out using a biopsy punch and placed on a monolayer of myoblasts.

The unconfined compression applied on the cylindrical gel using the Mach-1™ micromechanical system (Biomomentum Inc) with a flat platen of diameter 25.4 mm provided a uniform compressive stress on the central part of the myoblasts monolayer. The entire experiment was conducted at 37 °C and 5% CO_2 inside an incubator. A 20%-strain of the agarose gel generated about 125 Pa static compressive stress on the monolayer of myoblasts under the agarose gel. This loading methodology was adapted from an indentation protocol reported earlier (Yao et al., 2015).

2.3. Cyclic compressive stimulation

A cyclic ±0.2% gel compressive strain at 1 Hz was superposed on the 20% static compression (Fig. 1). A cyclic compressive stress of 125 ± 75 Pa at 1 Hz was generated on the myoblasts monolayer under the gel (Fig. 2). Such cyclic compressive stimulation was applied on the cells for 2 h as treatment.

Three groups of compressive stimulation were studied: (I) static loading (125 Pa) for 3 h; (II) cyclic compressive stimulation (125 ± 75 Pa) for 5 h; and (III) cyclic compressive stimulation (125 ± 75 Pa) for 2 h followed by static loading (125 Pa) for 3 h. Three samples were studied in each group. After the compressive loading, propidium iodide (PI) (Sigma, USA) at 10 μg/ml was applied to mark the dead cells by staining the nuclei of the damaged cells with red fluorescence under green light excitation. Fluorescence microscopy capturing the red PI signal from the damaged cells was performed in an inverted microscope (ECLIPSE Ti, Nikon). Images were taken at 40 × magnification with a CCD camera (DS-Fi1c-L3, Nikon Digital Sight). Nuclei stained by PI were identified and counted using ImageJ 1.46r (NIH) through gray value threshold setting. The total number of myoblasts in each cell culture dish was counted after fixing the cells with 4% paraformaldehyde followed by 0.1% Triton X-100 and PI staining at the end of each experiment. The percentage of cell death was obtained as the ratio of PI-stained myoblasts to the total myoblasts in each dish. One-way analysis of variance (ANOVA) with Tukey's post hoc analysis at 0.05 significance level was used to test the significance of the variances in the percentage of cell death among the three groups to study the effect of cyclic compressive stimulation on cell damage.

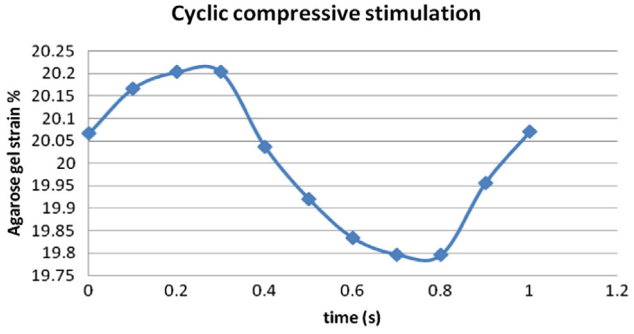

Fig. 1. Cyclic compressive stimulation with 0.2% stain amplitude on the baseline of 20% strain at 1 Hz.

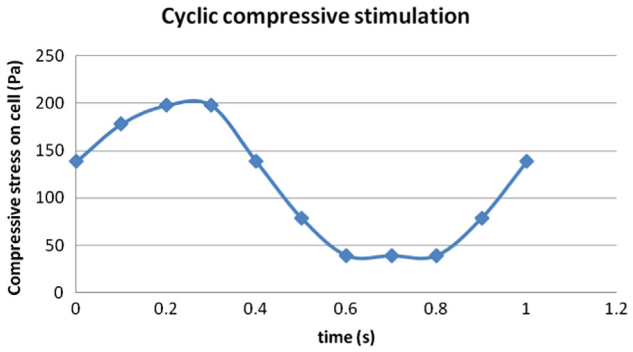

Fig. 2. The cyclic compressive stress acting on the myoblasts monolayer under the gel as caused by the applied cyclic strain.

2.4. Compressive stress damage threshold

The method to determine the compressive stress damage threshold described in Yao et al. (2015) was used in this study. After the myoblasts were treated with cyclic compressive stimulation for 2 h, agarose gel was removed and the myoblasts were rinsed with PBS for 3 times. A new 3 mm-thick agarose gel was fabricated with the method described above covering the whole myoblasts monolayer immerged in 1 ml culture media was loaded by a half-spherical indenter of 6.38 mm diameter in the Mach-1™ at 37 °C with 5% CO_2 at the rate of 0.01 mm/sec until the deformation at the center of gel reached 50%. Myoblasts with and without cyclic compressive stimulation were indented for 180 min. The compressive stress profile applied on the monolayer of myoblasts was estimated using ABAQUS 6.12 (Simulia, The Netherlands). The spatial profile of cell damage percentage was assessed by PI staining after indentation. The damage threshold of myoblasts was determined by correlating the cell damage percentage profile and the compressive stress profile acting on the myoblasts monolayer. With the criteria that the percentage cell damage within the damaged threshold circle was at least 3 times that in the peripheral region, the relation between the compressive damage threshold and indentation duration was determined. Independent *t*-test at 0.05 level of significance was used to determine the effect of cyclic compressive stimulation on the compressive stress damage threshold of cells.

2.5. Cell membrane resealing ratio

Cell wounding and repair were assessed by Fluorescein isothiocyanate (FITC)-dextran and PI (Belete et al., 2009; Caples et al., 2009; Doerr et al., 2005; Gajic et al., 2003; Leopold and Gefen, 2013; Orellana-Lezcano et al., 2005). When the cells are immersed in the FITC-dextran media, the FITC-dextran can enter a cell as long as its cell membrane is wounded. If the cell membrane reseals, the FITC-dextran would be trapped in the cell and show green fluorescence with blue excitation light. Thus FITC-dextran stained green cells are wounded but then healed. With PI introduced after the external insults, the cells wounded but not healed would be stained by PI showing red fluorescence with green excitation light. The cells not stained by FITC-dextran nor by PI are cells that remain intact during the external insults. With the above sequential using FITC-dextran and PI, cells could be separated into three categories: (I) wounded and healed (green); (II) wounded and not healed (red); (III) not wounded (no fluorescence). The cell membrane resealing ability could be quantified by the ratio of the number of healed cells (FITC-dextran) to the number of wounded cells (FITC-dextran + PI).

$$\begin{aligned}&\text{membrane resealing ratio}\\&=\frac{\text{resealed cell}}{\text{wounded cell}}\\&=\frac{\text{resealed cell}}{\text{nonresealed cell}+\text{resealed cell}}\\&=\frac{\text{FITC}-\text{Dextran stained cell}}{\text{PI stained cell}+\text{FITC}-\text{Dextran stained cell}}\end{aligned}\tag{1}$$

A uniform static pressure of 300 Pa was applied on the agarose gel of thickness 3 mm and diameter 14.8 mm with a monolayer of myoblasts underneath. This static loading was applied for 30, 70, 140, 160, 180, and 300 min ($N=3$ each). Culture medium with 0.1 mM FITC-dextran (molecular weight: 3000) was introduced to the culture dish before loading (Geddes et al., 2003; Leopold and Gefen, 2013). The Stokes' radius of FITC-dextran 3000 is 1.3 nm and the Stokes' radius of PI is 0.8 nm. Myoblasts with and without cyclic compressive stimulation were loaded in the presence of FITC-dextran. After loading, the media were removed and the cell culture dish was rinsed with PBS for 5 times to remove the residual FITC-dextran around the cells and in the dead cells. The green fluorescence signals were captured under the fluorescence microscope. PI was then introduced in the culture and red florescence signals were captured. The total number cells was counted as described earlier.

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