Int J Clin Exp Pathol 2015;8(10):12491-12499
www.ijcep.com /ISSN:1936-2625/IJCEP0015152

Original Article

Oxidative stress participates in quadriceps muscle dysfunction during the initiation of osteoarthritis in rats

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Received August 27, 2015; Accepted September 28, 2015; Epub October 1, 2015; Published October 15, 2015

Abstract: Osteoarthritis is the most common form of arthritis, affecting approximately 15% of the population. Quadriceps muscle weakness is one of the risk factors of osteoarthritis development. Oxidative stress has been reported to play an important role in the pathogenesis of various muscle dysfunction; however, whether it is involved in osteoarthritis-associated quadriceps muscle weakness has never been investigated. The aim of the present study is to examine the involvement of oxidative stress in quadriceps muscle dysfunction in the initiation of osteoarthritis in rats. Rat osteoarthritis was initiated by conducting meniscectomy (MNX). Quadriceps muscle dysfunction was evaluated by assessing muscular interleukin-6, citrate synthase activity, and myosin heavy chain IIa mRNA expression levels. Muscular oxidative stress was assessed by determining lipid peroxidation, Nrf2 expression, reactive oxygen species, and circulating antioxidants. Increased muscular interleukin-6 production as well as decreased citrate synthase activity and myosin heavy chain IIa mRNA expression were observed at 7 and 14 days after MNX. Biomarkers of oxidative stress were significantly increased after MNX. Muscular free radical counts were increased while glutathione and glutathione peroxidase expression were decreased in MNX-treated rats. We conclude that oxidative stress may be involved in the pathogenesis of muscle dysfunction in MNX-induced osteoarthritis.

Keywords: Muscle dysfunciton, osteoarthritis, oxidative stress, sesame oil, rats

Introduction

Osteoarthritis (OA) is the most common form of arthritis, affecting approximately 15% of the population. Due to its predilection for lower extremity joints such as the knee and hip, OA is the leading cause of lower extremity disability amongst older adults with an estimated lifetime risk for knee OA being approximately 40% in men and 47% in women. Therefore, osteoarthritis is now considered as a major public health problem worldwide [1].

Knee meniscectomy (MNX) is the most common procedure performed by orthopedic surgeons as a treatment of medial meniscal tears. Patients who have undergone MNX surgery show a marked muscle weakness of the ipsilateral limb [2-6]. Although muscle weakness has been considered as a secondary effect in knee OA historically, recent studies suggest that quadriceps muscle weakness may precede the onset of radiographic evidence of OA [7]. Moreover, quadriceps muscle weakness may be directly involved in the pathogenesis and development of OA [8]. The initiation of OA in the guinea pig is associated with the changes in the quadriceps skeletal muscle [9]. Improving quadriceps weakness may be a strategy for preventing OA development. However, mechanism involved in OA-associated quadriceps muscle weakness is still unclear.

Skeletal muscles are composed of striated subunits called sarcomeres, which are composed of the myofilaments actin and myosin. Myosin ATPase is localized to the globular head of the myosin heavy chain (MHC) [10]. MHC alteration plays a dominant role in muscle strength [11-15]. For example, decreased muscular MHC IIa mRNA and protein expressions are associated with muscle weakness in OA patients [16]. Cytokines and citrate synthase (CS) are involved in the loss of muscle mass and strength. Previous studies have shown a correlation between high levels of pro-inflammatory cytokine interleukin (IL)-6 and low muscle mass and strength [17, 18]. CS activity has been used as

an indicator of oxidative potential in skeletal muscle [19-21], and reported to be associated with fatigue resistance [22, 23].

Oxidative stress is imposed on cells as a result of one of two factors: an increase in oxidant generation and a decrease in antioxidant protection. If oxidant attacks continuously, oxidation of lipid constituents (lipid peroxidation; LPO) of membranes ensues, which impairs the function of cell organelles and eventually culminates in ultrastructural injury [24]. In addition, nuclear factor erythroid 2-related factor 2 (Nrf2) is a transcriptional activator that plays a critical role in cellular response to oxidative stress. Nrf2 is suppressed under a basal condition through Keap1-dependent degradation [25]. Oxidative stress initiates Nrf2 transcription of anti-oxidative genes and their proteins through inhibiting Keap1 [26]. Therefore, both LPO product and Nrf2 expression have been used as an important marker of oxidative stress [27].

Reactive oxygen species (ROS), such as superoxide anion, hydroxyl radical, and peroxynitrite, are produced in animals and humans under pathophysiologic conditions [28-30]. Superoxide anion can act with nitric oxide to generate peroxynitrite and hydroxyl radical, both of them are the important mediators of LPO [31-33]. On the other hand, glutathione peroxidase (GPx), localized in the cytosol and the inner membrane of mitochondria of animal cells, is a crucial enzyme in the biosynthesis of glutathione (GSH). As a potent free radicals scavenger, GSH prevents interactions of reactive intermediates with critical cellular constituents, such as phospholipids of biomembranes, nucleic acids, and proteins [34-37].

Although oxidative stress is believed to be responsible for many tissue changes, whether it involves in the pathogenesis of quadriceps muscle dysfunction during OA development has never been investigated. The aim of the present study was to investigate the involvement of oxidative stress in the pathogenesis of quadriceps muscle dysfunction in MNX-model of OA in rats.

Materials and methods

Animals

Male SPF Sprague Dawley rats weighing 200-300 g were obtained from our institution's Laboratory Animal Center. They were individually housed in a room with a 12-hour dark/light cycle and central air conditioning (25°C, 70% humidity), allowed free access to tap water, and fed a rodent diet from Richmond Standard, PMI Feeds, Inc (St Louis, MO), without a sesame oil supplement. The animal care and experimental protocols were in accordance with nationally approved guidelines.

Experimental designs

Experiment I: Time course study of MNX-induced osteoarthritis-like joint pain in rats. Rats were divided into two groups of five. Group I (Sham group), rats were received sham operation only; and Group II (OA group) rats received MNX operation only. Weight distribution of the ipsilateral hind paw was assessed 0, 7, and 14 days after sham of MNX operation.

Experiment II: The role of muscular oxidative stress in muscle dysfunction in rats with MNX. Rats were divided into three groups of five. All rats received MNX operation. Quadriceps muscle samples were collected at 0, 7, and 14 days after MNX from Group I, II, and III, respectively.

MNX surgery

MNX surgery was performed under 3.5% isofluorane inhalational anesthetics. Rats received cephalexin (Ceporex oral drops) (0.03 ml/100 g body weight) 1 h before and 12, 24, and 36 h after surgery. A small incision was made longitudinally down the medial side of the knee and a cauterizer was used to work through both the connective tissue and muscle layers until the medial collateral ligament, anchoring the medial meniscus to the tibial plateau, was identified. The ligament was grasped at the tibial end and cut until fully transected. The ligament was then transected again at the femoral end to remove the portion overlying the meniscus. The meniscus was freed from the fine connective tissue, allowing a full thickness, medial meniscal transection. Sham animals underwent the same surgical procedure with the omission of medial meniscal transection [2].

Measuring muscular protein concentration

The protein concentration in tissue homogenate was determined by using protein assay dye (Bio-Rad Laboratories, Hercules, CA, USA).

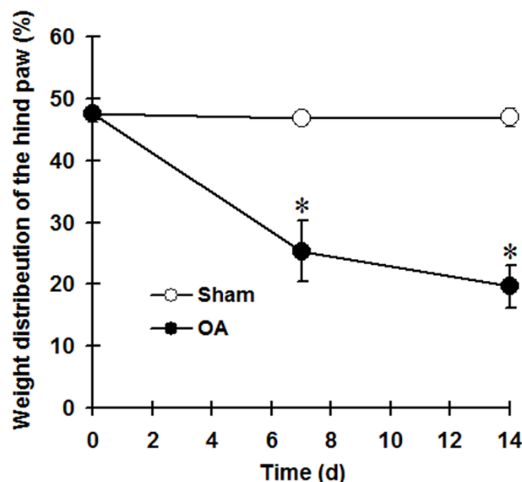

Figure 1. Time course of OA induction in rats. Rats were divided into two groups of five. Group I (Sham group), rats were received sham operation only; and Group II (OA group) rats received MNX operation only. Weight distribution of the ipsilateral hind paw was assessed 0, 7, and 14 days after sham of MNX operation. Data are means ± SD. *$P < 0.05$ compared with Sham group (Group I).

Measuring muscular IL-6 levels

IL-6 levels were quantitatively measured by using ELISA kits (Duo-Set; R&D Systems Inc., Minneapolis, MN). Briefly, sample was incubated with biotinylated rabbit antibody for 2 h, and then streptavidine-conjugated horseradish peroxidase was added for 20 min. The peroxidase reaction was initiated by adding 3,3',5,5'-tetramethylbenzidine/H_2O_2 (R&D Systems) for 30 min, and then stopped by adding 0.5 M H_2SO_4. The absorbance was measured at 450 nm [38].

Measuring muscular CS activity

Tissues were homogenized in 0.1 M Tris buffer containing 0.1% Triton X-100, pH 8.35. Briefly, the homogenates were frozen and thawed four times to disrupt the mitochondria. Added 4 l of homogenate to 200 l assay buffer (100 mM Tris buffer, 5 mM 5,5-dithiobis (2-nitrobenzoate), 22.5 mM acetyl-CoA, and 25 mM oxaloacetate, pH 8.35). The rate change in color was monitored at wavelength of 405 nm at 15-s intervals for a period of 3 min [39].

Measuring muscular MHC IIa mRNA expression

The total RNA was extracted from the muscular tissue using TRIZOL reagent (Invitrogen) in accordance with the manufacturer's instructions. Quantitative real-time polymerase chain reaction (RT-PCR) was performed by using an SYBR Green and Applied Biosystems Step One Real- PCR system (Life Technologies, CA). After reverse transcription, Quantitative PCR was performed 40 cycles under the following conditions: for 40 cycles, initial treatment at 95°C for 10 min, denaturing at 95°C for 15 s, and annealing at 60°C for 1 min without extension. Primer pairs were designed against MHC IIa (forward 5'-AAGATCAAATCATCAGTGCC-3'; reverse 5'-TCTAGCAGATATGTCTCGATG-3') and GAPDH (forward 5'-AACATCATCCCTGCCTCTACTG-3'; reverse 5'-CTCCGACGCCTGCTTCAC-3'). The amounts of MHC IIa mRNA expression were normalized with GAPDH mRNA value.

Measuring muscular LPO levels

Muscle homogenate (200 μl) was taken for LPO measurement by using a commercial assay kit (Lipid Peroxidase Assay Kit; Calbiochem-Novabiochem Co, Darmstadt, Germany) followed the manufacturer's instruction, and the spectrophotometer was read at 586 nm.

Western blotting

Nuclear extraction kit (Sigma, Inc., St. Louis, MO) was used to separate nuclear and cytosolic protein. Fifty micrograms of protein was loaded on SDS-PAGE, and then transferred to nitrocellulose sheets (NEN Life Science Products, Inc., Boston, MA). After blocking, the blots were incubated with Nrf2, GPx, or -actin antibody (dilution 1:1000) in 5% non-fat skim milk (using -actin as a loading control). After washed, the blots were incubated with secondary antibodies conjugated with alkaline phosphatase (dilution 1:3000) (Jackson ImmunoResearch Laboratories, Inc., Philadelphia, PA). Immunoblots were developed using bromochloroindolyl phosphate/nitroblue tetrazolium solution (Kirkegaard and Perry Laboratories, Inc., Baltimore, MD) [40].

Determining muscular hydroxyl radical, superoxide, and peroxynitrite levels

Briefly, tissue was homogenized in Tris-sucrose buffer (0.24 M sucrose in 20 mM Tris HCl buffer containing 1 mM EDTA (pH 7.4) (1:10; wt/vol). The homogenate was centrifuged at 400g at 4°C for 30 min. Superoxide, peroxynitrite, and hydroxyl radical were measured using a high-

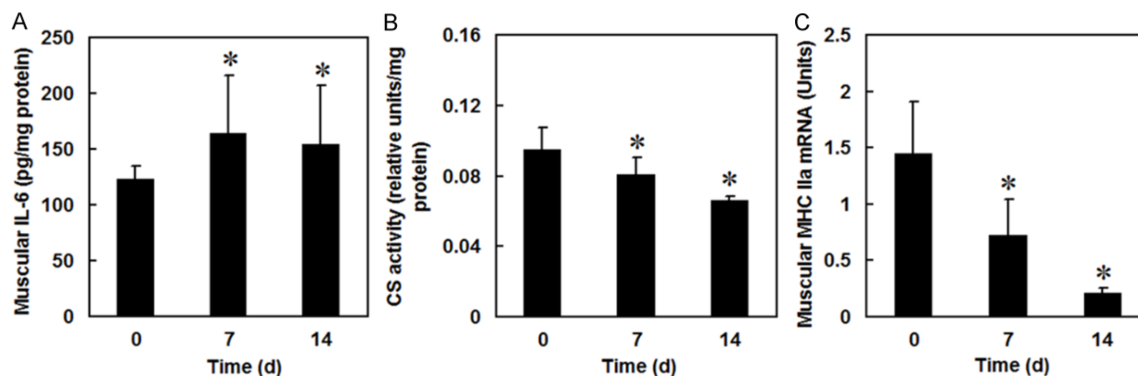

Figure 2. Quadriceps muscle dysfunction in MNX-induced OA. Rats were divided into three groups of five. Rat quadriceps muscle was collected 0 (Group I), 7 (Group II), and 14 (Group III) days after MNX, respectively. Muscular IL-6 production (A), CS activity (B), and MHC IIa mRNA expression (C) levels were determined at different time points. Data are means ± SD. $^{*}P < 0.05$ compared with day 0 group (Group I).

performance chemiluminescence (CL) analyzer (CLA-2100; Tohoku Electronic Industrial Co, Ltd, Rifu, Japan). Briefly, 400 μl of tissue homogenate were mixed with 200 μl of phosphate buffer solution in a stainless dish, and then the background CL count was read for 60 s. One hundred microliters of lucigenin, indoxyl [beta]-D-glucuronide, or luminol (17 mM dissolved in phosphate buffer solution, to determine superoxide anion or hydroxyl radical, respectively) was injected into the machine, and the CL is counted for another 1200 s at 10-s intervals. The data are analyzed using Chemiluminescence Analyzer Data Acquisition Software (Tohoku Electronic Industrial Co) [30].

Measuring muscular GSH levels

Muscle tissues were homogenized in ice-cold trichloroacetic acid (0.1 g of tissue plus 1 ml of 10% trichloroacetic acid). Briefly, after the homogenates had been centrifuged at 3,000 rpm for 10 min, 500 μl of supernatant was added to 2 ml of 0.3 M Na_2HPO_4 solution. A 200 μl solution of dithiobisnitrobenzoate (in 1% sodium citrate, 0.4 mg/mL) was added, and the absorbance at 412 nm was measured immediately [30].

Hindlimb weight distribution assessment

Hindlimb weight distribution was measured on an incapacitance meter (IITC, Inc., Woodland Hills, CA, USA), a behavioral analysis assay that measures weight bearing on the hindlimbs while the animal was in an induced rearing posture. In brief, an incapacitance meter consists of two scales and specialized caging to encourage a rearing posture. Weight on the left and right hindlimbs was acquired during 5-second intervals (five trials per rat). These data were converted into weight distribution by dividing the weight on the right limb by the total weight for both hindlimbs. Weight-distribution imbalance was determined at each time point by using a repeated-measures test, with balanced weight distribution represented by a right limb percentage weight near 50% [41].

Statistical analysis

Data were expressed as the means ± standard deviation (SD). One-way analysis of variance (ANOVA) followed by student's *t*-test analysis was used to make pairwise comparisons between the groups. Statistical significance was set at P < 0.05.

Results

Time course of joint pain in MNX-induced OA

To establish the rat model of MNX-induced OA, weight distribution of the hind paw was assessed after MNX surgery. The weight distribution was significantly decreased at 7 and 14 days after MNX in OA group compared with that in Sham groups (**Figure 1**).

Muscle dysfunction changes in MNX-induced OA

To examine the onset of muscle dysfunctionin MNX-induced OA, muscular IL-6 production, CS

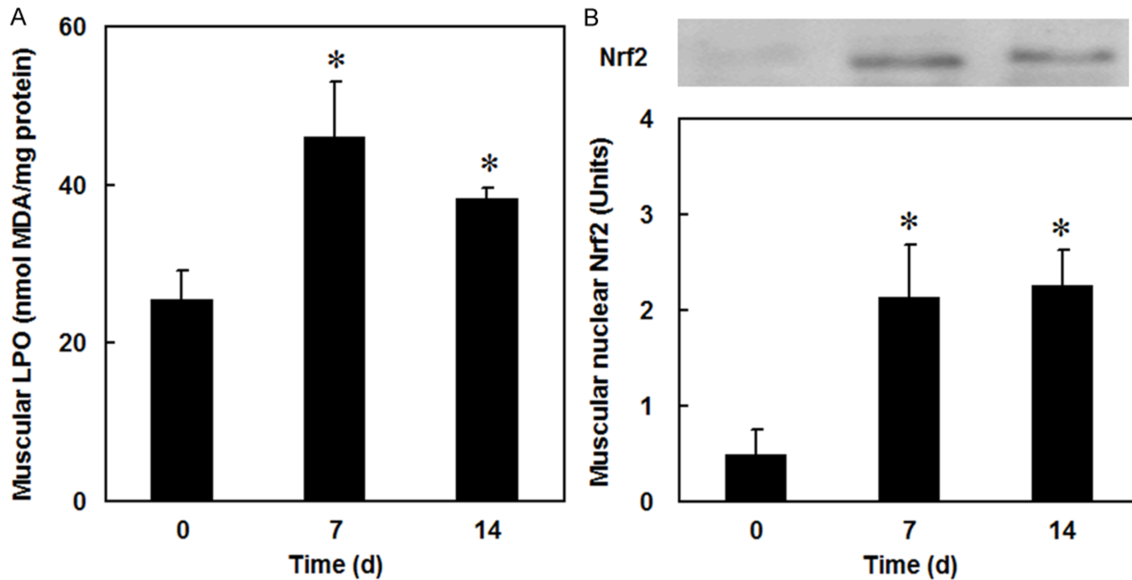

Figure 3. Muscular oxidative stress in MNX-induced OA. Rats were divided into three groups of five. Rat quadriceps muscle was collected 0 (Group I), 7 (Group II), and 14 (Group III) days after MNX, respectively. Muscular LPO level (A) and nuclear Nrf2 expression (B) were determined at different time points. Data are means ± SD. **P* < 0.05 compared with day 0 group (Group I).

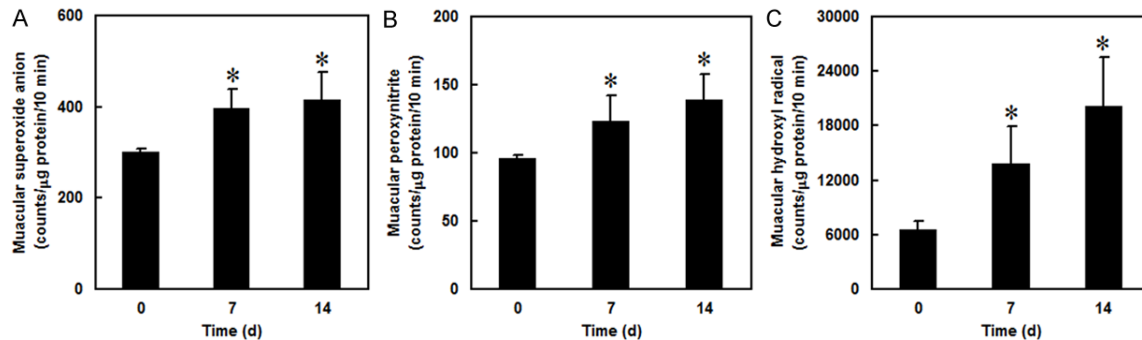

Figure 4. Muscular reactive oxygen species generation in MNX-induced OA. Rats were divided into three groups of five. Rat quadriceps muscle was collected 0 (Group I), 7 (Group II), and 14 (Group III) days after MNX, respectively. Muscular superoxide anion (A), peroxynitrite (B), and hydroxyl radical (C) generations were determined at different time points. Data are means ± SD. **P* < 0.05 compared with day 0 group (Group I).

activity, and MHC IIa mRNA levels were determined. Muscular IL-6 production (**Figure 2A**) was significantly increased while CS activities (**Figure 2B**) and MHC IIa mRNA levels (**Figure 2C**) were significantly decreased at 7 and 14 days after MNX surgery compared with them in control groups.

Role of muscular oxidative stress in OA-associated muscle dysfunction

To examine the role of oxidative stress in MNX-associated muscle weakness, muscular LPO and nuclear Nrf2 expression levels were determined after MNX surgery. Muscular LPO (**Figure 3A**) and nuclear Nrf2 expression (**Figure 3B**) were significantly increased at 7 and 14 days after MNX surgery compared with them in control groups.

Involvement of ROS generation and circulating antioxidant production in OA-associated muscular oxidative stress

To examine the mechanism involved in OA-associated muscle dysfunction, ROS includ-

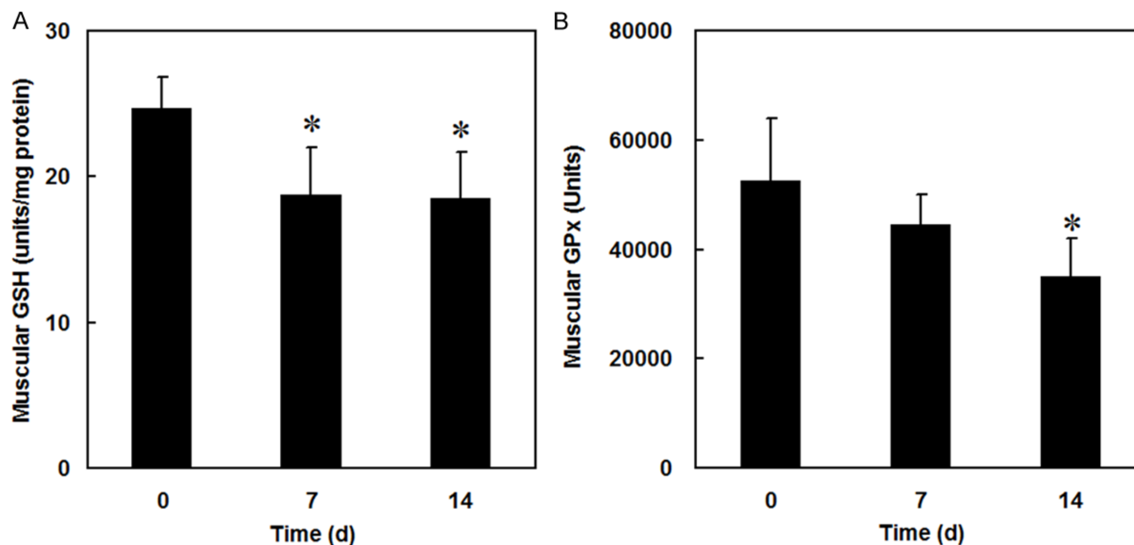

Figure 5. Changes in muscular circulating antioxidant levels in MNX-induced OA. Rats were divided into three groups of five. Rat quadriceps muscle was collected 0 (Group I), 7 (Group II), and 14 (Group III) days after MNX, respectively. Muscular GSH (A) and GPx (B) levels were determined at different time points. Data are means ± SD. $^{*}P < 0.05$ compared with day 0 group (Group I).

ing superoxide anion, hydroxyl radical, and peroxynitrite, as well as circulating antioxidant GSH and GPx were determined after MNX surgery. Muscular superoxide anion (**Figure 4B**), hydroxyl radical (**Figure 4B**), and peroxynitrite (**Figure 4C**) counts were significantly increased at 7 and 14 days after MNX surgery compared with them in control groups. In addition, both GSH level (**Figure 5A**) and GPx expression (**Figure 5B**) were significantly lowered at 7 and 14 days after MNX compared with control group.

Discussion

We have for the first time demonstrated that muscular oxidative stress is involved in muscle dysfunction in the development of OA. In the present study, increased cytokines as well as decreased CS activity and MHC IIa mRNA were found at 7 and 14 days after MNX. Muscular oxidative stress markers and reactive oxygen species production were significantly increased while circulating antioxidants were decreased after MNX. We suggested that muscular oxidative stress is involved in the quadriceps muscle dysfunction during the initiation and development of OA.

Muscle dysfunction can be detected in the early stage of MNX-induced OA. Previous studies suggest that muscle weakness may be a result of disuse after MNX [14]; however, recent evidences show that muscle weakness may participate in the pathogenesis and development of OA [8]. In the present study, increased IL-6 and as well as decreased CS activity and MHC IIa mRNA expression were found from 7 days after MNX, which indicated that muscle weakness could be found in the early stage of MNX-induced OA. Therefore, we suggested that muscle dysfunction may play an important role in the development of OA after MNX.

Inhibiting MHC IIa gene expression may be associated with muscular weakness after MNX. MHC typing changes have been suggested as one of the major causes of muscle weakness after MNX [14]. Decreased MHC IIa fiber is associated with lower muscle strength/muscle weakness in the quadriceps in patients with OA [42]. Beta2-adrenergic agonist which induces muscle hypertrophy of the quadriceps skeletal muscle without affecting MHC IIa expression, does not modulate disease severity in rat MNX model of osteoarthritis [2]. Therefore, it is likely that inhibiting MHC IIa gene expression may be involved in muscle dysfunction during OA development, at least partially.

Oxidative stress, resulted from the over-production of ROS and the decrease of circulating anti-

oxidant, may be involved in the pathogenesis of muscle dysfunctionin MNX-induced OA. Oxidation can alter the structure and function of lipids, proteins and nucleic acids, leading to cellular injury and even cell death [43]. Genetic evidence has shown that increased oxidative stress in skeletal muscle is sufficient to induce muscle atrophy [44, 45]. In the present study, increased muscular LPO and nuclear Nrf2 expression were found in MNX-treated rats. We suggest that muscular oxidative stress may be associated with muscle weakness after MNX.

In addition, oxidative stress is resulted from an increase of oxidants or a decrease of antioxidants, both of which may be involved in MNX-associated muscle weakness. Inhibiting circulating antioxidant expression in mice results in significant loss of skeletal muscle mass and muscle weakness [28]. Elevated ROS levels can contribute to muscle loss and weakness by oxidative damage, degradating contractile proteins, or activating calpain and ubiquitin proteolytic systems [46, 47]. In addition, overproduction of ROS alters the fiber type and muscle function by regulating MHC gene expression [48]. In the present study, muscular ROS generation was marked increased while GSH and GPx levels were reduced after MNX. Therefore, we suggested that the increase of ROS generation and the decrease of circulating antioxidant may be associated with MNX-induced oxidative muscular damage.

We concluded that oxidative stress may be involved in the pathogenesis and development of muscle dysfunction in MNX-induced OA model. Further, inhibiting muscular oxidative stress may be beneficial in preventing the quadriceps muscle dysfunction as well as the initiation and development of OA. However, more investigation will be needed to confirm this.

Acknowledgements

This study was supported by the National Science Council Taiwan [Grant 101-2314-B-675-002-MY2].

Disclosure of conflict of interest

None.

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