ISSN: 2230-9926

Available online at http://www.journalijdr.com

IJDR

International Journal of Development Research
Vol. 08, Issue, 06, pp.21025-21032, June, 2018

ORIGINAL RESEARCH ARTICLE OPEN ACCESS

IMPACT OF MATERNAL PROTEIN RESTRICTION ON MUSCLE FIBER AND NEUROMUSCULAR JUNCTION MORPHOLOGY OF EXTENSOR DIGITORUM LONGUS MUSCLE IN 21-DAY-OLD RAT OFFSPRING

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ARTICLE INFO

Article History:
Received 18th March, 2018
Received in revised form
26th April, 2018
Accepted 14th May, 2018
Published online 30th June, 2018

Key Words:
Morphology,
Morphometry, Protein Restriction, Extensor Digitorum Longus Muscle, Rat.

ABSTRACT

The fetal period is essential for the development of muscle fibers and neuromuscular junctions (NMJs). This study evaluated the effects of maternal protein restriction during pregnancy and lactation on muscle fibers and NMJs of extensor digitorum longus (EDL) muscle in 21-day-old rat offspring. Wistar rats were divided into a control group consisting of animals born to dams fed a normal protein diet (17% protein) during pregnancy and lactation, and a restricted group consisting of animals born to dams fed a low-protein diet (6% protein) during pregnancy and lactation. The pups were kept with their mothers throughout lactation (21 days). Samples of the EDL muscle were collected for the analysis of muscle fibers (hematoxylin-eosin and ultrastructure) and NMJs (nonspecific esterase) and for collagen quantification (Masson's trichrome). Restricted animals exhibited an increase of 18% in the percentage of intramuscular collagen and a reduction of 43% in the cross-sectional area of the muscle spindle. In the restricted group, the area, major diameter and minor diameter of NMJs were reduced by 42%, 21% and 23%, respectively. Protein restriction during the period studied interfered with the formation and maturation of muscle and NMJs, affecting offspring growth and development.

Citation: Leslie Cazetta Jeronimo, Heloisa Deola Confortim, Josiane Medeiros de Mello et al. 2018. "Impact of maternal protein restriction on muscle fiber and neuromuscular junction morphology of extensor digitorum longus muscle in 21-day-old rat offspring", *International Journal of Development Research*, 8, (06), 21025-21032.

INTRODUCTION

Maternal nutrition during pregnancy plays a key role in the adequate development of the fetus and placenta. The nutritional status of the mother is one of the extrinsic factors that programs nutrient partitioning and, finally, the growth, development and function of the main systems and organs of the fetus (Funston; Larson; Vonnahme, 2010).

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In mammals, much of the developmental environment is transduced through the mother, or more strictly through her phenotype as this can affect the transmission of external stimuli such as nutrition to her fetus or infant (Uller, 2008; Badyaev; Uller, 2009; Hanson, 2011). The principle whereby the nutritional, hormonal and metabolic environment provided by the mother permanently programs the structure and physiology of her offspring has already been established and is a manifestation of the general phenomenon of developmental plasticity (Barker, 2007). Fetal nutrient deficiency can result from many pregnancy conditions, including maternal malnutrition, reduced placental efficiency and adolescent pregnancy (Zhu *et al.,* 2006).

Nutrient restriction during pregnancy and lactation compromises overall fetal growth and development (Zambrano *et al.,* 2005), especially muscle tissue. Skeletal muscle is the most abundant tissue in the human body and can adapt to different situations. During the development of fetal skeletal muscle, mesenchymal stem cells differentiate into myogenic cells, adipogenic cells, and fibroblasts. Fibroblasts synthesize connective tissue that forms the endomysium, perimysium and epimysium of fetal skeletal muscle (Du *et al.,* 2010). Skeletal muscle has a lower priority in nutrient partitioning than the brain and heart, a fact that renders this tissue particularly vulnerable to nutrient deficiency (Zhu *et al.,* 2006; Funston *et al.,* 2010; Chaurasia *et al.,* 2016). Consequently, in the case of dietary protein deficiency, muscle tissue becomes the target of depletion (Nascimento *et al.,* 1990; Oliveira *et al.,* 1999), causing changes in the growth, function and differentiation of muscle fibers (Alves *et al.,* 2008). With the understanding of the role of nutrients in growth and development, the nutritional status of individuals, especially malnutrition, has become a subject of investigation (Leandro *et al.,* 2009). Deficient protein intake during critical periods of fetal development is known to trigger a series of metabolic and physiological changes as well as alterations in structural parameters (Barker, 1995). Although the fetal period is crucial for the development of skeletal muscle (Funston *et al.,* 2010), little is known about the influence of protein restriction on muscle spindles, intramuscular collagen and neuromuscular junctions (NMJs) of offspring skeletal muscle. Therefore, the objective of this study was to evaluate the morphological and morphometric alterations that occur in the muscle fibers and NMJs of extensor digitorum longus (EDL) muscle of 21-day-old rat offspring born to dams submitted to protein restriction (6%) during pregnancy and lactation.

MATERIALS AND METHODS

Animals

Male and female Wistar rats obtained from the Animal House of the Department of Anatomy, Institute of Biosciences, Universidade Estadual Paulista Júlio de Mesquita Filho (UNESP), Botucatu Campus, were used. The animals were maintained under standard light conditions (12/12-h light/dark cycle) at a temperature of about 23 ± 1ºC. The study was approved by the Ethics Committee on Animal Experimentation of UNESP (Approval No. 264-CEEA). At the beginning of the experiment, two females and one male of fertile age (12 weeks) were housed in maternity boxes overnight for mating. The next morning, the male was removed from the box and vaginal smears were obtained from the females to verify the presence of spermatozoa. The detection of spermatozoa in the vaginal smear was defined as day 0 of pregnancy. After the confirmation of pregnancy, the females were transferred to individual boxes. On day 0, 16 pregnant rats were divided into two groups: 1) dams fed a normal protein diet (17%) during pregnancy and lactations; 2) dams fed a low-protein diet (6%) during pregnancy and lactation. Both diets were isocaloric and were offered *ad libitum* to the two groups until the day of weaning (Table 1). On the day of birth, the offspring were weighed and eight pups per dam were kept during lactation to ensure the equal availability of food for all offspring. The pups remained with their mothers throughout the lactation period (21 days). After weaning, the pups were divided into two groups: control group (CG, n = 8) consisting of offspring born to dams fed the normal protein diet (17%) during pregnancy and lactation; restricted group (RG, n = 8) consisting of offspring born to dams fed the low-protein diet (6%) during pregnancy and lactation. Two male pups per litter were used, totaling 16 pups.

Collection of the Extensor Digitorum Longus Muscle: The rats were desensitized in a CO_2 chamber, weighed, and killed by decapitation in a guillotine. The animal was then positioned on a surgical table for dissection of the EDL muscle. The skin and tibialis anterior muscle were dissected from the right and left pelvic limbs for removal of the EDL muscle. The muscle was then weighed and measured with a digital caliper (Digimess®, São Paulo, Brazil). The right antimere was collected for histological analysis and the left antimere was cut for histochemical study of the NMJs and transmission electron microscopy (TEM) analysis of muscle fibers. Samples of visceral and epididymal fat were also collected and weighed.

Morphological and morphometric study of muscle fibers and collagen

The EDL muscle samples were fixed in Karnovsky's solution (1965) (2.5% in 0.1 M phosphate-buffered saline, PBS). After fixation, the fragments were washed in PBS to remove excess fixative and embedded in Paraplast. The muscle was embedded in the vertical position to permit subsequent cross-sectioning. The blocks were prepared at an embedding center (EasyPath Cygni®, São Paulo, Brazil). The muscle samples were cut into 5-µm thick sections with a microtome (RM2165, Leica®, Wetzlar, Germany). The sections were mounted on silanized slides and stained with hematoxylin-eosin (HE) for observation of general muscle fiber morphology. Muscle spindles were measured by capturing the images (1,000x magnification) of six spindles per animal in different sections. The following measurements were standardized: area and major diameter of the muscle spindle, average cross-sectional area of intrafusal fibers, and number of intrafusal fibers in the muscle spindle with largest diameter.
For quantification of the percentage of intramuscular collagen, the slides were stained with Masson's trichrome (Bancroft; Stevens, 1990). Six random images were obtained per animal at 400x magnification. The measurements were made using the Image-Pro Plus 6.0 software (Media Cybernetics, Maryland, USA).

Analysis of Muscle Fibers by Transmission Electron Microscopy: For ultrastructural study, the muscle was removed and cut into longitudinal fragments (width of approximately 1 mm), fixed in Karnovsky's solution (1965), and examined by TEM. For microscopy analysis, the samples were removed from the fixative and washed in 0.1 M PBS (pH 7.3). The material was immersed in osmium tetroxide for 2 h and washed in distilled water. After washing, the material was immersed in 0.5% uranyl acetate in distilled water for about 2 h, dehydrated in an increasing acetone series (50%, 70%, 90% and 100%), and embedded. The tissue blocks were then cut into ultrathin sections (approximately 90 nm). The sections were counterstained with a saturated solution of uranyl acetate in 50% alcohol for 20 min and with lead citrate for 10 min. The material was examined and photographed under a CM100 transmission electron microscopy (Philips®, The Netherlands).

Morphology and Morphometry of Neuromuscular Junctions: Fragments of the EDL muscle were removed, fixed in Karnovsky's solution (1965), kept at room

Table 1. Composition of the diet fed rats during pregnancy and lactation

Components*	Normal protein (17% of protein)	Low protein (6% of protein)
Casein (84% protein)**	202.00	71.50
Corn Starch	397.00	480.00
Dextrin	130.50	159.00
Saccharose	100.00	121.00
Soybean Oil	70.00	70.00
Fiber (microcellulose)	50.00	50.00
Mixture of Minerals ***	35.00	35.00
Mixture of Vitamins ***	10.00	10.00
L - cystine	3.00	1.00
Choline Chlorine	2.50	2.50

* Diet for gestation stage in rodents - AIN-93G; ** Values corrected according to the protein content of the casein; *** According to AIN-93G

Table 2. Body weight, visceral and epididymal fat weight, and weight and length of the EDL muscle in 21-day-old rat offspring born to control dams or dams submitted to protein restriction

Parameters	Group Control	Group Restricted
Body weight (g)	48.650 ± 6.874	21.910 ± 3.588***
Visceral and epididymal fat weight (g)	0.539 ± 0.084	0.164 ± 0.035***
EDL muscle weight (g)	0.015 ± 0.004	0.004 ± 0.002***
EDL muscle length (mm)	10.790 ± 1.128	7.785 ± 0.814***

Values expressed as mean ± standard deviation. *** Statistical significance set at $p < 0.001$

temperature, and cut longitudinally into several slices with a stainless-steel knife. The sections were then submitted to the nonspecific esterase reaction (Lehrer & Ornstein, 1959). The images of NMJs were captured at 200x magnification and the areas and major and minor diameters of 50 NMJs per animal were measured using the Image-Pro Plus 6.0 software.

Image Analysis

Muscle fibers were observed under a Primo Star microscope (***Zeiss***®, Oberkochen, Germany) equipped with a camera (AxioCam ERc5s) and analyzed using the AxioVision Rel.4.8 program (Zeiss®, Microimaging, Inc., Germany). The NMJs and collagen were photodocumented in images captured with an Olympus Bx60® microscope equipped with an Olympus DP71 camera (Tokyo, Japan) using the DP Controller 3.2.1 276 program.

Statistical Analysis

First, the Shapiro-Wilk test was applied to test the normality of the data. Body weight, visceral and epididymal fat weight, and EDL muscle length were compared by the Student *t*-test. Muscle weight, area and major and minor diameter of the NMJs were analyzed using the Mann-Whitney test. Significance was defined for $p < 0.05$ and the data are expressed as mean ± standard deviation.

RESULTS

Macroscopic Parameters

At 21 days of age, the body weight of RG animals was approximately 55% lower than that of CG animals ($p < 0.001$). In RG, reductions of 70% in visceral and epididymal fat weight ($p < 0.001$), of 73% in EDL muscle weight ($p < 0.001$) and of 28% in EDL muscle length ($p < 0.001$) were observed when compared to CG (Table 2).

Morphological and Morphometric Study of Muscle Fibers and Collagen

Fetal fibers, myotubes and central nuclei were detected in animals of CG (Figure. 1A) and RG (Figures. 1B, 1C, and 1D), but were more frequent in the latter. Analysis of the muscle spindle showed an average of five intrafusal fibers per spindle in CG and RG (Figures. 2A and 2B). The cross-sectional area of the muscle spindle was reduced by 43% in RG (Figure. 2D) when compared to CG. The two groups did not differ significantly in terms of the major diameter of muscle spindles, number of intrafusal fibers, or cross-sectional area of intrafusal fibers (Figures. 2C, 2E, and 2F). Analysis of collagen by staining with Masson's trichrome revealed an increase of 18% in RG compared to CG (Figure. 3).

Transmission Electron Microscopy Study of Muscle Fibers

Animals of CG exhibited areas with disorganized or loosely arranged myofibrils and a disorganized Z line (Fig. 4A and 4C). These features occurred in scattered muscle fibers and in foci along same fibers; however, normal-appearing fibers were found in neighboring areas. In RG, the muscle fibers contained areas of rarefied or loosely arranged myofibrils and a disorganized Z line (Fig. 4B). In addition, a larger number of lipid droplets was observed (Fig. 4D).

Morphological and Morphometric Analysis of Neuromuscular Junctions

In the groups studied, the NMJs of the EDL muscle exhibited the classical plaque-like morphology and had an oval, round or elliptical shape. Morphological variations such as open, irregular or compact junctions were a common finding and characterize the polymorphism of these structures (Fig. 5A and 5B). Reductions were observed in the area (42%), major diameter (21%) and minor diameter (23%) of NMJs in RG animals compared to CG (Fig. 5C, 5D, and 5E).

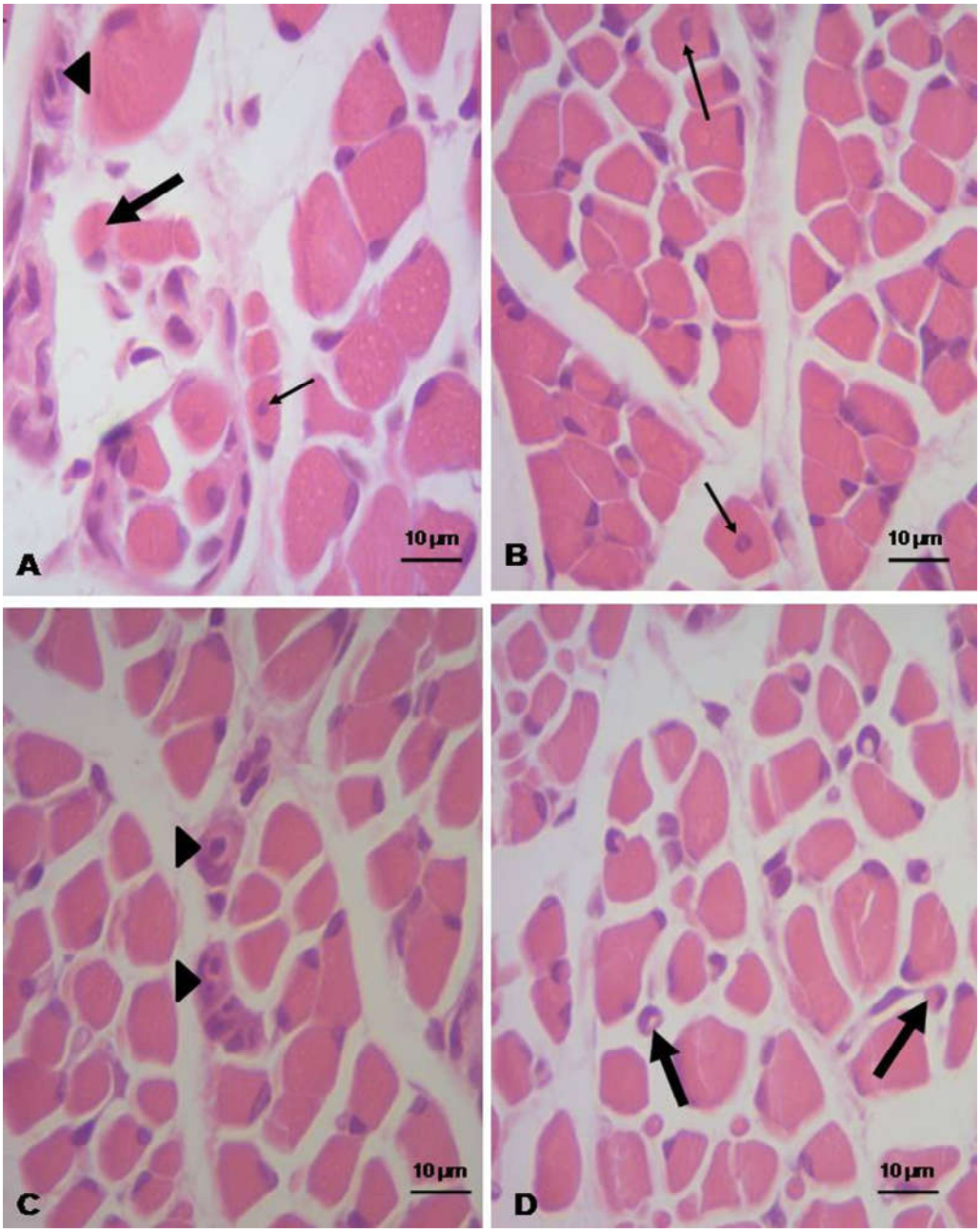


Figure 1. Photomicrographs of cross-sections of the EDL muscle in 21-day-old rat offspring born to dams fed a normal protein diet (control group) and a low-protein diet (6%, restricted group). HE. A: Control group. Central nucleus (thin arrow); fetal fiber (thick arrow), and myotube (arrowhead). B, C and D: Restricted group. Central nuclei (thin arrows); fetal fibers (thick arrows), and myotubes (arrowheads)

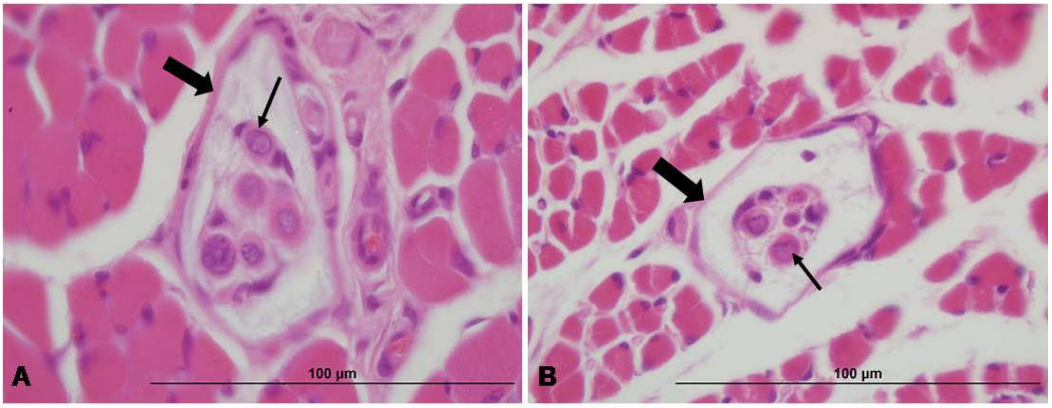
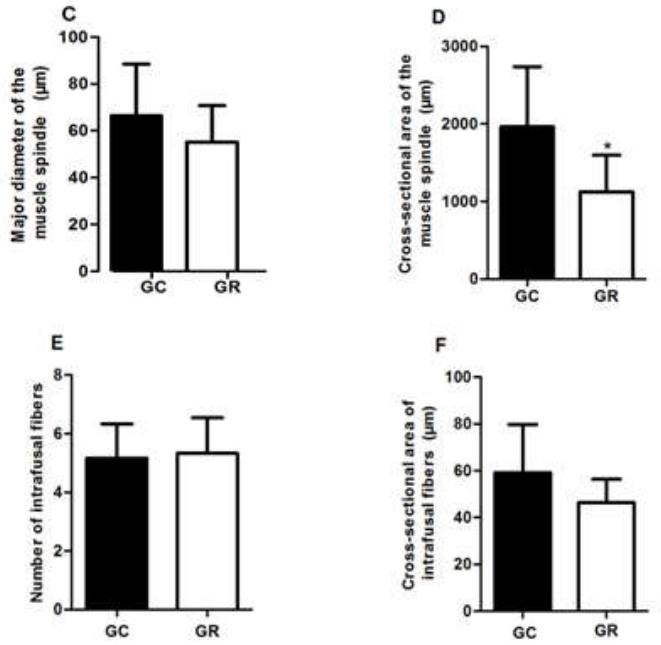


Figure 2. Photomicrographs of cross-sections of the EDL muscle in 21-day-old rat offspring. HE. A and B: Observe the capsule of the muscle spindle (thick arrow) and intrafusal fibers (thin arrow) in control (CG) and restricted (RG) animals, respectively



C, D, E and F: Major diameter and cross-sectional area of the muscle spindle and number and cross-sectional area of intrafusal fibers in CG and RG. Values are expressed as the mean ± standard deviation. Student *t*-test. * p < 0.001

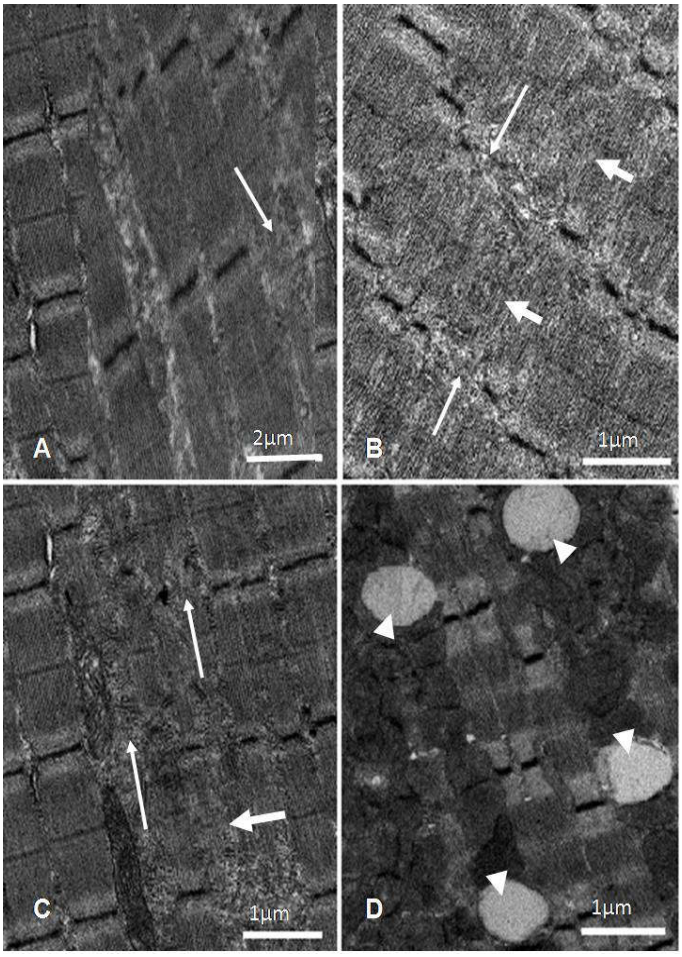


Figure 4. Electron photomicrographs of the EDL muscle in 21-day-old rat offspring. Longitudinal sections. A and C: Control group. Observe the muscle fibers with foci of sparse myofibrils (thick arrow) and disorganized Z line (thin arrow). B and D: Restricted group. Observe the muscle fibers exhibiting foci of sparse myofibrils (thick arrow) and disorganized Z line (thin arrow) and the presence of lipid droplets (arrowhead)

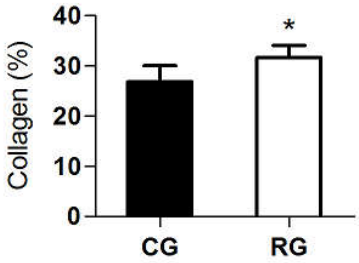
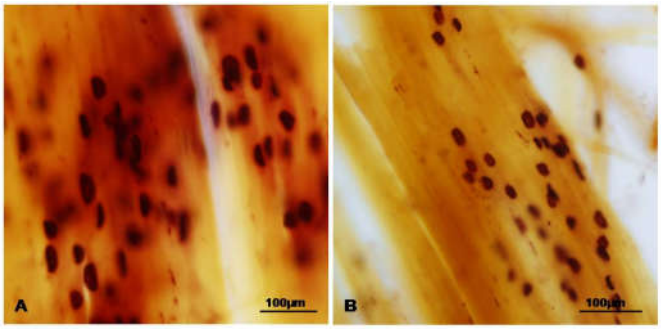


Figure 3. Percentage of collagen in EDL muscle of 21-day-old rat offspring of the control (CG) and restricted groups (RG). Value are expressed as the mean ± standard deviation. Student *t*-test. * p < 0.05



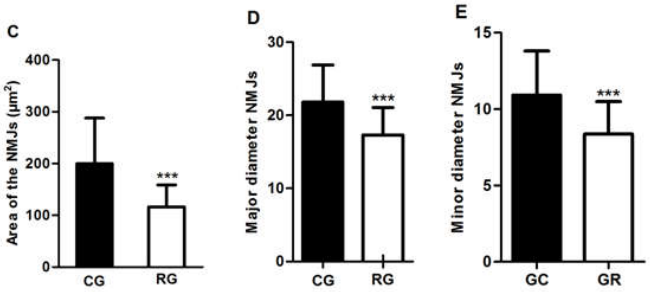


Figure 5. Photomicrographs of the neuromuscular junctions (NMJs) of EDL muscle in 21-day-old rat offspring. Longitudinal sections, nonspecific esterase reaction. A and B: Morphological features of the NMJs of control (CG) and restricted animals (RG) respectively. C, D and E: Area, major diameter and minor diameter of the NMJs of CG and RG animals. Values are expressed as the mean ± standard deviation. Student *t*-test. * p < 0.001

DISCUSSION

Deficient protein intake during critical periods of fetal development, such as the gestational period, can induce a series of metabolic and physiological changes such as weight loss, as well as alterations in structural parameters, (Barker, 2007). In the present study, a body weight reduction of 55% was observed in RG offspring compared to CG. This result shows that the maternal protein-restricted diet administered during pregnancy and lactation triggered malnutrition, which influenced the development and growth of the offspring at 21 days of age. Body weight loss is a clinical parameter that characterizes protein energy malnutrition (Torun and Chew, 1994) and might be related to factors such as reduced maternal metabolism, low umbilical blood flow, and inadequate placental transfer of nutrients (Harding, 2001; Belkacemi *et al.,* 2010). Consequently, maternal nutrition may have a direct effect on fetus growth and development through the deficient

availability and transfer of nutrients. However, contradictory results showing no significant body weight loss in animals submitted to maternal protein restriction have been reported (Oliveira *et al.,* 1999; Cabeço, 2011). These divergences can be attributed to the duration of protein restriction (pregnancy and lactation, only during pregnancy or lactation, after lactation), type of diet (high or low calorie/protein), and age of the animals. At 21 days of age, RG animals also exhibited a reduction of 70% in visceral and epididymal fat weight, of 73% in EDL muscle weight, and of 28% in EDL muscle length when compared to CG. According to Bedi *et al*. (1982), protein energy restriction after the lactation period can be restored with normalization of the nutritional state, while the changes resulting from protein restriction during pregnancy and lactation can cause permanent deficits. Malnutrition of rats during pregnancy and lactation was found to reduce the weight of EDL muscle (Toscano *et al.,* 2008) and of the tibialis anterior muscle (Ventruci *et al.,* 2004; Alves *et al.,* 2008). Like the EDL, the tibialis anterior muscle is predominantly composed of fast-twitch fibers. On the other hand, Cabeço (2011), using the same diet protocol but protein restriction only during pregnancy, found no differences in EDL muscle weight at 30 days and 16 weeks of age. The alterations in muscle weight and length observed in the present study were probably due to the amount of protein provided to the animals during pregnancy and lactation, which was sufficient to affect short-term skeletal muscle development. This evidence is consistent with the hypothesis that during nutritional deprivation the fetus supports the growth of vital organs such as the brain and heart at the expense of other tissues such as skeletal muscle (Zhu *et al.,* 2006; Toscano *et al.,* 2008; Funston *et al.,* 2010; Chaurasia *et al.,* 2016).

A large number of myotubes, central nuclei and fetal fibers we observed in the EDL muscle of both groups studied, confirming the immaturity of part of the muscle fibers at this age (Sarnat, 1982; Oliveira *et al.,* 1999; Alves *et al.,* 2008). An increase of 18% in collagen was observed in offspring of RG compared to CG. The development of fetal skeletal muscle mainly involves myogenesis, but also adipogenesis and fibrogenesis. Fibrogenesis is responsible for the formation of the endomysium, perimysium and epimysium of fetal skeletal muscle (Du *et al.,* 2010).

The increase in collagen and immaturity of part of the muscle fibers, which occurred to a greater extent in RG animals, are related to maternal protein deprivation since maternal malnutrition affects the development of fetal skeletal muscle (Zhu *et al.,* 2004; Yan *et al.,* 2013), delaying the growth of muscle cells in relation to connective tissue development (Oliveira *et al.,* 1999) and increasing fibrogenesis, with a consequent increase of muscle connective tissue in the offspring (Yan *et al.,* 2013).

The higher proportion of connective tissue between muscle fibers is an indicator of muscle hypoplasia (Alves *et al.,* 2008). Analysis of the muscle spindle revealed a reduction of 43% in the cross-sectional area of the spindle in RG animals at 21 days of age. Muscle spindles are mechanical receptors that detect variations in muscle length, as well as the speed in this variation, and are arranged in parallel to extrafusal muscle fibers (Barker *et al.,* 1973; Maier, 1997). We found no study that specifically reported the effects of malnutrition on the neuromuscular spindle. However, the alterations observed in this study were probably due to protein deprivation during a crucial period of skeletal muscle development, since the morphological and functional properties of muscle are determined during embryonic development and during the fetal and early postnatal period (Norman, 2012). In the absence of nutrients, the priority of skeletal muscle in nutrient partitioning is lower, a fact rendering these muscles vulnerable to nutritional deficiencies and compromising their structures (Zhu *et al.,* 2006).

Ultrastructural analysis of the EDL muscle in RG animals showed a higher frequency of areas with disorganized or loosely arranged myofibrils and disorganized Z lines and a larger number of lipid droplets when compared to CG. With respect to sarcomere and Z-line disorganization, similar results have been reported by Oumi *et al*. (2000) in rats submitted to protein restriction after lactation. The authors suggested that, although it is unknown which factors are involved in this disorganization, they are likely to be associated with protein metabolic disorder of muscle cells. Nascimento *et al*. (1990) also observed an increase of lipid droplets in 30-day-old animals submitted to maternal protein restriction.

The alterations observed in the present study might be related to the immaturity of part of the muscle fibers, which are still in the process of formation and sarcomere organization (Confortim *et al.,* 2016). However, in the present study, in addition to the immaturity of part of the muscle fibers, maternal protein deprivation also affected to a greater extent the development and maturation of muscle fibers in RG animals.

Protein restriction during pregnancy can alter the skeletal muscle phenotype in the offspring, but it remains unclear whether it also affects NMJs considering that maintenance of the muscle phenotype depends on the functional integrity of the NMJ (Castro *et al.,* 2017). In the present study, the area of NMJs was reduced by 42% and their major and minor diameter by 21% and 23%, respectively, in 21-day-old RG animals compared to CG. Confortim *et al*. (2016), using the same diet protocol and animals of the same age, also found alterations in NMJ morphology of soleus muscle. Castro *et al.* (2017) demonstrated that a low-protein diet (6%) administered during pregnancy compromised mRNA expression of the nAChR subunit in NMJs of the EDL muscle and promoted morphological changes in the NMJs of soleus muscle in 30-day-old offspring.

The intrauterine period is an important phase of NMJ and muscle fiber development. The larger number of immature muscle fibers observed in RG animals and the changes in NMJ morphology are probably related to the immaturity of the neuromuscular system. In this respect, malnutrition significantly affects the growth and maturation of the central and peripheral nervous system (Winick *et al.,* 1975; Chaves *et al.,* 1977; Alves *et al.,* 2008) and muscle dysfunction caused by malnutrition is the result of changes that occur across the motor unit (Nascimento *et al.,* 1990). Further studies are necessary to elucidate the changes in NMJs caused by maternal protein malnutrition.

In conclusion, the present results showed that a low-protein diet (6%) administered during pregnancy and lactation promoted a delay in the morphological differentiation of muscle fibers and NMJs of the EDL muscle in 21-day-old rat offspring, affecting the growth and development of the animals

due to the interference of protein restriction with the formation and maturation of muscle tissue.

Acknowledgement

The UNIOESTE granted by the infrastructure, the Electronic Microscopy Center of UNESP- Botucatu and technician Gelson Rodrigues of anatomy department for the greathelp in all steps of research.

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