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Ultrastructural Alterations in Equine Skeletal Muscle Associated with Fatiguing Exercise

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ABSTRACT. This study examined the effects of acute high intensity exercise on the ultrastructure of mitochondria and sarcoplasmic reticulum (SR), and the reversibility of these effects. Thoroughbred horses were run at a speed which elicited maximal oxygen uptake on a high-speed treadmill until fatigued. Muscle temperature and biopsy samples were collected at rest, immediately after exercise and at 30 and 60 min of recovery. When expressed as a percentage of total area, the area occupied by mitochondria and SR increased 3.5 and 1.6-fold respectively in samples collected immediately after exercise. Muscle pH and muscle temperature were 6.55 and 43°C respectively, but approached pre-exercise values by 60 min after exercise. The ultrastructural alterations in mitochondria and SR in the post-exercise biopsies and resolution of these changes in the recovery period are discussed and compared to a previously measured decline in SR function.

Key words. Horses; ultrastructure; fatigue; sarcoplasmic reticulum; mitochondria

INTRODUCTION

Several reports have described alterations in the ultrastructure of muscle and heart, including segmental distension of the sarcoplasmic reticulum (SR) and swelling of the mitochondria with disruption of cristae, after prolonged exercise.[1,17,25] Accompanying this structural disruption is evidence of alterations in SR and mitochondrial function.[6,9,19] Few data are available describing the effects of high-intensity, short-term exercise on skeletal muscle ultrastructure and SR function. The present study quantified the ultrastructural alterations in SR and mitochondria in skeletal muscle before and after a single bout of high intensity exercise. The ultrastructural findings are discussed and compared with a concurrent, previously reported decline in the rate and capacity of Ca^{2+} uptake and Ca^{2+}-stimulated ATPase measured in SR isolates.[7] Results demonstrate an increase in SR and mitochondrial area when expressed as a percentage of the total area measured, after a single bout of exercise to fatigue at the maximum O_2 uptake ($\dot{V}O_2max$).

MATERIALS AND METHODS

Subjects and exercise procedures

Eight Thoroughbred horses, aged 3–6 years, were used in the study. These animals were acclimated to run on a high-speed treadmill before the experiments, and their $\dot{V}O_2max$ measured.[20] $\dot{V}O_2max$ averaged 131.85 ± 12.83 ml kg^{-1} min^{-1}. On the day of the experiment, each horse ran for 5 min at 4 m s^{-1} (~40% of $\dot{V}O_2max$) as an initial warm up. The treadmill velocity was then rapidly increased to the speed that elicited $\dot{V}O_2max$. The horse ran at this speed until it could not keep pace with the treadmill. Heart rate was recorded with a cardiotachometer (PEH horse tester, Finland). Collection of muscle and blood samples. Muscle samples of approximately 1 g were collected from the middle gluteal muscle using the percutaneous needle biopsy technique[2] and

standard surgical procedures. Muscle biopsies were collected at a depth of 8 cm from a 5 cm^2 area approximately 18 cm caudal to the tuber coxae where this axis transected a line joining the tuber sacrale and tuber ischii. One portion of the sample was immediately quenched in liquid N_2 and stored at −80°C prior to metabolite and pH analyses. The remaining portion was minced and placed in fixative for subsequent ultrastructural analysis. After anesthetizing the overlying skin, muscle temperature was measured in the contralateral muscle at the time each muscle sample was collected. This was achieved by inserting a copper Constantan thermocouple (Bailey Instruments IT-14 probe attached to a Bailey Instruments BAT 8 thermometer) into the muscle through an 18-gauge needle. A catheter was placed in the jugular vein and blood samples (~5 ml) for lactate (La) determination were collected at rest and 5 min after exercise. Blood samples were placed in a tube containing potassium oxalate and NaF and kept on ice prior to extraction. Lactate was analyzed according to the method described previously.[7]

Preparation of muscle samples for electron microscopy

Portions of the muscle biopsies were minced into cubes of 0.5 mm and prefixed at 4°C for 3 to 4 hours in a solution of 2.5% glutaraldehyde in 0.05 M cacodylate buffer (pH 7.2–7.4) containing 0.5% dimethyl sulfoxide. They were rinsed twice with 0.1 M cacodylate with 3.5% sucrose (pH 7.2–7.4) and maintained in buffer overnight. Samples were postfixed for 1 to 3 hours in ice cold 1% osmium tetroxide buffered with 0.1 M cacodylate buffer containing 3.5% sucrose (pH 7.2–7.4). After postfixation, tissues were rinsed 3 times in the cacodylate buffer (5 min each), 3 times in distilled water (5 min each), then dehydrated in an ethanol series and embedded in Epon 812. Thin (0.05–0.09 μm) sections were cut, placed on 300 mesh copper grids, and poststained for 1 to 5 min each in uranyl acetate and lead citrate.

Ultrastructural analyses

Tissue sections were cut from one or more blocks of material embedded from each biopsy and the sections were oriented to the myofiber longitudinal axis. A minimum of 10 micrographs were obtained from each sample, with micrographs representing a given block separated by at least 4 grid holes (300 mesh grid). The majority of micrographs were taken at 12000× magnification with a Hitachi H-600 transmission electron microscope. Final magnification of tissue sections was 27000 to 32000×. Areas occupied by myofilaments, sarcoplasm, mitochondria, and SR were measured using Bioquant IV Digitizing Morphometry and Bone Morphometry software/hardware package (R & M Biometrics, Nashville, Tennessee) interfaced with an IBMXT computer, and were expressed as a percentage of total area. Allowance was made for the variation in the degree of contracture between fibers by measuring the sarcomere lengths in each micrograph. This length was then expressed as a percentage of uncontracted sarcomere length as estimated from sarcomere measurements obtained in a series of sections of relaxed fibers from fixed length surgical biopsies. All mitochondrial, SR, fibrillar, and interfibrillar areas were then adjusted to represent a percentage of the total area based on the average length of the sarcomeres present in an individual micrograph. Original (unadjusted) and adjusted measurements were recorded and analyzed.

RESULTS

Exercise time to fatigue

Run time to fatigue at 100% of VO_2max was 4.65 ± 1.06 (SD) min and heart rate increased from 35 beats min^{-1} at rest to 211 during maximal exercise.

Muscle and blood metabolites and muscle temperature and pH

There was no change in the concentration of adenosine triphosphate ([ATP]) of the mus-

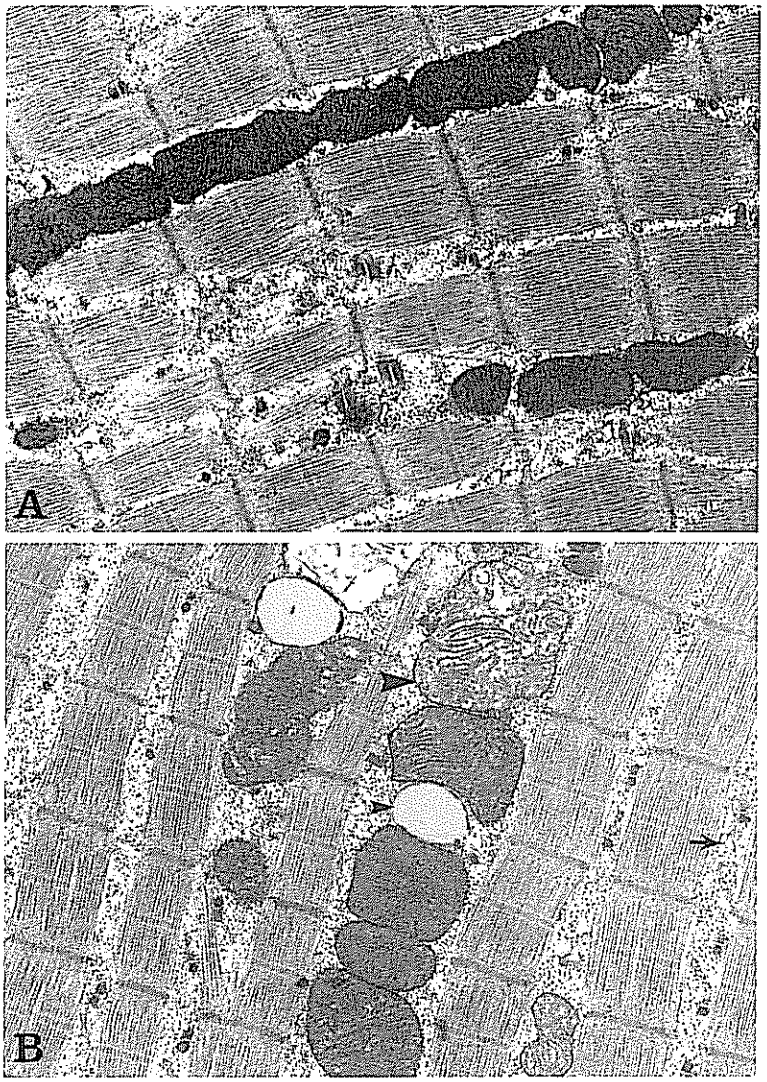

Fig 1. Electronmicrographs of portions of equine middle gluteal muscle biopsies collected prior to (A) and upon cessation of high intensity exercise (B). Note the varying degrees of dilation in mitochondria (large arrowhead). SR (arrow); lipid droplet (small arrowhead). Magnification ×27000.

cle at fatigue. The [creatine phosphate] (PCr) was 36% lower and the [glucose-6-phosphate] (G-6-P) of muscle nearly fourfold greater ($p<0.01$) than control values immediately after exercise, but both values were normal after 60 min of recovery. The blood [lactate] (La) increased from 1.59 at rest to 16.84 mol l^{-1} 5 min after exercise ($p<0.01$). Muscle pH declined from 7.00 at rest to 6.52 upon cessation of exercise ($p<0.01$) while muscle [La] increased from 0.15 mol mol^{-1} TCr in the pre-exercise samples to 0.75 mol mol^{-1} TCr at the end of exercise ($p<0.01$). The muscle [La] and pH returned toward rest values by 60 min of recovery (0.34 mol mol^{-1} TCr and 6.92 respectively). Muscle temperature rose from 37°C at rest to 43.0°C ($p<0.01$) immediately after the exercise bout and had returned to pre-exercise values by 60 min after exercise.

Ultrastructural analysis

The morphological changes noted in muscle sections were similar for all subjects. Most

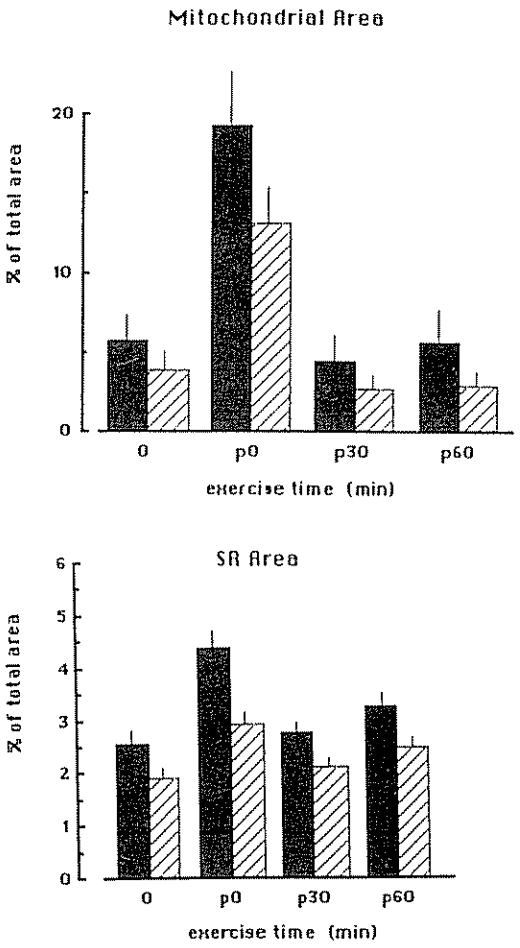

Fig. 2 Adjusted (□) and unadjusted (■) mitochondrial and SR areas measured in biopsies obtained at rest (O), immediately after exercise (PO), and at 30 (P30) and 60 min (P60) post-exercise expressed as a percentage of the total area measured

notably, mitochondria in the samples collected after the exercise were increased in size and had undergone substantial alterations in shape (Fig. 1). Mitochondria appeared intact but were easily distinguished as they were rounded and individual cristae were more prominent. The swelling in mitochondria often was not uniform, with adjacent mitochondria demonstrating marked variation in the degree of expansion of the inner compartment. There was also extensive, intermittent distension of longitudinal SR and swelling of the lateral sacs of the triads. Quantitatively, SR area increased 1.6-fold in all post-exercise samples whereas the area occupied by the mitochondria was increased 3.5-fold. Distended mitochondria occupying the interfibrillar spaces often obscured portions of the SR.

The dilation of SR and mitochondria diminished in all recovery biopsies. All variables measured were not significantly different from rest values by 60 min of recovery (Fig. 2).

DISCUSSION

The major findings of this study were substantial changes in the ultrastructure of the SR and mitochondria in skeletal muscle after a single bout of high intensity exercise. Although alterations in equine skeletal muscle ultrastructure have been reported previously, this was an attempt to accurately quantitate these changes. The ultrastructural changes paralleled the previously reported depression in SR function present in the same biopsies.[7] In this earlier report,[7] SR was isolated from portions of each of the biopsies. The initial rate and maximal capacity of Ca^{2+} uptake in SR declined to 57 and 49% of rest values, respectively, while the total ATPase activity was diminished by ~40% immediately after exercise. The presence of concurrent functional and structural alterations is further supported by the reports of 50% depression in mitochondrial respiration in muscle homogenates from horse.[11]

By 30 and 60 min of recovery, ultrastructure resembled that in rest samples. This recovery was similar to the return of normal function in SR[7] and mitochondria.[11] The apparent close association between the rate of functional and structural deterioration and of normalization during recovery agrees with changes observed in the myocardium after ischemia and reperfusion.[21] The return of normal ultrastructure preceded functional and metabolic recovery following fatiguing exercise[7,11] and ischemia.[21]

Reports of ultrastructural changes in skeletal and cardiac muscle mitochondria have produced considerable controversy. In fact, it has been suggested that all exercise-induced ultrastructural changes are fixation artifact.[10,15,22] Part of this controversy may be the result of dissimilar exercise protocols. One purpose of this study was to determine whether or not the observed ultrastructural changes were due to fixation artifact. Several procedures including sample preparation, fixation techniques, and alterations in temperature, pH, and buffer osmolality were tested to determine their effect on the quality of the electron micrographs produced.

The most convincing evidence that some ultrastructural changes may be artifactual is that changes depended on the method of fixation.[3,10,24] In a study of methodology, Gale[10] noted that there were ultrastructural changes in post-exercise biopsies fixed in veronal-buffered osmium that were absent when the samples were treated with a glutaraldehyde and osmium tetroxide fixation technique. Although this indicates varying sensitivity of the subcellular structures to different fixatives, the fact that there were differences between samples from the non-exercised control animals and those that had exercised is evidence that some change has been produced by the exercise.

After examining several fixation procedures, a method similar to that described by Gale[10] was used in the present study. One difference was the inclusion of a small quantity of dimethyl sulfoxide added to the glutaraldehyde fixative to aid penetration without altering ultrastructural appearance. Gale[10] reported that the combination of glutaraldehyde and osmium tetroxide postfixation produced no swelling in mitochondria after exhaustive exercise. However, unlike Gale's findings the number of dilated mitochondria in this study was increased in the post-exercise samples. This change was reversible because alterations were not present in the recovery samples. Mitochondrial swelling and dilation of SR were also a consistent post-exercise finding in numerous other studies including prolonged, exhaustive exercise,[25,23] eccentric exercise in man,[8] and short term, high intensity exercise in the horse.[17] If the changes in mitochondria and SR immediately post-exercise are indicative of membrane perturbations, such as altered lipid composition, these changes are reversible and do not affect the ultrastructure of these organelles in the recovery samples.

One explanation for the post-exercise swelling of mitochondria and SR is that their sensitivity to fluid or ion shifts during exercise alters their response to fixatives. This is plausible as mitochondrial osmotic pressure is sensitive to relatively small fluctuations in buffer osmolality.[13,18] However, such ultrastructural alterations are minimal in lower intensity exercise prior to fatigue when sufficient time should have elapsed to allow fluid or ion shifts to occur.

There are many studies describing similar ultrastructural changes in ischemic myocardium[5,16] and in a number of tissues exposed to hyperthermia.[4] One of the best descriptions of the alterations with myocardial ischemia is that of Schaper et al.[21] where the time course of changes in myocardial ultrastructure and cardiac function with ischemia and during reperfusion was followed. The early ischemic changes in the cardiac muscle resembled those in the present study. Further, when reperfused, the return of normal ultrastructure was time dependent and varied as a function of the ischemic period. With a short period of ischemia, ultrastructure was normal within a period of 30 min. Similarly, Herdson et al.[14] reported that in hearts subjected to temporary coronary occlusion ultrastructural changes were evident in the ischemic myocardium which were not evident in nonischemic areas of the same heart. These findings argue against changes in mitochondrial or SR ultrastructure being entirely the result of a fixation artifact. In the present study there was also resolution of ultrastructural alterations in the recovery samples accompanied by a normalization of muscle temperature and pH. As the ultrastructural changes were not evident in rest-

ing or recovery samples that were treated identically to those collected after the exercise, it is unlikely that either post-exercise or post-ischemia alterations in mitochondria and SR were artifactual. A similar conclusion can be reached for the changes observed in this study.

The data from this study indicates alterations in substrate concentrations, elevations in muscle temperature and blood and muscle [La], and a substantial decline in muscle pH were present following the fatiguing exercise. Although it is possible to speculate as to the factors which produced the ultrastructural alterations, it is not clear whether the mechanisms involved in the production of structural changes are also responsible for SR dysfunction. The effect of hyperthermia should be considered as it is one factor common to prolonged and high intensity exercise. Incubation of SR and mitochondria at high temperature diminishes function and could affect muscle ultrastructure by altering membrane fluidity and permeability.[4,12] However, it is unlikely that any single element is responsible for the structural and functional changes that occur in skeletal muscle during fatiguing exercise. It is more conceivable that several changes occur, all of which contribute to the observed changes in structure and function. If the ultrastructural changes result from alterations in membrane lability incurred by changes in substrates, pH, or temperature, then the increased areas measured may reflect an increased sensitivity to fixatives and buffers. If this is construed as an artifact, then it is a measurable and representative artifact which is not evident in recovery samples.

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REFERENCES

1. Banister, E. W., Tomanek, R. J. and Cvorkov, N. (1971) Ultrastructural modifications in rat heart: responses to exercise and training. Am. J. Physiol. 220, 1935–1940
2. Bergström, J. (1962). Muscle electrolytes in man. Scand. J. Clin. Lab. Suppl. 68, 11–100
3. Bowers, W. D. Jr, Hubbard, R. W., Smoake, J. A., Daum, R. C. and Nilson, E. (1974). Effects of exercise on the ultrastructure of skeletal muscle. Am. J. Physiol. 227, 313–316
4. Buckley, I. K. (1972) A light and electron microscopic study of thermally injured cultured cells. Lab. Invest. 26, 201–209
5. Burdette, W. J. and Ashford, T. P. (1965). Structural changes in human myocardium following hypoxia. J. Thorac. Cardiovasc. Surg. 50, 210–217.
6. Byrd, S. K., Bode, A. K. and Klug, G. A. (1989). Effects of exercise of varying duration on sarcoplasmic reticulum function. J. Appl. Physiol. 6, 1383–1389
7. Byrd, S. K., McCutcheon, L. J., Hodgson, D. R. and Gollnick, P. D. (1989). Altered sarcoplasmic reticulum function after high intensity exercise. J. Appl. Physiol. 67, 2072–2077
8. Friden, J. (1984) Changes in human skeletal muscle induced by long-term eccentric exercise. Cell Tissue Res. 236, 365–372
9. Fitts, R. H., Courtright, J. B., Kim, D. H. and Witzmann, F. A. (1982) Muscle fatigue with prolonged exercise: contractile and biochemical alterations. Am. J. Physiol. 242 (Cell Physiol. 11), C65–C73.
10. Gale, J. B. (1974). Mitochondrial swelling associated with exercise and method of fixation. Med. Sci. Sports 6, 182–187
11. Gollnick, P. D., Bertocci, L. A., Kelso, T. B., Witt, E. H. and Hodgson, D. R. (1990). The effect of high intensity exercise on the respiratory capacity of skeletal muscle. Pflügers Arch. 415, 407–413
12. Gwozdz, B., Dyduch, A., Grzybek, H. and Panz, B. (1978) Structural changes in brain mitochondria of mice subjected to hyperthermia. Exp. Path. Bd. 15, 124–126.
13. Hayat, M. A. (1981). Factors affecting the quality of fixation. *In Fixation for Electron Microscopy*. Academic Press, New York
14. Herdson, P. B., Sommers, H. M. and Jennings, R. B. (1965). A comparative study of the fine structure of normal and ischemic dog myocardium with special reference to early changes following temporary occlusion of a coronary artery. Am. J. Pathol. 46, 367–386
15. Hoppeler, H. (1986). Exercise-induced ultrastructural changes in skeletal muscle. Int. J. Sports Med. 7, 187–204.
16. Miller, D. R., Rasmussen, P., Klionsky, B., Cossman, F. P. and Allbritten, F. F. (1961) Elective cardiac arrest: Its effect on myocardial structure and function. Ann. Surg. 154, 751–763

17 Nimmo, M. A. and Snow, D. H. (1982) Time course of ultrastructural changes in skeletal muscle after two types of exercise. J. Appl. Physiol. 52, 910–913.
18 Pexeider, T. (1977) The role of buffer osmolarity in fixation for SEM and TEM. Experientia 32, 806–814.
19 Pierce, G. N., Kutryk, M. J. B., Dhalla, K. S., Beamish, R. E. and Dhalla, N. S. (1984) Biochemical alterations in heart after exhaustive swimming in rats. J. Appl. Physiol. 57, 326–331.
20 Rose, R. J., Hodgson. D. R., Kelso, T. B., McCutcheon. L. J., Reid, T.-A., Bayly, W. M. and Gollnick, P. D. (1988) Maximal O_2 uptake, O_2 debt and deficit, and muscle metabolites in Thoroughbred horses. J. Appl. Physiol. 64, 781–788.
21 Schaper, J., Mulch, J., Winkler, B. and Schaper. W. (1979) Ultrastructural, functional, and biochemical criteria for estimation of reversibility of ischemic injury: a study on the effects of global ischemia on the isolated dog heart. J. Mol. Cell. Cardiol. 11, 521–541.
22 Terjung, R. L., Klinkerfuss, G. H., Baldwin, K. M., Winder, W. W. and Holloszy, J. O. (1973) Effect of exercise on rat heart mitochondria. Am. J. Physiol. 225, 300–305.
23 Thomas, D. P. and Marshall, K. I. (1988) Effects of repeated exhaustive exercise on myocardial subcellular structures. Int. J. Sports Med. 9, 257–260.
24 Tomanek, R. J. and Banister, E. W. (1972) Myocardial ultrastructure after acute exercise stress with special reference to transverse tubules and intercalated discs. Cardiovasc. Res. 6, 671–679.
25 Warhol, E. R., Siegel, A. J., Evans, W. J. and Silverman, L. M. (1985) Skeletal muscle injury and repair in marathon runners after competition. Am. J. Pathol. 118, 331–339.

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Carnosine Accounts for Most of the Variation in Physico-Chemical Buffering in Equine Muscle

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ABSTRACT The ability of muscle to buffer protons may be a key determinant of sprint performance. Buffering capacity determined by titration (Bm_{titr}, mmol H^+ kg^{-1} d.m.) between the pH limits of 7.1 to 6.5, carnosine content (carn., mmol kg^{-1} d.m.) and % fibre section area were measured in muscle taken from up to six different sites in the middle gluteal muscle of four Thoroughbred horses and one pony at post mortem. Bm_{titr} and carnosine were positively correlated to % type II fibre section area ($r=0.57$, $p<0.01$ and $r=0.75$, $p<0.001$ respectively). Multiple linear regression analysis was used to estimate the carnosine content of individual fibre types when present at 100%. Type I fibres appear to have a low carnosine content whilst type IIa and IIb fibres would appear to have a similar carnosine content. The variability in Bm_{titr} was accounted for almost exclusively through variability in the specific buffering of carnosine (Bm_{carn}) calculated from the Henderson-Hasselbach equation. Non-carnosine buffering ($Bm_{non\text{-}carn}$) was constant at all fibre compositions.

Key words: Acidosis; horses; muscle fibres; middle gluteal.

INTRODUCTION

Sprinting mammals perform high intensity exercise of short duration resulting in the production and accumulation of large amounts of lactic acid. The energy providing pathways incur a rapid turnover of ATP, an accumulation of protons, and a decrease in intracellular pH. The buffering of protons occurs through the dynamic processes of bicarbonate exportation and proton loss into the blood, and within the muscle through phosphocreatine hydrolysis[8] and physico-chemical (static) buffering by proteins, dipeptides and inorganic phosphate. The ability of muscle to buffer protons may be a key determinant of sprint performance.[12]

The histidine dipeptides carnosine, anserine and ophidine have pKa values in the range 6.8 to 7.1, making them effective physico-chemical buffers over the normal physiological pH range. Based on their content in the muscle of various species, Davey[4] concluded that these dipeptides can contribute as much as 40% to the physico-chemical buffering in resting muscle. The higher content of histidine dipeptides in the muscles of animals exposed to prolonged periods of hypoxia, and those adapted for high speed running, suggests an adaptive response to the adverse effects of acidosis. In a comparison of the Thoroughbred horse, greyhound dog, and man, Harris et al.[7] concluded that the higher physico-chemical buffering (Bm_{titr}) in the horse and dog, compared with man, was due to the higher muscle content of histidine dipeptides.

In the present study, we have investigated the variability of carnosine with fibre type within the middle gluteal muscle of the horse and examined its contribution to muscle buffering capacity (Bm_{titr}).

MATERIALS AND METHODS

The left or right middle gluteal muscle of four Thoroughbred horses and one pony was