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ORIGINAL ARTICLE

Genistein attenuates genioglossus muscle fatigue under chronic intermittent hypoxia by down-regulation of oxidative stress level and up-regulation of antioxidant enzyme activity through ERK1/2 signaling pathway

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OBJECTIVE: This study aims to investigate the effects of genistein on contractile properties of genioglossus under chronic intermittent hypoxia (CIH) conditions and its relationship with oxidative stress, antioxidant enzyme, and ERK1/2 signaling pathway.

MATERIALS AND METHODS: Fifty female Sprague–Dawley rats were randomly divided into five groups 1 week after ovariectomy: the normal control group, the CIH group, the CIH with low-dose, medium-dose, and high-dose genistein groups. Rats in the latter four groups were exposed to CIH for 5 weeks. Twitch tension, tetanic tension, and fatigue resistance of genioglossus were investigated. Malondialdehyde (MDA) and mitochondrial reactive oxygen species (ROS), enzymatic activity of superoxide dismutase (SOD), glutathione peroxidase (GPx), catalase (CAT), and ERK1/2 were detected.

RESULTS: Muscle fatigue resistance and enzymatic activity of GPx, CAT, and SOD were reduced after CIH exposure and improved by different doses of genistein at different degrees. CIH increased the level of ROS and MDA, and they were returned to normal by genistein. The expression of phospho-ERK1/2 is opposite to the changes in muscle fatigue resistance.

CONCLUSION: Chronic intermittent hypoxia decreases fatigue resistance of genioglossus, and genistein treatment reverses the fatigability of genioglossus by down-regulation of oxidative stress level and up-regulation of antioxidant enzymatic activity probably through ERK1/2 signaling pathway.

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Keywords: chronic intermittent hypoxia; genioglossus muscle fatigue; genistein treatment; oxidative stress; antioxidant enzyme; ERK1/2 signaling pathway

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Introduction

Obstructive sleep apnea/hypopnea syndrome (OSAHS) is a clinical syndrome characterized as recurring episodes of upper airway (UA) obstruction leading to intermittent hypoxemia, hypercapnia, and disruption of sleep (McGinley *et al*, 2008). OSAHS takes a large toll on the physical, mental, social, and economic health of patients. It puts the patients at greatly increased risk for the development of pulmonary and systemic hypertension and cardiovascular morbidity with excessive daytime sleepiness, impaired cognitive performance, and behavioral changes (Cartwright, 2001; Jia and Liu, 2010).

Anatomic structure abnormality of UA and UA dilator muscles' dysfunction play an important role in the pathogenesis of OSAHS (Mezzanotte *et al*, 1996). Among the UA dilator muscles, genioglossus is one of the most important muscles in maintaining UA patency. OSAHS is found to affect 1.2% of women and 3.9% of men with an overall ratio of sleep apnea for men to women of 2:1–4:1 (Bixler *et al*, 2001). Sleep apnea is uncommon in premenopausal women but increases its prevalence after menopausal. These data combined indicate that menopausal is a significant risk factor for sleep apnea in women and that estrogen replacement treatment appears to be associated with reduced risk. We previously detected that estrogen improves genioglossus muscle dysfunction by the down-regulation of hypoxia-inducible factor-1 (HIF-1), which is responsible for sensing oxygen variation (Jia and Liu, 2010). However, long-term use of estrogen may increase the risk of many diseases such as invasive breast cancer, cardiovascular disease, stroke, and thromboembolic events (Rossouw *et al*, 2007).

Genistein is a kind of isoflavone phytoestrogen, which has many biological activities, such as antioxidant and anticancer, without side effect for long-term use (Yeh *et al*, 2007; Xu *et al*, 2009). It is promising that genistein could serve as drug therapy for OSAHS, as there is no satisfied treatment at present (Schmidt-Nowara *et al*,

1995; Sher, 2002; Kushida *et al*, 2006). OSAHS is featured as chronic intermittent hypoxia (CIH), associated with oxidative stress (Prabhakar *et al*, 2007). It is reported that oxidative stress marker was increased and antioxidant capacity was reduced in the blood of persons with OSAHS (Cofta *et al*, 2008). CPAP (continuous positive airway pressure) treatment could attenuate the oxidative stress level of OSAHS. Exogenous oxidant impairs UA muscle endurance in an animal model of sleep-disordered breathing (Dunleavy *et al*, 2008). These data combined indicate that oxidative stress is associated with the UA muscle dysfunction in patients with OSAHS. However, there is little study focusing on the change of oxidative stress status in genioglossus under CIH condition.

Skeletal muscle continually generates reactive oxygen species (ROS, including superoxide anion, hydrogen peroxide, and hydroxyl radical) at a slow rate that increases during muscle contraction. This activity-dependent increase in ROS production contributes to muscle fatigue and injury through depletion of intracellular ATP, alterations in calcium homeostasis, and lipid and protein oxidation (Reid, 2008). Therefore, we hypothesize that the increased level of oxidative stress is associated with the genioglossus muscle dysfunction under CIH condition. There are several enzymatic defending systems in organism serving as ROS scavenger such as superoxide dismutase (SOD), glutathione peroxidase (GPx), and catalase (CAT) (Valko *et al*, 2007). ERK1/2 MAPK signaling pathways regulate a variety of cellular activities including oxidative stress, proliferation, differentiation, survival, and death. It is determined that ERK1/2 MAPK signaling pathway is involved in the antioxidant activity of genistein (Borrás *et al*, 2006). Therefore, the purpose of this study is to investigate whether genistein has an influence on genioglossus muscle contractility properties under CIH conditions and its possible relationship with oxidative stress, antioxidant enzyme, and ERK1/2 signaling pathway.

Materials and methods

Materials

Sprague–Dawley (SD) rats were obtained from the Experimental Animal Center of Second Military Medical University (Shanghai, China). Pentobarbitone sodium was from Sigma-Aldrich Chemical Co. (St Louis, MO, USA). The DCFH-DA fluorescent probe was from Molecular Probes (Eugene, Oregon, USA). Genistein was from Tauto Biotech Co. (Shanghai, China). The malondialdehyde (MDA), SOD, GPx, and CAT detecting kit were from Beyotime Institute of Biotechnology (Jiangsu, China). The antibody of phospho-ERK1/2 and ERK1/2 were from Cell Signaling Technology (Danvers, MA, USA). The horseradish peroxidase (HRP)-conjugated secondary antibody (goat antirabbit IgG, 1:10 000 dilution) was from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The ECL (enhanced chemiluminescence) detecting system was from Beyotime Institute of Biotechnology.

Chronic intermittent hypoxia and sham exposure

The CIH rat model was established and refined according to our previous study to mimic the state of sleep apnea (Jia and Liu, 2010). Animals were housed in identical self-made plexiglas chambers. They were allowed free mobility, water and food provided *ad libitum*. The O_2 concentration in each chamber was continuously monitored, and the degree and timing of hypoxia were manipulated by computer-driven servo-controlled solenoids. The CIH exposure was divided into two phase: the hypoxia phase and reoxygenation phase, with each phase for 1 min. During the hypoxia phase, N_2 was delivered to the chambers to reduce the ambient fraction of inspired oxygen to 6–8% rapidly, and the nadir O_2 was lasted for 15–20 s per cycle. During the reoxygenation phase, O_2 concentration was turned up to 21% at maximum by rapid flushing with room air. The CIH cycle was repeated daily for 8 daylight hours for 5 weeks. Following the exposure, animals were returned to their usual cages in the animal housing facility with a 12–12 h light–dark cycle. Control animals were handled similarly as the CIH animals, except exposure to a continuous flow of room air. Data, as described later, were collected at the end of the 5 weeks, in the next morning after the last exposure, to minimize acute effects of intermittent hypoxia.

Blood gas tensions were monitored over the course of these experiments. Three additional animals were anesthetized with 3% pentobarbitone sodium, the right femoral artery was cannulated, and arterial blood samples were collected at the following time points: baseline (before intermittent hypoxia), during the nadir O_2 in the 3rd, 6th, and 9th hypoxia phase, and during the 3rd, 6th, and 9th reoxygenation phase. Results of blood gases were as follows: (1) at baseline: pH 7.25 ± 0.13, PO_2 95 ± 6 mmHg, and PCO_2 45 ± 4 mmHg; (2) during the hypoxia phase: pH 7.14 ± 0.08, PO_2 49 ± 6 mmHg, PCO_2 49 ± 6 mmHg; and (3) during the reoxygenation phase: pH 7.29 ± 0.11, PO_2 88 ± 9 mmHg, and PCO_2 42 ± 3 mmHg. The results indicate that our protocol led to moderate to severe hypoxia, in the range seen in patients with moderate to severe OSAHS (Alford *et al*, 1986).

Animal division and genistein administration

Fifty 8-week-old female SD rats (206–236 g) were allowed free mobility, and water and phytoestrogen-free diet with corn oil replacing soybean oil were provided *ad libitum*. The rats were ovariectomized under 3% pentobarbitone sodium anesthesia. They were randomly divided into five groups 1 week later: the normal control group (NC), the CIH group, the CIH with low-dose Gen group (LG), the CIH with medium-dose Gen group (MG), and the CIH with high-dose Gen group (HG). Daily intragastric administration was performed with three different dosage of genistein base on our preliminary experiment. The genistein were dissolved in 2.5 ml of dimethyl sulfoxide (DMSO) in the groups of LG (90 mg kg^{-1}), MG

Table 1 The changes of body weight and contractile properties in genioglossus muscles of five groups

Groups	*Body weight Pretreatment (g)*	*Body weight Posttreatment (g)*	*Twitch tension Newton cm^{-2}*	*Tetanic tension Newton cm^{-2}*
NC	219.00 ± 12.77	308.25 ± 5.85	1.06 ± 0.07	3.45 ± 0.05
CIH	219.20 ± 13.50	309.80 ± 6.91	1.06 ± 0.08	3.45 ± 0.04
LG	222.20 ± 11.60	306.80 ± 9.73	1.12 ± 0.05	3.47 ± 0.11
MG	224.40 ± 10.33	307.00 ± 5.92	1.11 ± 0.03	3.52 ± 0.05
HG	222.20 ± 14.00	290.60 ± 7.89[a]	1.13 ± 0.05	3.53 ± 0.07

CIH, chronic intermittent hypoxia; HG, high-dose Gen group; LG, low-dose Gen group; MG, medium-dose Gen group; NC, normal control group.
[a]Compared with CIH group, $P < 0.05$.

(180 mg kg^{-1}), and LG (270 mg kg^{-1}), and DMSO was used as placebo in the NC and CIH group. Changes in body weight during the research duration were taken into account in calculating the genistein dosage. The body weight in HG group was slightly lower than that in another four groups posttreatment (Table 1). All procedures used in this study were approved by University of Tongji Animal Care Committee, which serves to ensure that all international guidelines concerning animal experimentation are met.

Contractile properties

After 5 weeks exposure, the rats were anaesthetized with 3% pentobarbitone sodium. With rats under general anesthesia and supine, the animals were tracheostomized and placed on mechanical ventilation. The right femoral artery and vein were cannulated with the aid of a microscope to record arterial BP and to administer supplementary anesthetic, respectively. Core temperature was maintained at 37°C using a thermostatically controlled heating blanket and radiant heat. A ventral midline incision was made in the neck, and the left genioglossus was quickly removed for homogenate preparation and mitochondrial isolation. The right genioglossus was isolated for the contractile experiment according to our previous study (Jia and Liu, 2010). The tendon of the genioglossus was cut at the mandible, and the other end was left intact. The dissociated tendon was attached to an isometric force transducer that was mounted to a vertical micropositioner. The position of the force transducer could be adjusted by the micropositioner, thus altering the length of the muscle strips. The optimal length (Lo) (i.e., the length that produced maximal isometric twitch tension) was determined, and the muscle was held at this length for the remainder of the experiment. The isometric twitch tension (Pt) and the tetanic tension (Po) were measured in response to electrical field stimulation with platinum electrodes hooked onto the medial hypoglossal nerve that supplies motor output to the genioglossus. A single twitch was elicited by stimulating the hypoglossal nerve at 1.86 V, 1 Hz and 0.5 ms duration. The tetanic tension was induced by stimulation at 1.86 V and 50 Hz, which approximated the fusion frequency, and the stimulation lasted for 2 min. Specific tension was calculated in newton per square centimeter of strip cross-sectional area. Cross-sectional area was approximated by weighing the muscle strip after removal from the bath and blotting dry and dividing this by the product of the optimal length and muscle density, assumed to be 1.06 mg mm^{-1}. The fatigue resistance was calculated as the ratios between the fatigue tension measured at different time points and the maximal tension. The force transducer output was amplified and recorded on a computer via analog-to-digital conversion system (MedLab-U; MedEase Science and Technology Co., Nanjing, China). Data analysis was performed with the use of MedLab software (V6.0; MedEase Science and Technology Co.). During this experiment, the muscles were kept moist and at a constant temperature (37 ± 0.5°C) by constantly dripping warmed Krebs-Ringer solution onto it. After the contractile experiment, the muscles were removed and stored in liquid nitrogen for western blot analysis.

Mitochondria isolation and detection of ROS generation

The removed genioglossus was placed in a buffer containing 0.25 M sucrose, 1.0 mM EDTA, 5.0 mM HEPES, 0.2% fatty acid-free albumin, and 13 units ml^{-1} of collagenase (pH 7.4) at a weight-to-volume ratio of 1:10. Tissues were minced thoroughly and homogenized with a motor-driven Potter-Elveljem glass homogenizer at 0–4°C at low speed. The homogenate was filtered through four layers of medical gauze to remove connective tissue debris. A portion of the homogenate was used to determine lipid peroxidation and enzyme activity assays. The remaining homogenate was used to isolate mitochondria according to the methods described by Ji *et al* (1988). The final pellets containing mitochondria were suspended in 0.25 M sucrose (pH 7.4). Preparation of muscle mitochondria took 50 min after its removal.

Generation of ROS was evaluated in isolated mitochondria using 2′, 7′-Dichloroflu orescein-diacetate (DCFH-DA) as a probe, according to the previous study (Lebel and Bondy, 1990; Kim *et al*, 1996). Briefly, isolated mitochondria were incubated in the buffer containing (in mM) 130 KCl, 5 $MgCl_2$, 20 NaH_2PO_4, 20 Tris–HCl, and 30 glucose (pH 7.4), two malate, and two pyruvate at 37°C for 15 min to allow DCFH-DA to cross mitochondrial membrane. The solution was then centrifuged at 12 000 *g* for 8 min, and the supernatant containing excess DCFH-DCFH-DA not crossing the mitochondrial membrane was discarded. The mitochondrial pellets were resuspended, and 50 μl of the suspension (2 mg protein) was used for assay. DCF formation was followed at the excitation wavelength of 488 nm and emission wavelength of 525 nm for 30 min by using F-2000 fluorescence spectrometer (Hitachi, Tokyo, Japan). The rate of DCFH conversion to DCF was linear for at least 60 min, corrected with the auto-oxidation rate of DCFH without protein. All assays were carried out in duplicates. DCF production was proportional to the amount of protein added in a wide range. The units

were picomoles DCF formed per minute per milligram protein.

Lipid peroxidation assay

Peroxidative damage to cellular lipid constituents was determined by measuring MDA according to the previous study (Ohkawa *et al*, 1979). MDA concentration was measured in tissue homogenates after precipitation of protein with trichloroacetic acid. Thiobarbituric acid (TBA) reacts with MDA to form TBA reactive product, which was measured at 532 nm spectrophotometrically. An MDA solution freshly made by the hydrolysis of 1,1,3,3-tetramethoxy propane was used as standard. The results were expressed as nmol of MDA per mg protein.

Enzyme activity assays

Superoxide dismutase activity in genioglossus homogenates was assayed as previously described (Nishikimi *et al*, 1972; Kakkar *et al*, 1984). Proteins (5 μg) were mixed with sodium pyrophosphate buffer, phenazine methosulphate, and nitro blue tetrazolium. The reaction was started by the addition of NADH. The reaction mixture was then incubated for 90 s at 30°C and stopped by the addition of 1 ml of glacial acetic acid. The absorbance of the chromogen formed was measured at 560 nm. One unit of SOD activity was defined as the enzyme concentration required to inhibit chromogen production by 50% in 1 min under the assay conditions. CAT activity was measured in homogenates by the method of Bonaventura *et al* (1972). Proteins (5 μg) from the homogenate were mixed with 2 ml of 7.5 mM H_2O_2 and a time scan was performed for 10 min at 240 nm at 25°C. One unit of CAT activity was defined as the amount of enzyme decomposing 1 μmol of H_2O_2 per minute. GPx activity was assayed using H_2O_2 and NADPH as substrate as described previously (Pagalia and Valentine, 1967).

Western blot analysis

To prepare total tissue homogenates, genioglossus was solubilized with ice-cold Tris lysis buffer (PH 7.6) containing protease inhibitor and PMSF (phenylmethanesulfonyl fluoride). Equal protein amounts (50 μg) of genioglossus homogenates were electrophoresed through 12% sodium dodecyl sulfate-polyacrylamide gel and electroblotted onto polyvinylidene fluoride membranes (Millipore, Billerica, MA, USA). The membranes were blocked by incubation in PBS containing 5% non-fat milk and 0.05% Tween 20 at room temperature for 1 h and incubated overnight at 4°C with primary antibody of phospho-ERK1/2 after 15 min × 3 times wash by PBS with 0.05% Tween 20, and the detection was made with HRP-conjugated secondary antibodies and ECL System by Kodak IMAGE STATION 2000MM (Kodak, Rochester, NY, USA). Thereafter, the membranes were stripped by the strip solution, incubated with the antibody of ERK1/2, and detected subsequently by ECL System. The data from the Western blot analysis were expressed as ratio of phospho-ERK1/2 to ERK1/2.

Data analysis

All statistical analysis was performed with SPSS software packages (SPSS for windows XP, version 13.0; SPSS, Chicago, IL, USA). The mean and the standard deviation were determined for all dependent variables. ANOVA and the least significant difference *post hoc* test were used to identify differences between control and experimental groups. The statistical significance was determined at 0.05 levels of confidence.

Results

Contractile properties

No significant differences were found in the magnitude of twitch tension and tetanic tension of the five groups (Table 1). However, significant differences were detected in the fatigue resistance among them. The time course of decline in tetanic tension during continuous electrical stimulation at 50 Hz over the time of 100 s is shown in Figure 1. The relative decline of force was calculated as a percentage of the maximal tetanic tension. After CIH exposure, the tetanic tension was significantly decreased compared with NC group at each time point ($P < 0.05$). The relative decline of force was 89.00 ± 3.33% in muscles from the NC group and 50.02% ± 2.13% in muscles from the CIH group over the first 20 s ($P < 0.05$). Compared with the NC group (63.8 ± 2.08 s), the time duration for 50% decline of tetanic tension was reduced in the CIH group (19.9 ± 3.51 s) ($P < 0.05$), and it was increased by the different doses of genistein at different degrees with no significance between LG (29.1 ± 3.42 s) and MG (36.7 ± 4.90 s) groups ($P > 0.05$). However, the time duration for 50% decline of tetanic tension in HG (53.5 ± 2.57 s) group was still less than that in NC group ($P < 0.05$).

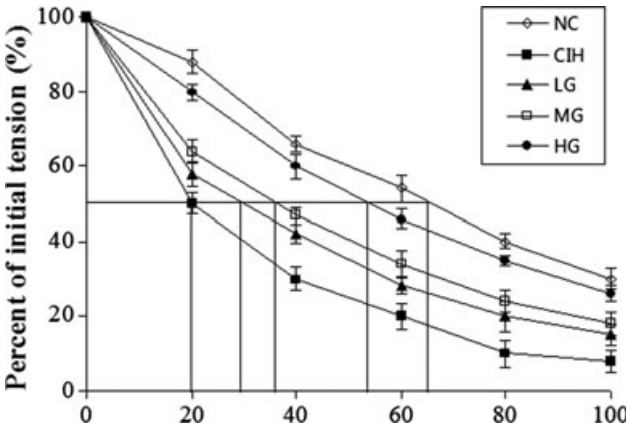

Figure 1 The fatigue resistance in the genioglossus of five groups. There was a significant decrease in the fatigue resistance in chronic intermittent hypoxia (CIH) group when compared with normal control (NC) group ($P < 0.05$). Different doses of genistein treatment significantly increased the fatigue resistance in different degree with no significant difference between low-dose Gen and medium-dose Gen groups ($P > 0.05$). The half time period of tetanic tension (i.e., the time taken to reach 50% of the initial force) was significantly decreased in CIH group when compared with NC group, while it was increased by genistein treatment

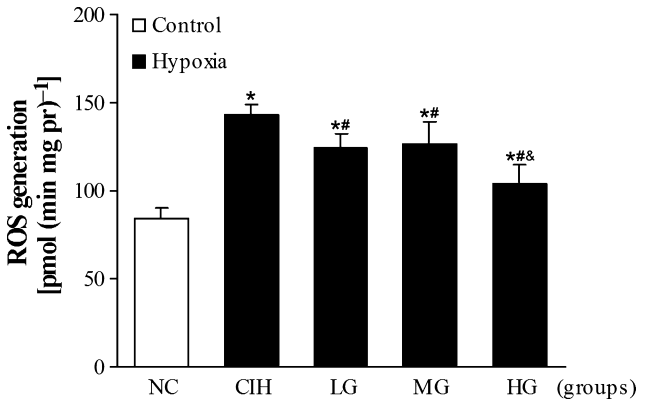

Figure 2 The level of mitochondrial reactive oxygen species (ROS) generation in genioglossus of five groups. The mitochondrial ROS generation was detected by DCFH-DA fluorescence probe. ROS generation was significantly increased after 5 weeks chronic intermittent hypoxia exposure (Figure 2) ($P < 0.05$). Different doses of genistein treatment significantly decreased the level of ROS ($P < 0.05$) in mitochondria at different degree with no significant difference between low-dose Gen and medium-dose Gen groups ($P > 0.05$). *compare to normal control (NC) group, $P < 0.05$; #compare to NC group, $P < 0.05$; & compare to NC group, $P < 0.05$

The level of oxidative stress in genioglossus

The mitochondrial ROS generation was detected by DCFH-DA fluorescence probe. ROS generation was significantly increased after 5 weeks CIH exposure (Figure 2) ($P < 0.05$). Different doses of genistein treatment significantly decreased the level of ROS ($P < 0.05$) in mitochondria at different degree with no significant difference between LG and MG groups ($P > 0.05$). Similarly, MDA was increased after 5 weeks CIH exposure (Figure 3) ($P < 0.05$). Different doses of genistein treatment significantly decreased the level of MDA at different degree ($P < 0.05$), and it was returned to normal in HG group.

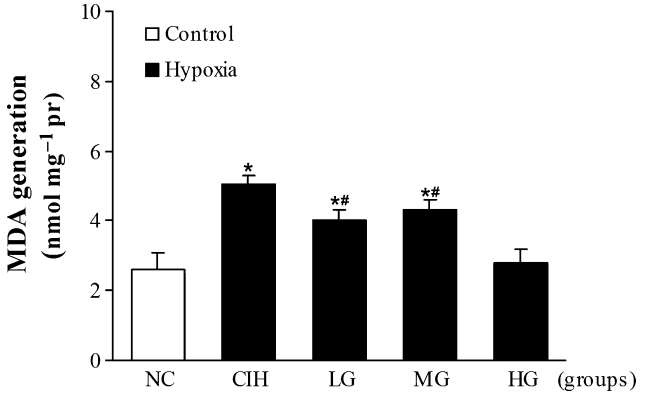

Figure 3 The level of malondialdehyde (MDA) generation in genioglossus of five groups. MDA was increased after 5 weeks chronic intermittent hypoxia exposure ($P < 0.05$). Different doses of genistein treatment significantly decreased the level of MDA at different degree ($P < 0.05$), and it was returned to normal in high-dose Gen group. *compared with normal control (NC) group, $P < 0.05$; #compared with NC group, $P < 0.05$

Table 2 Enzymatic activity of SOD, GPx, and, CAT of in genioglossus muscles of five groups

Groups	*SOD (U mg.pr^{-1})*	*GPx (U mg.pr^{-1})*	*CAT (U mg.pr^{-1})*
NC	23.02 ± 2.56[a]	13.04 ± 2.32[a]	30.53 ± 3.92[a]
CIH	16.43 ± 1.76	8.43 ± 1.22	21.63 ± 3.07
LG	17.71 ± 2.25	11.81 ± 1.70[a]	26.86 ± 1.57[a]
MG	17.96 ± 2.44	11.69 ± 1.84[a]	27.49 ± 1.76[a]
HG	22.34 ± 2.72[a]	12.16 ± 1.26[a]	28.31 ± 2.26[a]

CAT, catalase; CIH, chronic intermittent hypoxia; GPx, glutathione peroxidase; SOD, superoxide dismutase; HG, high-dose Gen group; LG, low-dose Gen group; MG, medium-dose Gen group; NC, normal control group.
[a]Compared with CIH group, $P < 0.05$.

The level of antioxidant enzymatic activity

The enzymatic activity of SOD, GPx, and CAT was significantly decreased after 5 weeks CIH exposure (Table 2) ($P < 0.05$). Different doses of genistein treatment increased the activity of GPx and CAT to normal level. No significant difference was found in the activity of SOD in CIH, LG, and MG groups ($P > 0.05$). However, high dose of genistein enhanced the activity of SOD to normal level.

The expression of phospho-ERK1/2 in protein levels

The expression of phospho-ERK1/2 was significantly decreased after 5 weeks CIH exposure in protein level (Figure 4) ($P < 0.05$). Different doses of genistein treatment significantly enhanced the expression of

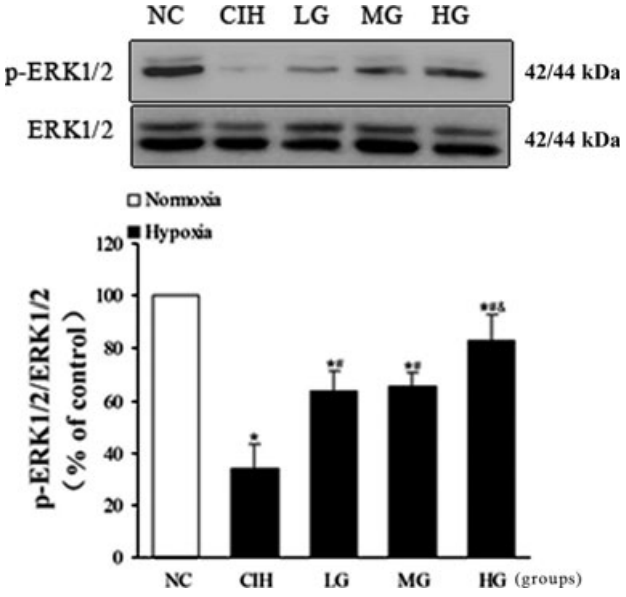

Figure 4 The protein expression of phospho-ERK1/2 relative to ERK1/2 in five groups. The expression of phospho-ERK1/2 was significantly decreased after 5 weeks chronic intermittent hypoxia exposure in protein level ($P < 0.05$). Different doses of genistein treatment significantly enhanced the expression of phospho-ERK1/2 ($P < 0.05$) with no significant difference between low-dose Gen and medium-dose Gen groups ($P > 0.05$). *compared with normal control (NC) group, $P < 0.05$; #compared with NC group, $P < 0.05$; & compare to NC group, $P < 0.05$

phospho-ERK1/2 ($P < 0.05$) with no significant difference between LG and MG groups ($P > 0.05$).

Discussion

The development of UA obstruction has been linked to an increase in the collapsibility of UA. The patency of UA is dependent on the equilibrium of the negative pharyngeal pressure caused by lung inflation and dilating force generated from UA dilator muscle. During wakefulness, an anatomically compromised UA may increase the genioglossus muscle activity to stabilize UA patency (Mezzanotte *et al*, 1992). However, this increase in muscle activity results in elevated fatigability and reduced muscle endurance (Blumen *et al*, 2004). Therefore, it increases the likelihood of UA collapse, especially during frequent and prolonged apnea, leading to a vicious cycle of further airway obstruction and muscle dysfunction. OSAHS is characterized as CIH; thus a CIH animal model was set up to investigate the pathogenesis of OSAHS. It was found in this study that CIH had no effect on genioglossus contractile properties instead of an increase in the muscle fatigue, which is coincident with the previous study (McGuire and Bradford, 1999). They confirmed that the muscle fatigue is not because of the fiber-type transition, because CIH had no effect on fiber-type distribution. To explore how muscle fatigue was caused by CIH, we detected the effect of CIH on HIF-1 expression previously (Jia and Liu, 2010). HIF-1 is a key player in the cellular response to changes in oxygen tension and regulates a variety of genes associated with cellular processes, including metabolism, angiogenesis, cell survival, and oxygen delivery. However, ROS appears to play an important role in both the hypoxic and non-hypoxic signaling processes, which control the activity of HIF (Kietzmann and Görlach, 2005). Therefore, a question was raised about the relationship between ROS and muscle fatigue under CIH condition.

Evidence is emerging that the pathological changes in patients with OSAHS is associated with increased level of oxidative stress. It is reported that oxidative stress marker, such as lipid peroxidation products, was increased in the blood of persons to a variable degree, depending on the severity of the syndrome (Cofta *et al*, 2008). Ling *et al* found that left ventricular dysfunction is related to increased oxidative stress and decreased antioxidant capacity (Chen *et al*, 2005). Therefore, we suppose that the oxidative stress in genioglossus would be increased by CIH exposure. There is little study focusing on the oxidative status in genioglossus at present. We found that CIH significantly increased the ROS generation and lipid peroxidation and decreased the activity of the antioxidant enzymes including SOD, GPx, and CAT in genioglossus. The result indicated that CIH damaged the redox homeostasis by inhibiting antioxidant defending system. Furthermore, the effect of exogenous oxidant on UA muscle has been explored by Dunleavy *et al* (2008). They found that intermittent hypoxia was associated with a decrease in sternohyoid muscle endurance, an effect that was exacerbated by treatment with buthionine sulfoxamine (BSO, an inhibitor of the rate-limiting enzyme in glutathione synthesis). Therefore, it could be concluded that the increased level of oxidative stress accounts for the UA muscle fatigue under CIH condition.

It is reported that muscle fatigue is regulated by ROS by modulating a variety of cellular processes including sarcolemmal function, calcium regulation, myofilament interaction, and mitochondrial metabolism (Reid, 2008). Andrade *et al* (1998) used murine flexor digitorum longus to test the effects of hydrogen peroxide on contractile function. They found 300 μM hydrogen peroxide caused a marked decrease in tetanic force without altering peak calcium concentrations, which could be reversed by a reducing agent [1 mM dithiothreitol (DTT)]. The data indicated that hydrogen peroxide induces muscle fatigue by decreasing calcium sensitivity of the myofilaments. Furthermore, they found exposure to hydrogen peroxide for prolonged periods or at higher concentrations caused peak tetanic calcium levels to decline. The rate of calcium reuptake into the SR (sarcoplasmic reticulum) was also diminished and resting calcium levels in the cytosol were increased. The loss of calcium regulation was generally not reversible by DTT. These findings suggest that excessive ROS may cause irreversible muscle fatigue and injury. The reason might be that excessive ROS are cytotoxic, causing lipid peroxidation, DNA damage and mutagenesis, depletion of intracellular ATP, alterations in calcium homeostasis, protein oxidation and apoptosis, and tissue necrosis (Chen *et al*, 2005).

Furthermore, we tested whether genistein treatment could reverse the decrement of genioglossus fatigue resistance caused by CIH. We found that different doses of genistein could reduce the genioglossus muscle fatigue in different degree. This was coincident with the previous study showing that daily treatment with the antioxidant N-acetyl cysteine blocked the deleterious effects of intermittent hypoxia on UA muscle function (Dunleavy *et al*, 2008). Skelly *et al* (2010) found that superoxide scavenger tempol-incubated muscles generated significantly higher forces compared with control muscles and showed improved performance in the early phase of the fatigue trial under hypoxic conditions. We also found high dose of genistein recover the activity of SOD, GPx, and CAT to normal level and reduced the level of oxidative stress. The result was consistent with the previous study (Lee, 2006). Lee detected that genistein supplements altered the SOD and CAT activities and reduced oxidative stress in the diabetic rats, resulting in a lower lipid peroxidation concentration. Genistein has been determined to be more effective than that of the antioxidant vitamins and estradiol in scavenging of ROS and lipid peroxidation (Exner *et al*, 2001; Lee *et al*, 2001; Sierens *et al*, 2001). Furthermore, we found low and moderate genistein have no effect on SOD, which indicates that CAT and GPx are more sensitive to genistein treatment. This might account for the stronger protection of high-dose genistein on muscle fatigue than low and moderate dose. These findings combined further verified the

important role of ROS in UA muscle fatigue. Moreover, excessive dose of genistein induces tissue injury by pro-apoptosis and inhibition of tyrosine protein kinase and DNA topoisomerase II activites. Therefore, further study is required to explore the safety dose of genistein for clinical application.

Phytoestrogen is considered to exert their effects by binding to estrogen receptors (ERs). There are mainly three members of phytoestrogen including: isoflavones, lignans, and coumestrans. Coumestrol (a kind of coumestrans) has a higher binding affinity for ERs than genistein (a kind of isoflavone) (Morito *et al*, 2001). Therefore, we suppose that coumestrol has a higher activity to improve genioglossus muscle fatigue. However, we previously found opposite result that genistein is more effective to improve fatigue resistance. This indicates that other signaling pathways may be involved in the protective effect of genistein on genioglossus. ERK1/2 signaling pathway regulates a variety of cellular activities including oxidative stress, proliferation, differentiation, survival, and death. It is determined that ERK1/2 signaling pathway is involved in the antioxidant activity of genistein (Borrás *et al*, 2006). We found that CIH remarkably decreased the level of ERK1/2 in genioglossus. However, different doses of genistein increased the level of ERK1/2 in different degree. The changes of ERK1/2 were parallel to that of ROS in genioglossus. Therefore, we conclude that genistein probably exert its protective effect on genioglossus muscle fatigue through ERK1/2 signaling pathway. However, further study is required to determine how ERK1/2 was involved in muscle fatigue.

In conclusion, CIH could decrease the fatigue resistance of genioglossus without effect on other contractile properties. Genistein treatment attenuates muscle fatigue of genioglossus by reduction of oxidative stress and up-regulation of antioxidant enzymatic activity probably through ERK1/2 signaling pathway.

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Author contributions

WH Ding: animal experiment, data analysis, article drafting.
YH Liu: experiment design, article revising.

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