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Immunohistochemistry versus traditional myofibrillar ATPase histochemistry for identification of muscle fibre types in horses

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Summary

Five young (2- to 3-years old) thoroughbred horses were intensively trained for 8 months on a high-speed treadmill. Biopsies were taken from the gluteus medius muscle at the beginning, after 4 months, and again at the end of the training programme. Serial sections of the samples were stained by myofibrillar ATPase (mATPase) histochemistry and immunohistochemistry using a number of monoclonal antibodies specific to selected myosin heavy chain (MyHC) isoforms. The histochemical and immunohistochemical categorization of a large number of fibres (N=2,078) was compared fibre by fibre. A high proportion of fibres examined (~20%) were mis-classified by traditional mATPase histochemistry. Many fibres histochemically identified as type IIB displayed both type IIa and type IIb MyHc isoforms, and nearly all type IIAB fibres in mATPase contained only the type IIa MyHC isoform by immunohistochemistry. Training had no significant effect on the number of fibres mis-classified by mATPase histochemistry. These data demonstrate a significant limitation in the mATPase histochemistry for assessing fibres containing fast MyHC isoforms.

Keywords: skeletal muscle, monoclonal antibody, muscle fiber, horse

Vergleich von immunohistochemischer und traditioneller myofibrillärer ATPase-histochemischer Nachweismethode zur Identifikation von Muskelfasern beim Pferd

Fünf Vollblutpferde, im Alter von 2 bis 3 Jahren, wurden auf einem Hochgeschwindigkeitslaufband 8 Monate lang intensiv trainiert. Zu Beginn des Trainings, nach 4 Monaten und am Ende der Trainingsperiode wurden Muskelbiopsien aus dem M. glutaeus medius aus einer Tiefe von 2 cm entnommen. In jedem Bioptat wurden die Muskelfasern anhand histochemischer und immunohistochemischer Methoden identifiziert. Dazu wurden zunächst von jedem Bioptat mehrere Schnitte angefertigt. Diese Schnitte wurden bei der histochemischen Identifikationsmethode angefärbt, um die Aktivität der myofibrillären Adenosin-Triphosphatase (mATPase) bei verschiedenen pH-Werten nachzuweisen. Bei der immunohistochemischen Methode wurden verschiedene monoklonale Antikörper auf die Schnitte aufgetragen, anhand derer verschiedene Isoformen der schweren Myosinkette (MyHC) nachgewiesen werden. Die histochemische und immunohistochemische Klassifizierung einer großen Anzahl Fasern (n=2.078) wurde Faser für Faser verglichen. Ein großer Anteil der untersuchten Fasern (ca. 20%) wurde anhand der traditionellen mATPase-Histochemie falsch klassifiziert. Viele Fasern, die histochemisch als Typ IIB klassifiziert wurden, wiesen die MyHC-Isoformen von sowohl Typ IIa- als auch von Typ IIb-Fasern auf. Fast alle histochemisch als Typ IIAB eingestufte Fasern enthielten laut immunohistochemischer Identifikation nur die MyHC-Isoformen der Typ IIa-Fasern. Der Anteil der mittels der mATPase-Histochemie falsch klassifizierten Fasern wurde durch das Training nicht signifikant beeinflußt. Diese Ergebnisse zeigen den begrenzten Aussagewert der mATPase-Histochemie bei der Bestimmung von Muskelfasern, in denen gleichzeitig MyHC-Isoformen vorkommen.

Schlüsselwörter: Skelettmuskel, monoklonale Antikörper, Muskelfaser, Pferd

Introduction

In horses, muscle fibres have been routinely categorized into three major types, designated types I, IIA and IIB, and the minor IIC, based upon the myofibrillar actomyosin adenosine triphosphatase (mATPase) histochemical reaction proposed by *Brooke* and *Kaiser* (1970). Using immunohistochemical methods, it has been suggested that the histochemical staining intensity of the mATPase reaction in a given muscle fibre is determined by its myosin heavy chain (MyHC) content (*Billeter* et al., 1981). In a companion study (*Rivero* et al. 1995), we have identified one slow- and two fast- (IIa, and IIx or IIb) MyHC isoforms in the adult equine skeletal muscle by using monoclonal antibodies and gel electrophoresis techniques.

Although a direct correlation between the histochemical reactivity for mATPase and the MyHC content of a given fibre has been established (*Staron* and *Pette,* 1986), the disadvantages of the mATPase technique have recently been highlighted in humans (*Klitgaard* et al. 1990; *Andersen* et al., 1994). These studies suggest that many fibres histochemically classified as type I or type IIB contain to some degree the fast IIa-MyHC, particularly in trained muscles. Moreover, the coexpression of multiple MyHC isoforms within a single fibre also occurs under normal conditions (*Biral* et al., 1988). In summary, as the dominant MyHC isoform determines the histochemical mATPase reaction of a fibre (*Danielli-Beto* et al., 1986), this method does not reveal subtle alterations in the expression of MyHCs in muscle fibres and, therefore, may not adequately characterize muscle fibre distribution in control or in trained horses. The main purpose of the present investigation was to ascertain the degree of association between the MyHC content of muscle fibres and histochemical muscle fibre type distribution by combining classical qualitative mATPase histochemistry and immunohistochemical analyses of MyHC.

Materials and methods

Six clinically healthy thoroughbred racehorses (5 mares and one gelding) were used in this study. Two horses were 3 years old and the other four were 2 years old at the beginning of the study.

Five of the horses were included in a training programme aimed at investigating the influence of intensity and duration of exercise on several physiological variables. The sixth horse was used as a control in order to evaluate changes associated with age. All the exercise workouts and standardized tests were done on a high-speed treadmill (Mustang 2200®, Kagra SA. Fahrwangen, Switzerland). The experimental period was extended for about 8 months. After a one-month acclimatization period, all 5 trained horses carried out 6 phases of exercise, varying in intensity and duration of exercise. Each phase consisted of 11 exercise workouts (once a day every second day), so each phase was extended for 21 days. Between two consecutive phases, horses were allowed to rest for one week. In this week, a standardized exercise test (SET) was performed for each horse in order to determine $V_{LA2.5}$ and V_{LA4}, or speeds which run over a defined period of time produce a concentration of lactate in blood of 2.5 and 4 mmol/l, respectively (*Lindner* et al., 1992). This was the parameter on which the intensity of exercise made in each phase of training was based. Of the 6 phases of training, 3 were made at an intensity of work of $V_{LA2.5}$ and the other 3 in V_{LA4}. In both sets of these phases the duration of each exercise session was 5, 15 or 25 minutes.

Muscle biopsies (75–150 mg) were obtained from the right gluteus medius muscle (depth: 2 cm) of each horse according to *Lindholm* and *Piehl* (1974). Biopsies were taken before, after 4 months of training, and again at the end of the 8-month training programme. Control horse biopsies were taken at the same time periods. After collection, muscle samples were frozen and stored as described elsewhere (*Rivero* et al., 1995, companion paper).

Frozen biopsy samples were thawed to –20 °C in a cryostat and serially sectioned for histochemistry (using 10 µm thick sections placed on cover slips) and immunohistochemistry (using 10 µm thick sections placed on gelatin coated slides)

Serial cross-sections were stained qualitatively for the demonstration of myofibrillar adenosine triphosphatase (mATPase) activity after alkaline (pH 8.75) and acid (pH.4.2 and pH 4.5) preincubation using a modification (*Nwoye* et al., 1982) of the *Brooke* and *Kaiser* (1970) method.

Serial sections were reacted with 4 different monoclonal antibodies specific to rat MyHC isoforms. Individual cross-sections were labeled for Slow, Fast, A4.74 and N2.261 monoclonal antibodies. The source and specificity of these monoclonal antibodies, as well as the immunohistochemical procedure employed, are described in the companion paper (*Rivero* et al., 1995).

A region of the cross sections containing between 100 and 135 fibres per muscle biopsy was randomly selected for further analysis. These fibres were numbered and classified at random using histochemical and immunohistochemical methods. Myofibres were classified into types I, IIC, IIA, IIAB and IIB (*Brooke* and *Kaiser,* 1970; *White* and *Snow* 1985) according to the mATPase staining characteristics at the different levels of preincubation acidity. The same fibres were allotted to types I, I+IIA, IIA, IIA+IIB (or IIA+IIX), and IIB (or IIX) according to their MyHC content as revealed by reactivity against monoclonal antibodies (see companion paper *Rivero* et al., 1995). To compare histochemical vs. immunohistochemical data, these five fibre types were also renamed as types I, IIC, IIA, IIAB, and IIB, respectively. The fibre type distribution of each muscle biopsy was established by counting and typing the relative frequency of the various fibre types in each sample.

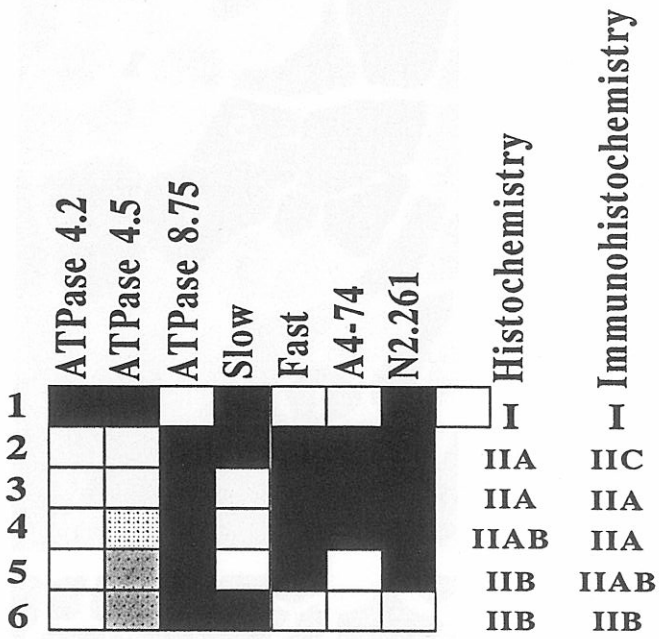

Fig. 1: Scheme showing the patterns of reactivity of the six most common fiber types identified by histochemistry and immunohistochemistry. These six fibre types are labelled in Fig. 2.

One-way analysis of variance (ANOVA) with repeated measurements was used to determine whether significant effects of training existed.

Results

Fig. 1 illustrates a scheme showing the pattern of reactivity of the six most common fibre types identified by histochemistry and immunohistochemistry. Based on histochemical analysis (mATPase reaction) the muscle fibres could be divided into five categories: I, IIC (not shown), IIA, IIAB, and IIB (Fig. 2A). A continuum in the staining intensity for mATPase after acid preincubation (pH 4.5) was observed between the type IIA and type IIB fibre population (Fig. 2A). All these fibres were identified as type IIAB. Five different fibre populations were also demonstrated immunohistochemically using specific monoclonal antibodies (Fig. 2B–D). Fibres that reacted exclusively with the Slow MyHC antibody were termed I. Fibres that were unreactive with the Slow MyHC antibody and reacted with Fast, A4.74, and N2.261 antibodies were called IIA, and those fibres that did not react with all monoclonal antibodies except the Fast MyHC antibody were called IIB. Two subgroups of fibres co-expressing two different MyHCs were also identified, and these were designed as IIC (fibres coexpressing types I and IIa MyHCs), and IIAB (fibres co-expressing both fast MyHCs).

Combined histochemical and immunohistochemical analyses demonstrated a certain degree of correlation, albeit not unequivocal, between mATPase staining intensities and myosin heavy chain (MyHC) content. Type I fibres were histochemically uniform and reacted with the Slow, A4.74, and N2.261 (e.g. fibres labeled '1' in Fig. 2). However, the fast-twitch fibre population was histochemically and immunohistochemically heteroge-

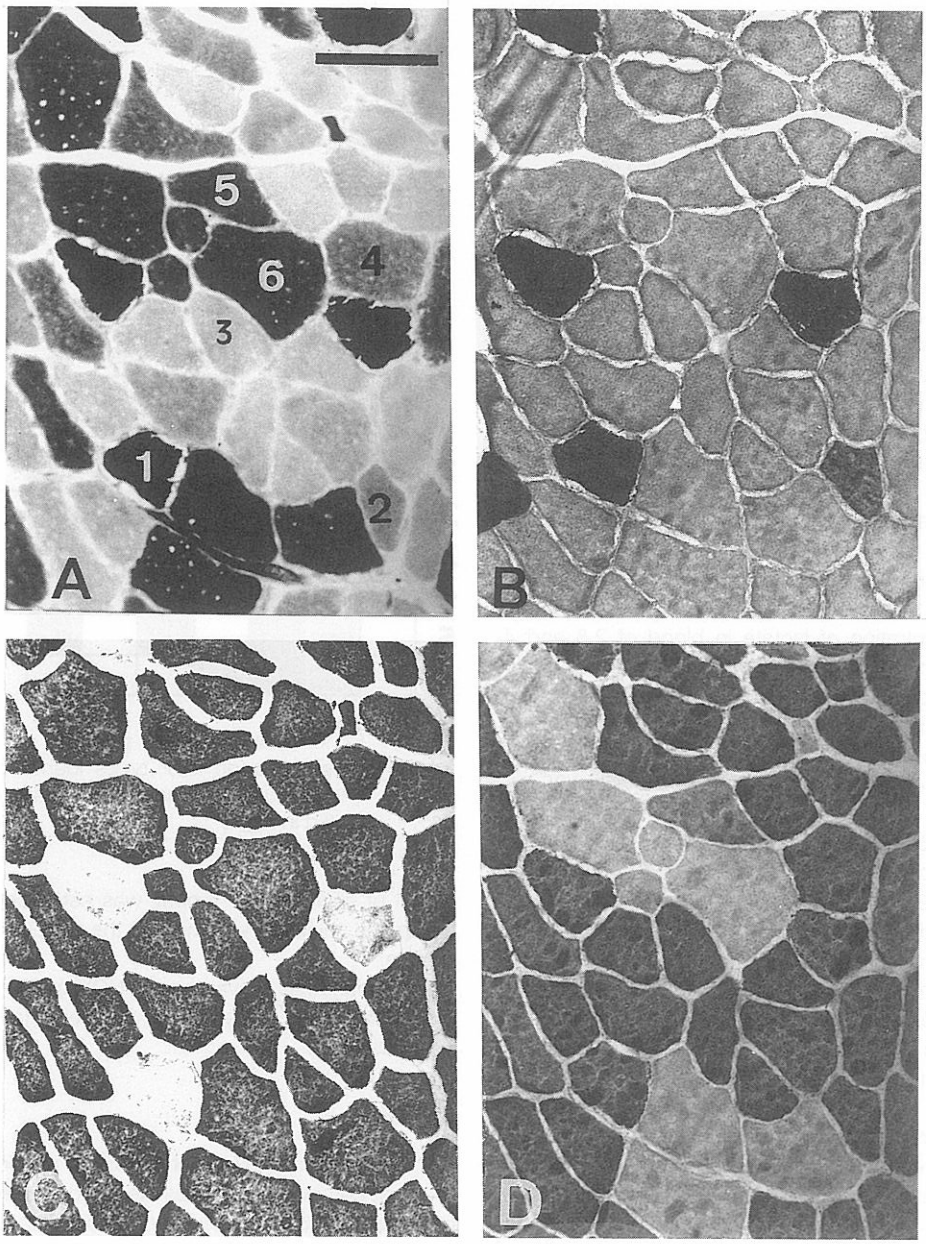

Fig. 2. Identification of fibre types by enzyme histochemistry and immunohistochemistry. **A**) Myosin ATPase after preincubations at pH 4.5. Immunohistochemical staining with monoclonal antibodies Slow **(A)**, Fast **(B)** and N2.261 **(C)**. The fibres labelled '1' to '6' correspond to those fibres illustrated in Fig. 1. Scale bar = 100 µm.

neous. Most fibres histochemically classified as IIA contained only IIa MyHC (e.g. fibre labeled '3' in Fig. 2). Similarly, a high percentage of fibres histochemically classified as IIB contained a mixture of IIa and IIb MyHCs (~24 % of IIB mATPase fibres), and were classified as type IIAB by immunohistochemistry (e.g. fibre labeled '5' in Fig. 2). Those fibres which stained intermediately between IIA and IIB mATPase fibres (type IIAB) had frequently a mixture of IIa and IIb MyHCs, but a high proportion of these hybrid fibres for mATPase were exclusively composed of IIa MyHC (~61%), so they were classified as IIa by immunohistochemistry (e.g. fibre labeled '4' in Fig. 1). Finally, some fibres stained more like IIC fibres by histochemistry contained only the slow-MyHC (not shown); conversely, a few fibres co-expressing both the slow- and the fast IIa-MyHCs were typed as I for mATPase (e.g. fibre labelled '2' in Fig. 2).

Almost no significant changes ($P>0.05$) in fibre type composition was recorded as a consequence of training (Tab. 1). Training had no significant effect on the number of muscle fibres mis-classified by qualitative mATPase activity in correspondence with their MyHC content revealed by immunohistochemistry (Tab. 2).

Discussion

Comparison between the histochemical and immunohistochemical data shows that ~20% of the fibres were misclassified by traditional mATPase histochemistry. Each MyHC isoform can be associated rather consistently with a specific mATPase-based muscle fibre type (*Staron* and *Pette,* 1986), except when two or more MyHCs coexist within the same fibre. It has been suggested that fibres coexpressing two or more MyHC isoforms will his-

Tab. 1: Percentage of the various fibre types identified immunohistochemically and by qualitative myosin ATPase of 5 horses (Training) and one control horse (Control) along the experimental period

	Immunohistochemistry			Histochemistry		
Month	0	4	8	0	4	8
Training (N=5)						
I	13.2 (4.2)	8.6 (6.1)	13.8 (11.4)	13.2 (4.2)	8.6 (6.1)	13.8 (11.4)
IIA	42 (7)	43.2 (7.2)	40.8 (13.7)	34.4 (8.5)	34.6 (5.3)	34.2 (10.2)
IIAB	9 (3.8)	13.4 (4.2)	11.8 (3.7)	10.2 (2.2)	15 (4.2)	6.2 (1.5)*
IIB	35.8 (4.8)	34.8 (5.4)	33.6 (11.5)	42.2 (6.4)	41.8 (4)	45.8 (8.2)
Control (N=1)						
I	12	7	5	12	7	5
IIA	38	42	35	32	34	27
IIAB	15	16	27	8	12	23
IIB	35	35	33	48	47	45

Values are mean (SD). N = number of horses. * Significantly different from second muscle biopsy ($P<0.001$). No significant ($P>0.05$) variations along the experimental period were found for the other muscle parameters

Tab. 2: Number and percent of muscle fibres mis-classified by qualitatitive mATPAse histochemistry in comparison with immunohistochemistry in all the 18 muscle biopsies examined

		Month				
Immunohisto-chemistry	Histochemistry	0	4	8	Total	Percent
		(N=684)	(N=698)	(N=696)	(N=2,078)	
IIAB	IIA	3	8	5	16	4
IIAB	IIB	49	59	65	173	45
IIA	IIAB	46	65	49	160	42
IIB	IIAB	3	5	3	11	3
IIA	IIB	10	3	4	17	4
IIB	IIA	2	1	0	3	1
I	IIC	3	0	0	3	1
Total		116	141	126	383	
Percent		17	20	18	18.5	

N = number of fibres examined

tochemically react according to the dominant isoform (*Klitgaard* et al., 1990). The finding that many fibres histochemically classified as type IIB coexpressed IIa and IIb MyHCs supports this assumption. Further some fibres histochemically identified as type I, coexpressed type I and type IIa MyHCs based on immunohistochemistry. On the other hand, nearly all fibres histochemically classified as type IIAB contained only IIa MyHC.

The observation that a large number of histochemically determined IIB fibres actually contain a varying amount of type IIa MyHC is in agreement with previous studies on human single fibres (*Andersen* et al., 1994). In this study, it was reported that nearly all histochemical type IIB fibres of sprinters (*Andersen* et al., 1994) coexpressed both IIa and IIb MyHC isoforms. The explanation for the finding that practically all histochemically typed IIAB fibres contained only IIa MyHC is unclear. It may be speculated that the mATPase histochemical staining is affected by factors other than the relative content of MyHC. The results of a former study of the mATPase activity of equine muscle fibre types by quantitative histochemistry (*White* and *Snow,* 1985), clearly showed a continuous range within the type II fibres with the two or three overlapping peaks. As this continuum of fibre types is difficult to quantify using a qualitative technique such as mATPase histochemistry, this method of classification of muscle fibres into four major types (5 if the type IIC are included) may have major limitations in objectively differentiating among the 3 fast subtypes (IIA, IIAB, IIB) based on MyHC content. Our observation of some fibres histochemically identified as type I, but coexpressing both I and IIa MyHC is similar to reported for human muscle (*Klitgaard* et al. 1990). *Klitgaard* et al. (1990) reported that in the 95% of fibres in endurance athletes that contained both I and IIa MyHCs the major fraction was the type I MyHC, perhaps explaining the histochemical staining profile of a type I fibre.

Another striking observation of the present study was that training had no significant effect on the correlation between mATPase activity and MyHC content of muscle fibres. In some studies, it has been argued that the 'mis-classification' of many fibres by mATPase histochemistry seems to be especially pronounced in trained individuals, because of the presence of a high number of fibres coexpressing multiple MyHCs (*Klitgaard* et al., 1990).

Surprisingly, no significant changes in muscle fibre type composition were observed in the current study after 4 and 8 months of training. In horses, as in humans and other mammals, the most common adaptation to training is an increase of the type IIA to type IIB histochemical fibre ratio, and an increase of both the oxidative capacity and capillary supply of muscle fibres (see *Snow* and *Valberg,* 1994 for a review). As the intensity and duration (both daily and in total) of exercise involved in the current study was higher than other previous studies in horses (*Snow* and *Valberg,* 1994), the limited training-related changes in muscle fibre type characteristics recorded in our study is difficult to explain. It

may be, however, linked to the relatively superficial sampling site of the gluteus medius muscle from which the samples were taken. In a recent study (*Rivero* et al., 1995), it was found that the adaptation of the gluteus medius muscle fibres to training was not homogeneous in superficial and deep sampling regions of this muscle. As these training-linked modifications were more marked in the deep region of the muscle compared with the superficial one, it was concluded that different functional demands are placed on different depths of the gluteus medius muscle. This lack of muscular response to training may also be partially explained because no real metabolic adaptation occurred in response to training, in spite of the relative intensity of the training being individually adjusted to each horse. On the other hand, the performance capacity of each individual horse did not improve throughout the training programme, as was shown by the constant values of V_{LA4} (*Lindner,* personal communication).

In conclusion, histochemical and immunohistochemical techniques applied to equine skeletal muscle establish a relationship between qualitative myofibrillar mATPase activity and MyHC content. However, a high percentage of fibres of the equine gluteus medius muscle are mis-classified by traditional mATPase histochemistry, since it does not adequately identify the hybrid fibre population coexpressing the two fast MyHC isoforms. Therefore, and despite the extensive use of mATPase histochemistry to distinguish skeletal muscle fibre types in horses, the present results do no support the use of this qualitative technique. The use of monoclonal antibodies against specific MyHCs seems to be a more sensitive, more reproducible and less subjective method for that purpose.

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