Postnatal muscle fibre composition of the gluteus medius muscle of Dutch warmblood foals; maturation and the influence of exercise

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Summary

The fibre type composition of the deep gluteus muscle was studied in biopsies of Dutch warmblood foals from birth until 48 weeks of age. Half the foals were given box rest, the other half received exercise consisting of an increasing number of gallop sprints. The muscle fibre types were determined using monoclonal antibodies discriminating against the following myosin heavy chain (MHC) isoforms: type I, Ila, IId, Cardiac-α and Developmental. During the first 48 weeks there was a consistent increase of fibres expressing Ila MHC, replacing fibres expressing IId MHC. This change was reflected in the presence of a quite large population of fibres co-expressing MHC Ila and IId. The difference between the exercised (training-) and non-exercised (boxrest-) group was small but suggested that the increase of Ila fibres was larger in the training group. It appeared that after birth a significant number of fibres co-express either Developmental and type Ila-MHC or Cardiac-α and type I-MHC. The Developmental isoform disappears during the first 10 weeks after birth and almost all the α isoform expression during the first 22 weeks. It is concluded that a fast turnover of fibre types takes place in the deep gluteus medius in the first months of life. Potentially, exercise could have an effect on the rate of change of these fibre types.

Keywords: horse, fibre type, immunohistochemistry, growth, myosin heavy chain, cardiac myosin
Introduction

Mammalian skeletal muscle fibres can be subdivided into different types. Histochemical staining for mATPase after acid and alkaline preincubation identifies type I, IIA and IIB fibres with increasing ATPase activity and speed of contraction (Brooke and Kaiser, 1970; Bárány, 1967). Another classification system (slow oxidative, fast oxidative/glycolytic and fast glycolytic) was used by Peter et al. (1972) in which the mATPase stain was combined with oxidative and glycolytic enzyme activities in muscles of guinea pigs and rabbits. Their study showed a correlation between these fibre types and oxidative capacity. The latter is indicative for resistance against fatigue. Although in most small animals the I and IIA fibres are highly oxidative and the IIB fibres have little oxidative capacity, this relationship does not hold for all muscles and species. For example, it is well known that high oxidative capacity also can be found in IIB fibres in muscles of both humans, horses and wild animals.

With help of monoclonal antibodies against myosin heavy chain (MHC), the main determinant of contraction speed, type I, IIA and IIB fibres were shown to express specific isoforms of MHC, e.g. MHC I, IIa and IIb. However, this correlation is not perfect. Some fibres designated as IIB with the ATP-ase method seem to express both IIa and IIb MHC’s and nearly all type IIA/B fibres contained only type IIa MHC isoform (Danieli-Betto et al. 1986; Rivero et al. 1996a). Furthermore, various other MHC isoforms were discovered in skeletal muscle, including type IId (or IIx) (Bár and Pette 1988, Schiaffino et al. 1989, Gorza 1990) and cardiac-α (Bredman et al. 1990). In fetal and muscle fibres from newly born individuals, the MHC isoform Developmental is expressed by the genome (d’Albis et al. 1986; Butler-Browne et al. 1988). Multiple expression of MHC’s is the rule in some muscles (Biral et al. 1988), but occurs particularly during growth and after a change in training regimen (Termin et al. 1989; Talmadge et al. 1995). All the isoforms differ in their ATP-ase reaction speed and consequently engender differences in sarcomere contraction speed. Contraction speed increases and oxidative capacity usually decreases in the order: I, cardiac-α, IIa, IId and IIb. The position of the Developmental type in this order is yet unknown.

In the horse, there has always been a great interest in the muscle fibre composition and the effect of exercise on muscle properties. Most of these studies were performed on adult or young adult animals. They show that trained horses have a larger proportion of type I and type IIA and a smaller proportion of type IIB fibres in the locomotory muscles (e.g. Henckel, 1983; Lovell and Rose, 1991).

In the first months after birth, mammalian muscle fibres undergo changes in innervation and MHC expression, so it can be expected that external factors like the amount of exercise in this period may have a strong effect on the muscle fibre composition and hence on the capacity for later athletic performance as it may create better possibilities for further adaptation during the conventional training period later in life.
This study is the first to describe the expression of MHC isoforms in the gluteus medius muscle of Dutch warmblood horses during the first year of life and the effect of exercise on this MHC expression.

Materials and methods

Foals
The investigation was performed in a group of 16 Dutch warmblood foals. They were bred, raised and trained for an experiment focusing on the effects of exercise at a very early age on the development of the equine musculoskeletal system (van Weeren, P.R. and Barneveld, A. (submitted). All foals were individually housed with the mare in 3 x 3.5 m box stalls. They were fed freshly cut grass ad libitum, along with the mother’s milk. One week after birth (day 7) the group was divided in 2 subgroups (boxrest and training group). The boxrest group consisted of 4 males and 4 females (mean weight at birth: 51 kg; at 22 weeks: 271 kg; at 48 weeks: 376 kg) and the training group of 2 males and 6 females (mean weight at birth: 53 kg; at 22 weeks: 256 kg; at 48 weeks: 367 kg).

Exercise protocol
The boxrest group was kept in the box stall for 24 h/day. The training group was kept in box stalls of the same size, but was given an increasing number of gallop sprints in a paddock of 48 x 15 m with a concrete floor covered by a sandy top layer. The exercise was given by two persons at the far ends of the paddock who chased the mares in between them. The foals would follow the mares. Exercise started the day when they were allotted to the training group (day 7) and consisted of 12 sprints. From day 8 the number of sprints was increased to 16 which remained so till day 24. From day 25 to day 38 they made 24 sprints and from day 39 till weaning at 5 months 32 and 16 sprints on alternating days. Exercise was given 6 days a week from Monday to Saturday. After weaning, the foals were joined in one single group which was kept in a loose house with access to a small paddock. None of these foals was trained, so all got the same exercise regimen.

Muscle biopsies
From each foal percutaneous muscle biopsies were taken by the same person, according to Lindholm and Piehl (1974) in the first week after birth (day 3 on average) and at the age of 2, 4, 8 and 22 weeks. From four horses (3 from the boxrest group, 1 from the training group) biopsies were also taken at the age of 48 weeks. The biopsies were taken from the deep gluteus medius muscle on an imaginary line drawn from the coxal tuber to the sacral tuber, at one third distance from the sacral tuber, perpendicular to the skin. They were taken as deeply as possible (until resistance from the iliac wing). Duplicate biopsies were not taken because in earlier studies the analysis of variance of gluteus medius muscle biopsies showed that the interindividual variation was greater than the intra-individual variation (Snow, 1983). The samples were rolled in talcum powder, mounted on cork blocks with the
use of OCT embedding medium and oriented in such a way that the fibres could be sectioned transversely. All samples were stored at -80°C.

**Antibodies**

To identify the fibre types according to their MHC content we used specific monoclonal antibodies (Mabs). They were prepared according to the procedure of Fazekas de St. Groth and Scheidegger (1980). Mab 249-5A4 (raised against human atrial myosin) reacts with cardiac-α MHC (de Groot et al. 1989; Wessels et al. 1991). Mab 219-1D1 (raised against chicken heart myosin) reacts with type I (Wessels et al. 1991; Bredman et al. 1991). Mab 332-3D4 (raised against rabbit eye muscle) reacts with type Ila and IIId. Mab 333-7H1 (raised against protein extract from muscle tissue of adult rabbit anterior tibialis) reacts with type Ila (Bredman et al. 1991). Mab 412-R1D5 (raised against myosin isolated from rabbit psoas muscle) reacts with type I, IIId and α (Bredman et al. 1991). Mab Developmental (raised against hind limb muscle of 7 days old rats (Novocastra Labs. Ltd., Newcastle upon Tyne, UK) recognises a Developmental type MHC present during the embryonic and neonatal period in the development of muscle (Butler-Browne et al. 1988).

**Immunohistochemical staining**

Transverse serial sections (10 µm) were made with a cryostat at -20°C. Overnight fixation took place at -20°C in a 35% methanol, 35% acetone and 5% acetic acid solution. The slides were rinsed in 0.01 M phosphate buffered saline (PBS; pH 7.4) followed by incubation in pronase (1:100 in PBS) for 30 minutes. After rinsing in PBS the slides were incubated in Teng-T (100 mM Tris, 50 mM EDTA, 1.5 M NaCl, 2.5 % gelatine and 0.5 % Tween 20; 1:10 in aqua dest.; pH 8.0) for at least 15 minutes, followed by rinsing in PBS and incubation overnight at room temperature with the Mabs at a dilution of 1:10 in PBS. After this incubation the slides were rinsed with PBS and then incubated for 90 minutes with a biotinylated horse anti mouse polyclonal antibody (1:100 in PBS; ABC-peroxidase staining kit Elite, Vector Labs., Burlingame, USA). The slides were again rinsed in PBS and subsequently incubated for 90 minutes with the components avidin (A; 1:100 in PBS) and biotin (B; 1:100 in PBS) of the ABC staining kit. Both components (A and B) were mixed at least 30 minutes before use. After rinsing the immunoreaction was visualised by incubation with 0.05 % 3,3'-diaminobenzidine tetrachloride (Sigma Chemical Co., St. Louis, USA) in 30 mM imidazole (Janssen Chemica, Beerse; Belgium) and 0.09 % H2O2 (Merck, Darmstadt, Germany). The slides were subsequently stained with Harris hematoxylin for 45 seconds.

This protocol was followed for the Mabs 249-5A4, 219-1D, 332-3D4 and 333-7H1. For Mab 412-R1D5 basically the same protocol was followed. The difference is that no pronase was used, the dilution of Mab 412-R1D5 was 1:25 and instead of imidazole, di-ammonium nickelsulphate (2.5 % in acetate buffer) was used. Before visualisation with 3,3'-diaminobenzidine tetrachloride the slides were rinsed in 0.1 M acetate buffer (pH 6.0; 5-10 minutes) and were stained in Harris for only one second.
For Mab Developmental the protocol differed at the following points: no fixation took place and the procedure started with the incubation with the Mab Developmental at 37 °C for 60 minutes (dilution 1:20 in PBS). From here the procedure was the same as for Mab 412-R1D5.

**Analyses**

In the sections, a group of at least 200 contiguous fibres (Snow and Guy, 1980) were used for fibre typing and calculation of fibre type composition. The muscle fibres were classified into type I, type IIa, type IId, type IIa/d and fibres also containing Cardiac-α or Developmental MHC isoforms on basis of their reactions with the Mabs.

Analysis of variance (ANOVA) was used to test the effects of age and exercise. Thereby, time was used as within subject factor and exercise as between subject factor. Only data up to 22 weeks were used for this analysis. The data from the 48 weeks old foals were used to demonstrate the continuity of the trends.

**Results**

**Muscle fibre characterisation**

The monoclonal antibodies against MHC used in this study allowed us to distinguish seven frequently occurring fibre types in the deep gluteus medius muscle of the juvenile horses (Table 1). Like Rivero et al. 1996b), we could not find IIb fibres using immunohistochemical methods. Using combined ATPase- and immunohistochemistry, ATPase IIB fibres appeared to contain MHC IId. In addition to the types mentioned in table 1 a few rarer (< 3 %) mixed fibre types were observed.

**Table 1. Fibre types in the equine gluteus medius muscle and their reactions with the panel of monoclonal antibodies that was used in this study.**

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Figure 1 illustrates the results of the immunohistochemical staining. Figure 1A shows the reaction with Mab 332-3D4 and demonstrates an overview of the fibre types occurring in this section. Fibres reacting positively with Mab 219-1D1 (Fig. 1B) were identified as fibres expressing MHC type I. Fibres reacting positively with 249-5A4 (Fig. 1C) were identified as fibres expressing MHC type α and are usually fibres also expressing type I (83 %) and in some cases IIa MHC. Fibres reacting positively with 333-7H1 (Fig. 1D) and negatively with 412- R1D5 (Fig. 1E) were identified as fibres expressing MHC
Fig. 1. Results of immunohistochemical staining. Transverse serial sections (10 µm) of a biopsy from the deep part of the gluteus medius muscle of a Dutch warmblood foal of 4 days old. A: Mab 332-3D4; B: Mab 219-1D1; C: Mab 249-5A4; D: Mab 333-7H1; E: Mab 412-R1D5; F: Mab Developmental. Original magnification: ×601.4 (bar = 16.63 µm).
Fig. 2. Scatterplot of fibre type IIa (A) and IIId (B) during aging (in weeks). Each marker is a biopsy taken from one horse. Line (–) markers are from the boxrest and cross (×) markers are from the training group.
Fig. 3. Mean frequencies of fibre type IIa (●), type IIId (■) and type IIa/d (▲) during aging (in weeks) from the boxrest (--- - - - -) and the training group (---). *p < 0.05.

Fig. 4. Mean frequencies (+/- SEM) of fibres expressing Developmental MHC during aging (in weeks) from the boxrest (--- - - - -) and the training group (---).
Fig. 5. Mean frequencies (+/- SEM) of type I/α and type I fibres during aging (in weeks) from the boxrest (---) and the training group (———).
type IIa, the ones reacting positively with 333-7H1 and 412- R1D5 were identified as fibres expressing MHC type IIa/d and the ones reacting positively with 412- R1D5 and 332-3D4 but negatively with 333-7H1 and 219-1D1 were identified as fibres expressing MHC type IId. Fibres reacting positively with Mab Developmental (Fig. 1F) were identified as fibres expressing MHC type Developmental and are usually fibres also expressing type IIa (53.3 %) or type IIa/d MHC (38.6 %).

Muscle fibre development

Fast fibres The frequencies of type IIa and IId fibres in individual biopsies of the deep gluteus medius muscle in the two groups of foals are plotted in Figure 2. The figure shows the range of interindividual variation. There are clear trends of an increase of the IIa frequency and a decrease of the IId frequency during growth.

The mean frequency of type IIa fibres is increasing in both groups during the first year of life (Fig. 3). The two other fast fibre types, IId and IIa/d decrease in mean frequency in both groups. Analysis applied on the data from 0 to 22 weeks (see material and methods) showed that the increase of type IIa fibres and the decrease of type IId fibres are statistical significant (p < 0.05). The decrease of IIa/d fibres groups is not statistically significant (p = 0.08).

Exercise did not significantly influence the rate of change of the fast fibre types. Figure 3 suggests a difference between the boxrest and the training group in the increase of IIa fibres. Although the increase in the training group is higher, the difference does not reach the level of statistical significance. Figure 3 shows that the percentages of IId fibres are higher in the boxrest group, but this difference was already present at birth and did not increase.

Fibres containing Developmental MHC Developmental MHC is present during the embryonal and neonatal period of skeletal muscle development in some IIa and IIa/d fibres and disappears in both boxrest and training group at the same time (Fig. 4), before 10 weeks of age.

Slow fibres Two slow fibre types are seen: fibres expressing solely type I MHC and fibres co-expressing type I and α MHC. Type I and type I / α fibres combined increase slightly in numbers during growth, from about 15 to 25 %, but this effect does not reach the level of statistical significance (p = 0.06). Strikingly, at birth almost all slow fibres are type I / α. In the first 22 weeks α disappears almost completely from these fibres (Fig. 5). No exercise effect was found on the disappearance of these fibres.

Discussion

Age effect

The age effect on muscle fibre composition depends on the type of muscle, the species, breed and even on sex. Suzuki and Cassens (1980) found in pigs that the percentage of type I muscle fibres increased from birth to 8 weeks of age in several muscles. In contrast, in
Postnatal muscle fibre composition

Hamsters it was found that the percentage of type I fibres decreases in the biceps brachii, and increases in the soleus (Goldspink and Ward, 1979). Lindholm and Piehl (1974) reported a higher percentage fast twitch, high oxidative fibres (FOG) in the middle gluteal muscle in adult horses compared with 6 months old foals. The percentage fast twitch, low oxidative fibres (FG) became lower over time. Others (Essen-Gustavson et al. 1983; Henckel, 1983) also found increasing percentages of fibre type IIA and decreasing percentages of fibre type IIB from 6 month up to older ages. On the other hand Bechtel and Kline (1987) reported that foals, during the first 6 months of life enhance their anaerobic, rather than their aerobic components of muscle metabolism.

In this study we showed that the age effect on deep gluteus medius muscle fibre composition in the first year of life is dramatic in both groups. There is a simultaneous increase of type IIa fibres and decrease of type IId fibres. This suggests that type IId fibres turn into IIa fibres. If this is the case, it should most probably happen via transitional fibres, expressing both type IIa and IId MHC (IIa/d). There is indeed a population of such fibres. Essé et al. (1980), investigating standardbred trotters, found that the increase of type IIA fibres and the decrease of IIB fibres continued to the age of 3-4 years. So, probably the transition in our study is not complete yet. Indeed, at the age of 11 months the frequency of type IIa/d fibres is still 10 % in both groups. Rivero et al. (1996a) found frequencies of 10-15 % in the gluteus medius of 2 and 3 years old horses. These fibres, coexpressing IIa and IId, may not only reflect fibre type transformation, but also may form a biologically important fibre type in horses in the first place.

The occurrence of Cardiac-α MHC in the gluteus medius muscle is a new finding. This myosin, characteristic for heart atrium, was already found in cranial muscles, like masticatory, extraocular and hyoid muscle, but not in trunk and limb (Bredman et al. 1990). Rivero et al. (1996b) claimed that this myosin was not present in equine gluteus medius muscle. This contradictory finding may be explained by the fact that the horses used in their study were not young enough to demonstrate the expression of Cardiac-α MHC. In contrast with earlier studies (Bredman et al. 1991), in this study Cardiac-α MHC did not appear as the only MHC in a muscle fibre, but always in combination with other MHC isoforms, usually with type I MHC.

The increase in the slow fibre (I plus I/α) frequency was not statistically significant. Except for the disappearance of fibres co-expressing MHC I and Cardiac-α, the slow fibre population is stable in the first year of life. However, the loss of Cardiac-α from these fibres probably implies that they become slower contracting (van Buren et al. 1995; Kwa et al. 1995).

Another age effect is the disappearance of Developmental MHC expression in the first 2 months after birth. In an electrophoretic study d’Albis et al. (1986) showed that Developmental MHC was present after birth in mice and rats. In rats these isoforms also persisted until the age of 2 months.

Our foals were not trained after weaning (22 weeks). Nevertheless, the trends of type I and IIa increase and IId decrease continued until the age of 48 weeks. In older, less active, standardbred trotters (>10 years) it was found that the IIA/IIB fibre ratio was still
high (Essén et al. 1980). Therefore it may be assumed that the muscle fibre composition created in the early phase of life is the basis for further adaptations during the conventional training period later in life.

**Effect of exercise**
The gluteus medius, as one of the main propulsive muscles of the body, is expected to be well adaptable to exercise. Earlier studies show that trained horses, especially trained for endurance, have a larger proportion of type I and type IIA and a smaller proportion of type IIB fibres (now called type IId) in the locomotory muscles (e.g. Rivero et al. 1995). However, in our study exercise did not significantly influence the rate of change of the fibre types. The question arises if our exercise protocol provided enough stimulation to induce changes in the muscle. The paper of Suwannachot et al. (1999) in this issue shows a significant increase in the total concentration of Na⁺,K⁺-ATPase in the deep gluteus medius muscle of the training group. This means that the exercise was intensive enough to initiate some reaction in this muscle. The mean difference in fibre composition is subject to a quite large intra-individual (e.g. Essen-Gustavsson et al. 1989; Lopez-Rivero et al. 1992) and interindividual variation (e.g. Snow 1983). Exercise induced effects will therefore only be measurable if they are large. If the effect is small it can only be demonstrated using larger groups. The higher percentages of IId fibres in the boxrest relative to the training group at birth must be explained by the small group size.

In conclusion this study is the first to demonstrate the presence of Cardiac-α and Developmental MHC in equine skeletal muscle. It also demonstrates a high degree of MHC co-expression, especially occurring during the first months after birth. Furthermore, the results of the present study show that there is an age effect in the MHC expression. As type I and type IIA fibres generally have a better fatigue resistance, the muscle apparently becomes increasingly resistant against fatigue. This conclusion is opposite to the one drawn by Bechtel and Kline (1987). The transition of fibre types can possibly be influenced by exercise. However, we have yet not been able to demonstrate that exercising young foals can produce such a change in fibre composition.

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References


Chapter 2


Postnatal muscle fibre composition