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MORPHOLOGICAL CHANGES OF EXTENSOR DIGITORUM LONGUS MUSCLE IN THE OFFSPRING OF OBESE RATS

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ABSTRACT

Objective: To evaluate the morphology and morphometry of muscle fibers and neuromuscular junctions (NMJs) of the extensor digitorum longus muscle (EDL) in the offspring of obese ratsinduced by a cafeteria diet. **Methods:** Three-week-old female Wistar rats were randomly divided into two groups: a control (CTL) group that received a standard diet and an obese (OB) group that received a cafeteria diet. The animals were mated at 23 weeks of age. After birth, only males were used for the experiment. The offspring of the first generation (F1) were designated CTL-F1 and OB-F1 according to the treatment of the mothers and received the standard diet. The animals were euthanized at 17 weeks of age and the EDL muscle was collected and routinely processed for morphological and morphometric analysis by light microscopy and transmission electron microscopy. **Results:** The body weight, retroperitoneal and periepididymal fat weight and capillary/fiber ratio were higher in the OB-F1 group compared to CTL-F1. However, a reduction in the number of nuclei, connective tissue, ultrastructural parameters, and NMJ area and largest diameter o was observed in the OB-F1 group. **Conclusion:** Feeding mothers an obesogenic diet throughout life induces morphological and morphometric changes in the skeletal muscle fibers and NMJs of their offspring in later life.

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INTRODUCTION

Obesity is a multifactorial disease that has reached epidemic proportions in Brazil and in the world (De Lorenzo, 2016; IBGE, 2010). In addition to a sedentary lifestyle and the habit of consuming hypercaloric diets, a new concept has emerged that helps understand the obesity epidemic. This concept, known as "Developmental origins of health and disease" (DOHad), postulates that insults during fetal life can lead to the development of diseases in adult life (Heindel and Vandenberg, 2015). According to this concept, a hypercaloric maternal diet during pregnancy and lactation increases the risk of offspring developing obesity, metabolic syndrome and type 2 diabetes mellitus through metabolic programming (BARKER, 2004).

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Skeletal muscle tissue is the most abundant tissue in the body that has important physical and metabolic functions (LE *et al*., 2014). The morphofunctional properties of skeletal muscle are determined during embryogenesis and the fetal period is therefore crucial to its development. Maternal obesity has been shown to compromise fetal muscle development by affecting the differentiation of mesenchymal cells (DU *et al*., 2010). However, there are no data in the literature on the effects of maternal obesity on skeletal muscle fiber morphology in the offspring. The objective of the present study was to evaluate the morphology and morphometry of muscle fibers and neuromuscular junctions (NMJs) of the extensor digitorum longus (EDL) muscle in the offspring of obese rats induced by a cafeteria diet.

MATERIALS AND METHODS

The study was conducted at the Laboratory of Endocrine Physiology and Metabolism, Sectorial Animal House, Center

for Biological and Health Sciences, and at the Experimental Laboratory of Morphology, Universidade Estadual do Oeste do Paraná, Cascavel Campus, in collaboration with the Center for Electron Microscopy, Universidade Estadual Paulista "Júlio de Mesquita Filho" (UNESP), Botucatu Campus. All adopted procedures were approved by the Ethics Committee on Animal Use of Universidade Estadual do Oeste do Paraná, and were conducted in accordance with animal research guidelines.

Experimental procedure – dams: First, 27 female Wistar rats at 3 weeks of age were randomly divided into two groups. The control group (CTL; n = 13) received standard rat chow (3.8 kcal/g: 70% carbohydrate, 20% protein, and 10% fat) (BioBase, Brazil) and water *ad libitum*. The obese group (OB; n = 14) received, in addition to the standard diet, a cafeteria diet (5.4 kcal/g: 38.5% carbohydrate, 15% protein, and 46.5% fat) (Goularte *et al*., 2012), with some modifications (Table 1), and 350 ml/day degassed Coca-Cola (Coca-Cola, Brazil) or Guaraná. The animals were maintained at a room temperature of 23±2°C under a 12h photoperiod. After 20 weeks of feeding the diets, the dams of the two groups were mated at a ratio of two females per male.

Experimental procedure – offspring

Animals: Six pups per litter were maintained at birth. After 30 days, the offspring were separated from the dams and only males were used for the experiments. The offspring of the first generation (F1) were designated CTL-F1 (n = 7) and OB-F1 (n = 7) according to the treatment of the mothers. All animals received the standard diet from weaning until euthanasia.

Evaluation of weight and obesity and collection of extensor digitorum longus muscle: The animals were euthanized at 17 weeks of age. The body weight and retroperitoneal and periepididymal fat weight were used for the evaluation of obesity. For collection of EDL muscle, the skin of the hind limb was separated and the tibialis anterior muscle was removed for dissection of the EDL. The length (mm) of the muscle was measured with a digital caliper (Digimess®, São Paulo, Brazil) and the muscle was weighed. The muscle was then cut into fragments with a stainless-steel blade for subsequent histological study, histoenzymological analysis of muscle fibers, and histochemical study of NMJs.

Study of muscle fibers: For the study of muscle fibers, the distal fragments of the right antimere of the EDL muscle were removed and kept for 30-40 min at room temperature (KHAN, 1977). For tissue preservation, the material was covered with neutral talc (MOLINE *et al*., 1964), frozen in liquid nitrogen for 2 min, and stored in a biofreezer at -80°C. The frozen muscle fragments were transferred to a cryostat chamber (LUPETEC CM 2850 Cryostat Microtome) at -30°C and kept for 30 min. Next, one end of the fragments was fixed to a metal support with Jung Tissue Freezing Medium (Leica, Germany) and cut transversely at intervals of 7 µm. The sections obtained were submitted to specific staining methods and reactions for muscle fiber analysis as described below.

Histological study: Cross-sections of the EDL muscle were stained with hematoxylin-eosin (HE) according to the technique of Junqueira *et al*. (1983) to determine the number of nuclei, number and area of muscle fibers, number of capillaries, and the capillary/fiber ratio. Ten microscopic fields (40X objective) were analyzed per animal.

For nucleus count, fiber count and fiber area, only intact fibers in the microscopic fields were considered. For capillary count and capillary/fiber ratio, muscle fibers covering the upper and right margins were included in the count, while those present at the lower and left margins were excluded from the analysis. The counts were performed individually by two trained evaluators and the mean of the values obtained was used for analysis (Fernandes *et al*., 2012).

Histoenzymological and morphometric study: The oxidative and glycolytic metabolism of muscle fibers was evaluated by the nicotinamide adenine dinucleotide-tetrazolium reductase (NADH-TR) reaction as described by Pearse (1972) and modified by Dubowitz *et al*. (1973). For morphometry, the cross-sectional area of the EDL muscle obtained from the histoenzymological study was measured and the different muscle fiber types (I, IIa, and IIb) were quantified. Two microscopic fields per animal were randomly chosen (20X objective).

Study of connective tissue: For connective tissue analysis, the proximal fragments of the right antimere of the EDL muscle were fixed in buffered formalin and stored in 70% alcohol for subsequent histological processing consisting of dehydration in an increasing alcohol series, clearance in xylene, and embedding in paraplast. The embedded muscles were cut with a microtome into 7-µm sections and stained with Masson's trichrome for the measurement of connective tissue percentage (endomysium and perimysium). This percentage was calculated by the number of pixels (BOSI *et al*., 2008) in 10 random microscopic fields per animal (40X objective).

Ultrastructural study of muscle fibers: For ultrastructural analysis of muscle fibers, distal fragments of the left antimere of the EDL muscle were removed and fixed in Karnovsky solution (KARNOVSKY, 1965). The material was cut into longitudinal sections of approximately 1 mm, washed in 0.1 M phosphate buffer, pH 7.3 (15 min), and post-fixed in 1% osmium tetroxide in the same buffer (2 h). Next, the sections were washed in distilled water (3 times for 5 min), incubated with 0.5% uranyl acetate in aqueous solution (2h), dehydrated in an increasing acetone series, and immersed in a mixture of resin and 100% acetone (12 h) for subsequent resin embedding. The desired fields were selected in semi-thin sections (0.5 µm) and ultrathin sections (90 nm) were cut with an ultramicrotome (Ultracut UCT, Leica®, Germany). The ultrathin sections were stained with a saturated solution of uranyl acetate (20 min) and lead citrate (10 min) for subsequent analysis.

Histochemical and morphometric study of neuromuscular junctions: For morphological and morphometric analysis of NMJs, proximal fragments of the left antimere of the EDL muscle were removed, immersed in Karnovsky solution (Karnovsky, 1965) at room temperature, and cut longitudinally into three or four pieces with a stainless-steel blade. The sections obtained were submitted to the nonspecific esterase reaction (Lehrer; Ornstein, 1959). For morphometric analysis, the area and largest and smallest diameters of 100 NMJs per animal were measured on the microscopic images (20X objective).

Image analysis: The morphological and morphometric analyses of muscle fibers and NMJs were performed on images captured with an Olympus Bx60® microscope coupled

to an Olympus DP71 camera (Tokyo, Japan) using the DP Controller 3.2.1 276 program. For ultrastructural analysis, the material obtained was examined and photographed under a transmission electron microscope (CM100, Philips®, The Netherlands). The Image Pro Plus 6.0® program (Media Cybernetics, Maryland, USA) was used for the analyses.

Statistical analysis: The ultrastructural data were analyzed descriptively. The remaining data were submitted to statistical analysis using the GraphPad Prism® program (La Jolla, USA), considering the results of the Kolmogorov-Smirnov test for normality. Normally distributed data were compared by the Student *t*-test, while data following a non-normal distribution were analyzed by the nonparametric Mann-Whitney test. A p value $p \leq 0.05$ was considered significant.

RESULTS

Macroscopic parameters: As can be seen in Table 2, animals of the OB-F1 group exhibited an increase of 10% in body weight ($p = 0.05$), of 88% in retroperitoneal fat ($p < 0.0$), of 43% in periepididymal fat ($p = 0.016$), and of 3% in the Lee index ($p = 0.035$) compared to the CTL-F1 group. There was no significant difference in naso-anal length ($p = 1.0$), EDLmuscle weight ($p = 0.278$), or EDL muscle length ($p = 0.256$) between the groups studied (Table 2).

Morphology and morphometry of muscle fibers and collagen: The general architecture of muscle fibers of the EDL muscle was preserved in the groups studied (CTL-F1 and OB-F1), with the observation of multinucleated polygonal fibers with peripheral nuclei organized in fascicles and capillaries intermingled with endomysial and perimysial connective tissue (Figure 1A and 1B). Morphometry of HE-stained muscle fibers revealed no significant differences in the area ($p = 0.972$) or number of fibers ($p = 0.152$) between the groups studied. However, there were 19% fewer nuclei in the OB-F1 group compared to CTL-F1 ($p = 0.03$), but no significant difference was found in the nucleus/fiber ratio ($p = 0.605$). Regarding capillary, no difference was observed in the number of capillaries ($p = 0.871$), but the capillary/fiber ratio was increased by 15% in the OB-F1 group when compared to CTL-F1 ($p = 0.004$) (Table 3).

Connective tissue was analyzed by staining with Masson's trichrome (Figure 1C and 1D). Analysis of its percentage revealed a 32% reduction in the OB-F1 group compared to CTL-F1 ($p = 0.0002$) (Table 3). The muscle fibers were classified as proposed by Brooke and Kaiser (1970) into type I, IIa and IIb fibers. The NADH-TR reaction evidenced type I fibers (smaller diameter and intense oxidative activity), type IIa fiber (intermediate diameter and intermediate oxidative activity), and type IIb fibers (larger diameter and weak oxidative activity) in the two groups (Figure 2A and 2B). No significant differences in the area or number of the different fiber types were found between the groups studied (Figure 2C and 2D).

Ultrastructural analysis: Ultrastructural assessment by transmission electron microscopy showed a well-defined morphology. The muscle fibers were organized in sarcomeres following the striated pattern of light and dark bands (I-band and A-band, respectively) in the CTL-F1 and OB-F1 groups (Figure 3A and 3B). Increased myofibrillar disorganization and increased disorganization and dissolution of the Z-line were observed in the OB-F1 group (Figure 3B).

Analysis of neuromuscular junctions: The NMJs of the groups studied were polymorphic and had a round, oval and elliptical shape (Figure 4A and 4B). Morphometric analysis showed a reduction of 17% in NMJ area ($p < 0.001$) and of 12% in the largest diameter ($p < 0.001$) in the OB-F1 group when compared to CTL-F1. The smallest diameter was similar in the two groups ($p = 0.118$) (Figure 4C, 4D, and 4E).

DISCUSSION

Pregnancy is a physiological state characterized by a complex anatomical and functional interaction between the mother and fetus (LEIVA *et al*., 2011). Human and animal studies have shown that exposure to disturbances during this period can affect fetal development, resulting in metabolic abnormalities (DESAI *et al*., 2015; SHARPLES *et al*., 2016). This concept, known as metabolic programming, is associated with epigenetic alterations (DESAI *et al*., 2015). However, the underlying mechanisms are still unclear. In the present study, an increase in the Lee index, body weight and retroperitoneal and periepididymal fat weight was observed in OB-F1 animals when compared to the CTL-F1 group. Studies have shown that maternal obesity during pregnancy causes excess weight in animals (ZHENG *et al*., 2014) and children (PIRKOLA *et al*., 2010; RIBEIRO *et al*., 2015). These findings might be related to placental and endothelial alterations (PARLEE and MACDOUGLAD, 2014). The placenta of obese mothers contains inflammatory markers that promote vasodilation and overproduction of nitric oxide in the endothelium, increasing its permeability. This placental dysfunction is the key mechanism in the future development of metabolic syndrome (LEIVA *et al*., 2011). Skeletal muscle has the ability to remodel itself and adapt in response to functional demands and diseases such as obesity (KEMP *et al*., 2009). Adult rats receiving a hypercaloric diet exhibit a reduction in muscle weight and in muscle fiber cross-sectional area (LE *et al*., 2014; LEE *et al*., 2015). However, studies on offspring are still sparse and the present investigation is the first to evaluate the effects of maternal obesity on the morphology and morphometry of muscle fibers and NMJs in adult offspring. Bayol *et al*. (2005) demonstrated a reduction in the number of muscle fibers and cross-sectional fiber area in offspring born to obese mothers. In the present study, the area and number of muscle fibers were similar in the two groups, but a reduction in the number of nuclei was observed in the OB-F1 group. The number of nuclei is related to cell volume and is responsible for a specific but not fixed amount of sarcoplasm. A reduction in cell volume requires less protein and myofibrils respond by eliminating their nuclei (Teixeira and Duarte, 2001). Although no difference in muscle fiber area was observed, it may have recovered its volume until adult age. One of the determinant factors of muscle fiber integrity is capillarity. In the present study, the capillary/fiber ratio was increased by 13% in the OB-F1 group. This increase is related to a greater supply of oxygen to the muscle fiber, which is important for maintaining the metabolic activities of the muscle (DEGENS and ALWAYS, 2006). Hypercaloric diets require greater oxygen supply and the increase in capillarityis probably an adaptation to insufficient oxygen transport (SILVENNOINEN *et al*., 2013). Structural support and protection of muscle tissue are the functions of the extracellular matrix, which is mediated by connective tissue (CARMELI *et al*., 2004).

Table 1. Nutritional composition of the ingredients of the cafeteria diet

	Energy (kcal/100 g)	Carbohydrates (g/100 g)	Protein (g/100 g)	Fat (g/100 g)	Sodium (mg/100 g)
Standard chow (BioBase, Brazil)[a]	295	70	20	10	0
Salami (Sadia, Brazil)[b]	415	0.56	35	30	2,000
Bread roll (Nutrella, Brazil)[b]	332	64	6.8	6	356
Bacon-flavored snack (Troféu, Santa Helena, Brazil)[b]	496	56	7.6	16	1,036
Mixed sausage (Sadia, Brazil)[b]	320	0	18	28	1,574
Chocolate cake (Renata, Selmi, Brazil)[b]	381	55	4.1	16.2	173.3
Corn snack (Cheetos, Pepsico, Brazil)[b]	125	17	1.6	5.6	99
Mortadella (Frimesa, Brazil)[b]	317	9.5	13.2	16	2,112.5
Marshmallow (Fini, Brazil)[b]	330	80	5	0	47.5
Corn flour biscuits (Zadimel, Brazil)[b]	440	80	11	5	333
Chocolate wafers (Bauduco, Brazil)[b]	523	63	5	27	177

[a]Data provided by the manufacturer. [b]Data obtained from the packaging.

Table 2. Macroscopic parameters of rat offspring born to control dams (CTL-F1) and to obese dams (OB-F1) at 17 weeks of age

Parameter	CTL-F1 (n = 7)	OB-F1 (n = 7)
Body weight (g)[a]	382.1± 24.71	420.0 ± 40.11*
EDL muscle weight (g)[a]	0.16 ± 0.01	0.18 ± 0.01
EDL muscle length (mm)[a]	26.15 ± 3.30	24.45 ± 1.87
Retroperitoneal fat (g)[a]	2.29 ± 0.83	4.31 ± 0.82 **
Periepididymal fat (g)[a]	3.51 ± 0.67	5.01 ± 1.24*
Naso-anal length (cm)[b]	22.93 ± 0.53	23.0 ± 0.28
Lee index[a]	315.7 ± 8.11	325.9 ± 7.92*

Values are expressed as the mean ± standard deviation. [a]Student *t*-test. [b]Mann-Whitney test.
* $p \leq 0.05$; ** $p < 0.001$.

Table 3. Microscopic parameters of the EDL muscle of rat offspring born to control dams (CTL-F1) and to obese dams (OB-F1) at 17 weeks of age

Parameter	CTL-F1 (n = 7)	OB-F1 (n = 7)
Fiber area (μm^2)	1,37 ± 232.0	1,38. ± 295.7
Number of fibers	149.9 ± 33.71	125.9 ± 24.35
Number of nuclei	229.9 ± 41.36	185.3 ± 24.86*
Nucleus/fiber ratio	1.54 ± 0.19	1.49 ± 0.20
Number of capillaries	294.9 ± 35.59	291.7 ± 35.68
Capillary/fiber ratio	1.23 ± 0.09	1.42 ± 0.09**
% Connective tissue	6.73 ± 0.87	4.57 ± 0.68***

Values are expressed as the mean ± standard deviation. Student *t*-test. * $p < 0.05$; ** $p < 0.005$; ***$p < 0.0005$.

A reduction in the percentage of connective tissue was observed in EDL muscle of OB-F1 animals. This finding might be associated with an increase in matrix metalloproteinases (MMPs), particularly MMP-2 and MMP-9, enzymes involved in the degradation of collagen (CHEN and LI, 2009). MMPs have been found to be increased in inflammatory processes (CARMELI *et al*., 2009) and it has been shown that maternal obesity is associated with inflammatory responses in the offspring (TONG *et al*., 2009).

Obesity also interferes with the different muscle fiber types, with the observation of a smaller number of type I fibers and a predominance of type II fibers in obese individuals. Fibers type II participate in the storage of lipids in skeletal muscle (TANNER*et al*., 2002). There are no reports in the literature investigating the effects on different fiber types in the offspring. Thus, this is the first study demonstrating a similar area and similar number of the different fiber types in rat offspring born to obese and control dams. Moreover, myofibrillar disorganization and Z-line disorganization and dissolution were observed in the OB-F1 group.

These findings might be explained by the protein deficit of the cafeteria diet administered during pregnancy and lactation, which may compromise normal myofibrillar arrangement (Jeronimo *et al*., 2016). Myofibrillar disorganization, Z-line disorganization and lipid accumulation were also found in the diaphragm of obese rats (Ulsenheimer *et al*., 2017). Obesity is characterized by chronic inflammation and elevated levels of inflammatory markers such as interleukin 6 (IL-6). Excessive expression of this marker is implicated in the negative regulation of insulin-like growth factor 1 (IGF-1) (MAGGIO *et al*., 2013). IGF-1 plays an important role in the growth and differentiation of skeletal muscle, as well as in the neuromuscular system (Caroni, 1993). In the present study, the area and largest diameter of NMJs were reduced in the OB-F1 group. It is suggested that the increase of IL-6 may reduce IGF-1 signaling in the NMJ, consequently affecting its development. This study demonstrated damage to skeletal muscle tissue in offspring of dams exposed to an obesogenic diet. This knowledge is important for the development of strategies to combat obesity in order to prevent its consequences in the offspring.

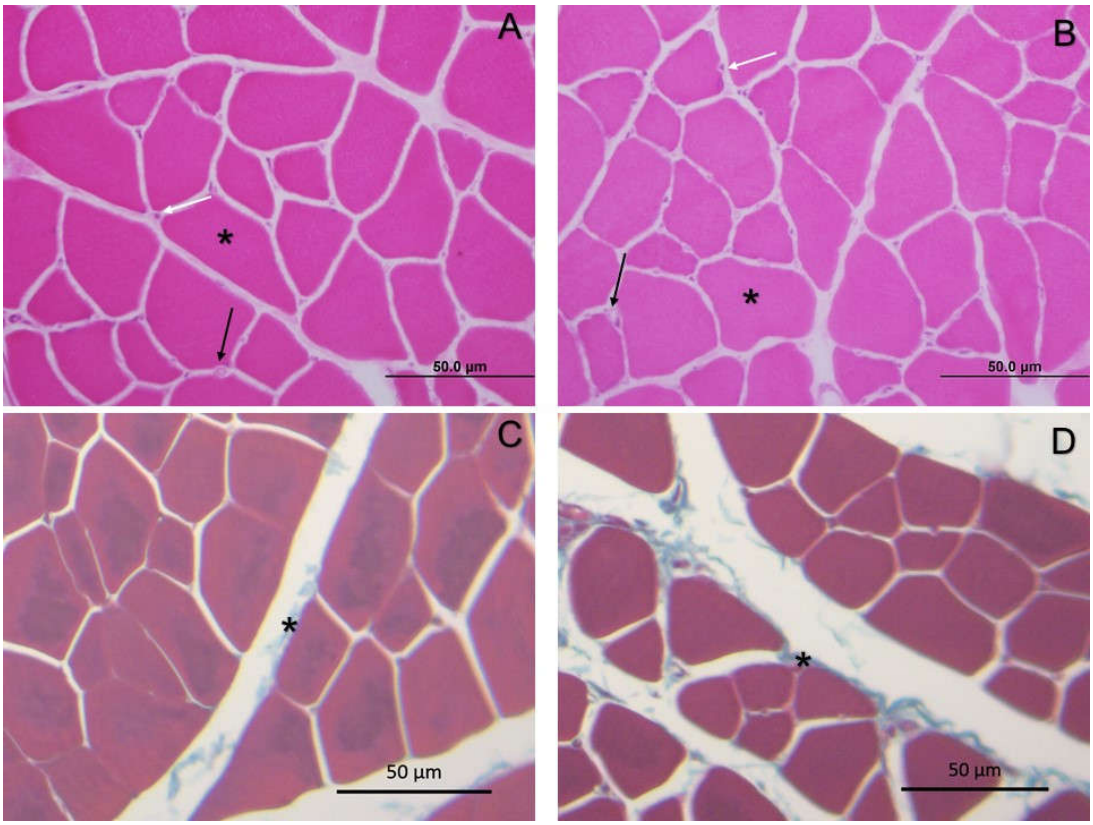

Figure 1. Photomicrographs of the extensor digitorum longus muscle of Wistar rats at 17 weeks of age. Cross-section. A and B: Muscle fibers (asterisk), peripheral nuclei (white arrow), and blood capillaries (black arrow) in rat offspring born to control dams (CTL-F1) and to obese dams (OB-F1), respectively. HE. C and D: Perimysium (asterisk) in CTL-F1 and OB-F1, respectively. Masson's trichrome

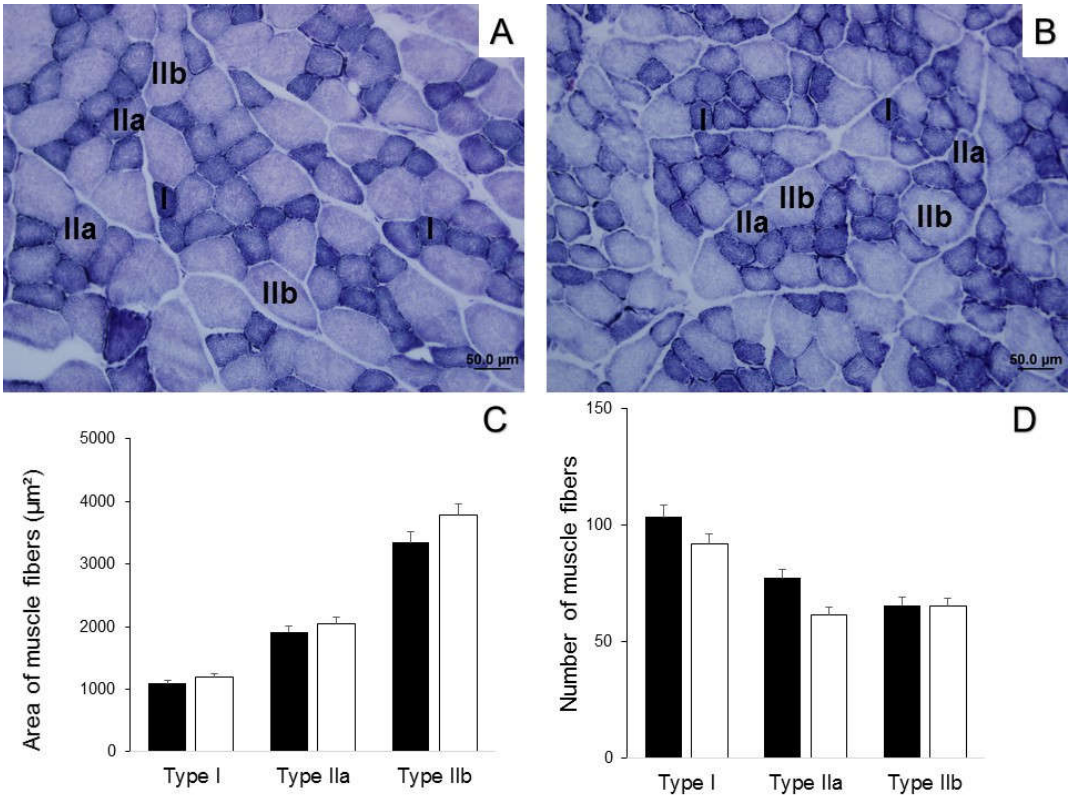

Figure 2. Photomicrographs of the extensor digitorum longus muscle of Wistar rats at 17 weeks of age. Cross-section, NADH-TR reaction. A and B: Types I, IIa and IIb muscle fibers in rat offspring born to control dams (CTL-F1) and to obese dams (OB-F1), respectively. C and D: Area and number of the different muscle fibers types in animals of the CTL-F1 and OB-F1 groups. Values are expressed as the mean ± standard deviation. Student *t*-test

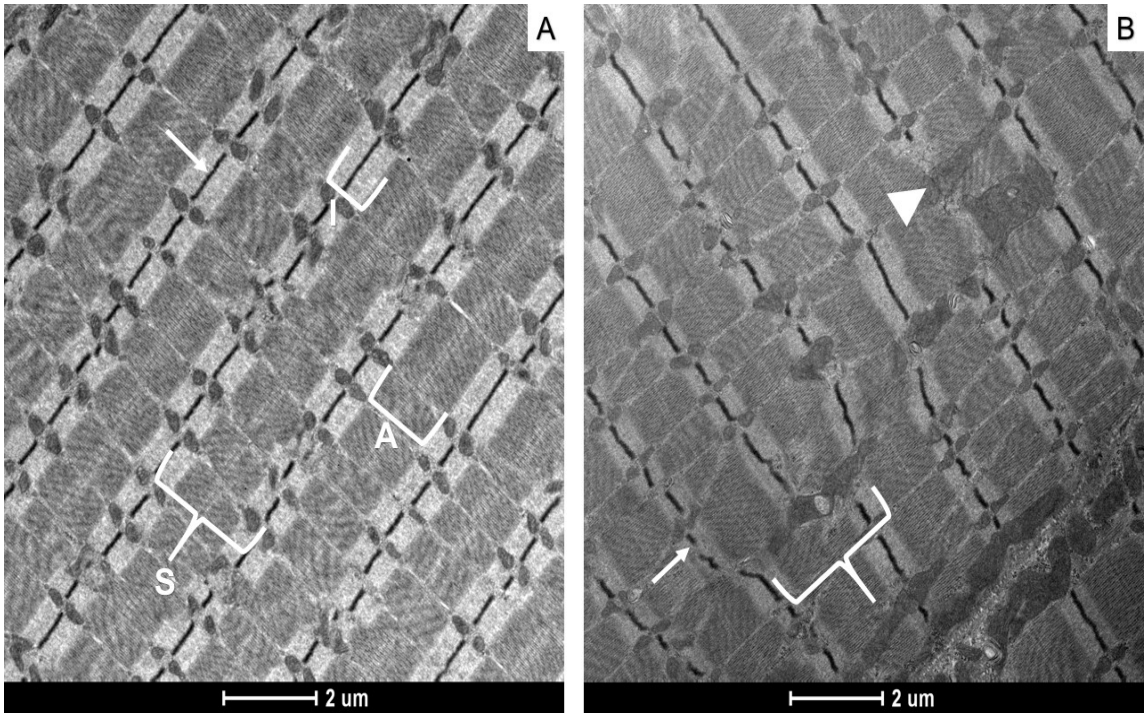

Figure 3. Electron micrographs of the extensor digitorum longus muscle of Wistar rats at 17 weeks of age. Longitudinal section. A: Preserved muscle fiber with organized sarcomere (S), A-band (A), I-band (I), and Z-line (white arrow). Rat offspring born to control dams (CTL-F1). B: Muscle fiber showing disorganization of the Z-line (white arrow), myofibrillar disorganization (key), and dissolution of the Z-line (arrowhead). Rat offspring born to obese dams (OB-F1).

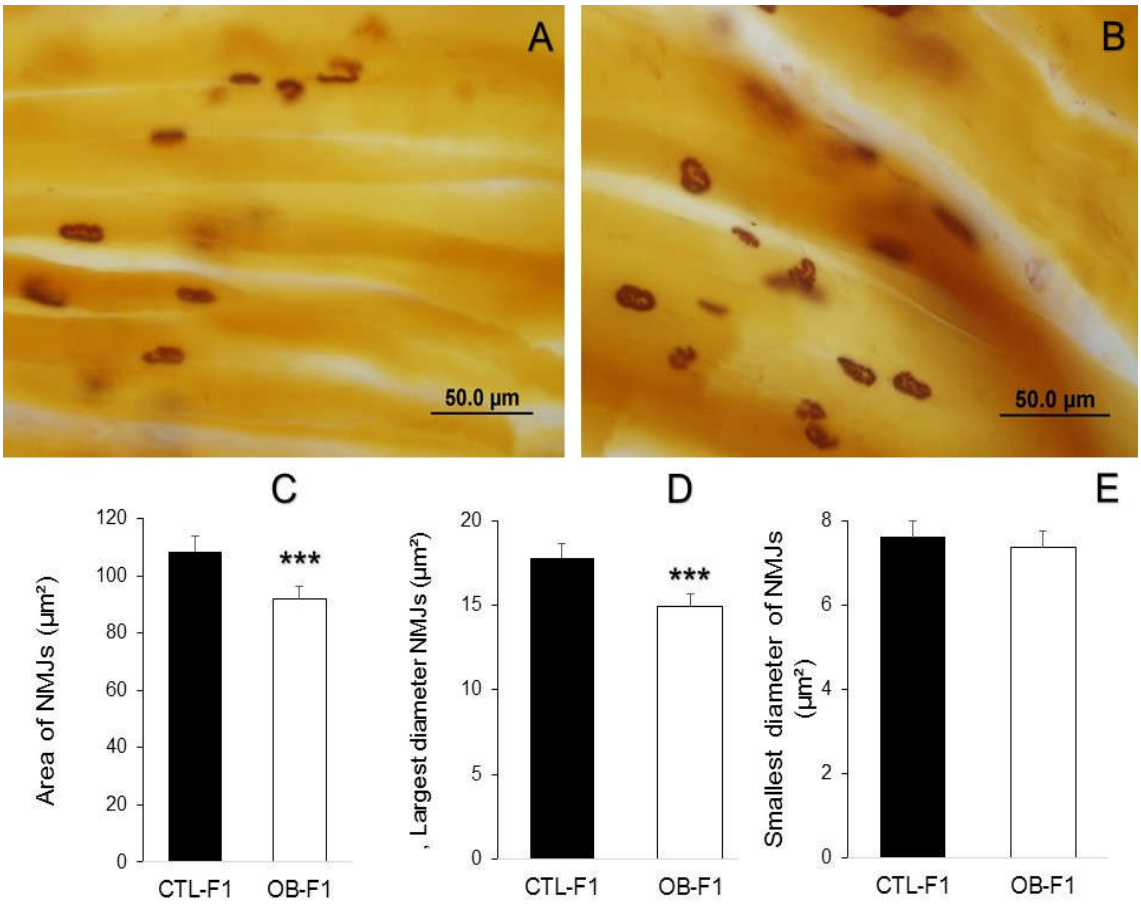

Figure 4. Photomicrographs of the neuromuscular junctions (NMJs) of the extensor digitorum longus muscle of Wistar rats at 17 weeks of age. Longitudinal section, nonspecific esterase reaction. A and B: Observe the morphological characteristics of NMJs in rat offspring born to control dams (CTL-F1) and offspring born to obese dams (OB-F1), respectively. C, D and E: Area, largest diameter and smallest diameter of NMJsin animals of the CTL-F1 and OB-F1 groups. Values are expressed as the mean ± standard deviation. Student *t*-test. *** p< 0.0006

In conclusion, the results indicate that feeding mothers an obesogenic diet throughout life induces morphological and morphometric changes in the skeletal muscle fibers and NMJs of their offspring in later life, including a reduction in the number of nuclei, collagen and NMJ area and largest diameter, and an increase in capillary/fiber ratio. Maternal obesity also altered muscle fiber ultrastructure in the offspring. These findings highlight the importance of obesity prevention since this condition may not only affect obese individuals but also their offspring.

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