

Sarcoplasmic Reticulum Ca^{2+} -ATPase Activity and Glycogen Content in Various Fiber Types after Intensive Exercise in Thoroughbred Horses

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To find a new parameter indicating muscle fitness in Thoroughbred horses, we examined time-dependent recovery of glycogen content and sarcoplasmic reticulum (SR) Ca^{2+} -ATPase activity of skeletal muscle after intensive treadmill running. Two repeated 50-sec running sessions (13 m/sec) were performed on a flat treadmill (approximately 90% $\dot{V}\text{O}_2\text{max}$). Muscle samples of the middle gluteal muscle were taken before exercise (pre) and 1 min, 20 min, 60 min, and 24 hr after exercise. Muscle fiber type composition was determined in the pre muscle samples by immunohistochemical staining with monoclonal antibody to myosin heavy chain. SR Ca^{2+} -ATPase activity of the muscle and glycogen content of each muscle fiber type were determined with biochemical analysis and quantitative histochemical staining, respectively. As compared to the pre value, the glycogen content of each muscle fiber type was reduced by 15–27% at 1 min, 20 min, and 60 min after the exercise and recovered to the pre value at 24 hr after exercise test. These results indicate that 24 hr is enough time to recover glycogen content after short-term intensive exercise. The mean value of the SR Ca^{2+} -ATPase activity showed a slight decrease (not significant) immediately after exercise, and complete recovery at 60 min after exercise. There were no significant relationship between the changes in glycogen content of each muscle fiber type and SR Ca^{2+} -ATPase. Although further studies are needed, SR Ca^{2+} -ATPase is not a useful parameter to detect muscle fitness, at least in Thoroughbred horses.

Key words: glycogen recovery, sarcoplasmic reticulum Ca^{2+} -ATPase, Thoroughbred

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Introduction

Thoroughbred race horses frequently compete in a race, which they run at the average speed of more than 60 km/hr for several minutes. Such extensive exercise causes muscle fatigue, and the accumulation of fatigue damage raises the risk of injury [22]. Therefore, it is important for Thoroughbred horse trainers to understand muscle conditions after exercise and

recovery time course. Up to now, quantitative evaluation of substrate (e.g. glycogen, ATP) has been used to examine muscle fatigue because exhaustion of substrate is a main reason for muscle fatigue [6]. Furthermore, it is suggested that enzyme activity in sarcoplasmic reticulum (SR) could become a physiological indicator of muscle fatigue, because muscle contraction and relaxation are directly affected by changes in SR Ca^{2+} uptake [33].

Skeletal muscle SR regulates intracellular free Ca^{2+} concentration and plays a key role in muscle contraction and relaxation. Several studies in horse [2,

34], human [4, 10, 15, 31], and rat [11, 28, 36] have demonstrated that exercise-induced muscle fatigue disturbs SR Ca²⁺ handling properties, and the major protein responsible for Ca²⁺ uptake is SR Ca²⁺-ATPase. Exercise-induced changes in Ca²⁺ uptake results mainly from a shift of catalytic activity of SR Ca²⁺-ATPase [11, 17]. However, no consistent results were obtained in studies of the change in SR Ca²⁺-ATPase activity after exercise. Factors that have an impact on SR Ca²⁺-ATPase activity have not been fully elucidated.

Studies have demonstrated a relationship between muscle fatigue and reduction in muscle glycogen during exercise [1, 7, 8, 26]. Other studies have reported that reduction in SR glycogen influences Ca²⁺ uptake and/or SR Ca²⁺-ATPase [13, 14]. However, in Thoroughbred horses, there is little information about muscle glycogen and SR Ca²⁺-ATPase during the recovery period after exercise.

Knowledge about the recovery time course of muscle glycogen and SR Ca²⁺-ATPase is important when we consider training load and/or interval between trainings. However, in horses, there are only two reports [2, 21] that have examined glycogen content and SR Ca²⁺-ATPase activity, and no study has been done to determine the relationship between changes in the SR Ca²⁺-ATPase activity and glycogen content of each muscle fiber type after exercise. Therefore, in this study, we examined the recovery time course of glycogen content of each muscle fiber type and SR Ca²⁺-ATPase activity of skeletal muscle after intensive treadmill running in Thoroughbred horses.

Materials and Methods

Animals, exercise test, and muscle samples

Six male Thoroughbred horses that weighed 476 ± 32 (436–531) kg were used in this study. Before the exercise test, all horses stayed on the pasture in the daytime and were accustomed to running on a treadmill for a short time.

To measure $\dot{V}O_2$ max of each horse, incremental exercise tests were performed on both flat and 10% inclined treadmills. After walking for 2 min at a speed of 1.8 m/sec, the speed was increased to 3.6 m/sec and maintained for 5 additional minutes. Following this warm-up period, the speed was then increased to 6 m/sec for 1 min, to 8 m/sec for 1 min, and continuously increased by intervals of 1 m/sec for 1 min until

exhaustion. We measured O₂ and CO₂ concentrations, temperature and relative humidity (Vice Medical, Chiba, Japan) in open flow system. All instrument signals were stored on a computer with an analog-to-digital converter, and then calculated using a software analysis package (DATAQ Instruments, Akron, OH). The average $\dot{V}O_2$ of the last 15 sec of each running speed was determined a $\dot{V}O_2$ for the speed, and $\dot{V}O_{2max}$ was determined at a leveling off point by regression line analysis. We used the values of $\dot{V}O_{2max}$ during 10% inclined exercise test to calculate the running speed at flat examination because treadmill performance was limited. Although a running speed corresponding to 90% $\dot{V}O_{2max}$ was determined based on the regression line for each horse, the speeds for all horses were predicted within small range (12–14 m/sec). Therefore, we adopted a speed of 13 m/sec as a speed corresponding to 90 % $\dot{V}O_{2max}$ for all horses.

The exercise test consisted of a warm-up (3 min of walking, 1 min of trotting and 3 min of walking), two repeated running tests (50 sec of galloping at 13 m/sec separated by 10 min of walking), and then a cool down (10 min of walking) on a flat treadmill. Muscle biopsy samples were obtained from the middle gluteal muscle (Bergstrom *et al.*, 1967) at the same depth (5 cm) before the exercise test and 1 min, 20 min, 60 min, and 24 hr after the exercise test. A portion of each muscle sample was immediately frozen in liquid N₂ and stored at –80°C for later histochemical and biochemical analyses. The remaining portion of the sample was immediately used for measurement of SR Ca²⁺-ATPase activity.

All procedures used in this study were approved by the Animal Experiment Committee of the Equine Research Institute, Tochigi Branch, Japan

Histochemical analysis

Frozen pieces of the muscle sample were cut with a freezing microtome (Leica CM510) into 8 transverse sections of 8 μ m thickness. Four sections were used for the immunohistochemical assay to identify fiber type and the others were used for Periodic acid-Schiff (PAS) stain to estimate glycogen content.

Based on previous studies [23, 29, 35], four transverse sections were reacted for immunohistochemical analysis with anti-mouse IgG or IgM. The sections were allowed to warm to room temperature and then pre-incubated in normal horse serum in phosphate buffer at 25°C for 10 min. The primary monoclonal antibody was then applied: 1) BA-D5 (1:1000), which specifically labels MHC-I, for the

detection of MHC-I; 2) SC-71 (1:1000), which specifically labels MHC-IIa, for the detection of MHC-IIa; 3) BF-35(1:1000), which specifically labels MHCs-I and IIa, for the detection of MHC-IIx; and 4) BF-F3 (1:1000), which specifically labels MHC-IIb, for the detection of MHC-IIb. The specificity of these monoclonal antibodies has been previously demonstrated in horses [24, 25]. The sections were incubated at 25°C for 180 min, then washed with phosphate buffer five times and reacted with a horseradish peroxidase-labeled secondary antibody (1:1000) at 25°C for 180 min, and then washed with phosphate buffer again. Diaminobenzidine tetrahydrochloride was used as a chromogen to localize peroxidase in all primary antibodies. To avoid interbatch variation, all samples from each horse were processed simultaneously. On the basis of examination of the immunohistochemical staining images, muscle fibers were classified as type I, IIA, IIA/IIX and IIX fibers. The optical density of PAS staining (OD-PAS) and muscle fiber area were measured in at least 25 muscle fibers of type I and IIA/IIX fibers, and 50 muscle fibers of type IIA and IIX fibers. Type I/IIA fibers were excluded from the result, because little of this fiber type existed in all muscle samples (less than 0.1%).

The other sections were reacted for glycogen with the PAS procedure [32]. The sections were incubated in 0.5% periodic acid for 5 min at room temperature (25°C), rinsed in distilled water and placed in Schiff's reagent for 15 min at room temperature. After washing, microscopic images of muscle fibers were obtained by use of a personal computer and image-processing system (Leica DC 100). During the analysis, optical intensity remained constant. To measure the OD-PAS in muscle fibers, luminosity was expressed at 256 Gy. Luminosity was calibrated by use of 4 filters with differing transmissivity (100, 16, 8, and 2%). The OD-PAS were measured in at least 25 muscle fibers of type I and IIA/IIX fibers, and 50 muscle fibers of type IIA and IIX fibers.

Biochemical analysis

SR Ca^{2+} -ATPase activity was measured spectrophotometrically according to the methods of previous studies [19, 30]. Muscle pieces of ~100 mg wet weight were homogenised with 0.9 ml of cold homogenising buffer consisting of 40 mM Tris-HCl and 300 mM sucrose. The assay mixture (pH 7.5) was composed of 20 mM N-2 hydroxyethylpiperazine-N'-2-ethanesulfonic acid, 0.005% (vol/vol) Triton X-100, 1

mM EGTA, 200 mM KCl, 15 mM MgCl_2 , 0.5 mM CaCl_2 , 10 mM sodium azide, 0.4 mM NADH, 10 mM phosphoenol pyruvate, 18 U/ml pyruvate kinase and 18 U/ml lactate dehydrogenase. Homogenates were centrifuged at 3,000 rpm for 10 min at 0°C, and supernatant including SR was isolated as the sample. After the addition of a 20 μl aliquot of the homogenate, the assay mixture was preincubated for 2 min. The reaction was started by adding ATP to give a final concentration of 4 mM and the absorbance signal was recorded for 1.5 min. Finally, the CaCl_2 concentration was increased to 20 mM in order to selectively inhibit SR Ca^{2+} -ATPase activity. The remaining activity was defined as basal ATPase activity. SR Ca^{2+} -ATPase activity was calculated as the difference between total ATPase and basal ATPase activities. During this procedure, the assay mixture was continually stirred and the temperature was maintained at 37°C.

Statistical analysis

Significant differences between the pre-experiment condition and each experimental time point were analysed using a paired t-test (Stat View-J 5.0). In all cases, significance was established at $P < 0.05$. All values were reported as mean \pm standard deviation.

Results

Muscle fiber composition

Table 1 showed muscle fiber composition in the Thoroughbred middle gluteal muscle before the exercise test. Fiber area of Type IIX fiber was the largest in the all fiber types (Type IIX = $5,196 \pm 628$, Type IIA/X = $3,909 \pm 466$, Type IIA = $3,239 \pm 576$, Type I = $2,923 \pm 578 \mu\text{m}^2$). Type IIA and Type IIX fibers were the highest in number and area percentages, respectively ($45.0 \pm 5.1\%$, $45.2 \pm 5.4\%$). There was no type IIB fiber in all muscle. This result is consistent with past study [24]. Although there were some variations (less than 5% and 12% in composition and area, respectively), similar values were obtained in another muscle samples at 1, 20, 60 min and 24 hr after exercise.

Recovery of glycogen content in each muscle fiber type

The time-dependent recovery of glycogen content of each muscle fiber type is represented in Fig. 1. Glycogen content of Type IIX fiber was the highest of all fiber types before exercise test (OD-PAS in Type

Table 1. Mean value of area and composition in each fiber type of the Thoroughbred middle gluteal muscle
Values are means \pm standard deviation.

	Type I	Type IIA	Type IIA/IIX	Type IIX
Fiber area (μm^2)	2,923 \pm 578	3,239 \pm 576	3,909 \pm 466	5,196 \pm 628
Fiber type in percentage number (%)	14.5 \pm 2.7	45.0 \pm 5.1	6.7 \pm 0.9	33.9 \pm 2.7
Fiber type in percentage area (%)	10.7 \pm 1.6	37.4 \pm 6.7	6.7 \pm 0.8	45.2 \pm 5.4

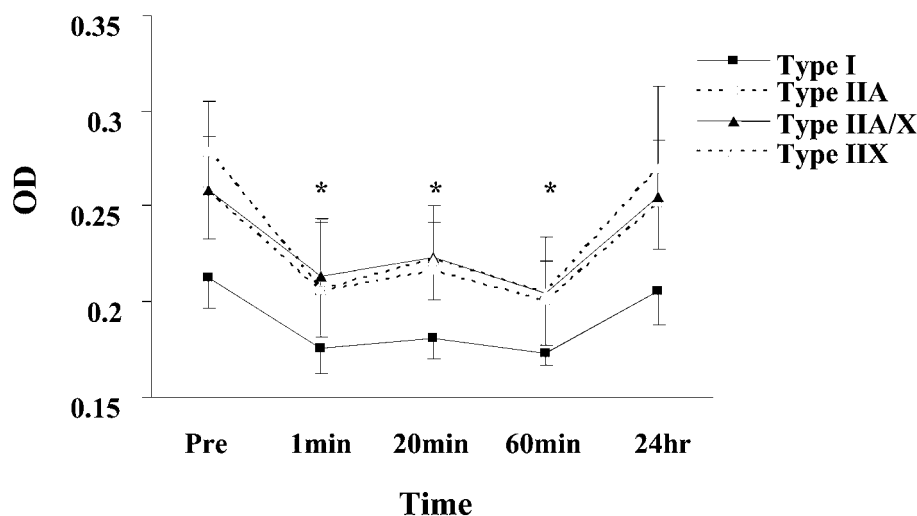


Fig. 1. Glycogen content pre- and post-exercise test in each muscle fiber type of the Thoroughbred middle gluteal muscle. Significant reductions (*: $P < 0.05$) were found at 1 min, 20 min and 60 min after exercise in all fiber types, but not at 24 hr after exercise as compared to pre-exercise values.

IIX=0.28 \pm 0.03, OD-PAS in Type IIA/X=0.26 \pm 0.03, OD-PAS in Type IIA=0.26 \pm 0.02, OD-PAS in Type I=0.21 \pm 0.01). As compared to the pre-exercise value, glycogen content of all muscle fiber types significantly ($P < 0.05$) decreased 1 min after exercise test (Type I=82 \pm 4% Type IIA=79 \pm 5% Type IIA/X=82 \pm 6% Type IIX=74 \pm 7%). Significant glycogen depletion remained 20 and 60 min after the exercise test (Type I=84 \pm 5 and 80 \pm 4%, Type IIA=82 \pm 5 and 77 \pm 4%, Type IIA/X=85 \pm 5 and 78 \pm 5%, Type IIX=79 \pm 6 and 73 \pm 4%). The glycogen content of each muscle fiber type was restored up to the pre-exercise value 24 hr after the exercise test (Type I=97 \pm 8%, Type IIA=98 \pm 9%, Type IIA/X=99 \pm 10%, Type IIX=97 \pm 14%).

Recovery of SR Ca^{2+} -ATPase activity

Changes in SR Ca^{2+} -ATPase activity before the exercise and during the recovery period are shown in Fig. 2. The mean value of SR Ca^{2+} -ATPase activity at pre-exercise, 1 min, 20 min, 60 min and 24 hr were 17.61 \pm

2.02, 16.75 \pm 1.10, 16.87 \pm 1.72, 18.34 \pm 1.68, 18.58 \pm 2.66 $\mu\text{mol}/\text{min}/\text{g}$ muscle, respectively. Although a decreased tendency of SR Ca^{2+} -ATPase activities at 1 and 20 min after the exercise was found, there were no significant differences as compared to the pre-exercise value and SR Ca^{2+} -ATPase activity had returned to pre-exercise value by 60 min after exercise. When focusing on changes of SR Ca^{2+} -ATPase activity in each horse, after the exercise, a decreased tendency of the activities was found in four horses, but an increased tendency of the activities was found in two horses.

Relationship between changes in glycogen content and SR Ca^{2+} -ATPase

Correlations between the changes in glycogen content of each muscle fiber type and SR Ca^{2+} -ATPase activity during the recovery period are shown in Fig. 3. The degree of changes in each parameter at 1 min, 20 min, 60 min, and 24 hr were represented as standardized value based on the pre-exercise value. In

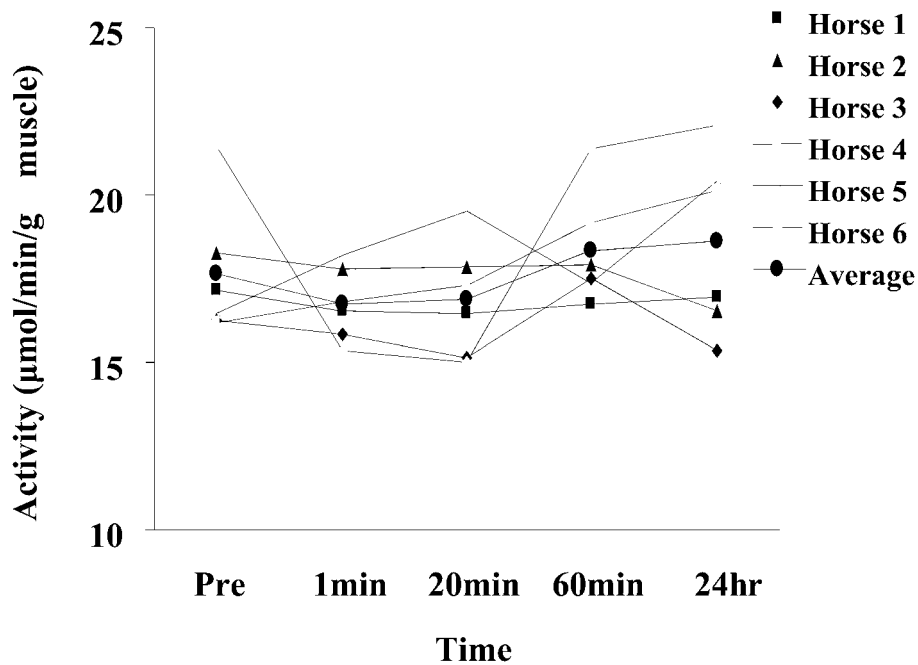


Fig. 2. Sarcoplasmic reticulum (SR) Ca²⁺-ATPase activity of the muscle pre- and post-exercise test in each Thoroughbred. No significant changes were found in the mean value after exercise test as compared to pre-exercise value.

all muscle fiber types, there were no significant correlations between the changes in muscle glycogen content and SR Ca²⁺-ATPase activity.

Discussion

The purpose of this study was to examine time-dependent recovery of glycogen content and SR Ca²⁺-ATPase activity of skeletal muscle after intensive treadmill running in Thoroughbred horses. Furthermore, we examined a relationship between changes in the SR Ca²⁺-ATPase activity and glycogen content of each muscle fiber type. These results might provide information to estimate muscle fatigue.

Past studies [12] have clearly demonstrated that there is a relevant relationship between aerobic exercise and depletion in glycogen. Byrd *et al.* [2] reported that muscle glycogen was decreased by 24% after $\dot{V}O_2$ max running that was performed until fatigue in Thoroughbred horses and a recovery period of 60 min restored this to the pre-exercise value. Pratt *et al.* [21] reported that muscle glycogen was decreased by 44% after increment running (4 m/sec for 60 sec each speed) until fatigue on a 3% slope in Standardbred

horses, and after a 24-hr recovery period the values were only 67% of the pre-exercise values. Willson *et al.* [34] reported that muscle glycogen was decreased by 28% after running at 7–10 m/sec (for 2 min each speed) on a 7% slope in Quarter horses. In the present study, we performed two repeated running periods (each for 50 sec at 13 m/sec on a flat treadmill). Glycogen content was decreased by 18–27% and a recovery period of 24 hr restored glycogen to the pre-exercise values in all fiber types. The results of this and past studies suggest that, in horses, reduction in muscle glycogen might depend on exercise time rather than exercise intensity. It is possible that muscle glycogen contents completely recover at 24 hr after a relatively short exercise time (i.e., exercise giving rise to only a 30% reduction in muscle glycogen) but incompletely recover at 24 hr after a relatively long exercise time (i.e., exercise giving rise to more than a 50% reduction in muscle glycogen).

The mean value of SR Ca²⁺-ATPase activity did not significantly change after exercise. On the other hand, previous studies [3, 13] demonstrated that SR Ca²⁺-ATPase activity and/or Ca²⁺ uptake changed by means of glycogen depletion after electrical stimulation. Lees *et al.* [13] reported that the release of glycogen

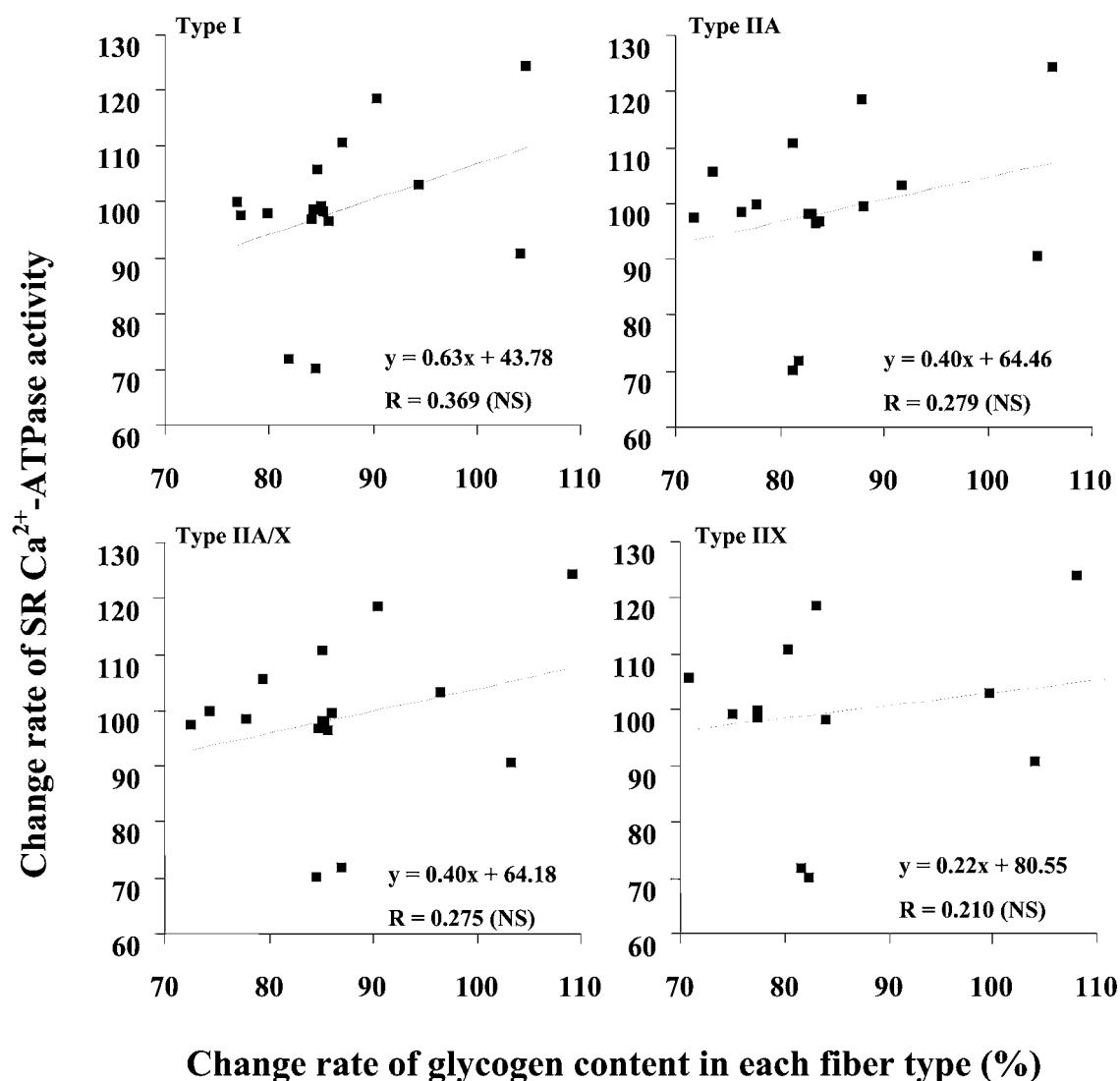


Fig. 3. Relationships between change rates of glycogen content in each fiber type and SR Ca²⁺-ATPase activity. Change rates at 1 min, 20 min, 60 min and 24 hr after exercise are expressed relative to pre-exercise value (%). No significant correlation between glycogen content and SR Ca²⁺-ATPase activity was noted.

phosphorylase from the SR membrane by SR glycogen depletion may exert the effects on the conformation of the ATP binding site of SR Ca²⁺-ATPase. Thus, the change in SR Ca²⁺-ATPase activity could be nearly related to SR glycogen depletion rather than muscle glycogen depletion. In the present study, although glycogen content of each muscle fiber type decreased by 18–27%, we did not measure SR glycogen depletion. However, muscle glycogen depletion of these levels could not cause large glycogen depletion in SR. In fact, Mishima *et al.* [18] reported that 30% reductions in muscle glycogen did not influence SR Ca²⁺-ATPase

activity. These results suggest that muscle glycogen depletion after a short running performed in real training programs has no influence on SR Ca²⁺-ATPase activity in horses.

Because middle gluteal muscle from Thoroughbred horses is composed of more than 80% of Type II fibers, we expected that there is a significant relationship between glycogen content in Type II fibers and SR Ca²⁺-ATPase activity. Previous studies [3, 5, 9] reported that after muscle contraction, SR Ca²⁺-ATPase activity changes in muscle composed of mainly Type II fiber. However, contrary to our expectations, there was no

significant relationship between glycogen content in type II fibers and SR Ca²⁺-ATPase activity. In this study, intensive treadmill running tended to decrease SR Ca²⁺-ATPase activity in four Thoroughbred horses and increase SR Ca²⁺-ATPase activity in two, and the mean value of SR Ca²⁺-ATPase activity did not significantly change. Previous studies also demonstrated inconsistent results in SR Ca²⁺-ATPase activity after exercise including increases [31], decreases [2, 11, 15, 34, 36], or no changes [10, 28]. Various factors that influence SR Ca²⁺-ATPase activity were reported, e.g., increase in phosphorylated (recruited or activated) SR Ca²⁺-ATPase activity [28], activation of SR Ca²⁺-ATPase activity by NO through cGMP mediated signaling cascades [16], increase in SR Ca²⁺-ATPase activity by increasing HSP 72 [20], change in SR Ca²⁺-ATPase isoform [31], decline in pH by increasing lactate [2], sharp rise in body temperature [2], and oxidative damage by increasing reactive oxygen species [27]. Although it is not clear which factors affect SR Ca²⁺-ATPase activity, it is possible that multiple factors are involved.

In summary, the glycogen content of each muscle fiber type was significantly reduced by 15–30% after exercise test and recovered to the pre-exercise value in all muscle fiber types 24 hr after the exercise test. No significant changes were found in the SR Ca²⁺-ATPase activity and there were no significant relationships between the changes in glycogen content and SR Ca²⁺-ATPase during the recovery period. Although further studies are needed, the SR Ca²⁺-ATPase is not a useful indicator to detect muscle fitness, at least in Thoroughbred horses.

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