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DNA DAMAGE INDUCED BY EXERCISE IN MIDDLE GLUTEAL MUSCLE OF THOROUGHBREDS HORSES

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ABSTRACT

It is well known that two classical consequences of severe exercise are soreness and stiffness. Both consequences develop in the days following severe exercise. The objective of the present work was to establish the association between DNA damage, the energetic metabolism and the effects of the generation of oxygen free radicals in middle gluteal muscle of horses, measured after a severe exercise bout on a treadmill. Four thoroughbreds, ranging in aged from 3 to 4 years old were used for this study. The test consisted in warming-up, severe exercise, and cooling down. Venous blood samples were withdrawn via a catheter from the jugular vein before and at 5 min, 30 min, 45 min and 24 h post-exercise. Muscle biopsy samples were obtained from the middle gluteal muscle before and at 45 min and 24 h after exercise. All statistical analyses were performed using Student's t-tests for unpaired data. ATP concentration significantly decreased ($p < .05$) after 45 min of severe exercise and subsequently increased by 24 h. Plasma CK activity increased significantly ($p < .05$) after 45 min and 24 h of exercise. Both free MDA and protein-bound MDA concentrations significantly increased ($p < .001$) after 45 min of exercise, and both of them returned almost to the pre-exercise values after 24 h of exercise. The percentage of TUNEL positive cells increased significantly ($p < .001$) so as the plasma hypoxanthine and uric acid level during exercise. It was strongly suggested that the generation of free radicals was developed during exercise. The mechanism of exercise-induced myopathy could be the cell membrane damage by lipid peroxidation and DNA fragmentation in the working muscle cells by free radicals generation.

RESUMEN

Dos consecuencias clásicas del ejercicio severo son el dolor y la rigidez. Ambas consecuencias ocurren en los días siguientes al ejercicio. El objetivo de este trabajo fue establecer la asociación entre daño en el ADN, el metabolismo energético y la generación de radicales del oxígeno en el músculo gluteo medio de caballos sometidos a ejercicio severo. Se utilizaron 4 caballos de entre 3 y 4 años de edad, que fueron sometidos a ejercicio en cinta ergométrica. Se obtuvieron muestras de sangre por punción yugular antes, a los 5, 30, 45 minutos y 24 horas pos ejercicio. Se realizaron biopsias del músculo gluteo medio antes, a los 45 minutos y 24 horas pos ejercicio. Se realizaron múltiples determinaciones y mediciones y en todos los casos el análisis estadístico se realizó utilizando el test de t para muestras no apareadas. La concentración de ATP disminuyó significativamente a los 45 minutos de ejercicio severo ($p < 0, 05$) y se recupero a las 24 hs. La actividad plasmática CK se incremento significativamente ($p < .05$) a los 45 minutos y 24 h de realizado el ejercicio. Tanto el MDA libre como el fijado a proteínas aumentaron sus concentraciones significativamente ($p < .001$) a 45 minutos, y ambos volvieron a los valores de pre ejercicio a las 24 horas. El porcentaje de células tunnel positive y los niveles plasmáticos de hipoxantina y ácido úrico se incrementaron signioficativamente durante el ejercicio ($p < .001$). Los datos obtenidos sugieren la generación de radicales libres durante el ejercicio y la producción de daño en el ADN. El mecanismo por el cual el ejercicio induce miopátias puede deberse al daño de membranas celulares por peroxidación de lípidos y a la fregmentación del ADN, debida a la producción de radicales libres.

Introduction

It is well known that two classical consequences of severe exercise are soreness and stiffness. Both consequences develop in the days following severe exercise. This delayed onset muscle soreness is considered to be mainly resulted from an imbalance between energetic utilization and generation in working skeletal muscle. Degradation of high-energy phosphates [36] and decreased mitochondrial respiratory control [21] were demonstrated in exercised muscle, indicating the energetic impairment. Moreover, the severe exercise is also known to induce the generation of oxygen free radicals in muscle [21,29] by the mitochondrial electron transport chain [5], xanthine/xanthine oxidase reaction [8], and activated neutrophils [12,28], which infiltrated in working muscle. Oxygen free radicals lead to loss of membrane integrity and cellular functions by the lipid peroxidation of polyunsaturated lipids [34,36] followed by the inactivation of enzymes and increase of $Ca^{2+}i$, which in turn could activate various degradative pathways in working muscle cells [6,30]. All of them induced myofibers death that usually explained in terms of muscle necrosis [9,32]. Otherwise, some researchers suggested that the apoptotic pathway also induced myofibers death after severe exercise [38, 42, 43, 45, 47].

The objective of the present work was to establish the association between DNA damage, the energetic metabolism and the effects of the generation of oxygen free radicals in middle gluteal muscle of horses, measured after a severe exercise bout on a treadmill.

Materials and Methods

Chemicals: All chemicals used were of analytical grade obtained from Wako Pure Chemical (Osaka, Japan) unless otherwise indicated. All standard compounds for HPLC analysis were purchased from Sigma (St. Louis, MO, U.S.A.). Fluo-3 AM ester and PI were obtained from Molecular Proves (Eugene, OR, U.S.A.).

Animals: Four thoroughbreds, two mares and two geldings, weighing 557 ± 16 kg, ranging in age from 3 to 4 years old with a mean age of 3.5 years were used for this study.

Exercise and sampling protocol: All horses performed the exercise test on a treadmill (Mustang-2200, Kagra, Switzerland). The test consisted in warming-up, severe exercise, and cooling down as follows.

Warming up: 3 min of walk at 1.8 m/sec on a treadmill and 3 min of trot at 3.6 m/sec on a sloped treadmill (5%).

Severe exercise: run at 16 m/sec on a sloped treadmill (5%) until the horses could not keep the pace with the treadmill belt.

Cooling down: 5 min of walk at 1.8 m/sec on a treadmill.

Venous blood samples were withdrawn via a catheter from the jugular vein at before, and at 5 min, 30 min, 45 min and 24 h post-exercise. The samples obtained were placed on ice in heparinized glass test tubes and centrifuged at 1500 x g for 10 min, and plasma was removed. Plasma was stored at -80°C until assay.

Muscle biopsy samples were obtained from the middle gluteal muscle before, and at 45 min and 24 h after exercise using a 6 mm diameter modified Bergstrom needle (Lindholm and Piehl et.al.)[25].The biopsy was performed 16 cm from the highest point of the tuber coxae along a straight line to the tail head at a depth of 6 cm. The middle gluteal muscle samples were frozen in liquid nitrogen and stored at -150°C until assay.

Cell injury: Plasma creatine kinase (CK) activity was measured for evaluating the cell membrane injury by a model 736-20 automatic analyser (Hitachi) with commercial assay reagents (Wako Pure Chemical, Osaka, Japan).

Analysis of purines nucleotides: Purine nucleotides were extracted from muscle biopsy samples by the method of Lazzarino et. al. [23]. Briefly, frozen muscle wafers were crushed, deproteinized by adding 1 ml of ice-cold 0.6 mM $HClO_4$, and homogenized in a glass homogenizer on ice for 1 or 2 min. Extracts were neutralized with 300 µl ice-cold 2.0 M K_2CO_3 and centrifuged (4°C, 15,000 x g, 2 min). The supernatant was filtrated by a 0.22-µm syringe filter (ADVANTEC,

Tokyo, Japan) and stored at -80°C until HPLC assay. For plasma purine nucleotides, the 100 µl of plasma was mixed with 200 µl of 0.6 mM $HClO_4$. Extracts were neutralized with 60 µl ice-cold 2.0 M K_2CO_3 and centrifuged (4°C, 15,000 x g, 2 min). The supernatant was filtrated by a 0.22-µm-syringe filter (ADVANTEC, Tokyo, Japan) and stored at -80°C until HPLC assay.

The intracellular and plasma concentrations of ATP, ADP, AMP, GTP, GDP, IMP, HX and Uric acid were measured with an HPLC system (JASCO, Tokyo, Japan). The system consisted of two model PU-980 pump, an AS-950 sampler, a CO-965 column oven, a UV-970 detector and an LCSS-905 integrator. The chromatographic analysis was performed at a flow rate of 1.2 ml/min on a 250x4.6 mm Pegasil ODS column (Senshu, Tokyo, Japan) maintained at 40°C. The mobile phase consisted of 10 mM tetrabutylammonium hydroxide, 25 mM KH_2PO_4, 1.0% methanol, pH 6.5 (solvent A), and 5 mM tetrabutylammonium hydroxide, 100 mM KH_2PO_4, 30% methanol, pH 4.5 (solvent B). A linear gradient from 0 to 100% of buffer B was formed between 10 and 50 min. A 50 µl of sample aliquot was injected and the eluant was monitored at 266 nm, 0.64 AUFS. Total procedure time was 50 min. The initial conditions were restored after over 20 min of washing with buffer A. Calibration curves for the quantitative measurements of the biological samples were carried out by analysing standard solution with different known concentrations.

Measurement of malondialdehyde (MDA) and protein-bound MDA: A piece of the tissue specimen (100 to 150 mg) was homogenized with 2.5 ml of ice-cold 0.1 M phosphate buffer (pH 7.4, containing 10 mM ethylendiamine-tetraacetic acid) in a glass homogenizer on ice for 1 min. The 1.2 ml of the homogenate was used for the extraction of whole lipids and free MDAs.

The whole lipids and free MDAs were extracted with the Bligh-Dyer lipid extraction method [4] according to the report of Schmedes and Hølmer [44]. In brief, the 1.2 ml of the tissue homogenate was placed in a screw-capped glass test tube. The 4.5 ml of ice-cold chloroform-methanol mixture (1:2, v/v) containing 0.02% tert-buthylhydroxytoluene (Sigma, St. Louis, MO, U.S.A.) was added and mixed vigorously for 1 min. Then, the 1.5 ml of ice-cold chloroform and 1.5 ml of ice-cold 1.15% KCl were added. The mixture was vigorously mixed again for 1 min. Finally, the mixture was centrifuged at 2,300 x g for 10 min. The aqueous phase (upper layer) including free MDAs was collected and inmediately used for assay. The Chloroform phase (lower layer) including whole lipids was collected and filtrated with a membrane filter (Dismic 13Jp, ADVANTEC, Tokyo, Japan) and stored at -20°C until assay.

For the preparation of protein-bound MDAs, an alkaline pre-treatment of the sample [24] was performed prior to the Bligh-Dyer extraction. In brief, 1.0 ml of the tissue homogenate was placed in a screw-capped glass test tube. The 0.1 ml of 0.5 N NaOH was added, and the tightly capped test tube was incubated at 60°C for 30 min for the release of protein-bound MDAs. After cooling in tap water, the mixture was neutralized to pH 7.8 with 0.1 ml of 0.5 N HCl. Then, the 1.2 ml of the mixture was subjected to the Bligh-Dyer extraction as described above. The aqueous phase (upper layer) including protein-bound MDAs with free MDAs was collected and immediately used for assay.

The concentration of free MDA and protein-bound MDA was determined with a thiobarbituric acid (TBA) method as described in chapter I. The MDA value was obtained with the standard curve prepared in the each assay. The concentration of protein-bound MDA was subtracted the free MDA concentration from the MDA concentration in a sample of the alkaline hydrolysis.

Detection of fragmented DNA: TUNEL method: DNA fragmentation was examined on the paraffin sections by the modified TUNEL method proposed by Gavrieli et. al. [15], using a commercial apoptosis detection kit (Apop Tag, Oncor, Gaithersburg, MD). The procedure was as follows: multiple fragmented DNA 3‘-OH ends on the paraffin sections were labelled with digoxigenin-dUTP in the presence of terminal deoxynucleotidyl transferase (TdT). Peroxidase-

conjugated anti-digoxigenin antibody was then reacted with the sections. TUNEL positive nuclei were visualized by peroxidase-diaminobenzidine (DAB) reaction. The sections were then counterstained with methylgreen. The ratio of TUNEL-positive cells to all the muscle cells was calculated on three section/animal under light microscope. The mean ± SD of 4 animals was expressed as % at 45 min and 24 h after exercise, respectively.

Gel electrophoresis: Muscle tissue samples after 45 min and 24 h of severe exercise were homogenized with the addition of 1 ml 0.1 M PBS and centrifuged at 400 x g for 5 min. The supernatant was discard and tissue was lysed with 20 µl of lysis buffer (50 mM Tris-HCl (pH 7.8), 10 mM EDTA-4Na and 0.5% (w/v) sodium-N-lauroylsarcosinate) and 2 µl of proteinase K (10 mg/ml). The samples were incubated at 50°C for 90 min and then treated with RNase A (10 mg/ml) for 30 min at 50°C. Spin drops on the wall of microtubes were performed a 1.5% agarose gel electrophoresis for detection of fragmented DNA.

Measurement of XO activity in muscle tissue: Xanthine Oxidase activity was measured by the method described by Corte and Stirpe [10] with slight modifications. The muscle tissues were homogenized for 20 sec in 50 mM potassium phosphate buffer (pH 7.0) containing 10 mM dithioerythritol, 1 mM phenylmethylsulfonyl fluoride, and 0.1 mM ethylenediaminetetraacetic acid (EDTA) at 4°C. The homogenized samples were then centrifuged at 20,000 x g at 4°C for 10 min. Thirty microliters of the supernatant was added to a reaction mixture of 1 mM xanthine, 2 mM EDTA, and 200 µl/ml bovine serum albumin (BSA). The reaction was allowed to proceed at 25°C for 5 min and then stopped by the addition of 1 ml of 0.5 mM HCl. The absorbances of samples and of the XO standards were determined spectrophotometrically at 295 nm (Beckman DU 650, Fullerton, CA, U.S.A.). The final values are represented as mU/mg of muscle tissue.

Statistical analysis: All statistical analyses were performed using Student's t-tests for unpaired data.

Results

Purine nucleotides: Among the concentrations of purines nucleotides in muscle tissue, ATP concentration significantly decreased ($p < .05$) after 45 min of severe exercise and subsequently increased by 24 h without reaching the pre-exercise level. A parallel and significant ($p < .05$) reduction was also observed in the levels of GDP. The level of IMP significantly augmented ($p < .001$) after 45 min of exercise, while no remarkable changes of GTP and AMP levels were observed. In addition, intracellular hypoxanthine concentration significantly decreased ($p < .05$) after 45 min of exercise (Fig. 1). In plasma, significant increases of both hypoxanthine and uric acid levels ($p < .05$) were founded after 30, and after 45 min and 24 h of exercise, respectively (Fig. 2).

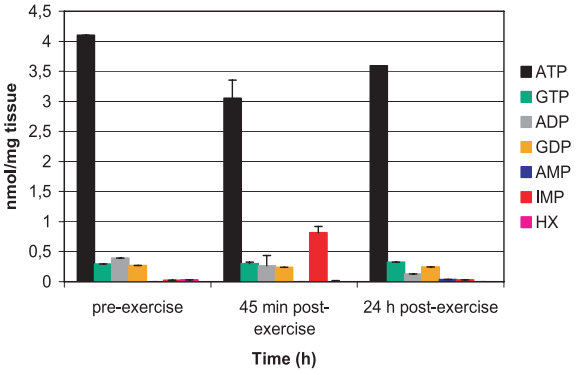
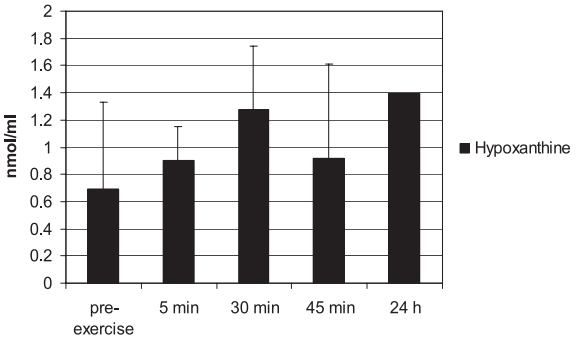


Fig. 1. Changes of purine nucleotides in the middle gluteal muscle tissue from horses after severe exercise. Bars indicate standard deviation of the mean and asterisks show the significance level to the initial level (* $p < .05$; ** $p < .001$).



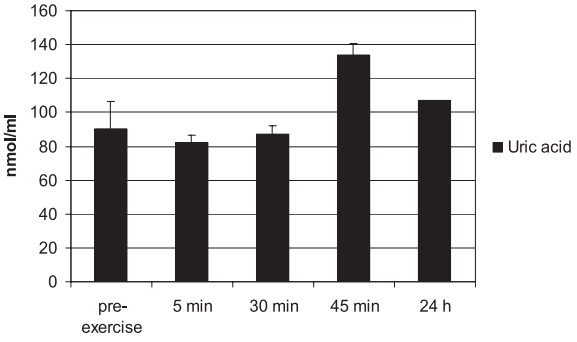


Fig. 2. Changes of plasma hypoxanthine (a) and uric acid (b) levels in horses after severe exercise. Bars indicate standard deviation of the mean and asterisks show the significant level to the initial level (* p< .05).

Plasma CK activity: For evaluation of the membrane permeability and/or damage after severe exercise, plasma CK activity was measured (Fig. 3). Plasma CK activity increased significantly (p< .05) after 45 min and 24 h of exercise.

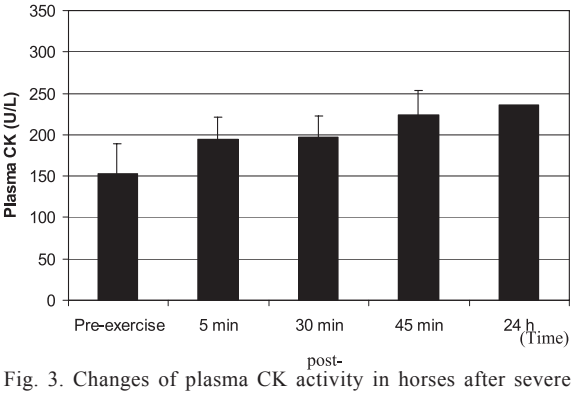


Fig. 3. Changes of plasma CK activity in horses after severe exercise. Bars indicate standard deviation of the mean and asterisks show the significant level to the initial level (* p< .05).

Lipid peroxidation: Lipid peroxidation was determined as final products MDA. Figure 4 shows the changes of the free MDA and protein-bound MDA levels before, and after 45 min and 24 h of exercise. Both free MDA and protein-bound MDA concentrations significantly increased (p< .001) after 45 min of exercise, and both of them returned almost to the pre-exercise values after 24 h of exercise.

Xanthine oxidase activity: Xanthine oxidase activity in muscle tissue was analyzed by the spectrophotometric method. The activity in muscle tissue was approximately 200 mU/g tissue (Fig. 5).

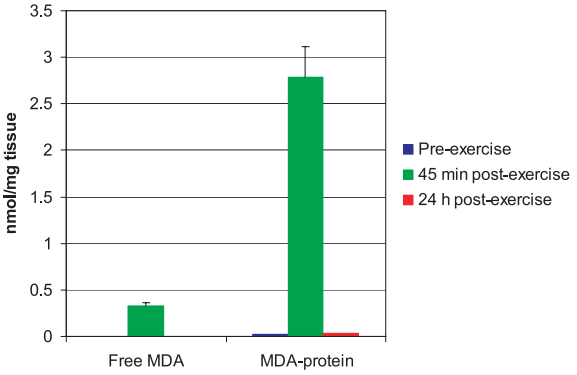


Fig. 4. Changes of free MDA and protein-bound MDA levels in the middle gluteal muscle tissue from horses after severe exercise. Bars indicate standard deviation of the mean and asterisks show the significant level to the initial level (** p< .001).

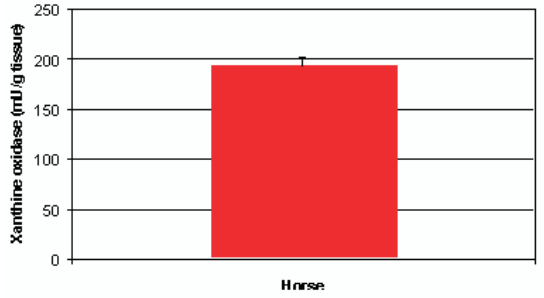


Fig. 5. Xanthine oxidase activities in the middle gluteal muscle tissue from horses. Bar indicate standard deviation of the mean.

Fragmented DNA: The change of the TUNEL positive cells percentage detected in muscle tissue from severe exercised horses are shown in Fig. 6. After 45 min of exercise, the TUNEL positive cell percentage, reached 55.9%, while at 24 h post-exercise it decreased to 44.9%. In both sampling time, the percentage of TUNEL positive cells increased significantly (p< .001) compared to that in the before exercise.

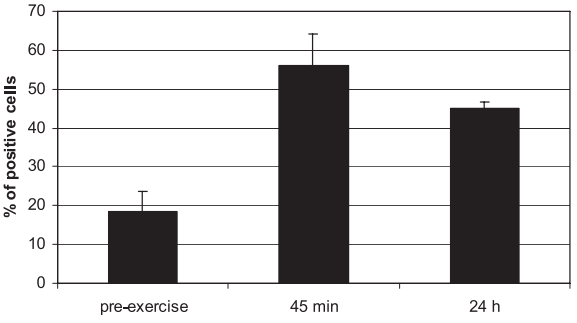


Fig. 6. Changes of the percentage of TUNEL positive cells in the middle gluteal muscle tissue from horses after severe exercise. Bars indicate standard deviation of the mean and asterisks show the significant level to the initial level(** p< .001).

On the result of gel electrophoresis, the fragmented chromosomal DNA was detected in muscle samples from exercised horses after both 45 min and 24 h of exercise (Fig. 7).

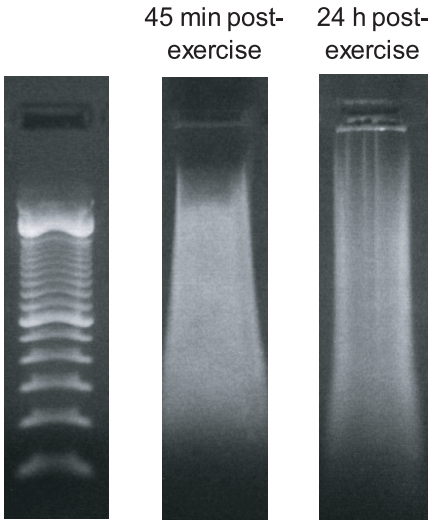


Fig. 7. Typical patterns of gel electrophoresis for fragmented DNA in the middle gluteal muscle tissue from horses after severe exercise. Fragmented DNA was detected in both samples after 45 min and 24 h of exercise, respectively.

Discussion

It has been widely accepted that severe exercise induced an imbalance between energetic utilization and generation in working skeletal muscle. The exercise also enhanced the generation of oxygen free radicals in muscle [21,29], which induced loss of the membrane integrity and cellular dysfunctions by the lipid peroxidation of membrane polyunsaturated lipids [34,36]. In addition, subsequent Ca^{2+}i increase in the muscle cells could activate various degradative pathways, leading to a muscle fibber damage [6, 30].

In this study, the middle gluteal muscle from exercised horses showed a remarkable ATP decrease with a consequent IMP increase after 45 min of severe exercise. The rate of ATP utilization during intensive exercise usually exceeded the ability of the muscle cells to rephosphorylate ADP. The accumulation of ADP within the cells activated adenylate kinase (AK), which catalysed the reaction (2 ADP ® ATP + AMP). Thus, the reaction led to the formation of AMP, which was deaminated by AMP deaminase ($AMP + H_2O$ ® $IMP + NH_3$). Therefore, adenine nucleotides in the muscle cells were degradated with the consequent accumulation of IMP during severe exercise. The major pathway for the removal of IMP was the second part of the purine nucleotide cycle, which allowed for the reamination of IMP to AMP within the cells [27]. However, there were various evidences suggesting that this reamination did not occur during intensive muscular contraction [22,33]. The accumulation of IMP was demonstrated in the muscle cells during exercise [18, 40], since IMP was unable to pass through the cell membrane. The reduction of the high-energy phosphates, observed in the middle gluteal muscle in this study, was the initiating event to induce muscle fibber injury according to the hypothesis reported by many researchers. The reduction of ATP reduced Ca^{2+}-ATPase activity in the SR or sarcolemma, resulted in the down regulation for removal of Ca^{2+}i from the cytoplasm. Finally, cytosolic Ca^{2+} levels increased, by which degradative pathways were activated.

In this study, a significant increase of plasma hypoxanthine was observed in severe exercised horses, followed by an increase of plasma uric acid level with a delay of approximately 15 min. The reason of the delay was considered as follows: hypoxanthine was produced in the working muscle cells and released into the blood stream during exercise [35]. Released hypoxanthine was taken up by the liver, which contained a relatively high level of xanthine oxidase activity compared to that in other organs, and was converted to uric acid. The uric acid was rapidly released into the blood stream [3]. In the present study, the xanthine oxidase activity was detected in the middle gluteal muscle tissue. This result supported previous reports of Jarasch et. al. [19] who found that the conversion of hypoxanthine to uric acid by xanthine oxidase was mainly produced at the capillary endothelial cells and at the smooth muscle cells in vessel walls [19], in which xanthine oxidase located. Therefore, the oxidation of hypoxanthine to uric acid was considered to occur in the vicinity of the muscle cells.

Both of free MDA and protein-bound MDA increased significantly after 45 min of exercise. The increase of MDA in muscle tissue was demonstrated by Alessio and coworkers, Alessio and Goldfarb, and Matsuki and coworkers [1,2,31]. The increase of TBARS level was also observed in skeletal muscle of rats after moderate and high-intensity treadmill running, however, Duncan and coworkers [11] reported that no significant changes in the concentration of TBARS in rat skeletal muscle after exhaustive exercise. Since the TBARS, and also MDA were easily passed through the cell membrane, the concentration of TBARS in the working skeletal muscle did not change in exhaustive exercise [41]. Severe damage might be developed in the cell membrane during exhaustive exercise, compared to that in moderate and high intensity one.

It is well accepted that lipid peroxidation was induced by free radicals. During severe exercise, the working skeletal muscle potentially was subjected to at least two major sources of oxygen radicals. The most common source is semiquinones in the electron transport chain of mitochondria [20]. It was proposed that 3-5% of the total oxygen supply to mitochondria produced superoxide radicals [13]. Thus, the generation rate of superoxide radicals might increase during exercise, since the higher circulation was reported in exercise to enhance the oxygen supply [20]. A second plausible source of oxygen radicals is hypoxanthine-xanthine-uric acids pathway by xanthine oxidase, which was mainly located in the vessel walls. Calcium dependent proteases are also known to convert xanthine dehydrogenase to xanthine oxidase, which generated oxygen free radicals [39]. The generation rate of oxygen radicals by xanthine oxidase was dependent on the concentrations of the substrate hypoxanthine. Hypoxanthine produced in the muscle cells was easily passed through the cell membrane to be a substrate for xanthine oxidase. In the present study, significant increase of plasma hypoxanthine and also uric acid level, the final product of hypoxanthine by xanthine oxidase, were observed in horses during exercise. Therefore, it was strongly suggested that the generation of free radicals was developed during exercise, resulted in the increase of TBARS and MDA levels in the working muscle. In addition, the site of the free radical generation was reported to be an attracting site for neutrophils through activation of a chemoattractant [37]. Indeed, neutrophils were found to adhere to the vessel walls in the injured muscle tissue [16, 46] suggesting the extent damage was occurred by further radical generation from neutrophils.

The release of cytoplasmic enzymes including creatine kinase (CK), aspartate transaminase (AST), and L-lactate dehydrogenase (LDH) were considered to be an available parameter for the working muscle injury during exercise [26]. Among them, CK activity was the most specific and sensitive indicator to detect and monitor for the muscle injury in horses [11]. In this study, plasma CK activity increased significantly after 45 min and 24 h of exercise. The increase of plasma CK, therefore, was resulted from the increase of cell permeability by lactic acidosis, which was commonly observed in exercised horses, and/or resulted from the cell membrane damage by lipid peroxidation [6, 30].

Death of adult myofibers in neuromuscular disorders and also exercise-induced myopathy is explained in terms of muscle necrosis [14,48]. However, the mechanism of the cell death has not been clearly elucidated. To investigate the contribution of apoptosis to exercise-induced myopathy, time course analysis of DNA fragmentation was carried out in the middle gluteal muscle during exercise by TUNEL staining. The number of TUNEL positive cells significantly increased after exercise. In addition, DNA fragmentation was detected by gel electrophoresis in the middle gluteal muscle, indicating that the apoptosis was developed during severe exercise. The trigger for apoptosis was proposed that excessive free radicals damaged macromolecules, such as proteins, lipids and DNA [42]. Free radical generation during severe exercise was considered to be one of the most important mechanisms to up-regulate pro-apoptotic and/or down-regulate anti-apoptotic genes [7,17].

In conclusion, it was suggested that the mechanism of exercise-induced myopathy was considered to be the cell membrane damage by

lipid peroxidation and DNA fragmentation in the working muscle cells by free radicals generation.

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