Assessment of reactive oxygen species production in cultured equine skeletal myoblasts in response to conditions of anoxia followed by reoxygenation with or without exposure to peroxidases

Justine D. Ceusters, DVM, MSc; Ange A. Mouithys-Mickalad, PhD; Geoffroy de la Rebière de Pouyade, DVM, PhD; Thierry J. Franck, PhD; Dominique M. Votion, DVM, PhD; Ginette P. Deby-Dupont, PhD; Didier A. Serteyn, DVM, PhD

Objective—To culture equine myoblasts from muscle microbiopsy specimens, examine myoblast production of reactive oxygen species (ROS) in conditions of anoxia followed by reoxygenation, and assess the effects of horseradish peroxidase (HRP) and myeloperoxidase (MPO) on ROS production.

Animals—5 healthy horses (5 to 15 years old).

Procedures—Equine skeletal myoblast cultures were derived from 1 or 2 microbiopsy specimens obtained from a triceps brachii muscle of each horse. Cultured myoblasts were exposed to conditions of anoxia followed by reoxygenation or to conditions of normoxia (control cells). Cell production of ROS in the presence or absence of HRP or MPO was assessed by use of a gas chromatography method, after which cells were treated with a 3,3′-diaminobenzidine chromogen solution to detect peroxidase binding.

Results—Equine skeletal myoblasts were successfully cultured from microbiopsy specimens. In response to anoxia and reoxygenation, ROS production of myoblasts increased by 71%, compared with that of control cells. When experiments were performed in the presence of HRP or MPO, ROS production in myoblasts exposed to anoxia and reoxygenation was increased by 228% and 183%, respectively, compared with findings for control cells. Chromogen reaction revealed a close adherence of peroxidases to cells, even after several washes.

Conclusions and Clinical Relevance—Results indicated that equine skeletal myoblast cultures can be generated from muscle microbiopsy specimens. Anoxia-reoxygenation–treated myoblasts produced ROS, and production was enhanced in the presence of peroxidases. This experimental model could be used to study the damaging effect of exercise on muscles in athletic horses. (*Am J Vet Res* 2012;73:xxx–xxx)

Horses have a very high maximal oxygen uptake (200 mL/kg/min), compared with values in other species,[1] because of an exceptionally high mitochondrial mass in skeletal muscles.[2] The main function of mitochondria is ATP synthesis, coupled with the reduction of oxygen to water. Under physiologic conditions, mitochondria have a basal production of superoxide anion that is correlated to a leak of electrons at the level of the respiratory chain complexes. Within the mitochondria, the principal producer of superoxide anions is complex I, but complexes II and III also have important roles.[3,4] It is estimated that 2% to 3% of oxygen is partially reduced in mitochondria into superoxide anions, which undergo spontaneous or enzymatic dismutation to yield hydrogen peroxide.[5] This physiologic production of ROS regulates intracellular signaling processes.[6,7] However, under pathological conditions, production of ROS can exceed its basal level and the mechanisms of

Abbreviations

CK	Creatine kinase
DAB	3,3′-diaminobenzidine
GC	Gas chromatography
HRP	Horseradish peroxidase
KMB	α-Keto-γ-(methylthio) butyric acid
MPO	Myeloperoxidase
ROS	Reactive oxygen species

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From the Center for Oxygen Research and Development, Institute of Chemistry (Ceusters, Mouithys-Mickalad, de la Rebière de Pouyade, Franck, Deby-Dupont, Serteyn), and the Department of Clinical Sciences, Large Animal Surgery, Faculty of Veterinary Medicine (de la Rebière de Pouyade, Votion, Serteyn), University of Liège, Sart Tilman, 4000 Liège, Belgium.
Dr. Ceusters was supported by a Fund for the Research in Industry and Agriculture scholarship.
Address correspondence to Dr. Ceusters (J.ceusters@ulg.ac.be).

cellular protection can be affected, leading to an oxidant-antioxidant imbalance called oxidative stress.

A pathological process that results in oxidative stress is ischemia followed by reperfusion. To better understand this phenomenon, numerous studies[8–10] including evaluations of in vitro and in vivo experimental models involving anoxia and reoxygenation have been reported to mimic ischemia-reperfusion injury. During reoxygenation, ROS overproduction (a process critical in the prevention of necrosis of the tissue) occurs and is due partly to mitochondrial alterations.[11] Furthermore, mitochondria represent both the main source and the target of ROS.[11,12] In horses, ischemia-reperfusion injuries develop in many clinical situations, such as gastrointestinal tract injuries or laminitis.[13,14]

The muscular function of athletic horses has to be optimal to ensure the success of their sporting careers. Unfortunately, intense exercises such as endurance rides, 3-day eventing, and sprint racing can extend muscle function to its limit. Recently, studies[15,16] in horses have revealed that intense exercise induces stimulation and degranulation of polymorphonuclear neutrophils; those cells release MPO and elastase, 2 key enzymes involved in inflammation. In 1 study,[16] plasma activities of these enzymes in horses that completed an endurance race were significantly higher than the values in those horses before the race; in some instances, the postrace values were as high as those encountered in horses with acute inflammation (eg, gastrointestinal tract diseases or laminitis).[17,18] In another study,[19] increases in MPO and elastase activities in the muscular tissue of the horses at 2 hours after a 120-km endurance race were significantly correlated to an increase in CK activity, a marker of muscular damage.[20]

On the other hand, it has been established that intense muscular work generates considerable amounts of ROS and consequently induces oxidative stress in humans[21,22]; similar changes occur in horses during intense[23,24] and endurance exercise.[25,26] In human athletes, muscle deoxygenation during incremental exercise has been demonstrated.[27] At rest, intracellular oxygen returns to physiologic concentration, mimicking a kind of reperfusion phenomenon. The increase of oxygen flux through the mitochondrial electron transport chain is probably the main source of ROS production.[28]

The relationship between ischemia-reperfusion injuries, ROS production by mitochondria, muscle damage, and inflammatory reaction in horses still remains largely unexplored. Therefore, the purpose of the study reported here was to culture equine myoblasts from muscle microbiopsy specimens, examine the cultured cells for the expression of specific muscular proteins and capacity for fusion into multinucleated myotubes, and investigate myoblast production of ROS in conditions of anoxia followed by reoxygenation in the presence and absence of HRP or MPO. Exposure to peroxidases was used to mimic a muscular inflammatory response involving neutrophils or the infiltration of neutrophil enzymes.

Materials and Methods

Microbiopsy specimens—The microbiopsy technique was approved by the Ethics Committee of the Faculty of Veterinary Medicine of the University of Liège. Microbiopsy procedures were performed on standing, awake horses. Microbiopsy specimens were obtained from 1 triceps brachii muscle (long head, at the intersection of a vertical line extending from the tricipital crest and a line between the scapulohumeral and radiohumeral joints) of each horse. In each of 2 horses, 1 sample was collected from 1 forelimb. In each of 3 horses, 2 samples were collected from 1 forelimb in a single procedure.

Microbiopsy specimens were collected with a 14-gauge microbiopsy needle and a microbiopsy pistol.[a] Briefly, the hair over the sample collection site (1 cm^2) was shaved, and the skin was desensitized via SC injection of 0.5 mL of mepivacaine and aseptically prepared. Each sample (approx 20 to 40 mg of tissue) was collected at a depth of 5 cm in the long head of the triceps brachii muscle, through a skin incision made with the tip of a No. 11 scalpel blade. For collection of a second sample from each of 3 horses, the microbiopsy needle was reintroduced into the incision at a different orientation (same depth) after the first sample collection. Closure of the skin incision was not necessary, and the entire microbiopsy procedure was completed within 15 minutes. Immediately after collection, each sample was placed in growth medium composed of Dulbecco modified Eagle medium[b] with glucose[b] (1 g/L), L-glutamine[b] (580 mg/L), pyruvate[b] (110 mg/L), 20% fetal bovine serum,[b] 2% penicillin-streptomycin,[b] 0.5% amphotericin B,[b] and 1% HEPES.[b] Microbiopsy specimens were kept in growth medium at 4°C until use.

Cell cultures—Culture preparation was performed by use of sterile equipment, under a streamline flow hood.[c] Microbiopsy specimens were placed in 20 mL of growth medium preheated to 37°C for 30 minutes, washed twice (1 min/wash) in 10mM PBS solution (pH, 7.4) containing 137mM NaCl and 2.7mM KCl, and immersed again in 20 mL of growth medium preheated to 37°C for 2 hours. Then, each microbiopsy specimen was cut into 2 equal parts with a sterile No. 22 scalpel blade, and each was placed into separate 1.9-cm^2 wells of multiwell culture dishes[d] coated with 0.2% type A porcine gelatin[e] in PBS solution. One hundred microliters of growth medium was added to each well, and culture dishes were incubated at 37°C under controlled atmosphere (5% CO_2 and 21% O_2). The growth medium was changed every day during the first 2 days of culture and 3 times/wk thereafter.

When a halo of cells was visible around the tissue, the muscle sample was transferred to another 1.9-cm^2 well[d] coated with 0.2% porcine gelatin; the cells that had separated from the microbiopsy were grown to 80% confluence. Cells were then passaged by use of trypsin-EDTA[b]; cells from the same microbiopsy specimen (2 wells) were mixed in an 80-cm^2 culture dish.[d] Finally, cells from one 80-cm^2 culture dish[d] were passaged by use of trypsin-EDTA, with appropriate dilution, in 175-cm^2 culture dishes.[d]

Characterization of cells in culture—To induce fusion of myoblasts into myotubes, differentiation medium (Dulbecco modified Eagle medium with 1% equine serum) was used. Myoblastic cells were plated at

a density of 10^6 cells/well (6 × 9.6-cm^2 dish[d]) and covered with 2 mL of differentiation medium. The medium was changed every 2 days during the period required for formation of myotubes.

Light microscope observation—Confluent cells in multiplication (myoblasts, passage 2 to 8) were stained with a Romanowsky-type stain[e] to examine their morphological characteristics in culture. To observe the evolution of the fusion process of myoblasts into myotubes, culture cells were also stained with a Romanowsky-type stain[e] at 2, 14, and 45 days after induction of fusion. To assess this process, percentage of fusion was calculated as the number of nuclei in myotubes divided by the total number of nuclei, multiplied by 100. Counts were made manually at 45 days after fusion was induced on a representative portion of each well. All these observations were made by the same person (JDC) by use of a light microscope[f] equipped with a digital camera.[g]

Immunocytochemical evaluation—To confirm the type of cells in culture, nearly confluent cells in multiplication (myoblasts, passage 2) and fused cells (myotubes, 45 days after induction of fusion; data not shown) in 9.6-cm^2 wells[d] were probed with antibodies against desmin, myosin, or muscle-specific actin that can be detected via fluorescence microscopy after addition of fluorescein isothiocyanate–conjugated secondary antibody.

The culture medium was discarded by aspiration, and after being washed 3 times (3 min/wash) with PBS solution (1 mL/well), cells were treated with 2.5% glutaraldehyde[h] (1 mL/well; 5 minutes at room temperature [approx 20°C]), fixed with 4% methanol[i] (1 mL/well; 5 minutes at 4°C), and washed again 3 times (3 min/wash) with PBS solution (1 mL/well) prior to the addition of 5% bovine serum albumin[j] (1 mL/well). The cell preparations remained at room temperature for 20 minutes and underwent 3 additional washes (3 min/wash) with PBS solution (1 mL/well). One milliliter of primary antibody[k] (1:20 in PBS solution) was added to each well. For the controls, 1 mL of PBS solution was used instead of the primary antibody. Following an incubation of 2 hours at 37°C, cells were washed again 5 times with PBS solution (1 mL/well; 3 min/wash) before further incubation with 1 mL of the secondary antibody[k] (1:20 in PBS solution) at 37°C for 30 minutes. Finally, cells were washed 5 times (3 min/wash) with PBS solution (1 mL/well) and observed by use of fluorescence microscopy.[f]

Anoxia-reoxygenation procedure and ROS measurement—Anoxic conditions were generated by enclosing the cell plates into a sandwich system as previously described.[29] With this system, it was possible to incubate and test the cells directly in the multiwell dishes, avoid trypsinization of the cells, and ensure that the individual cell compartments were sealed but still accessible via needle puncture for the addition of reagent or sampling from a syringe. Reactive oxygen species production was quantified by use of the GC technique previously described,[30] which measures the amount of ethylene generated by the oxidation of KMB[k] by ROS. The production of ethylene is proportional to the production of ROS by the cells.

Cells at passage 3 to 6 suspended in growth medium were seeded at a density of 2 × 10^6 cells/well in multiwell dishes (6 × 9.6-cm^2 wells[d]) and used the next day. The growth medium was discarded, and cells were rinsed with assay medium composed of Dulbecco modified Eagle medium containing glucose (1 g/L) and pyruvate (110 mg/L) without phenol red.[b] Control cells (which were maintained in normoxic conditions) were covered with 2 mL of assay medium and incubated for 2 hours at 37°C at ambient oxygen atmosphere with 5% CO_2. For cells undergoing the anoxia-reoxygenation experimental treatment, assay medium was deoxygenated by bubbling it with nitrogen for 15 minutes under mild agitation before use. Two milliliters of deoxygenated assay medium with KMB (final concentration, 170 µg/mL) was added to the cells, and the dish (without the cap) was placed on the bottom of the plate of the sandwich system.[29] After flushing with a mixture of 95% N_2 and 5% CO_2, the dish was covered with a rubber membrane before closing the upper part of the sandwich onto the dish, which was then sealed. The dishes in the sandwich systems were maintained in anoxic conditions at 37°C for 2 hours. Then, reoxygenation was performed by injecting 2 mL of medical-grade oxygen into each well through the holes in the upper plate. At the same time, KMB was added to the control cells. Control cell dishes were then placed in a sandwich system and sealed. Two milliliters of medical-grade oxygen were injected in each well. A further incubation period of 30 minutes was performed at 37°C for the control and anoxic-reoxygenated cells. During this period, the ethylene eventually produced by control cells was retained inside the well by the sandwich device.

After incubation, the cell dishes inside the sandwich system were refrigerated at 4°C for 30 minutes to halt the KMB reaction. The dishes were then warmed to room temperature, and 500 µL of the gas-phase was sampled with a gas syringe and analyzed via GC[l] on a filled-column porous polymer–adsorbent matrix[m] at 80°C, with injection temperature and flame ionization detection at 120°C. The amount of ethylene produced was calculated from the area of the ethylene peak of the chromatogram. Data were expressed as mean ± SEM. The production of ROS was proportional to the amount of ethylene produced by cells.

Effects of HRP and MPO on ROS production—To study the catalytic effect of peroxidases on ROS production and their reaction with KMB, similar experiments were performed on normoxic control and anoxic-reoxygenated cells in the presence of HRP[j] and equine MPO. Horseradish peroxidase was used at the final concentration of 45 µg/mL; MPO was purified as described by Franck et al[31] and used at the final concentration of 2 µg/mL. The enzymes were added immediately after KMB addition. Data were expressed as mean ± SEM. Production of ROS was proportional to the amount of ethylene produced by cells, represented by the area under the peak of ethylene of the chromatogram.

Peroxidase interaction with cells—After GC measurements were completed, the sandwich devices were opened; cells were rinsed 3 times (1 min/wash) with PBS solution and treated with a DAB chromogen so-

lution[m] to detect the presence of peroxidase within or on the surface of the cells. One milliliter of the DAB solution[n] (1 drop of DAB chromogen/mL of substrate buffer) was added to each well (normoxic control cells or anoxic-reoxygenated cells that were or were not exposed to MPO or HRP); after 10 minutes of incubation at room temperature, the cells were gently rinsed 3 times (3 min/wash) with distilled water and observed via light microscopy.

Statistical analysis—Normality of the data was assessed by use of a Shapiro-Wilk normality test. Statistical analysis for comparisons of ROS production and the effect of peroxidases between experimental groups was performed by use of 2-way ANOVA followed by a Bonferroni posttest to compare data. Each condition was compared to normoxic control cells without addition of peroxidase. The 2-way ANOVA considered 3 sources of variation: effect of peroxidase (no peroxidase, addition of HRP, or addition of MPO), effect of oxygen concentration (cells maintained in ambient oxygen atmosphere with 5% CO_2 [normoxic conditions] or cells exposed to anoxia and reoxygenation), and interaction between the 2. Statistical software[o] was used for the analyses. Values of $P < 0.01$ were considered significant.

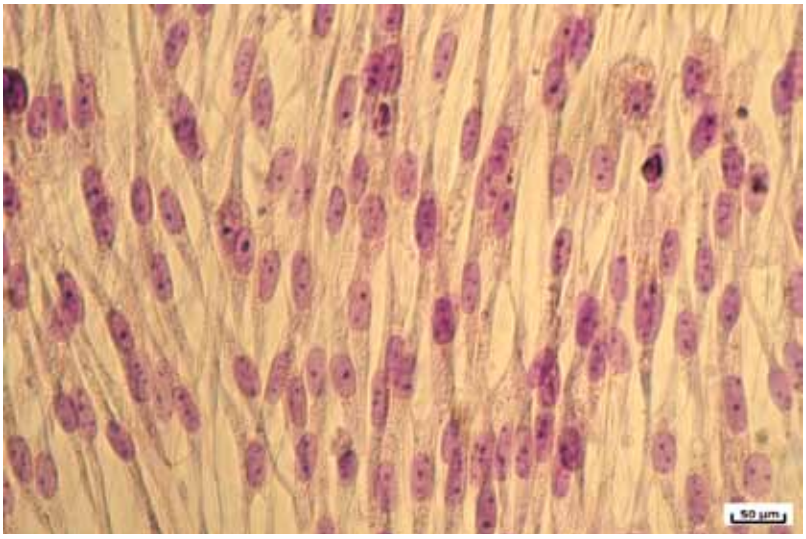

Figure 1—Representative photomicrograph of a culture of myoblasts derived from a microbiopsy specimen obtained from a triceps brachii muscle of a horse. The microbiopsy specimen was collected with a 14-gauge microbiopsy needle and a microbiopsy pistol at a depth of 5 cm in the muscle; half of the sample was used to generate this culture. Notice the confluent myoblasts (passage 1). Cells have a spindle shape, a large centrally positioned nucleus, and many nucleoli (a sign of an active synthesis of proteins). Romanowsky-type stain; bar = 50 µm.

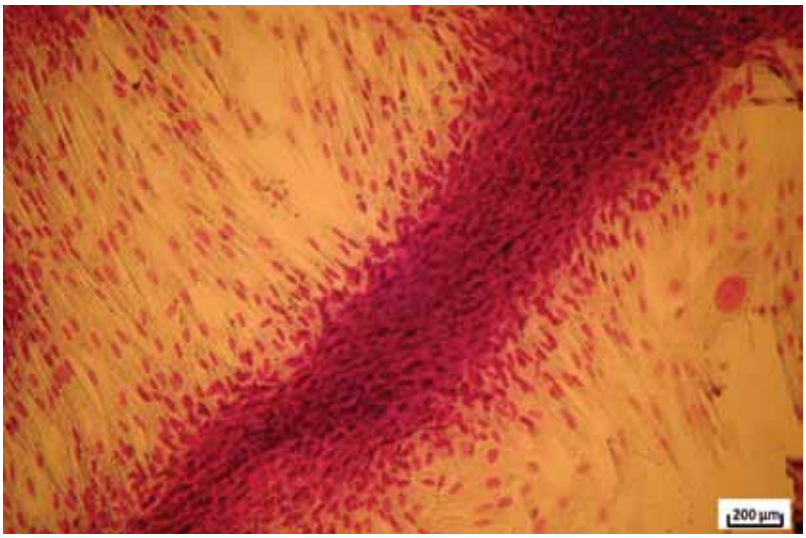

Figure 2—Representative photomicrograph of a culture of cells derived from a microbiopsy specimen obtained from a triceps brachii muscle of a horse. After 2 days in differentiation medium (used to induce fusion of myoblasts into myotubes), cells have formed a syncytium with loss of visible individual cell limits. Romanowsky-type stain; bar = 200 µm.

Results

Microbiopsy procedures and initiation of cultures—The microbiopsy technique allowed the acquisition of a sufficient amount of muscular tissue to easily initiate myoblast cultures. No contamination was observed, either during sample collection or treatment in the laboratory, thereby validating the working conditions. Because each microbiopsy specimen was approximately 20 to 40 mg and because each specimen was cut into 2 pieces, cultures were started with 10 to 20 mg of tissue.

After 3 or 4 days in culture, cells first started to appear around the muscle samples in the medium. After 9 or 10 days, the number of cells was sufficient for transplanting the explants to obtain a new cell strain. When the cells that had separated from the explants reached 80% confluence, the culture was divided. Each microbiopsy specimen was transplanted 3 times, providing 3 primary cultures (first, second, and third cell strains). Thus, 6 primary cultures were obtained for 1 microbiopsy specimen and could be divided until passage 8 without observable loss of cell morphology. These results are indicative of an excellent rate of cellular division from a very small amount of tissue.

Cell development and morphological characteristics—Morphologically, cells in culture were initially star shaped and then became spindle shaped because of the elongation of cellular bodies (**Figure 1**). The nucleus was quite large and had a central position in the cell as well as many nucleoli, a sign of an active synthesis of proteins. During growth, cells lined up progressively and started to form some spindles in various orientations. The cells were not multiplied beyond 8 passages because this number coincided with an increasing rate of cell adherence to the dish, which rendered trypsinization difficult. Furthermore, by working with cells that had not reached passage 8, the overgrowth of fibroblasts (which may contaminate the culture) was limited.

Fusion into myocytes—Two days after replacing growth medium with differentiation medium, cells started to gather and form syncytia, with loss of visible individual limits (**Figure 2**). After 2 weeks of culture with differentiation medium, the first myotubes were observed. Four weeks later (ie, 6 weeks of culture), numerous multinucleated myotubes, with characteristic striations of skeletal muscles, were present (**Figure 3**). On the basis of the calculated percentage of fusion, we estimated that the experiments were performed with approximately 75% of muscle cells.

Immunocytochemical analysis—During the cellular multiplication period, first, second, and third cell strains expressed myosin, desmin, and muscle-specific actin (**Figure 4**), thereby confirming their myoblastic type. Furthermore, the expression of these 3 specific proteins was also assessed for multinucleated cells: during the fusion process, unfused cells as well as cells that were fused into myotubes expressed the 3 proteins.

Cell strains used for experiments—Two microbiopsy specimens were collected from 1 forelimb of each of 3 horses and used to initiate 12 primary cultures/horse. For the 2 other horses, only 1 microbiopsy specimen was collected and used to initiate 6 primary cultures/horse. Each culture was characterized with regard to expression of myosin, desmin, and muscle-specific actin, even during multiplication or the fusion period. Myoblastic type was also assessed by morphological characteristics and capacity to fuse into myotubes. Once the myoblastic identity of cells in culture was confirmed, the cell strains in the multiplication state were then used for further experiments.

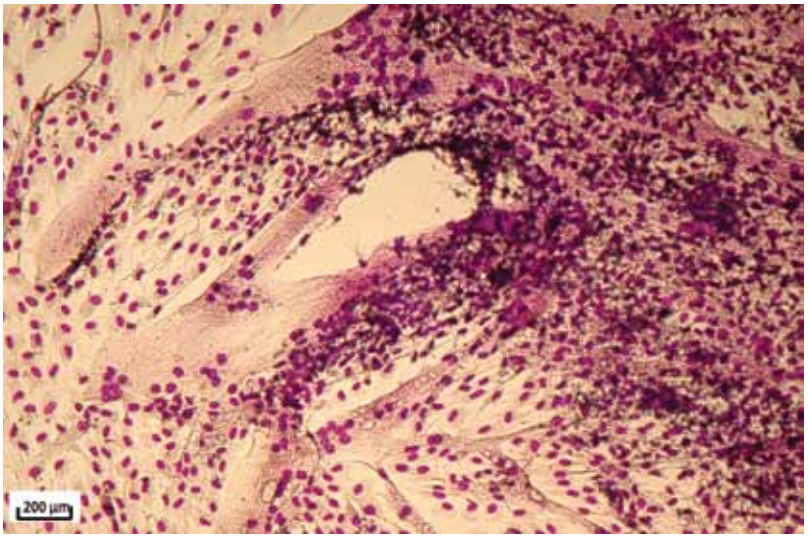

Figure 3—Representative photomicrograph of a culture of cells derived from a microbiopsy specimen obtained from a triceps brachii muscle of a horse. After 45 days of culture in differentiation medium, myotubes have formed. Romanowsky-type stain; bar = 200 µm.

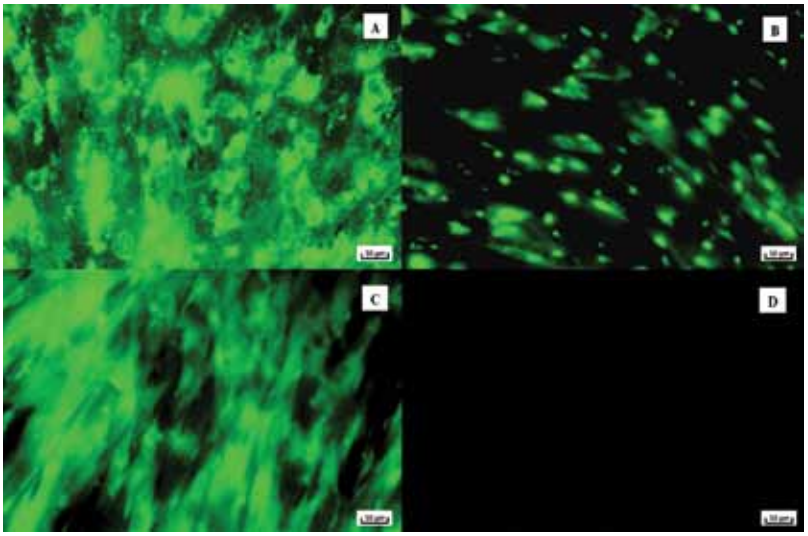

Figure 4—Representative fluorescence photomicrographs of cells cultured from a microbiopsy specimen obtained from a triceps brachii muscle of a horse after labeling of cells with antibodies against myosin (A), desmin (B), and muscle-specific actin (C). Negative control cells without addition of primary antibody (D) are also illustrated. Labeling patterns, which were similar for each culture started via cell transplantation, confirm the cells are of a myoblastic type. In each panel, bar = 50µm.

ROS measurement after anoxia and reoxygenation—Reactive oxygen species production was quantified by use of the GC technique previously described, which measures the amount of ethylene generated by the oxidation of KMB by ROS. The production of ethylene by cells is then proportional to their production of ROS.

Sources of variation considered included peroxidase effect and oxygen concentration. When performing a 2-way ANOVA, interaction between these 2 sources of variation was found to account for approximately 3.42% of the total variance ($P = 0.095$). The peroxidase effect accounted for approximately 18.34% of the total variance ($P < 0.001$) and the oxygen concentration for approximately 30.06% of the total variance ($P < 0.001$).

Compared with the normoxic control cells, the ethylene production by the cells exposed to anoxia and reoxygenation was increased by 71 ± 19%. Interestingly, the ethylene production by cells exposed to anoxia and reoxygenation was further increased when a peroxidase was used to catalyze the reaction. Ethylene production of the anoxic and reoxygenated cells was increased by 228 ± 26% ($P < 0.001$) following exposure to HRP and by 183 ± 37% ($P < 0.01$) following exposure to MPO, compared with findings for control cells that were not exposed to a peroxidase (**Figure 5**).

The addition of HRP or MPO to control cell preparations also increased their ethylene production, but this increase was not significant. Compared with ethylene production by normoxic control cells without peroxidase treatment, the addition of HRP to the cell preparations increased the production of ethylene by

63 ± 17%, and addition of MPO to the cell preparations increased the production of ethylene for 41 ± 25% (Figure 5).

Interaction of peroxidases with the cells—In parallel to the ROS assays, the ability of peroxidases (MPO or HRP) to bind to cells was investigated. Normoxic or anoxic-reoxygenated cells that were exposed to MPO or HRP and to which DAB chromogen solution was added developed a brown coloration, which persisted even after washing 3 times with PBS solution; this indicated that HRP and MPO were bound to the cells (**Figure 6**). Normoxic or anoxic-reoxygenated cells that were not exposed to a peroxidase had no coloration following similar treatment with DAB chromogen solution.

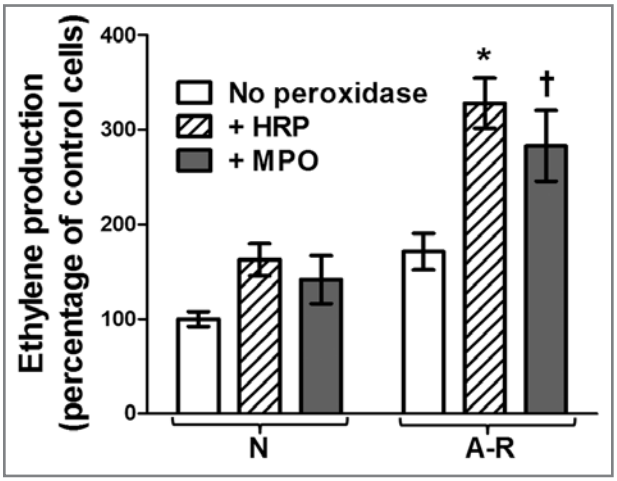

Figure 5—Effect of anoxia followed by reoxygenation (A-R) or conditions of normoxia (ambient oxygen atmosphere with 5% CO_2; N) with or without subsequent peroxidase exposure on ethylene production by equine skeletal myoblasts in culture (derived from microbiopsy specimens obtained from triceps brachii muscles of 5 horses). Normoxic or anoxic-reoxygenated cultured myoblasts were treated with HRP (final concentration, 45 µg/mL), MPO (final concentration, 2 µg/mL), or no peroxidase. Ethylene production is proportional to the ROS production of the cells and is expressed as the mean ± SEM percentage of the value for normoxic cells with no peroxidase (control cells). *Value is significantly ($P < 0.001$) different from that of the normoxic control cells. †Value is significantly ($P = 0.01$) different from that of the normoxic control cells. The numbers of GC measurements for each group were as follows: normoxic cells with no peroxidase, with HRP, or with MPO, n = 12, 14, and 8, respectively; anoxic-reoxygenated cells with no peroxidase, with HRP, or with MPO, n = 10, 15, and 8, respectively.

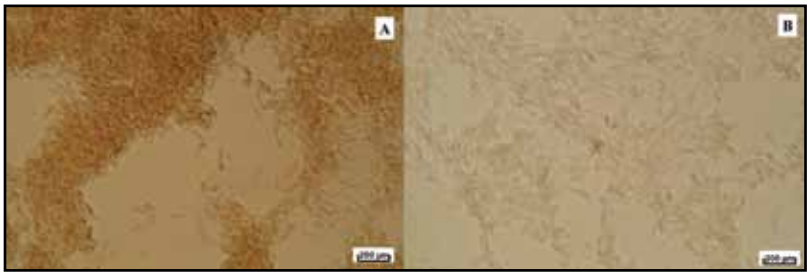

Figure 6—Representative photomicrograph of a culture of myoblasts derived from a microbiopsy specimen obtained from a triceps brachii muscle of a horse with or without subsequent HRP exposure and treatment with DAB solution (to detect the presence of a peroxidase). A—Anoxic-reoxygenated cells that were exposed to HRP. B—Anoxic-reoxygenated cells that were not exposed to HRP. Similar staining patterns were obtained for normoxic cells that were or were not exposed to HRP and for normoxic or anoxic-reoxygenated cells that were or were not exposed to MPO. In each panel, bar =200µm.

Discussion

The primary objective of the present study was to assess the use of equine muscular microbiopsy specimens to initiate cell culture. Collection of percutaneous needle biopsy specimens is well described in the literature; by use of that technique, 50 to 200 mg of muscle is collected. Although that procedure does not induce adverse effects,[32] owners of horses may be reluctant to give permission for percutaneous needle biopsy procedures, especially in healthy horses or when repeated biopsies are required for longitudinal follow-up. The method of sample collection used in the present study was tolerated well by horses; local anesthesia was administered, but tranquilization of the horses was not required. A study performed by Votion et al[33] revealed that microbiopsy performed by veterinarians in clinical practice is feasible. Furthermore, the absence of adverse effects permits consideration of this method of sample collection for use on high-performance horses, even during competitions.[33]

Biopsy specimens from various muscles (eg, semimembranous[34,35] and biceps femoris[36–38] muscles) have been used to generate cultures of equine skeletal muscle cells. Often, cultures of equine skeletal muscle cells are used to study a particular pathophysiologic mechanism, such as myopathies; therefore, the pathological process of interest has to be considered when selecting the muscle from which specimens are collected, because some myopathies develop only in certain types of muscular fibers.[39] Because the present study was not associated with investigation of any particular form of myopathy, we chose the triceps brachii muscle for sample collection because of its easy access and mixed fiber types.[40]

Two methods for the initiation of equine skeletal muscle cell cultures—the explant method[35] and enzymatic digestion[34,38]—have been described. Because the amount of tissue collected by use of the microbiopsy technique was insufficient to apply the enzymatic digestion technique, the explant method was used on the basis of a previous study by Lentz et al.[35] With that method, the authors cut each biopsy specimen into several explants of approximately 5 to 6 mm^3 and then cut each explant into several pieces of 1 mm^3 after a few days in culture because the small size facilitated the migration of cells out of the pieces of muscle. In the present study, we cut each microbiopsy specimen only once into 2 explants of 2 mm^3 to minimize the risk of contamination during cutting. The volume of medium added to the explants was adjusted to ensure complete immersion of the explants to avoid their drying out and furnish adequate growth factors to allow adhesion of the tissues to the dish, thereby facilitating the migration of cells.

Mesenchymal-type cells were obtained without any contamination from all explants in the present study. Moreover, the cells expressed myosin, muscle-specific actin, and desmin. Because muscle-specific actin and myosin were expressed by the first cells that separated

from the explants, an assumption regarding their muscular nature could be made. Desmin is expressed from the onset of the activation of satellite cells[41] and can thus be considered as the earliest characteristic marker of the myoblastic origin of cells found in culture. However, because this protein can also be found in myofibroblasts, the capacity of the cells in each culture and subculture to fuse into multinucleated myotubes has to be verified. Cells in all the cultures and subcultures that we initiated from microbiopsy specimens had a capacity to fuse into myotubes in differentiation medium, and cells in the myoblastic or myotube states expressed desmin, muscle-specific actin, and myosin. On the basis of these observations and the calculated percentage of fusion, we estimated that skeletal muscle cells composed approximately 75% of the cell preparations with which our experiments were performed. The cellular yield of the microbiopsy technique was high: 6 primary cultures were obtained from 1 microbiopsy specimen by use of a simplified growth medium without any growth factors, and the 6 cultures were maintained until the eighth passage, yielding a sufficient number of cells for repeated experiments. For the experiments in the present study, we chose to work with cells in the multiplication state (ie, myoblasts) because of their easy handling. Trypsinization and counting of myoblasts are easy procedures, unlike trypsinization and counting of myotubes. Moreover, because cells were in a multiplication state, maintenance of their culture during experiments was uninterrupted.

Another objective of the present study was to establish a method by which equine skeletal myoblasts could be exposed to conditions of anoxia and subsequent reoxygenation to enable investigation of ROS production in the presence or absence of peroxidase enzymes, thereby mimicking muscular inflammatory response. In general, horses are outstanding athletes, and their performance during strenuous exercise is dependent on an optimal muscular function. In the 1970s, an increase in lipid peroxidation after exercise in rats and humans[42,43] was demonstrated. Numerous studies[44–47] have revealed a relationship between oxidative stress and strenuous exercises in horses, and there is evidence that the generation of ROS and nitrogen species can be predominantly attributed to contractions of myocardial and skeletal muscles.[48]

In humans, skeletal muscle oxygenation decreases during exercise, and the magnitude of this response is dependent on exercise intensity.[27,49,50] At the cessation of exercise, the blood flow to hypoxic tissues resumes and results in their reoxygenation; this situation mimics the ischemia-reperfusion phenomenon, which is known to cause excessive production of free radicals.[11,51] The increase of oxygen flow and the subsequent increase of mitochondrial electron flux consequently induce ROS production. In addition to this mechanism, exercise-induced free radical generation may also be regulated by changes in intracellular oxygen pressure, hydrogen ion generation, norepinephrine autoxidation, peroxidation of damaged tissue, and xanthine oxidase activation.[52] We extrapolated the findings in human athletes to athletic horses, and direct assessment of ROS production in equine skeletal myoblast cultures that were exposed to conditions of anoxia and reoxygenation revealed evidence of a significant increase of in ROS, compared with cells maintained in an ambient oxygen atmosphere with 5% CO_2. The increase in ROS production by cells exposed to anoxia and subsequent reoxygenation can be attributed to mitochondrial alterations.[11]

In human athletes, a systemic inflammatory response due to strenuous exercise has been detected.[53] This inflammatory response induces neutrophil degranulation and respiratory burst, with subsequent ROS production as well as an increase in the plasma activity of the neutrophil oxidant enzyme, MPO.[54–57] An increase in plasma MPO activity following exercise has also been identified in horses,[15] and more recently, our group detected increases in counts of neutrophils and concentrations of 2 neutrophil enzymes, MPO and elastase, in plasma and muscles of endurance and eventing horses following competition.[16,19] Furthermore, significant correlations were established between blood elastase and CK activities in horses after intense exercise[16] and between plasma CK activity and muscular MPO and elastase activities in endurance horses.[19] High activities of these 2 enzymes in muscles may contribute to the muscle damage induced during endurance races, as indicated by the high plasma CK activity. Therefore, in the anoxic-reoxygenated cell cultures in the present study, we added MPO to investigate its enhancing effects on ROS production and compared the effects of MPO with those of HRP, another peroxidase that is known to catalyze the reaction between KMB and ROS.[29] We observed that similar increases in ROS production were achieved with the peroxidases, although the concentration of MPO was lower (22.5-fold decrease) than that of HRP. This major catalytic effect could be explained by the equine origin of the MPO, which resulted in increased specificity of the reaction, and by the capacity of MPO to produce hypochlorous acid (a chlorination agent) and other reactive species that are able to perform nitration and nitrosation. Chemical modifications induced by MPO could alter the electron transport chain in mitochondria. Furthermore, after exposure of cells to conditions of anoxia and reoxygenation, peroxidase treatment, and 3 washes, MPO or HRP was present on the surface of the cells, indicative of a strong interaction between the enzymes and the cells. The peroxidases also increased ROS production by the cells that were not exposed to anoxia and reoxygenation, but these increases remained modest, compared with the increases detected in the anoxic-reoxygenated cells.

In horses engaged in strenuous exercise, alterations of mitochondria and inflammatory reaction seem to be localized in muscles. Recently, an investigation[58] by our group highlighted that after an endurance race, active MPO is present in equine skeletal muscle and that its activity increases in association with an increase of plasma CK activity, an indicator of muscle damage. These results reflect findings of recent studies,[16,19] which indicated that the concentrations of 2 inflammatory markers, MPO and elastase, increase both in plasma and muscle and are correlated with an increase in plasma CK activities after an endurance race in horses. Furthermore, in all the horses participating in the endurance race, a significant negative correlation was

found between CK activity and mitochondrial complex I activity and there was evidence of a possible negative relationship between MPO activity and mitochondrial complex I activity, suggesting a potential link of MPO activity with mitochondrial function and muscle damage.[58] In another study[33] by our group, significant alterations in mitochondrial respiratory function in response to endurance training and endurance racing were identified in muscle microbiopsy specimens via high-resolution respirometry.

On the basis of these findings in horses undergoing intense exercise, it appears that damaging conditions combine to induce mitochondrial alterations, perhaps through the action of neutrophils and their degranulated enzymes, which could be responsible for oxidative damage occurring in skeletal muscle. Because mitochondria are one of the most important sources of ROS and because the increase of oxygen flow to hypoxic muscles and the subsequent increased mitochondrial electron flux induce a consequent ROS production, we hypothesized that mitochondria themselves became a target and a vicious circle was initiated (ie, increase in ROS production in parallel with an increase of oxidative mitochondrial damage).[59]

Results of the present study have indicated that the technique of microbiopsy sample collection is less invasive than conventional muscular biopsy procedures, and the use of cultures of equine skeletal myoblasts appears to be a useful experimental system in which to study equine muscle cells in vitro. By use of cultured myoblasts and their exposure to conditions of anoxia and subsequent reoxygenation and the activity of a neutrophil oxidant enzyme, it was possible to investigate the combined effects of neutrophils and anoxia-reoxygenation processes on oxidative stress and potential mitochondrial alterations in muscle tissue of horses. The method described in this report may provide an in vitro tool with which to better understand underlying pathophysiologic mechanisms involved in exercise-induced muscle damage in athletic horses in vivo.

a. PRO-MAG Ultra Automatic Biopsy Instrument and Biopsy Needles, Angiotech Europe, Lausanne, Switzerland.
b. Gibco, Invitrogen, Merelbeke, Belgium.
c. DLF 460R, Clean Air Techniek BV, JA Woerden, The Netherlands.
d. Nunc A/S, Roskilde, Denmark.
e. Diff-Quick, Dade Behring, Anderlecht, Belgium.
f. Axioskoop, Zeiss, Germany.
g. D70, Nikon, Japan.
h. Acros Organic, Geel, Belgium.
i. Merck, Darmstadt, Germany.
j. Roche, Mannheim, Germany.
k. Sigma-Aldrich, Steinheim, Germany.
l. Varian 450-GC Gas Chromatograph, Varian, Sint-Katelijne-Waver, Belgium.
m. Porapak T80/100, Sigma-Aldrich, Steinheim, Germany.
n. Liquid DAB+ Substrate Chromogen System, Dako, Everlee, Belgium.
o. GraphPad Prism, version 5.03, SAS Institute Inc, Cary, NC.

References

1. Poole DC. Current concepts of oxygen transport during exercise. *Equine Comp Exerc Physiol* 2004;1:5–22.
2. Hoppeler H, Weibel ER. Limits for oxygen and substrate transport in mammals. *J Exp Biol* 1998;201:1051–1064.
3. Turrens JF, Alexandre A, Lehninger AL. Ubisemiquinone is the electron donor for superoxide formation by complex III of heart mitochondria. *Arch Biochem Biophys* 1985;237:408–414.
4. Turrens JF. Mitochondrial formation of reactive oxygen species. *J Physiol* 2003;552:335–344.
5. Chance B, Williams GR. Respiratory enzymes in oxidative phosphorylation. *J Biol Chem* 1955;217:383–393.
6. Dröge W. Free radicals in the physiological control of cell function. *Physiol Rev* 2002;82:47–95.
7. Valko M, Leibfritz D, Moncol J, et al. Free radicals and antioxidants in normal physiological functions in human disease. *Int J Biochem Cell Biol* 2007;39:44–84.
8. Serteyn D, Mouithys-Mickalad A, Franck T, et al. La nature chimique et la réactivité de l'oxygène. *Ann Med Vet* 2002;146:137–153.
9. Serteyn D, Pincemail J, Mottart E, et al. Direct approach for demonstrating free radical phenomena during equine post-anesthetic myopathy: preliminary study. *Can J Vet Res* 1994;58:309–312.
10. Schneider N, Mouithys-Mickalad A, Lejeune JP, et al. Synoviocytes, but not chondrocytes, release free radicals after cycles of anoxia/re-oxygenation. *Biochem Biophys Res Commun* 2005;334:669–673.
11. Navet R, Mouithys-Mickalad A, Douette P, et al. Proton leak induced by reactive oxygen species produced during in vitro anoxia/reoxygenation in rat skeletal muscle mitochondria. *J Bioenerg Biomembr* 2006;38:23–32.
12. Brady NR, Hamacher-Brady A, Westerhoff HV, et al. A wave of reactive oxygen species (ROS)-induced ROS release in a sea of excitable mitochondria. *Antioxidants Redox Signal J* 2006;8:1651–1665.
13. Blikslager AT. Treatment of gastrointestinal ischemic injury. *Vet Clin North Am Equine Pract* 2003;19:715–727.
14. Moore RM, Eades SC, Stokes AM. Evidence for vascular and enzymatic events in the pathophysiology of acute laminitis: which pathway is responsible for initiation of this process in horses? *Equine Vet J* 2004;36:204–209.
15. Art T, Franck T, Gangl M, et al. Plasma concentrations of myeloperoxidase in endurance and 3-day event horses after a competition. *Equine Vet J Suppl* 2006;(36):298–302.
16. Lejeune JP, Sandersen C, Votion D, et al. Effect of intensive exercise on plasmatic neutrophil elastase level in eventing and endurance horses. *Equine Vet J* 2010;42(suppl 38):12–16.
17. de la Rebière de Pouyade G, Riggs LM, Moore JN, et al. Equine neutrophil elastase in plasma, laminar tissue, and skin of horses administered black walnut heartwood extract. *Vet Immunol Immunopathol* 2010;135:181–187.
18. Grulke S, Franck T, Gangl M, et al. Myeloperoxidase assay in plasma and peritoneal fluid of horses with gastrointestinal disease. *Can J Vet Res* 2010;72:37–42.
19. Serteyn D, Sandersen C, Lejeune JP, et al. Effect of a 120 km endurance race on plasma and muscular neutrophil elastase and myeloperoxidase concentrations in horses. *Equine Vet J* 2010;42(suppl 38):275–279.
20. Volfinger L, Lassourd V, Michaux JM, et al. Kinetic evaluation of muscle damage during exercise by calculation of amount of creatine kinase released. *Am J Physiol* 1994;266:R434–R441.
21. Urso ML, Clarkson PM. Oxidative stress, exercise, and antioxidant supplementation. *Toxicology* 2003;189:41–54.
22. Ramel A, Wagner KH, Elmadfa I. Correlations between plasma noradrenaline concentrations, antioxidants, and neutrophil counts after submaximal resistance exercise in men. *Br J Sports Med* [serial online]. 2004;38:e22. Available at: bjsportmed.com/content/38/5/e22.full. Accessed MONTH DAY, YEAR.
23. Art T, Kirschvink N, Smith N, et al. Cardiorespiratory measurements and indices of oxidative stress in exercising COPD horses. *Equine Vet J Suppl* 1999;(30):83–87.
24. de Moffarts B, Kirschvink N, Art T, et al. Effect of oral antioxidant supplementation on blood antioxidant status in trained Thoroughbred horses. *Vet J* 2005;169:65–74.
25. Hargreaves BJ, Kronfeld DS, Waldron JN, et al. Antioxidant status and muscle cell leakage during endurance exercise. *Equine Vet J Suppl* 2002;(34):116–121.
26. Williams CA, Kronfeld DS, Hess TM, et al. Comparison of oxidative stress and antioxidant status in endurance horses in three 80-km races. *Equine Comp Exerc Physiol* 2005;2:153–157.

27. Grassi B, Quaresima V, Marconi C, et al. Blood lactate accumulation and muscle deoxygenation during incremental exercise. *J Appl Physiol* 1999;87:348–355.
28. Ji LL. Antioxidants and oxidative stress in exercise. *Proc Soc Exp Biol Med* 1999;222:283–292.
29. Dupont G, Mouithys-Mickalad A, Serteyn D, et al. Resveratrol and curcumin reduce the respiratory burst of *Chlamydia*-primed THP-1 cells. *Biochem Biophys Res Commun* 2005;333:21–27.
30. Mouithys-Mickalad A, Deby-Dupont G, Nys M, et al. Oxidative process in human promonocytic cells (THP-1) after differentiation into macrophages by incubation with *Chlamydia* extracts. *Biochem Biophys Res Commun* 2001;287:781–788.
31. Franck T, Grulke S, Deby-Dupont G, et al. Development of an enzyme-linked immunosorbent assay for specific equine neutrophil myeloperoxidase measurement in blood. *J Vet Diagn Invest* 2005;17:412–419.
32. Snow DH, Guy PS. Percutaneous needle muscle biopsy in the horse. *Equine Vet J* 1976;8:150–155.
33. Votion DM, Fraipont A, Goachet AG, et al. Alterations in mitochondrial respiratory function in response to endurance training and endurance racing. *Equine Vet J* 2010;42(suppl 38):268–274.
34. Ward TL, Valberg SJ, Gallant EM, et al. Calcium regulation by skeletal muscle membranes of horses with recurrent exertional rhabdomyolysis. *Am J Vet Res* 2000;61:242–247.
35. Lentz LR, Valberg SJ, Herold LV, et al. Myoplasmic calcium regulation in myotubes from horses with recurrent exertional rhabdomyolysis. *Am J Vet Res* 2002;63:1724–1731.
36. Greene EA, Raub RH. Procedures for harvesting satellite cells from equine skeletal muscle. *J Equine Vet Sci* 1992;12:33–35.
37. Ericksson S, Welter C, Calkins D, et al. Fusion of equine myogenic satellite cell strains in vitro. *East Or Sci J* 1998;14:10–14.
38. Byrne KM, Vierk J, Dodson MV. In vitro model of equine muscle regeneration. *Equine Vet J* 2000;32:401–405.
39. Nollet H, Deprez P. Hereditary skeletal muscle diseases in the horse. A review. *Vet Q* 2005;27:65–75.
40. Ryan JM, Cobb MA, Hermanson JW. Elbow extensor muscles of the horse: postural and dynamic implications. *Acta Anat (Basel)* 1992;144:71–79.
41. Morgan JE, Partridge TA. Muscle satellite cells. *Int J Biochem Cell Biol* 2003;35:1151–1156.
42. Dillard CJ, Litov RE, Savin WM, et al. Effects of exercise, vitamin E, ozone on pulmonary function and lipid peroxidation. *J Appl Physiol* 1978;45:927–932.
43. Brady PS, Brady LJ, Ullrey DE. Selenium, vitamin E and the response to swimming stress in the rat. *J Nutr* 1979;109:1103–1109.
44. Kinnunen S, Hyyppä S, Lappalainen J, et al. Exercise-induced oxidative stress and muscle stress protein responses in trotters. *Eur J Appl Physiol* 2005;93:496–501.
45. Kinnunen S, Hyyppä S, Lehmuskero A, et al. Oxygen radical absorbance capacity (ORAC) and exercise-induced oxidative stress in trotters. *Eur J Appl Physiol* 2005;95:550–556.
46. Fazio F, Casella F, Gianetto C, et al. Serum homocysteine and oxidative stress evaluation during exercise in horse. *Pol J Vet Sci* 2009;12:169–174.
47. Ducharme NG, Fortier LA, Kraus MS, et al. Effect of a tart cherry juice blend on exercise-induced muscle damage in horses. *Am J Vet Res* 2009;70:758–763.
48. Powers SK, Jackson MJ. Exercise-induced oxidative stress: cellular mechanisms and impact on muscle force production. *Physiol Rev* 2008;88:1243–1276.
49. Belardinelli R, Barstow TJ, Porszasz J, et al. Changes in skeletal muscle oxygenation during incremental exercise measured with near infrared spectroscopy. *Eur J Appl Physiol Occup Physiol* 1995;70:487–492.
50. Bambhani Y, Maikala R, Esmail S. Oxygenation trends in vastus lateralis muscle during incremental and intense anaerobic cycle exercise in young men and women. *Eur J Appl Physiol* 2001;84:547–556.
51. Di Meo S, Venditti P. Mitochondria in exercise-induced oxidative stress. *Biol Signals Recept* 2001;10:125–140.
52. Bailey DM. What regulates exercise-induced reactive oxidant generation: mitochondrial O_2 flux or PO_2? *Med Sci Sports Exerc* 2001;33:681–682.
53. Suzuki K, Totsuka M, Nakaji S, et al. Endurance exercise causes interaction among stress hormones, cytokines, neutrophil dynamics, and muscle damage. *J Appl Physiol* 1999;87:1360–1367.
54. Camus G, Pincemail J, Ledent M, et al. Plasma levels of polymorphonuclear elastase and myeloperoxidase after uphill walking and downhill running at similar energy cost. *Int J Sports Med* 1992;13:443–446.
55. Bury TB, Pirnay F. Effect of prolonged exercise on neutrophil myeloperoxidase secretion. *Int J Sports Med* 1995;16:410–412.
56. Camus G, Poortmans J, Nys M, et al. Mild endotoxaemia and the inflammatory response induced by a marathon race. *Clin Sci (Lond)* 1997;92:415–422.
57. Morozov VI, Pryatkin SA, Kalinski MI, et al. Effect of exercise to exhaustion on myeloperoxidase and lysozyme release from blood neutrophils. *Eur J Appl Physiol* 2003;89:257–262.
58. Franck T, Votion DM, Ceusters J, et al. Specific immuno-extraction followed by enzymatic detection (SIEFED) of myeloperoxidase and mitochondrial complex I in muscular microbiopsies: preliminary results in endurance horses. *Equine Vet J* 2010;42(suppl 38):296–302.
59. Zorov DB, Juhaszova M, Sollott SJ. Mitochondrial ROS-induced ROS release: an update and review. *Biochim Biophys Acta* 2006;1757:509–517.

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