Original article

Effects of protein malnutrition on muscle fibers of the brachial biceps and medial pterygoid of Wistar rats

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Abstract

Protein malnutrition is a public health problem, and in childhood, it can lead to muscle deficits. Here, our objective was to evaluate the effects of malnutrition upon the muscle fibers in the medial pterygoid and braquial biceps. Ten just weaned rat pups that had been born to parents fed a nourished or malnourished diet (N = 5 per group) were studied. The medial pterygoid and braquial biceps muscles were removed and cross-sectioned, and histological staining with picrosirius and histochemistry reaction with nicotinamide adenine dinucleotide - tetrazolium reductase (NADH-tr) were performed. The samples stained with picrosirius were observed under polarized light, and from the qualitative analysis, we observed that type I collagen fibers were only present in the braquial biceps muscles of the nourished animals. The NADH-tr reaction indicated that the pterygoid muscle specimens from the malnourished pups lacked intermediate muscle fibers. The cross-sectional area of the muscle was lower in the malnourished group than in the nourished group. The density of muscle fibers was higher in the malnourished group than in the nourished group. The consequences of malnutrition were visible when comparing the muscles. We concluded that the differences in daily muscle action along with the differences in embryological origin are instrumental in establishing the results.

Keywords: skeletal muscle, fiber types, postnatal development, malnutrition, protein deprivation.

1 Introduction

Muscle tissue is the most abundant tissue in the human body. It is composed of diverse types of muscle fibers, which are bounded by the sarcolemma. The cytoplasm of muscle cells is filled with myofibrils formed by proteins. Muscle fibers can be classified into subtypes based on their nicotinamide adenine dinucleotide-tetrazolium reductase (NADH-tr) histochemical reactivities, as follows: type I slow oxidative fibers (high reactivity), type IIB fast twitch glycolytic fibers (weak reactivity) and type IIA fibers (intermediate reactivity) (BARNARD, EDGERTON, FURUKAWA et al., 1971; DUBOWITZ, BROOKE and NEVILLE, 1972;). Each type of fiber reacts and adapts uniquely to different stimuli, including hormones (BAHI, FORTIN, SERRURIER et al., 2005) age and growth (DESCHENES, 2004; CANEPARI, ROSSI, PELLEGRINO et al., 2005; HUSAM, FERRINGTON and THOMPSON, 2005), muscle architecture (LINDSTEDT, MCGLOTHLIN, PERCY et al., 1998; BACH, BEIER, STERN-STAETER et al., 2004; BLEMKER and DELP, 2005; KNIGHT and KAMEN, 2005), daily functional activity and muscle diseases (KRIVICKAS, ANSVED, SUH et al., 2000; LAING, CLARKE, DYE et al., 2004; CARROLL, GALLAGHER, SEIDLE et al., 2005; KARAKELIDES and NAIR, 2005; RAO and KOUL and INUWA, 2005). The muscle fiber may adapt by altering its type or its diameter (GUTH, SAMAHA and ALBERS, 1970; HUGHES and BLAU, 1992; HÄMÄLÄINEM and PETTE, 1993; SMERDU, KARSCH-MIZRACHI, CAMPIONE et al., 1995; MACCOMAS and WHITE, 1996; STARON, 1997; SCOTT, STEVENS and BINDER-MACLEOD, 2001).

Members of the UN have affirmed their commitment to end hunger (FERREIRA and FRANÇA, 2002). Child malnutrition is responsible for half of the 10.4 million annual deaths of children worldwide (WORLD..., 2003). In Brazil, child malnutrition is a serious public health problem (FERREIRA, 2000; MONTE, 2000), and there is evidence that the country is currently in transition from a higher to a lower prevalence of malnutrition. The effects of early malnutrition on muscle development may be permanent, causing an irreversible deficit in the number of muscle fibers. However, when malnutrition occurs at later stages of human development, there are no permanent deficits in the number of muscle fibers because the number has already been determined (MONTGOMERY, 1962; ENESCO and PUDDY, 1964). Muscle fibers do not proliferate, and the only way to increase muscle tissue is to increase the thickness of the fibers through the emergence of new myofibril proteins.

Malnutrition has been reported to cause changes in the type and cross-sectional areas of muscle fibers. For instance, in rats undernourished during pre- and postnatal periods, the cross section areas of the gastrocnemius muscle fibers were lower than in control animals (NASCIMENTO, MADI, SILVA et al., 1990).

The amount of force that a muscle fiber can produce is proportional to its cross-sectional area (KORFAGE, HELMERS, MATIGNON et al, 2009). This area may increase with the amount of resistance experienced during

contraction (EDGERTON, ZHOU, OHIRA et al., 1995; MCCALL, BYRNES, DICKINSON et al., 1996). Therefore, fiber type, composition and cross-sectional area can be used to characterize the functional properties of muscles.

In a study of the soleus muscle in rats with postnatal protein malnutrition, the malnourished group was found to have smaller muscles than controls due to two factors: loss of the number of muscle fibers and hypertrophy of remaining fibers (IHEMELANDU, 1985). In a study examining mice subjected to a 40% diet reduction, there was a decrease in the number of muscle fibers in the braquial biceps and extensor digitorum longus, but not in the soleus muscle (predominantly type I fibers), indicating that different types of muscle fibers respond differently to malnutrition (DWYER and STICKLAND, 1992).

We observed that type II brachial biceps muscle fibers were the most susceptible to the effects of malnutrition. Malnutrition resulted in a decrease in the number of these fibers, which was related to their embryological origin. This decrease occurs only in presence of motor innervation, myogenesis becoming the primary fibers (type I) preserved (KELLY and RUBINSTEIN, 1986). To examine whether the above-mentioned changes are a systemic phenomenon, this study examined the medial pterygoid and the biceps muscles. The medial pterygoid muscle is of embryonic origin from the first pair of gill arches, and its main functions to lift the jaw during protrusion movements. The biceps muscles are of mesodermal origin, which form the myotomes of the somites, and in rats these muscles are used for locomotion.

The aim of this study was to investigate whether changes occur during the development of collagen components and the types of muscle fibers in nourished and malnourished animals. We assessed morphometric factors, such as the density and cross-sectional area of each muscle fiber type and related them to development. We hypothesized that the malnutrition protocol and postnatal care may adversely affect the training of the muscles under study and interfere with muscle strength. Additionally, we hypothesized that by comparing nourished and malnourished groups, the embryology, movement type, and daily activity of these muscles may be decisive in their morphological development.

2 Material and methods

2.1 Experimental model

Male and female Wistar rats (Rattus norvegicus), weighing between 280 and 320 g, were used to generate the rat pups examined in this study. During mating, two groups of rats were offered different diets without restriction. The nourished group was fed a protein (20% casein) diet, and the malnourished group was fed a low protein diet (5% casein). Both diets were prepared using laboratory biochemicals (Rhost Industry and Trade , Ltd.) in accordance with the American Institute of Nutrition (REEVES, NIELSEN and FAHEY, 1993). The experimental procedure was approved by Ethical Comitee for animal research of the Biomedical Sciences Institute of the University São Paulo. After mating, the females were separated into individual cages and maintained on their respective diets until weaning their pups at 21 days of life. At this time, five pups were euthanized from each group with an overdose of Hypnol. The braquial biceps and medial pterygoid were removed, protected with neutral talc, and immersed in liquid nitrogen to freeze the samples instantly.

2.2 Histological staining and histochemistry

We obtained serial sections of the muscles with 10 μm thicknesses. The sections were stained with picrosirius to visualize the collagen fibers (JUNQUEIRA, BIGNOLAS and BRETANI, 1979). The sections were also histochemically stained with NADH-tr to characterize the muscle fibers.

2.3 Morphometric study

The total areas of the braquial biceps and medial pterygoid were estimated for both the nourished and malnourished groups by averaging the sizes of 14 sections from each animal, which were chosen at random. The sections were projected onto a sheet of paper from a binocular microscope with camera lucida (Zeiss) and a calibrated object micrometer (Zeiss). The projected areas were outlined with the aid of a planimeter (type-30 OTT_Planimeter) and measured in mm^2.

To estimate of percentage of fibers in transverse sections of the braquial biceps and medial pterygoid (number of fibers per mm^2), 25 sections were randomly selected from the nourished and malnourished groups and stained with NADH-tr. The fiber percentage values were measured using computerized imaging equipment (Zeiss-KS300), which was attached to a binocular microscope objective (100×), within a predetermined, 0.064 mm^2 field.

The diameters of the muscle fibers were determined in the biceps and medial pterygoid muscles for both the nourished and malnourished groups. These measurements were made in another 25 sections that were each reacted with NADH-tr. From each section, the diameters of 50 fibers of each type were measured using the equipment mentioned above, but using a 40x lens.

2.4 Statistical analysis

All data are reported as means ± standard errors. Weights, areas, total fiber densities and percentages of each fiber type were compared between the nourished and malnourished groups using the Student's t-tests (ZAR, 1984). The densities and areas of the fibers in the braquial biceps and medial pterygoid were analyzed using analysis of variance (ANOVA) with two factors: group (nourished and undernourished) and fiber type (oxidative and glycolytic intermediate, for only the biceps muscle), followed by multiple comparisons using the Tukey method (ZAR, 1984). The level of significance was 5%.

3 Results

3.1 Qualitative analysis

After picrosirius staining, type I collagen fibers were observable under polarized light in only nourished animals in the biceps (Figure 1a). Type I collagen fibers were not observed in the biceps of malnourished animals or in the pterygoid muscles of nourished or malnourished animals. The NADH-tr reaction was observed in oxidative (high reactivity), glycolytic (weak reactivity) and intermediate (intermediate reactivity) fibers in the biceps muscles from

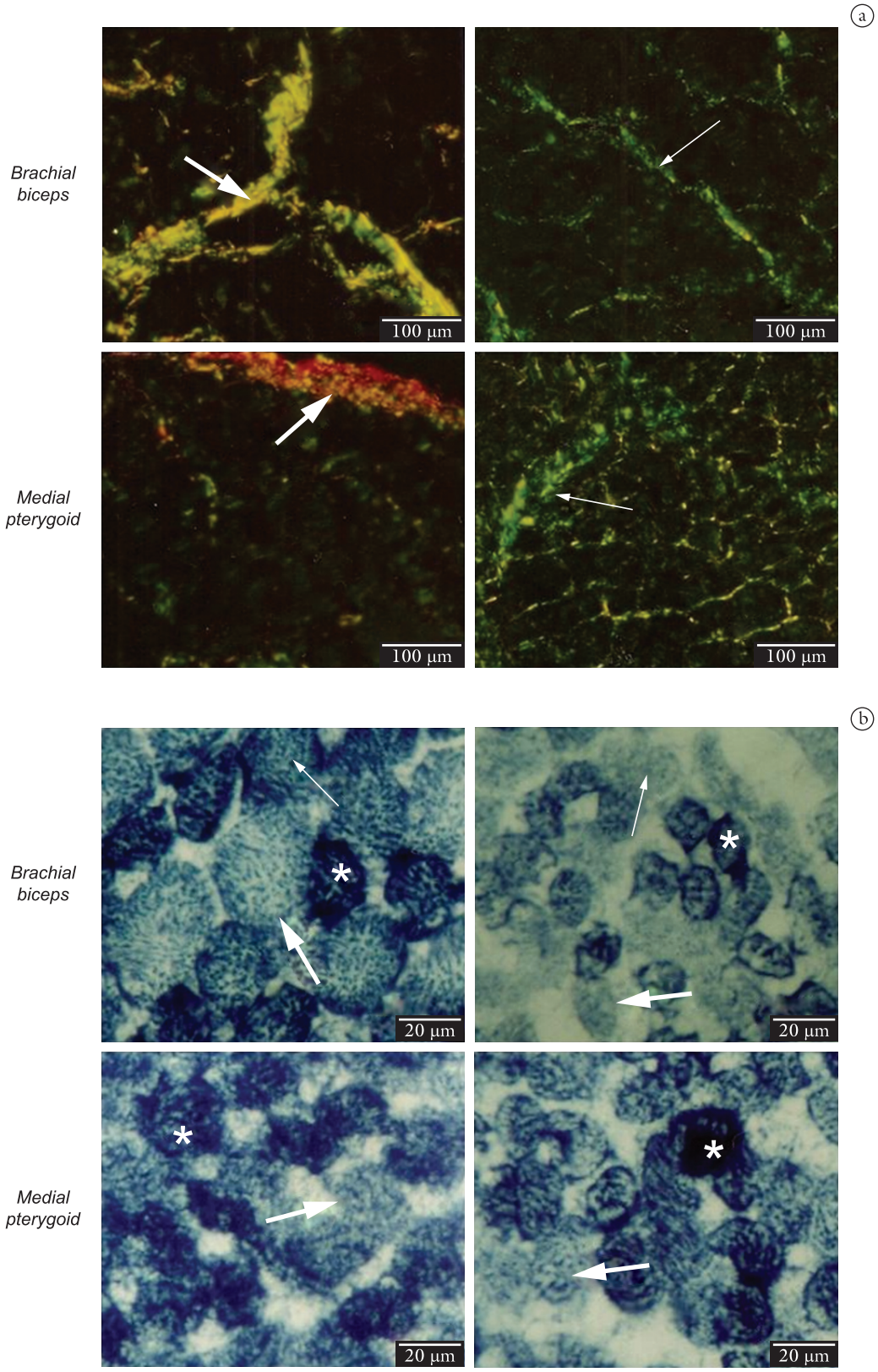

Figure 1. Photomicrographs of brachial biceps and medial pterygoid muscle sections. a) Representive collagen fibers from a nourished rat (type I collagen fibers, large arrows, in the perimysium) and a malnourished rat (type III collagen fibers,small arrows, in the perimysium). b) Representative NADH-tr histochemical reaction in sections from a nourished rat and a malnourished rat. Asterisks, oxidative fibers; small arrows, intermediate fibers; and large arrows, glycolytic fibers.

both groups (Figure 1b). In the pterygoid, it was only possible to see the Oxidative and glycolytic fibers.

In the biceps of nourished animals, the fibers showed clear outlines and reactivity could be related to the area of the fibers. The intermediate fibers stood out as the largest, while the oxidative and glycolytic fibers were the smallest. The malnourished animals also had three types of fibers; however, the areas were smaller than in the nourished animals. In pterygoid, this differentiation was inconspicuous in size for both the visually nourished group as to malnourishment. A vague outline of muscle fibers was observed.

3.2 Quantitative analysis

The mean body weight of the nourished animals was 48.3 ± 8.8 g, which was three times higher than that of the malnourished animals (15.5 ± 2.4 g). Despite the fact that the braquial biceps and medial pterygoid are of different embryological origins and had different cross-sectional areas (Table 1), their areas decreased in similar proportions (58.4% and 53.5% respectively) with protein restriction. The areas of the braquial biceps and medial pterygoid muscles were 2.3 times larger in the nourished animals than in the malnourished animals.

In the brachial biceps muscles of animals in the nourished and malnourished groups, there was a higher density of oxidative and intermediate fibers, respectively (Table 1). All three fiber types were observed in greater numbers per mm^2 (density) in the malnourished animals, but there were only significant differences in the number of oxidative and intermediate fibers between the groups ($p < 0.05$).

It was not possible to identify the intermediate fibers in the medial pterygoid muscle specimens and there was no significant difference in the density of oxidative fibers between the nourished and malnourished groups. In contrast, the density of glycolytic fibers was greater in the malnourished group than in the nourished group ($p < 0.05$). By analyzing these densities without comparing the different groups, the malnourished group had a higher density of glycolytic fibers than the nourished group ($p < 0.05$).

Similar values were observed in the biceps for all three fiber types in both the malnourished and nourished groups. In pterygoid muscle, the differences between the number of oxidative and glycolytic fibers were smaller, as shown in Table 1.

Independent of fiber type and diet group, the medial pterygoid muscle had muscle fiber areas that were larger than the braquial biceps. The nourished pterygoid fibers and malnourished glycolytic fibers were larger than the oxidative fibers. Intermediate fibers made up the largest area of the biceps muscle in both the nourished and malnourished groups. For both the pterygoids and the biceps, all fibers types had smaller areas in the malnourished group than in the nourished group ($p's < 0.001$) (Figure 2).

4 Discussion

Because the skeletal muscle is the most important reservoir of the body's proteins (OLDFORS, MAIR and SOURANDER, 1983), we chose to evaluate the effects of malnutrition in this tissue. The high endemicity of malnutrition in developing countries affects mainly the child population.

4.1 Experimental model

The present study was performed to evaluate muscles of different embryological origins. Following analysis in a pilot study of the pterygoid during embryological development, we observed that it did not have the unique morphological characteristics of skeletal muscle of somatic origin. In the biceps muscle, it was possible to distinguish three types of muscle fibers using NADH-tr reactions (glycolytic, oxidative, and intermediate), whereas only two types of muscle fibers were distinct (glycolytic and the oxidative) for the medial pterygoid. Here, muscles of different embryonic origin were compared in rats with protein malnutrition.

Various forms of malnutrition have been used in previous studies, such as reducing the food supply (WILSON, ROSS and HARRIS, 1988; DWYER and STICKLAND, 1992; OLIVEIRA, OLIVEIRA, SCHMIDT et al., 1999), reducing

Table 1. Comparison of muscle fiber properties between muscles from nourished and malnourished rats.

	Brachial biceps		Medial pterygoid	
	Nourished	**Malnourished**	**Nourished**	**Malnourished**
Weight (g)	48.3 ± 8.8	15.5 ± 2.4*	48.3 ± 8.8	15.5 ± 2.4*
Muscle area (mm^2)	5.74 ± 2.21	2.39 ± 0.53*	7.25 ± 1.00	3.37 ± 0.23*
Fiber densities, n_o/mm^2 (%)				
Oxidative	4544 ± 544 (50.3 ± 4.4)	7534 ± 611* (51.4 ± 2.4)	7125 ± 882 (62.9 ± 11.4)	5384 ± 1.175 (40.4 ± 7.4)*
Intermediate	2522 ± 468[a] (28.1 ± 5.6)[a]	4131 ± 393[a*] (28.3 ± 3.0)[a]	-	-
Glycolytic	1975 ± 662[a] (21.5 ± 5.2)[a]	3009 ± 872[a] (20.3 ± 5.3)[ab]	4347 ± 1.666[a] (37.1 ± 11.4)[a]	7969 ± 1734[a*] (59.6 ± 7.4)[a*]
Total	9041 ± 975	14675 ± 1.257*	11472 ± 1.320	13353 ± 2.029
Fiber areas (μm^2)				
Oxidative	188.9 ± 44.2	107.7 ± 18.2*	204.1 ± 33.7	149.3 ± 11.5*
Intermediate	321.3 ± 110.1[a]	204.9 ± 72.8[a*]	-	-
Glycolytic	230.7 ± 84.7[b]	110.0 ± 35.6[b*]	261.4 ± 48.8[a]	188.9 ± 22.0[a*]

[a]$p < 0.05$ vs. oxidative. [b]$p < 0.05$ vs. intermediate. *$p < 0.05$ vs. nourished.

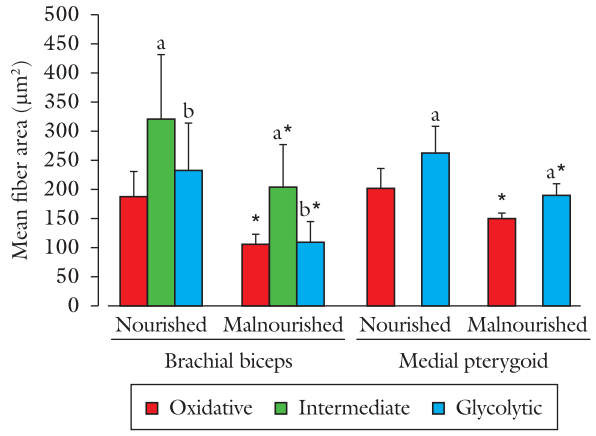

Figure 2. Relative mean fiber areas of oxidative, intermediate and glycolytic muscle fiber types in the brachial biceps and medial pterygoid muscles of nourished and malnourished rats. [a]p < .05 vs. oxidative, [b]p < .05 vs. intermediate; *p < .05 vs. nourished.

the level of protein by various percentages (OLDFORS and SOURANDER, 1985, 1986; TANAKA, HAYAKAWA, ZYO et al., 1992) and simulating regional diets (PAIXÃO, ALÉSSIO, MARTINS et al., 2005). The nutritional model used in this study involves a severe reduction in the supply of protein casein. Milk is mainly composed of casein (up to 80%), and casein is an indispensable food for individuals, especially the young. Casein is involved in the structural functions of cells, and it extends the growth and development of the individual. The diet was in accordance with the diet for rodents AIN93G with 5% casein for malnourished animals and 20% casein for the nourished control animals. This protocol was efficient, which can be confirmed by the reduction in body weight and decreased cross-sectional area of the muscles and muscle fibers in the malnourished animals.

4.2 Qualitative analysis

The histologic evaluation demonstrated the predominance of type I collagen fibers in the biceps muscles of nourished animals and type III collagen fibers in the biceps muscles of malnourished animals. In contrast, in the pterygoid, although both types of collagen were observed, there was a predominance of collagen type III in both nourished and malnourished animals. It is possible that these differences between the muscles of different embryological origins and between the biceps of animals from different nutritional models did not occur because of one aspect of tissue regeneration, but instead, are due to an apparent delay in the maturation of muscle collagen.

4.3 Quantitative analysis

In this study, the nourished rats had a mean body weight that was three times higher than that of the malnourished rats. These data are similar to those obtained previously in protein malnutrition models (GLORE and LAYMAN 1983; NASCIMENTO, MADI, SILVA et al., 1990; NAKAGAWASAI, YAMADERA, SATO et al., 2006). Our findings are in agreement with a prior study reported by Philbrick and Hill (1974) that employed a different protein malnutrition model; they reported a 20% weight loss in the malnourished animals and a weight gain of 300% in the nourished animals. Thus, it appears that weight loss is a deleterious consequence of low protein diet.

Along with the differences in body weights, the cross-sectional areas of muscles from animals in the nourished group were higher than those in the malnourished group. From this data, one can infer that the muscular action of nourished muscles is more efficient. However, the density of the biceps muscle fibers was significantly higher in the undernourished group than in the nourished group, but this difference was not observed for the medial pterygoid. These data coincide with those described by Guth, Samaha and Albers (1970), who reported a decrease in the number of biceps muscle fibers in newborn Wistar rats.

We observed larger muscle fibers sizes in the nourished group, in agreement with Tanaka, Hayakawa, Zyo et al. (1992) who studied the quadriceps muscle of nourished and malnourished rats and reported decreased fiber size in malnourished rats, but no significant change in the number of fibers. In evaluating the anterior soleus and tibialis anterior of nourished and malnourished rats, a previous report noted a slowdown in growth reflected in muscle fiber size, but no change in muscle fiber quantity (TIMSON and DUDENHOEFFER, 1985). This phenomenon can be justified by the fact that the total number of muscle fibers remains the same during the life of an animal (ROWE and GOLDSPINK, 1969). Hypertrophy of existing fibers can occur as a result of an increase in the number of myofibrils, which are essential integral proteins (GOLDSPINK and ROWE, 1968). Therefore, it is possible that the reduction in profile area of the muscle fibers observed in this study is due to a loss of myofibrils resulting from protein restriction.

In a prior study in which we studied 21-day-old rat pups at a stage when they had just began chewing, we observed a greater number of oxidative fibers in the nourished group in the pterygoid, which suggests that these animals performed little or no chewing (MAEDA, HANAI and KUMEGAWA, 1981). It is possible that the difficulty in distinguishing between the types of fibers in the medial pterygoid was due to the fact that masticatory function in rats begins only after the 30th day of extrauterine life (MAEDA, HANAI and KUMEGAWA, 1981). The behaviors of the developing muscle fibers of different embryonic origins was reported in a study of masticatory muscles and biceps in human fetuses, in which the researchers noted that differentiating between the muscles was only possible after the 16th week (RINGQVIST, RINGQVIST and THORNELL, 1977). After this time, the biceps and masticatory muscle fibers could be distinguished (RINGQVIST, RINGQVIST and THORNELL, 1977). In an evaluation of the masseter muscle in pigs, the distinction between the two types of fibers is not possible until the 6th day, and during this time, it was possible to distinguish three types of muscle biceps fibers these animals (HORAK, 1995). This finding was corroborated by Ringqvist, Ringqvist and Eriksson et al. (1982) , who noted that glycolytic fibers were correlated with a strong bite and indicated that these fibers are smaller than the oxidative fibers. We did not make a similar observation in this study, since the animals had performed little or no chewing according to the appearance of the glycolytic fibers with larger sizes.

Malnutrition generally produces more pronounced effects on glycolytic fibers because oxidative fibers are relatively resistant to environmental influences (OLIVEIRA, OLIVEIRA, SCHMIDT et al., 1999). This differential sensitivity held true for the biceps muscle which is characterized by the presence of oxidative and intermediates fibers in higher percentages. In the medial pterygoid muscle, however, glycolytic fibers were observed in the highest percentage in the undernourished group.

Skeletal muscle fibers may respond to stimuli by changing in size or from one type to another, and this plasticity allows skeletal muscle tissue to adapt to different functional demands (OLIVEIRA, OLIVEIRA, SCHMIDT et al., 1999). These conversions most commonly occur between IIA and IIB fibers (glycolytic, and intermediate, respectively). The conversion of types II fibers to type I (oxidative) fibers is possible only in denervated muscles activated by electrical stimulation (SIECK, LEWIS and BLANCO, 1989). Authors who have examined the effects of malnutrition in different types of muscle fibers (OLDFORS and SOURANDER, 1986; SIECK, LEWIS and BLANCO, 1989; LEWIS, LORUSSO, ZHAN et al, 1996; OLIVEIRA, OLIVEIRA, SCHMIDT et al., 1999) verified that there is a decrease in the prevalence of type II fibers and an increase in IIB fibers in malnourished glycolytic fibers. In a study that assessed mitochondrial production of H_2O_2 by type II myofibers, researchers observed that type IIB fibers possess unique properties that potentiate mitochondrial superoxide production, which can lead to a potential mechanism for changes in skeletal muscle mitochondrial dysfunction (ANDERSON and NEUFER, 2006).

We observed that type II fibers had a larger diameter and that the total density of biceps muscle fibers was greater in malnourished animals than in the nourished animals. As the fibers were preferentially converted to type II, it is possible that this conversion explains the decrease in fiber size and thus the increase in the number of fibers per mm^2 in the muscle.

5 Conclusion

Studying the deleterious effects of malnutrition on skeletal muscle is crucial given that this condition affects millions of children worldwide. When established following intrauterine life, malnutrition causes the muscles to have reduced cross sections, which interferes with the efficiency of muscular action and reduces quality of life. Because the systemic consequences of malnutrition were somewhat similar in the braquial biceps and medial pterygoid, we can deduce that the differences in muscle action are due to the differences in their embryological origins. The effects of malnutrition can be irreversible depending upon its duration and intensity. Future studies should focus on the recovery of muscle tissue following malnutrition and evaluate whether and under what conditions re-nourishment can reverse muscle damage.

Acknowledgement: The authors thank Marta Maria da Silva Righetti for histologicals procedures and Rosana Duarte Prisco for statistical analysis.

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Received May 18, 2011
Accepted September 9, 2011