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# Effects of aging and maternal protein restriction on the muscle fibers morphology and neuromuscular junctions of rats after nutritional recovery

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ABSTRACT

Changes in the nutritional status of mothers may predispose their offspring to neuromuscular disorders in the long term. This study evaluated the effects of maternal protein restriction during pregnancy and lactation on the muscle fibers and neuromuscular junctions (NMJs) of the soleus muscle in the offspring of rats at 365 days of age that had undergone nutritional recovery. Wistar rats were divided into two groups: control (CG) – the offspring of mothers fed a normal protein diet (17%) and restricted (RG) – offspring of mothers fed a low protein diet (6%). After lactation, the male pups received standard chow *ad libitum*. At 365 days, samples of soleus muscle were collected for muscle fiber analysis (HE staining, NADH-TR reaction and ultrastructure), intramuscular collagen quantification (picrosirius red staining) and NMJs analysis (non-specific esterase technique). The cross-sectional area of type I fibers was reduced by 20% and type IIa fibers by 5% while type IIb fibers increased by 5% in the RG compared to the CG. The percentage of intramuscular collagen was 19% lower in the RG. Disorganization of the myofibrils and Z line was observed, with the presence of clusters of mitochondria in both groups. Regarding the NMJs, in the RG there was a reduction of 10% in the area and 17% in the small diameter and an increase of 7% in the large diameter. The results indicate that the effects of maternal protein restriction on muscle fibers and NMJs seem to be long-lasting and irreversible.



## 1. Introduction

Experimental studies show a close relationship between the conditions offered in the intrauterine environment and the appearance of disease in the offspring, a phenomenon known as fetal programming (Ozanne, 2001; Barker et al., 2002; Bellinger and Langley-Evans, 2005; Fidalgo et al., 2012). The earlier intrauterine protein restriction occurs during development, the more deleterious its effects becomes to the offspring (Patrício et al., 1984). Changes in the nutritional status of mothers predispose the offspring to cardiovascular (Fowden et al., 2006), urinary (Assis and Fidelis, 2011), digestive (Gurmini et al., 2005), respiratory (De Andrade et al., 2012) and muscular changes (Mallinson et al., 2007; Toscano et al., 2008; Cabeço et al., 2012; Dal Pai Silva and Carvalho, 2007).

The muscles constitute the body's largest reservoir of protein and, coordinated by the nervous system, they are responsible for the locomotion, respiration and functional autonomy of the individual (Nascimento et al., 1990; Pierine et al., 2009). The neuromuscular junctions (NMJs) are synapses that occur between motoneurons and skeletal muscle fibers (Wu et al., 2010) that allow muscle contraction and the performance of motor functions (Delbono, 2003). During the fetal period, the NMJs and muscle fibers are formed, and their physiological and biochemical properties are determined (Sanes and Lichtman, 1999; Nissen et al., 2003; Cabeço et al., 2012). Following birth, there may be growth (hypertrophy) (Dal Pai Silva and Carvalho, 2007) or reduction (atrophy or hypertrophy) (Powers et al., 2007) in the muscle fibers areas according to the stimuli to which they are exposed. Besides, the muscle fibers

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can be classified into types I, IIa and IIb (Brooke and Kaiser, 1970). The type I fibers are smaller, the type IIa are medium-sized and the type IIb are larger. The first type fiber cited has slow twitching while the last two ones are characterized by fast twitching. The NMJs have different morphological characteristics in accordance with the type of muscle fibers they innervate. The NMJs of type I muscle fibers are small and rounded or slightly elliptical in shape; those of type IIb muscle fibers are large and elliptical in shape; lastly, the NMJs of type IIa fibers exhibit structural features that are intermediate to those seen in the previously described fibers (Ogata and Yamasaki, 1985).

The characteristics of striated skeletal muscles can be altered in the short and long term by nutritional conditions (Brito et al., 2006) and aging (Robinson et al., 2012). More specifically, protein restriction imposed during development can structurally and functionally modify muscles (Mallinson et al., 2007; Toscano et al., 2008; Cabeço et al., 2012). As the NMJs are closely interconnected to the skeletal muscles, it is believed that such structures can also undergo concomitant changes.

The focus of the present study was the soleus muscle, which is considered a postural muscle because is predominantly composed of type I muscle fibers, have a higher oxidative metabolism and slow contraction (Kelly and Rubinstein, 2003; Toscano et al., 2010.). The NMJs associated with this muscle are predominantly small and have a rounded shape (Ogata and Yamasaki, 1985). Studies in the literature have shown that the soleus muscle is more sensitive to the effects of maternal protein restriction when compared to other muscles (Toscano et al., 2008; Cabeço et al., 2012; Da Silva Aragão et al., 2013). However, few studies have evaluated the effect of maternal protein restriction on skeletal muscle in aging after nutritional recovery (Bedy et al., 1982; Ozanne et al., 2003).

Economic changes and the consequent increase in life expectancy in developing countries have drawn attention to the effects of aging, and currently a number of topics are being studied in this age group of the population (Moriguti et al., 2001; Chai et al., 2011). In this context, the aim of this study was to evaluate the morphological, morphometric and ultrastructural alterations in the muscle fibers and characterize the NMJs of the soleus muscle of the offspring of rats submitted to protein restriction during pregnancy and lactation, after nutritional recovery and aging.

## 2. Materials and methods

### 2.1. Animals

The study was approved by the Ethics Committee on Animal Experimentation of the Paulista State University (Universidade Estadual Paulista Júlio de Mesquita Filho – UNESP) (No. 264-CEEA). Male and female Wistar rats obtained from the Animal House of the Department of Anatomy, UNESP, were maintained under standard conditions of light (12-h light/dark cycle) and temperature (23 ± 1 °C). At the beginning of the experiment, two females and one male of reproductive age (12 weeks) were housed in boxes overnight for mating. The male was removed from the box in the following morning and a vaginal smear was obtained from the females. The detection of sperm in the vaginal smear was defined as day 0 of pregnancy, when the females were transferred to individual boxes. On day 0, female rats were divided into two groups: (1) dams fed a normal protein diet (17%) during pregnancy and lactation, and (2) dams fed a low-protein diet (6%) during pregnancy and lactation. The normal protein (17% protein) and low-protein (6% protein) diets were isocaloric and were administered *ad libitum* until the day of weaning of the offspring (Table 1). After this period, the offspring were provided with standard rodent chow (17% protein).

**Table 1**
Composition of the experimental diets.

| Ingredients* | Normal-protein diet (17% protein) | Low-protein diet (6% 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 |
| Mineral mix*** | 35.00 | 35.00 |
| Vitamin mix*** | 10.00 | 10.00 |
| L-Cysteine | 3.00 | 1.00 |
| Choline chlorine | 2.50 | 2.50 |

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

On the day of birth, male pups were separated from female pups. Additionally, to guarantee an equal availability of food, eight pups were maintained per dam during lactation (21 days). After this period the male pups received a normal protein diet until 365 days of age. Then, the male pups were divided in two experimental groups: the control group (CG) – the pups of mothers fed a normal protein (17%) diet during gestation and lactation ($n = 5$) and restricted group (RG) – the pups of mothers fed a low protein diet (6%) during pregnancy and lactation ($n = 8$).

### 2.2. Collecting the soleus muscle

At 365 days of age, the animals of the two groups were weighed and then euthanized in a $CO_2$ chamber, followed by decapitation using a rodent guillotine. The skin of the hind limbs (left and right antimere) was elevated and the gastrocnemius muscle was removed for exposure and dissection of the soleus muscle. The length (mm) of the soleus muscle from its origin until insertion was measured with a digital caliper (Gigimess®, Brazil) and the muscle was weighed.

The soleus muscles were sectioned into three fragments with a stainless steel blade for histological, histoenzymological, ultrastructural and immunohistochemical studies. In addition the liver and fat (epididymal, retroperitoneal and visceral) were collected and weighed.

### 2.3. Histological and morphometric analysis

The soleus muscle samples were fixed in Karnovsky's solution (Karnovsky, 1965) and washed in phosphate-buffered saline (PBS) to remove excess fixative. The muscle samples were embedded in paraplast in the vertical position. Blocks were prepared using an embedding center (Cygni, EasyPath®, Brazil). Next, the muscle fragments were cut into 5-μm thick sections with a microtome (RM2165, Leica®, Germany). The sections were mounted on previously silanized slides, dried for 1 h at 60 °C, and submitted to deparaffinization, hydration and staining with hematoxylin–eosin (HE) (Junqueira and Junqueira, 1983). The stained slides were then immediately dehydrated, cleared, and mounted in Permount (Fisher Scientific®, USA).

For the quantification of nuclei and muscle fibers and area measurement of these fibers, 10 images in random fields per animal were examined and captured at 400× magnification. Photomicrographs were obtained at 50× magnification (necessary to comprise the entire diameter of the muscle) for the measurement of total muscle area. These images were merged using appropriate software for subsequent measurement of the muscle area.