



**UNIVERSIDADE ESTADUAL PAULISTA
“JÚLIO DE MESQUITA FILHO”
FACULDADE DE MEDICINA**

Eduardo Klöppel

**Impacto transgeracional do diabetes materno sobre a morfofisiologia
mitocondrial e expressão proteica do
músculo esquelético de ratas fêmeas adultas**

Tese apresentada à Faculdade de
Medicina, Universidade Estadual
Paulista “Júlio de Mesquita Filho”,
Câmpus de Botucatu, para obtenção do
título de Doutor(a) em Tocoginecologia
(Especialidade: Bioquímica).

Orientadora: Profa. Dra. Débora Cristina Damasceno

**Botucatu
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ATA DA DEFESA PÚBLICA DA TESE DE DOUTORADO DE EDUARDO KLOPPEL, DISCENTE DO PROGRAMA DE PÓS-GRADUAÇÃO EM TOCOGINECOLOGIA, DA FACULDADE DE MEDICINA - CÂMPUS DE BOTUCATU.

Aos 18 dias do mês de outubro do ano de 2021, às 14:30 horas, por meio de Videoconferência, realizou-se a defesa de TESE DE DOUTORADO de EDUARDO KLOPPEL, intitulada **Impacto transgeracional do diabetes materno sobre a morfofisiologia mitocondrial e expressão proteica do músculo esquelético de ratas fêmeas adultas**. A Comissão Examinadora foi constituída pelos seguintes membros: Profa. Dra. DEBORA CRISTINA DAMASCENO MEIRELLES DOS SANTOS (Orientador(a) - Participação Virtual) do(a) Depto. de Ginecologia e Obstetrícia / FM/Botucatu - Unesp, Profa. Dra. DANIELA CARVALHO DOS SANTOS (Participação Virtual) do (a) Depto de Biologia Estrutural e Funcional / IB/Botucatu - Unesp, Profa Dra CAMILA RENATA CORREA CAMACHO (Participação Virtual) do(a) Unidade de Pesquisa Experimental / FM/Botucatu - Unesp, Prof. Dr. TIAGO RODRIGUES (Participação Virtual) do(a) CCNH/Mogi das Cruzes / Universidade Federal do ABC, Profa. Dra, ANA IZABEL SILVA BALBIN VILLAYERDE (Participação Virtual) do(a) Universidade do Contestado - Campus de Mafra/SC. Após a exposição pelo doutorando e arguição pelos membros da Comissão Examinadora que participaram do ato, de forma presencial e/ou virtual, o discente recebeu o conceito final **APROVADO**. Nada mais havendo, foi lavrada a presente ata que, após lida e aprovada, foi assinada pelo(a) Presidente(a) da Comissão Examinadora.

Profa. Dra. DEBORA CRISTINA DAMASCENO MEIRELLES DOS SANTOS

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ΕΠΙΓΡΑΦΕ

**“Não se conhece completamente
uma ciência enquanto não se
souber da sua história”.**

Auguste Comte

DEDICATÓRIAS

Dedico essa tese ao processo das coisas,
processo da vida.

Como diria o mestre **Paulo freire**:

“Não há transição que não implique um ponto
de partida, um processo e um ponto de chegada.
Todo amanhã se cria num ontem através de um
hoje.

De modo que o nosso futuro se baseia num
passado e se corporifica num presente.
Temos de saber o que fomos e o que somos para
sabermos o que seremos”.

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CAPÍTULO I

CHAPTER 1

Este manuscrito será formatado para submissão à revista *International Journal of Molecular Sciences* (Fator de Impacto = 5,923)

This manuscript will be formatted for submission to the International Journal of Molecular Sciences (Impact factor = 5.923)

**TRANSGENERATIONAL EFFECTS OF MATERNAL HYPERGLYCEMIA ON
INSULIN RESISTANCE AND ULTRASTRUCTURAL MORPHOLOGY OF
SOLEUS MUSCLE IN ADULT FEMALE RATS**

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ABSTRACT

Maternal diabetes has a negative impact on offspring phenotype, which contributes to an impaired metabolism in adulthood. Nonetheless, the mechanisms under these outcomes are still unclear. In this study by utilizing a rat model, we aimed to investigate the impact of maternal diabetes on the metabolism of adult female offspring in relation to mitochondrial dynamics, and ultrastructure of skeletal muscle. For this, female offspring coming from diabetic mothers (FOD) were obtained through pregnancy of *Sprague-Dawley* rats with streptozotocin neonatally-induced diabetes. Control mothers received citrate buffer during their neonatal life. At adulthood, FOD and Control rats were submitted to oral glucose tolerance test (OGTT) for inclusion criteria in the groups. At 150 days of life, the FOD and Control rats were sacrificed for collection of blood and muscle samples. The FOD rats presented a higher insulin and triglyceride levels, and abnormal OGTT. These animals also showed decreased activation of molecular markers of insulin signaling, and impaired mitochondrial function and ultrastructure in soleus muscle. Thus, the transgenerational impact of maternal diabetes caused fetal plasticity, which led to insulin resistance and glucose intolerance at adulthood, and the mitochondrial network of skeletal muscle is shown to have an important role during the prediabetic state.

Keywords: Skeletal muscle; insulin resistance; hyperglycemia; mitochondrial dynamics; AMPK; DOHaD; rat.

1. INTRODUCTION

Evidence has proved that environmental exposure in early life to different nutritional conditions is related to the incidence of multiple diseases in adulthood, which was recognized into a new arm of the scientific knowledge, called as the developmental origins of health and disease (DOHaD) [1]. There are experimental, clinical, and epidemiological studies demonstrating the consequences of programmed changes induced by diseases like diabetes, dyslipidemia, and insulin resistance leading to an impaired fetal metabolism, which plays a pivotal role in the fetal origin of adulthood diseases [2–5].

In fact, it is estimated that every year about 21.3 million of births worldwide are affected by abnormal blood glucose level during pregnancy [6]. In this matter, it has been explained for decades that the “thrifty” genotype of rapid insulin trigger which allows primitive man to be able to store ingested food and survive a low-nutrient environment, is now one of those responsible for the worldwide diabetic pandemic, also when associated with the diabetic intrauterine environment [7]. This “thrifty” genotype is now associated it to a sedentary lifestyle with a higher caloric intake and increased longevity of modern man [8]. There disturbances may interfere in the metabolism of the offspring during the whole developmental period, leading to an impaired metabolic adaptation which increases the risks of diabetes and other diseases of adulthood.

Moreover, studies has been focused on the role of mitochondrial metabolism and dynamics among the emerging mechanisms involved in the etiology and pathophysiological progression of insulin resistance and diabetes [9, 10]. The imbalance in mitochondrial metabolic processes has been pointed as having an important role diabetes, mainly when associated to the mitochondrial fission and fusion through outer membrane-bound proteins like Mitofusin 2 (MFN2) and mitochondrial fission factor (MFF) and to post-translational modification (PTM) effectors which controls mitochondrial homeostasis and mitophagy, through adenosine monophosphate-activated protein kinase (AMPK) [11–14]. Supplementary, AMPK is presented as a heterotrimeric holoenzyme, responsible for regulation of many biological processes like oxidative stress, mitochondrial function, and cell survival [15]. In the other hand, oxidative stress and insulin resistance can affect the downstream subtracts involved in cell survival and metabolism, such as MAPK (Mitogen Activated Protein Kinases) which are enzymes highly conserved among eukaryotes, and involved in diverse

metabolic processes of controlling proliferation and cell death. There are four classical subfamilies of MAPK in humans, including ERK1 and ERK2 (MAPK 3/1) [16]. Additionally, the Phosphatidylinositol 3-kinase (PI3K), is a critical effector on insulin action and plays key regulatory function on cell metabolism and insulin response [17].

Besides mitochondrion function on energetic metabolism and oxidative stress, emerging evidence points the especial role of skeletal muscle and its mitochondrial network on diabetes etiology and development mainly due to its high glycolytic and oxidative activity [18–22]. Equivalently, some authors showed that the soleus muscle is an adequate tissue to access these alterations due its high mitochondrial activity and network [20, 23].

Our research team has been studied the influence of the maternal diabetes on the offspring at different periods of life in order to better understand the effects of maternal diabetes on fetal programming/plasticity. It was verified that the diabetic mothers presented higher rates of embryo fetal losses at day 18 of pregnancy, which was associated changes in the endocrine pancreas [24]. Our laboratory have performed several studies to evaluate the diabetic repercussions in animals models, and one of these studies shows the negative correlation between the maternal hyperglycemia/oxidative stress levels and the number of fetal pancreatic islets in fetuses from mildly diabetic rats . Another one has demonstrated oxidative stress in the liver and blood in the perinatal period (at postnatal days 5 and 15 of life) of pups coming from diabetic mothers. Additionally, the female adult offspring (120 days of life) presented glucose intolerance, impaired function of the β -cells and a low-grade inflammatory in the blood as response to a hyperglycemic intrauterine environment. However, insights about the mechanistic ways of these outcomes remains unclear and needs to be elucidated. To our knowledge, our study is the first to investigate the effects of the maternal hyperglycemia in adult offspring's skeletal muscle and mitochondrial metabolism despite the organelle being widely involved in the diabetes-induced consequences. Thus, this study aimed to investigate if diabetic intrauterine environment during embryo fetal period increased the prevalence of insulin resistance in adult female rats (150 postnatal days) and if the skeletal muscle, more specifically the structural and molecular dynamics of mitochondrial network of soleus muscle, could play a role on this phenotype.

2. RESULTS

2.1 Criteria of inclusion in the experimental groups

From criteria inclusion by OGTT in the parenteral generation, all rats (future mothers) that received STZ presented glycemic levels higher than 200 mg/dL during OGTT and were considered as diabetic, while all control rats presented glycemia inferior to 140 mg/dL, characterizing a normoglycemic status (data not shown). In relation to female pups from control and diabetic dams at adulthood, all (100%) control pups were considered glucose tolerant. For FOD group, 66.6% of the rats had glycemia greater than 140 mg/dL during OGTT being considered glucose intolerant and included in the experimental group (data not shown).

2.2 Biochemical analyses – at adulthood

2.2.1 OGTT and AUC

The adult FOD rats presented higher glycemic levels at time point 30 minutes of OGTT when compared to the control female pups (Figure 1A). The AUC levels were also statistically higher in FOD group (Figure 1B).

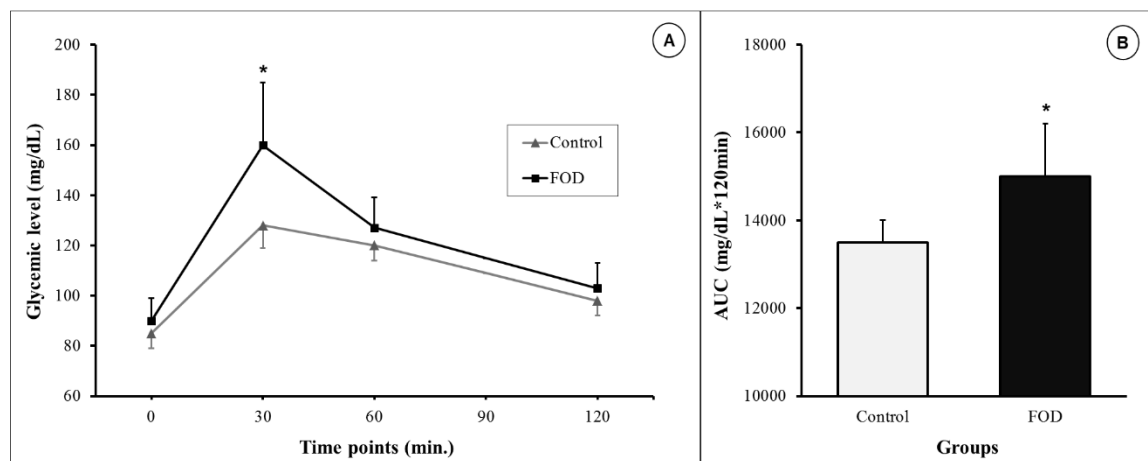


Figure 1. Blood glucose levels by oral glucose tolerance test (OGTT) (A), and Area under the curve (AUC) (B) of the female adult pups from control and diabetic mother (FOD) rats.

Values expressed as mean $\pm$ standard deviation ($n=10$ animals/group).

* $p < 0.05$ - compared to the Control group (Gamma Distribution test).

2.2.2 Insulin metabolism and HOMA measurements

The FOD group presented increased serum insulin concentration and HOMA-IR level (Figure 2A and 2C), but not in HOMA-beta and fasting blood glucose concentration (Figure 2B and 2D, respectively).

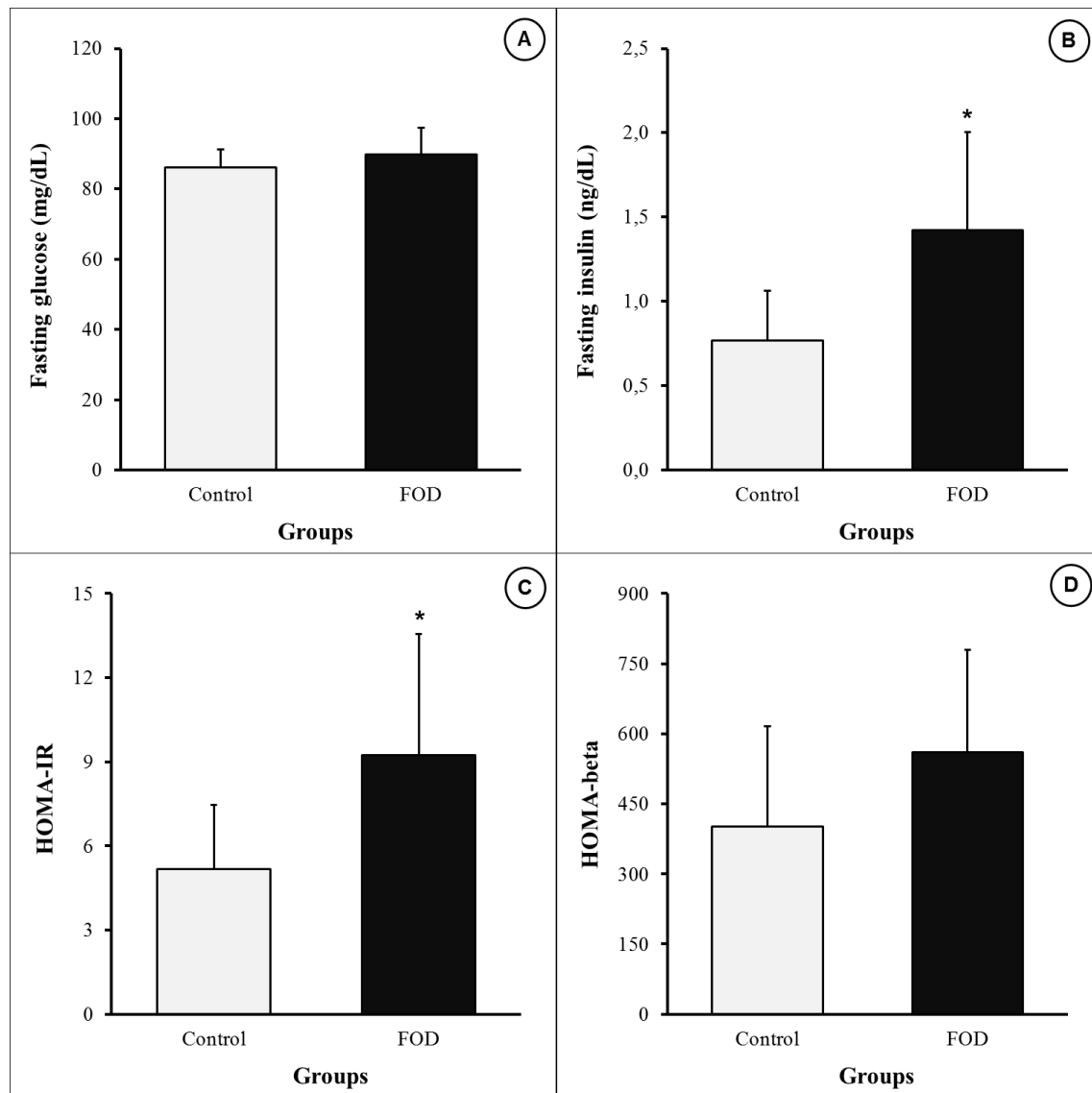


Figure 2. Serum fasting glucose (a) and insulin (B) concentrations, HOMA-IR (C) and HOMA-beta (D) of female adult pups from control and diabetic mother (FOD) rats.

Values expressed as mean $\pm$ standard deviation ($n=10$ animals/group).

* $p<0.05$ - compared to the Control group (Gamma Distribution test).

2.2.3 Body weight, TyG index and lipid profile

The FOD rats had increase in body weight (Figure 3A), serum triglyceride concentration (Figure 3B) and TyG index (Figure 3D). However, the serum cholesterol

concentration presented no significant differences between the groups analyzed (Figure 3C).

2.2.4 Oxidative stress biomarkers

The levels of TBARS and reduced thiol groups (-SH) were not changed in the experimental groups (Figure 4A and 4B).

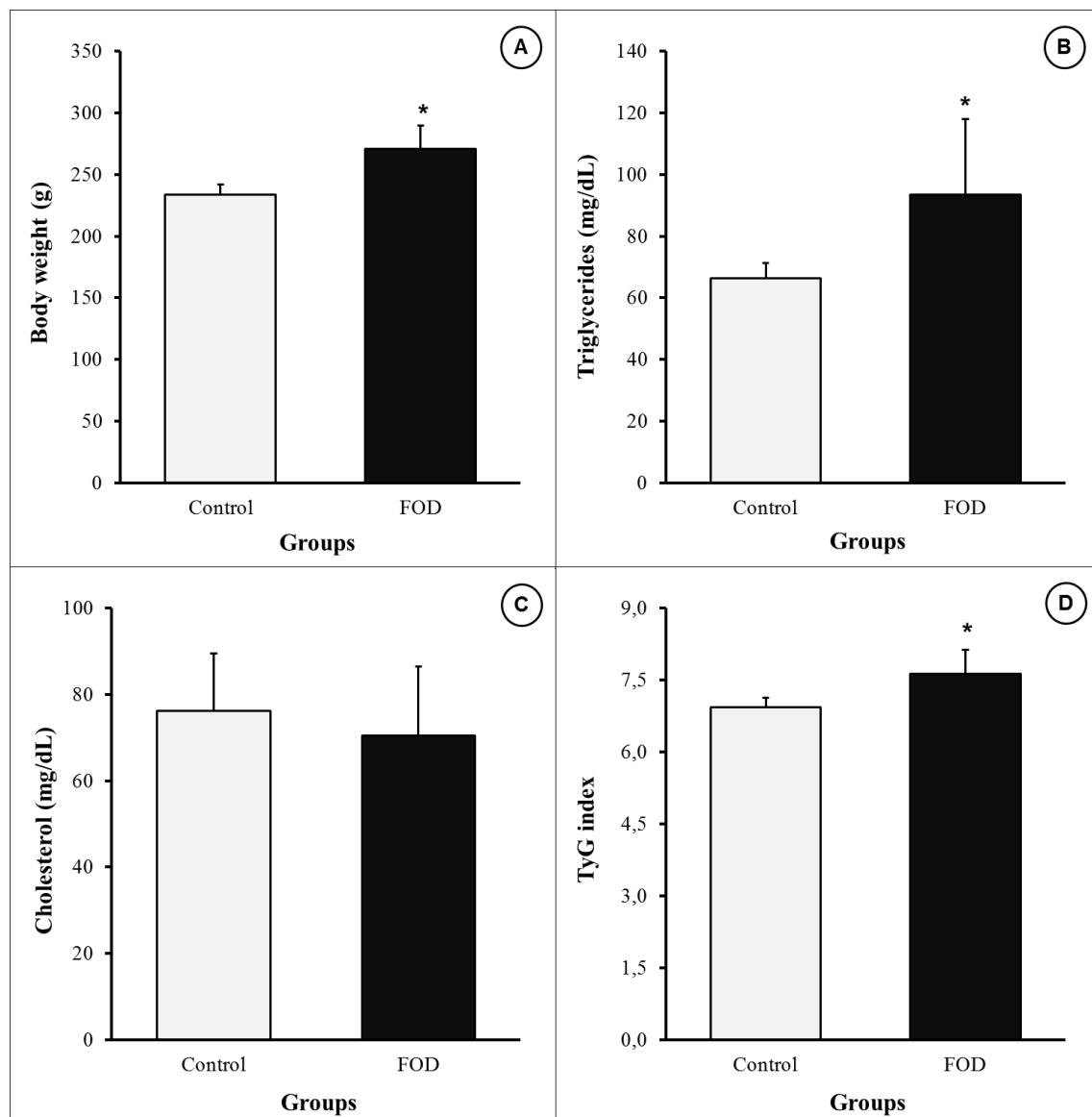


Figure 3. Body weight (A), Triglyceride (B), Cholesterol (C) and Triglyceride-glucose index (TyG) (D) of female adult pups from control and diabetic mother (FOD) rats.

Values expressed as mean $\pm$ standard deviation ($n=10$ animals/group).

* $p < 0.05$ - compared to the Control group (Student's t -test for body weight and Cholesterol concentrations, and Gamma Distribution test for Triglyceride concentrations and TyG Index).

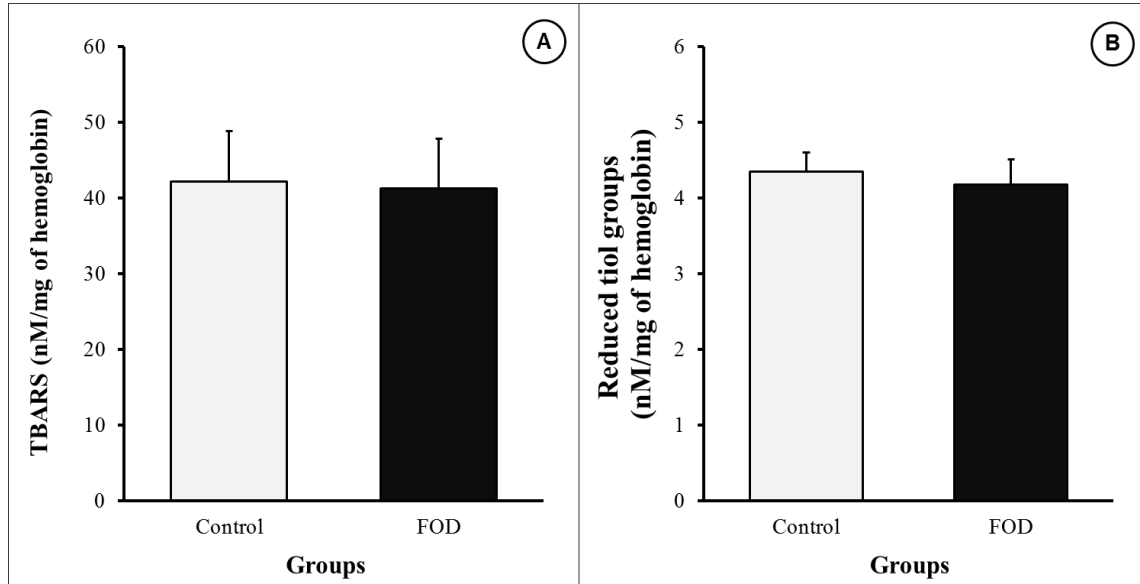


Figure 4. Thiobarbituric acid reactive substance (TBARS) (A) and reduced thiol group (-SH) concentrations (B) of the female adult pups from control and diabetic mother (FOD) rats.

Values expressed as mean $\pm$ standard deviation ($n=10$ animals/group).

$p > 0.05$ – not statistically significant difference.

2.3 Western blotting analyses

The FOD group had statistically lower protein expression of PI3K (phosphatidylinositol 3-kinase – p110- subunit) (Figure 5A), MAPK (mitogen activated protein kinases – ERK1/2) (Figure 5B), AMPK (adenosine monophosphate-activated protein kinase) (Figure 5C), and mitochondrial fission factor (MFF) (Figure 5D) in the soleus muscle when compared to the control adult pups. However, the levels of Mfn2 (mitofusin 2) (Figure 5E) presented no difference between experimental groups.

2.4 Structural and Ultrastructural Morphological Analyses

2.4.1 Central nuclei analysis in the soleus muscle fibers and quantification of type of slow and fast fibers in the soleus muscle

The morphological analysis demonstrated that the percentage of centrally located nuclei counting in the muscle fibers of the soleus muscle was higher in FOD adult pups than the control one. The experimental groups were not statistically different regarding the percentage of fast or slow type fibers in soleus muscle (Figure 6).

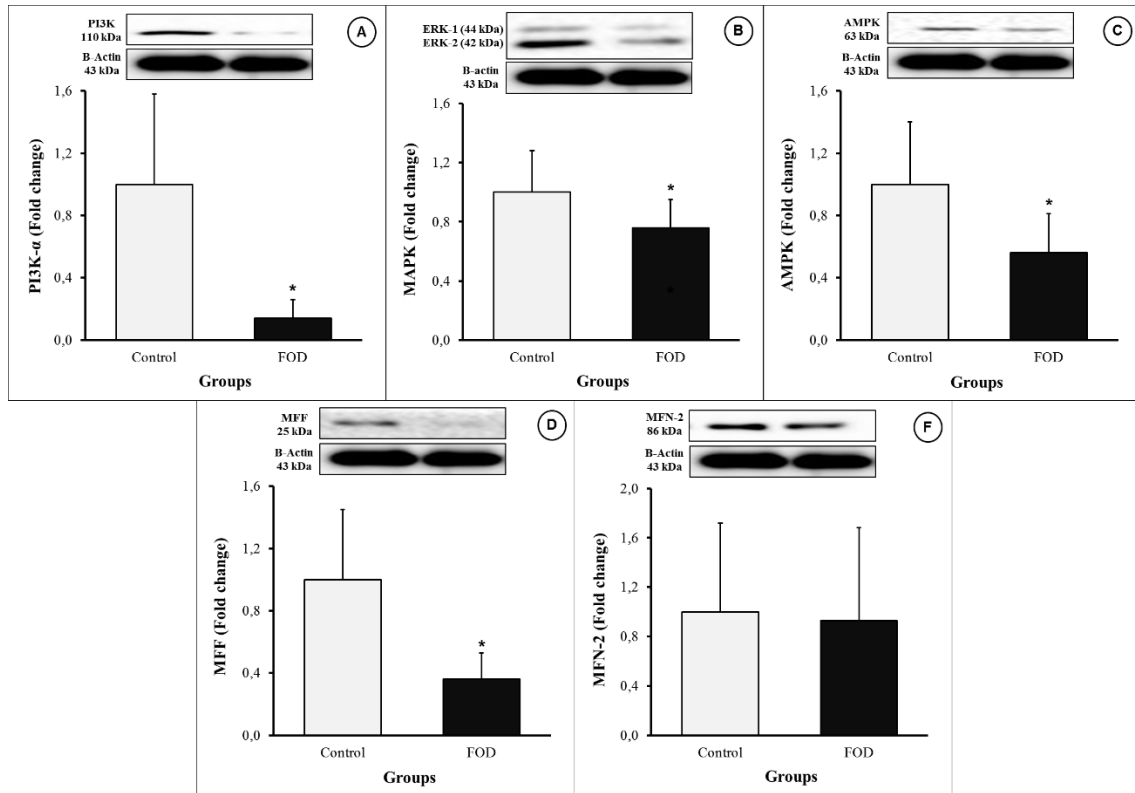


Figure 5. Immunoblot analyses of protein expression of PI3K-α (A), MAPK (ERK-1/ERK-2) (B), AMPK (C), mitochondrial fission factor (MFF) (D) and mitofusin-2 (Mfn-2) (E) in soleus muscle samples of female adult pups from control and diabetic mother (FOD) rats.

Values expressed as mean $\pm$ standard deviation ($n = 6$ animals/group).

* $p < 0.05$ - compared to the Control group (Gamma Distribution test)

2.4.2 Ultrastructural analysis of soleus muscle

The figure 7 shows the descriptive ultrastructural analysis performed in the soleus muscle of control (Figures 7A, 7B, and 7C) and FOD (Figures 7D, 7E, and 7F) female offspring. In Control group, the transmission electron microscopy showed integral muscle cells, with myofibrils organized in sarcomeres aligned in a regular pattern, oval or elongated intermyofibrillar mitochondria with a moderately electron-dense matrix and well-preserved parallel cristae. In the region of the T tubules, the

cisterns of sarcoplasmic reticulum forming the triads were also seen close to the mitochondria with rare lipid drops. In contrast, the FOD rats presented some small modification in the cellular ultrastructure when compared to the characteristics described in Control group. The sarcomeres were slightly disorganized amidst the intermyofibrillar mitochondria. The presence of mitochondria with aberrant morphology from the rarefaction of the matrix to the destruction of the mitochondrial cristae was observed in FOD rats. Despite the intact ultrastructure, the T tubules and Z lines region presents an irregular pattern when compared to the Control group. The FOD group also showed the cisterns of sarcoplasmic reticulum with a mild dilation close to the mitochondria.

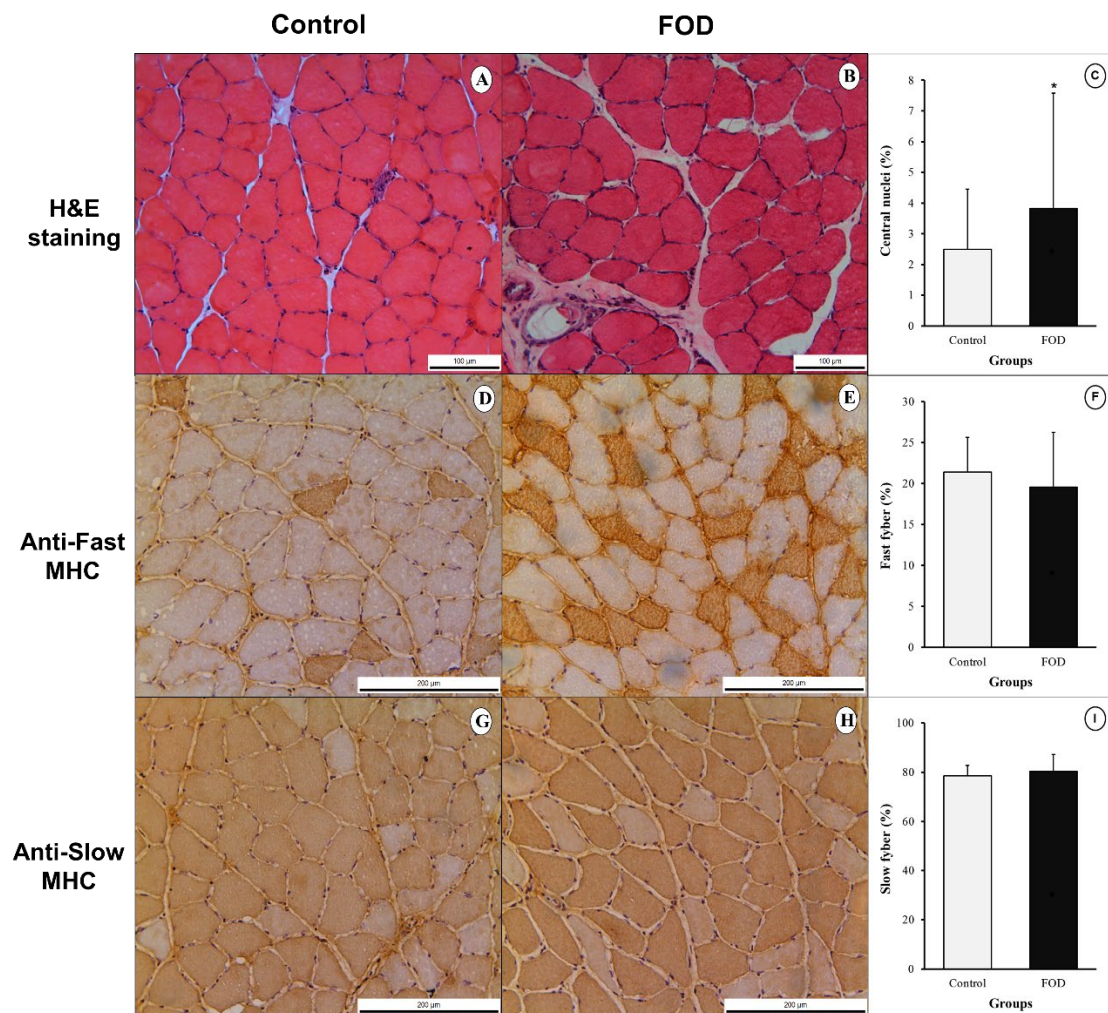


Figure 6. Histological sections of the soleus muscle stained with hematoxylin and eosin (A and B) and immunohistochemistry of fast (D and E) and slow (G and H) fibers. Graphs of the percentages of central nuclei (C), fast (F) and slow type (I) fiber number in the female adult pups from control and diabetic mother (FOD) rats.

Values expressed as mean $\pm$ standard deviation ($n=1$ sample/animal, 4 counted randomly fields of each animal, and 6 animals per group).

* $p<0.05$ - compared to the Control group (Gamma Distribution).

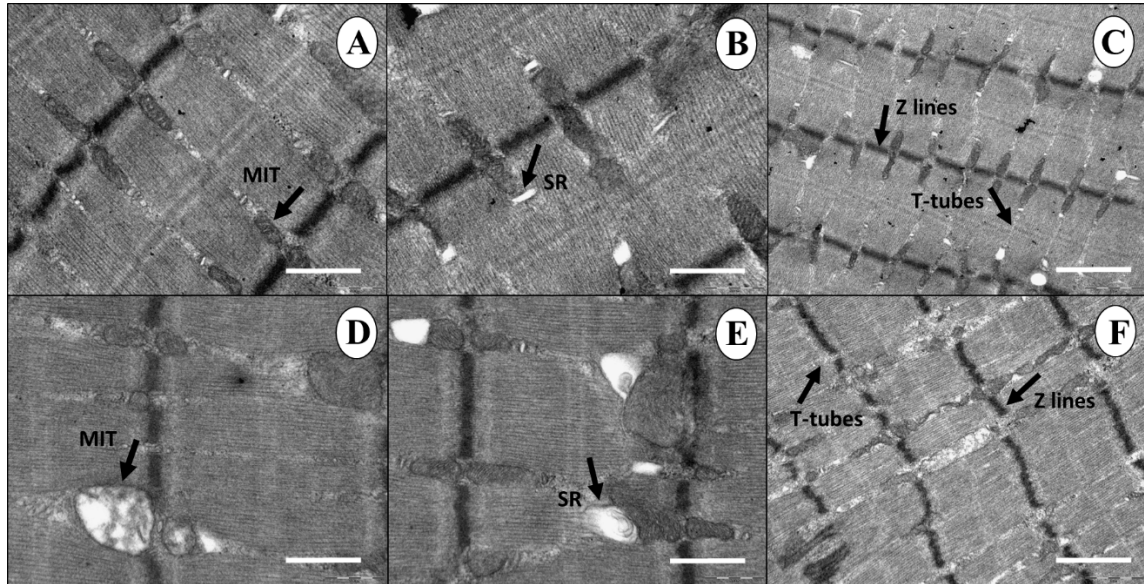


Figure 7. Transmission electronic microscopy of soleus muscle showing mitochondrial cristae conformation (MIT), sarcoplasmic reticulum (SR) cistern size, Z lines and T-tubes organization of the female adult pups from control (A, B and C) and diabetic mother (FOD) (D, E and F) rats.

Scale bars: 500 nm (A, B, D, E); 1 μ m (C, F).

3. DISCUSSION

The impaired beta (β)-cell function is recognized as a major cause of diabetes. However, the mechanisms of diabetes development are not fully understood [25]. In order to access these mechanisms, preclinical studies have committed on experimental models of diabetes development that could give tools for human clinical practice [26]. Based on DOHaD concept of fetal programming and adaptation to an inadequate intrauterine environment [27], studies about the diabetes-induced transgenerational effects on adult offspring are ongoing in our laboratory. In these investigations we verified that an unfavorable intrauterine environment induced by maternal hyperglycemia led to alterations in pancreatic beta (β)-cells in embryos, fetuses, neonates, and adult daughters. These (β)-cells showed an impairment on insulin synthesis homeostasis, which caused the glucose intolerant state of these animals. However, the periphery pathophysiological mechanisms involved are not clear so far, especially the role of proteins linked to the insulin signaling pathway and its repercussions on skeletal muscle cells in the face of this glycemic change at 150 days of life. Thus, the present study was carried out for a better understanding and relationship between these proteins and also to highlight the role of certain enzymes that have been studied in the onset of diabetes and metabolic diseases.

According to the results obtained in this study, we were able to confirm that fetal programming induced by maternal diabetes caused the emergence of diseases in adult life in female offspring of rats, confirmed by hyperlipidemia, insulin resistance, and glucose intolerance at 150 days postnatal. The increase in maternal glucose concentration during pregnancy leads to fetal hyperglycemia, which stimulates insulin synthesis, causing fetal hyperinsulinemia [28]. Early embryos exposed to elevated glucose levels experienced higher tricarboxylic acid (TCA) metabolites, changing alterations in mitochondrial physiology [29]. Diabetes models confirmed that mitochondrial bioenergetics and function are impaired in various tissues [30]. Then, a study about mitochondrial status in adult offspring of diabetic dams may bring us mechanistical insights on the fetal programmed insulin resistance in adult female rats.

This study showed that even daughters which mothers have mild hyperglycemia presented hypertriglyceridemia associated to insulin resistance. Moreover, insulin resistance in skeletal muscle is manifested long before the hyperglycemia becomes evident [31,32]. Furthermore, adult daughters showed reduced expression of different

proteins (PI3K, AMPK, ERK1/2 and MFF). These factors are directly or indirectly related to a damaged mitochondrial fusion causing morphological changes in the mitochondria-endoplasmic reticulum associated membrane (MAM) connection, possibly leading to ER stress. Thus, the association between reduced expression of insulin signaling pathway proteins, MAM disturbance and structural abnormalities of myofiber nuclei contributed to the impairment of mitochondrial and cellular homeostasis in the skeletal muscle, outlining the resistance insulin state. In normal conditions, the binding of insulin to the α -subunits of its receptor stimulates the tyrosine activity of the β -subunit that phosphorylates several intracellular proteins leading to the activation of PI3K and MAPK (ERK1\2) signaling pathways [32,33]. In order to address the major catalytic subunit of PI3K downstream after insulin stimulation, the p110 subunit was accessed once its lower expression is related to impaired insulin signaling, as evidenced in our study by its diminished protein expression, which might partially explain the hyperinsulinemia and glucose intolerance in the experimental group. Li et al. [34] demonstrated that p110 α is the dominant catalytic isoform of PI3K with effector activity in the muscle's mass control and insulin sensitivity, in conjunction with major role in mitochondrial homeostasis in the skeletal muscle. In its turn, the mitochondrion is a very mobile organelle that is able to divide (fission) or fuses (fusion) itself in response to a wide range of favorable or adverse conditions. [35]. The literature shows that the loss of p110 α subunit of PI3K in the muscles causes a decrease in the mitochondrial fission and an increase on mitochondrial fusion, leading to larger and more elongated mitochondria. In addition, the loss of p110 α reduced insulin signaling due to a decreased PI3K activity, which turned to an enhanced mitochondrial oxidative capacity with increased muscle NADH content, and higher muscle free radical production [34]. Therefore, the reduced PI3K p110 subunit expression suggests its relation to lower MFF expression, compromising mitochondrial dynamics, as observed in the elongated mitochondrial pattern in skeletal muscles in our study.

Furthermore, there was a decrease in ERK 1/2 expression. ERK1/2 are members of MAPK family, which is mainly linked to proliferation and cellular differentiation [36]. The tight regulation of these proteins is crucial for determining of survival or death cell [37]. Likewise, ERK-MFF/FIS1/Drp1 axis is a mitochondrial fission controller and is essential for the reprogramming process to cell [38]. The reduction in the protein expression of ERK1/2 and MFF can be related to a diminished metabolic ability of the cell to self-adapt and in activate the mitochondrial fission process of the muscle fibers

of the studied rats, further supporting the alteration in the mitochondrial dynamics of the muscle tissue.

Our results demonstrated AMPK, known as the guardian of mitochondrial homeostasis [39], presented a lower protein expression, confirming the abnormal signaling pathway and compromised mitochondrial health in the soleus muscle of female offspring of diabetic dams. Besides, AMPK is also a protector against insulin resistance by endoplasmic reticulum (ER) stress [40]. ER stress is associated to insulin resistance in skeletal muscle, as it was observed in our study. Because AMPK is a redox stress sensor, it phosphorylates substrates, increases catabolism to generate more ATP, and stimulates mitophagy and biogenesis [41]. This rapid AMPK-activated mitochondrial fission serves as an adaptive response to external and internal insults aiming to maintain their quality and function [42]. In mammals, loss of AMPK results in a defective mitophagy [43]. Different AMPK isoforms are capable to sense the cellular energetic *in vivo* and control the mitochondria-ER interaction during mitophagy, and for that reason, the discovery of a mitochondrial AMPK pool has local importance for the quality and control of the mitochondrial network. This fact highlights the importance of targeting mitochondrial plasticity for the treatment of conditions like diabetes [44]. Toyama et al. [45] found that MFF phosphorylation by AMPK is a critical step for mitochondrial fission in response to both cellular and mitochondrial stress. This finding corroborates our data since AMPK and MFF protein expression were reduced and consequently caused abnormal mitochondrial fission. This might be related to the others morphological and functional mitochondrial abnormalities.

The mitochondria and the ER present some independent biological functions, but are not independent structures. These organelles are not only related to lipid synthesis and transport, Ca^{2+} signaling, ER stress, mitochondria shape, motility, bioenergetics, mitophagy, and cell death, but also to the molecular plasticity in regulation of metabolic mechanisms at the ER-mitochondria interface. It has been found that the physical connection between the ER and mitochondria MAM ([46], has several proteins that are directly involved in mitophagy and are related to ER and mitochondria linkage, as such as the dynamic and complex subcellular signaling, regulating a variety of cellular processes [47]. In the present study, the ultrastructural analysis demonstrated a slight change in the ER-mitochondria connection, suggesting an ER stress, which might be also related to reduced AMPK expression leading to alteration of insulin signaling in the muscle fiber. In addition, the disrupted organelle crosstalk at MAM interfaces also may

alter insulin signaling [48], contributing for insulin resistance in the experimental group. In relation to nuclei positioning, a higher number of cells with central nuclei is another morphological alteration verified in the skeletal muscle of these rats at day 150 of life. This characteristic differs of the muscle fiber morphology, since those are multinucleated and the position of their nuclei is peripheral, close to the plasma membrane [49].

There is a report on the prevalence of centrally positioned nuclei in the myofibers of patients suffering from different muscle diseases [50]. However, the importance of nuclear positioning for disease pathogenesis and muscle weakness is unclear. As such, there are few studies on the role of nuclear positioning in muscle function or disease. This is explained in part by the prevailing hypothesis that is used to explain centrally positioned nuclei: central nuclei are considered merely a marker of ongoing myofiber repair. Several mechanisms of nuclear movement or nuclear positioning are attributed to a cytoskeletal network and myofibril organization. In this way, new research paths arise considering the questions of where the different organelles are located and why they are located in this way, how they become located and if the organization of different organelles is connected. It is relevant to consider centrally located nuclei as more than merely a marker of ongoing muscle repair and as a phenotype that can influence muscle function and health [50]. Abnormal nuclear positioning has previously been linked to muscle dysfunction [51]. Then, our results supports that this finding could also be an indicator of muscular degeneration related to several molecular and morphological alterations in the skeletal muscle. Literature suggests that both satellite cells and muscle-resident mesenchymal progenitors present roles in maintaining skeletal muscle homeostasis and defects in either cell population could contribute to the pathogenesis of muscle diseases [52,53].

Is now well recognized that the best way to prevent and treat insulin resistance is with diet control and regular physical exercise, which can change lipid deposition and activate intracellular pathways involved in energy expenditure and mitochondrial homeostasis during muscle contraction. This leads to a more favorable distribution in adipose and muscular tissues. But is also good to remember that there are still many issues to be determined on the most appropriate training intensity to cause these changes before exercise can be faithfully prescribed as "precision medicine" to combat the IR and its progression for Type 2 diabetes [54].

Besides all the efforts to help the patients living with diabetes, to determine the pathophysiological mechanisms and etiology of diabetes is still a very complex puzzle in many aspects. In order to reach some of these mechanisms, this is the first study to experimentally demonstrate that the adaptive responses caused by hyperglycemia in the intrauterine environment is involved to the insulin resistance and mitochondrial impairment on skeletal muscle of adult female offspring. Further, we highlighted the importance of AMPK, PI3K and MAPK protein levels during the regulation of mitochondrial dynamic (fission/fusion), and the connection between its molecular and ultrastructural muscular fiber compounds. In the other hand, there are some limitations to be pointed out; it is necessary to understand how the downregulation of these proteins effectively modify the downstream pathways involved in the metabolic control of energy expenditure and cell adaptation. And also if these mitochondrial adaptive changes associated to maternal hyperglycemia are reversible. This could help develop future therapeutic strategies by the elucidation of other proteins. The understanding of these mechanisms could not only help clarify the important regulators of the mitochondrial homeostasis in skeletal muscle, but also might help to provide more specific targets that could be used to better clarify some of the pathophysiological mechanisms of diabetic syndrome.

In summary, this study demonstrated the negative transgenerational effects of maternal hyperglycemia on insulin resistance, mitochondrial homeostasis and ultrastructural morphology of soleus muscle in adult female offspring in rat model. These data add a new understanding of the mechanisms of mitochondrial adaptation to the hyperglycemic environment and insulin sensitivity on diabetes etiology.

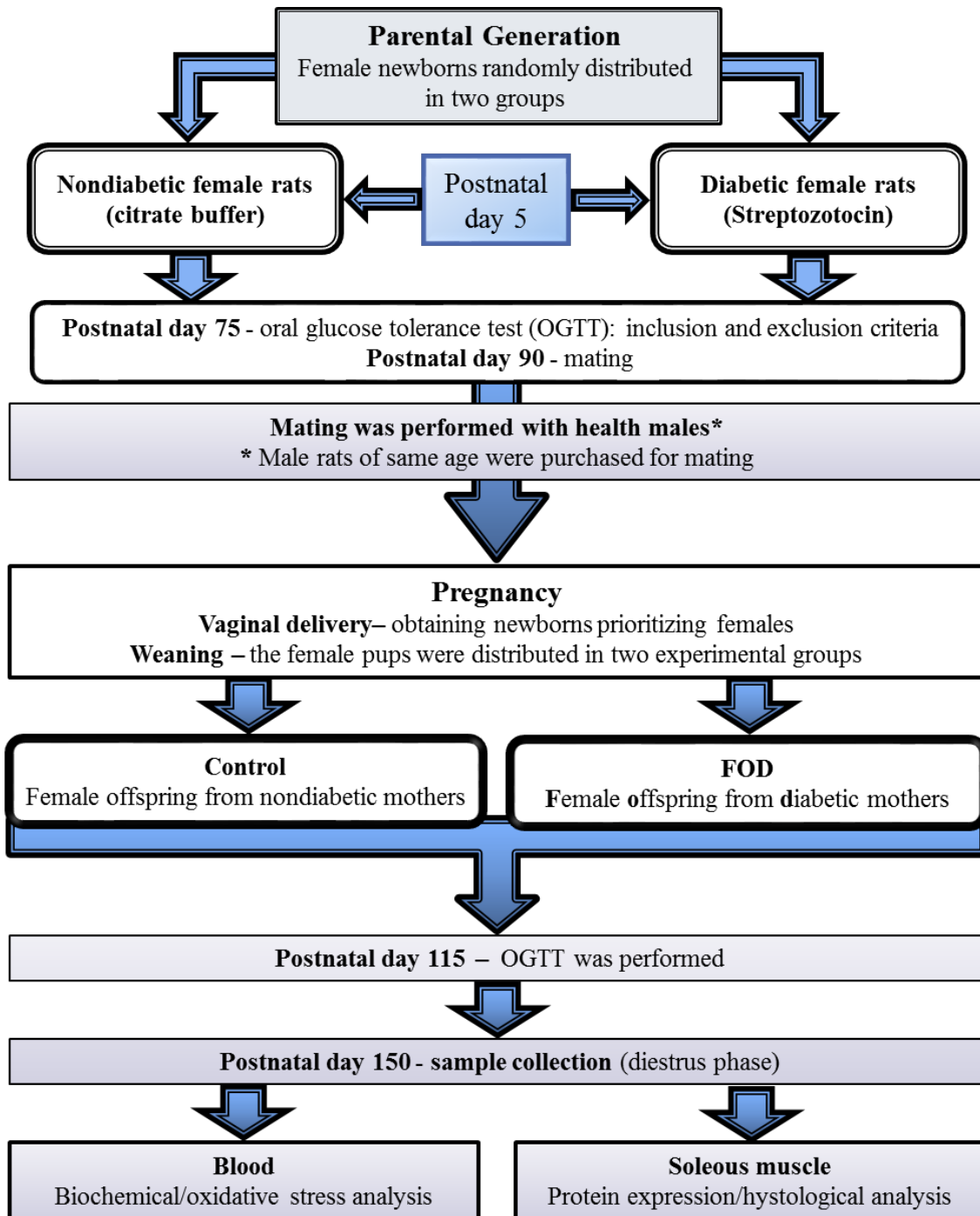
4. METHODS AND MATERIALS

4.1 Animals and experimental design

All procedures in this study were approved by the Animal Experimentation Ethics Committee of our institution under the register number: 1326/2019. Sprague-Dawley rats (one month of life) were acquired by CEMIB/Unicamp-Brazil and maintained in controlled conditions in our vivarium until mating. The sequence of the experimental design is shown in the figure 8. Our experimental sequence was performed in two steps: five days after birth, the female pups were injected with 70 mg/Kg of

streptozotocin (beta-cytotoxic drug) by subcutaneous (s.c.) route for composition of future mothers. The nondiabetic (Control) rats received citrate buffer (vehicle) within the same conditions as the diabetic group. The non-used male pups were anaesthetized (Thiopentax[®], 120 mg/kg dosage) and sacrificed by decapitation. The female rats were maintained in polypropylene cages of a maximum of three rats/cage under controlled lighting (lights on from 7 am to 7 pm at 22–23°C). Oral glucose tolerance test (OGTT) was used as the inclusion criterion at adulthood (75 days of life). Rats given STZ that presented glycemic levels equal to or greater than 200 mg/dL in one or more time points were considered as diabetic, while the animals that presented lower glycemia were not used in this study. For the Control group, female pups with glycemic levels lower than 140 mg/dL during OGTT were included [55]. The included female rats were mated with nondiabetic male rats (3 females: 1 male) for obtaining of their female pups for vaginal delivery, and composition of two experimental groups: Female offspring from nondiabetic mothers (Control) and female from diabetic mothers (FOD). All the female pups were maintained with their mother until the weaning (day 30 of postnatal life) in a maximum of 8 pups per mother, equivalent to functional nipple numbers. The female pups were housed and kept in polypropylene cages of a maximum of three rats/cage under controlled lighting (lights on from 7 am to 7 pm at 22–23°C), and the male pups were analyzed for another researcher team.

At adult life (145 postnatal days), the female rats were submitted to OGTT for inclusion criteria similarly to their mothers. For Control group, female rats with glycemic levels lower than 140 mg/dL during OGTT were used. For FOD, were included those rats that presented glycemic levels equal to or greater than 140 mg/dL in one or more time points during OGTT, being considered rats with glucose intolerance, and those with glycemia greater 200 mg/dL as diabetic rats. At day 150 of life, all the included animals were weighted, anesthetized with Thiopentax[®] (120 mg/kg, intraperitoneal route) and killed by decapitation. One part of the blood samples was collected in dry tubes and centrifuged at 1,600 ×g for 10 min at 4°C. After, the serum was obtained and stored in a freezer at –80 °C and processed for biochemical analysis. Another aliquot of total blood was processed to obtain washed erythrocytes as previously described for de Souza [56]. Soleus muscle samples from the right paw were collected, weighted and immediately frozen in liquid nitrogen for Western Blotting and frozen tissue morphology. And samples from the left paw were collected in glutaraldehyde for ultrastructure analysis.



4.2 Biochemical determinations

4.2.1 Oral glucose tolerance test - OGTT

All female rats were submitted to the OGTT according to Gallego [57] while glycemic values obtained during the two hours test were calculated in order to obtain the total area under the curve (AUC) [58].

4.2.2 Insulin measurement

Serum insulin levels were measured by spectrophotometry using a commercial kit from Cayman[®] following the manufacturer instructions.

4.2.3 Lipid profile

Triglyceride and total cholesterol levels were measured in the serum by spectrophotometry - using commercial kits from Wiener[®] and following the instructions of the manufacturer.

4.2.4 HOMA-IR, HOMA-beta and Triglyceride-Glucose index (TyG) calculations

HOMA-IR and HOMA-beta calculations were made as follows by Matthews et al. [59] and Wallace et al. [60], respectively:

HOMA-IR: $[\text{fasting insulin } (\mu\text{IU/ml}) \times \text{fasting glucose } (\text{mmol/ml})]/22.5$

HOMA-beta: $[20 \times \text{fasting insulin } (\mu\text{IU/ml})]/[\text{fasting glucose } (\text{mmol/ml}) - 3.5]$

In order to obtain the TyG (triglyceride-glucose index) values, the formula proposed by Simental-Mendía et al. [61]:

$\text{TyG} = [\text{fasting triglycerides } (\text{mg/dL}) \times \text{fasting glucose } (\text{mg/dL})]/2$

4.2.5 Oxidative stress biomarkers

Thiobarbituric acid (TBARS) and Reduced thiol groups (-SH) concentrations were measured in washed erythrocytes samples following procedures modified from de Souza et al. [56].

4.3 Western blotting

The samples of frozen muscle tissue were homogenized in RIPA Lysis Buffer extraction (Millipore®, USA) added with protease inhibitor (Sigma Chemical Company®, USA). Subsequently, they were centrifuged at 4000 rpm at 4°C for 20 min to remove the insoluble material. The determination of protein concentration was performed by method of Bradford [62]. Aliquots containing 70 µg of total proteins samples (n=6 animals per group) were separated on polyacrylamide gel (SDS-PAGE). Following the electrophoresis, the proteins were transferred to nitrocellulose membrane (Millipore, USA). The nonspecific binding of proteins was blocked by incubation the membrane with 5% non-fat milk diluted in TBS-T buffer for 1h at room temperature. The membranes were incubated for 16 hours at 4°C with primary antibodies: PI3K (Santa Cruz®, USA, #sc-134766, 1:500), MAPK (Erk1/2) (CellSignaling®, USA, #4695, 1:800), AMPK (Abcam®, USA, #ab32047, 1:800), MFF (CellSignaling®, USA, #84580, 1:800), MFN-2 (Abcam®, USA, #ab56889, 1:800), and β -Actin (Abcam®, USA, #ab8224, 1:800). After washing in TBS-T, the membranes were incubated for 90 min with an HRP-conjugated secondary antibody and specific for each primary antibodies (CellSignaling®, USA, #7074, #7076, 1:10,000). The reaction was developed by an ECL reagent kit (Bio-Rad®, USA) and the images of the bands were captured using a CCD camera (a G: BOX system (Syngene®, Cambridge, UK). The integrated optical densities (IODs) of the targeted protein bands were measured using ImageJ software (National Institutes of Health, USA). Expression levels were normalized by the values obtained from the internal control (β -actin) and the results were normalized from fold change and expressed as mean $\pm$ SD.

4.4 Morphological analyses

4.4.1 Central nuclei number in analysis of the soleus muscle fibers

The samples of frozen muscle (n=6 animals per group) were cut into 10-mm thick cross-sections in a cryostat (Leica CM 1800, Germany). The cross-sections were fixed for 10 min on glass slides for microscopy with cold acetone and then stained with hematoxylin and eosin (H&E) (n=6 animals samples per group). The slides were examined using a light microscopy and subsequently photographed (DMR, Leica coupled with digital camera CCD-IRIS/RGB, Sony, Germany) for the analysis of centrally located nuclei [63].

4.4.2 Immunohistochemical analysis

The frozen sections of muscle (n=6 animals per group) were hydrated for 10 min followed by endogenous peroxidase block using a 3% H₂O₂ solution embedded in methanol. After washing, the sections were incubated with 3% of fetal bovine serum solution at 37°C. Then, the slides were incubated with antibodies against WB-myosin heavy chain fast (WB-MHCf, Novocastra, 1:120) or WB-MHC slow (WB-MHCs, Novocastra, 1:180) overnight at 4°C. Next day, the samples were washed three times with 0.01 M PBS and incubated with 3,3-diaminobenzidine tetrahydrochloride (DAB) (Sigma-Aldrich) for 5 minutes, washed with 0.01 M PBS for 10 minutes and stained with hematoxylin for 1 minute. The visualization of fast and slow fibers was done using a light microscopy (DMR, Leica coupled with a CCD-IRIS/RGB digital camera, Sony), and the fiber type were counted and quantified using the image processing and analysis Java software ImageJ (National Institutes of Health, USA) [64].

4.4.3 Ultrastructural analysis of muscle samples

For TEM, the samples (n=6 animals per group) were fixed in 2% glutaraldehyde solution in 0.1 M phosphate buffer (pH 7.4) for 24 h at room temperature and then postfixed in 1% osmium tetroxide in the same buffer for 2 h. After being washed with distilled water, the samples were contrasted with an aqueous solution of 0.5% uranyl acetate for 2 h at room temperature, dehydrated in a graded acetone series (50%, 70%, 90% and 100%), and embedded in Araldite resin. Ultrathin sections were contrasted with uranyl acetate and lead citrate and were then observed and photographed with a Jeol JEM – 100CXII accoupled to Hamamatsu ORCA-HR digital camera.

4.5 Statistical analysis

The effect size was determined considering the variability of our biomarkers and the two experimental groups based on previous experiments conducted in our laboratory. Based on this effect size, the sample calculation was 10 rats per group for biochemical analysis and OGTT, while it was 6 rats per group for morphological analysis and Western Blotting quantifications. The calculation of the sample size defined by a specialist in Biostatistics in order to obtain results in animal research

compatible with the aimed translational sampling. All data are presented as means $\pm$ standard deviation (SD) and were analyzed by the Biostatistics from Research Support Office of our Institution. The minimum significance limit of 5% ($p < 0.05$) was considered. For the comparisons between glycemic means by oral glucose tolerance test (OGTT), the area under the curve (AUC) during OGTT, serum insulin and triglyceride concentrations, and central nuclei quantification, the Gamma Distribution test was used in view of the asymmetric distribution of the data. For TBARS, immunohistochemical and Western blotting analysis, reduced thiol Groups and total cholesterol, the Student's t-test was used.

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CAPÍTULO II

CHAPTER 2

**PROHIBITIN-MODULATED MITOCHONDRIAL METABOLISM IN
YOUNG AND ADULT OFFSPRING AS ADAPTATIVE RESPONSE AFTER
INTRAUTERINE DIABETIC EXPOSURE: A PROTEOMIC STUDY IN
LIVER OF FEMALE RATS.**

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ABSTRACT

The concept of intrauterine plasticity presupposes those physiological adaptations of offspring in early life can predict a phenotype for long-term. Maternal diabetes is one condition that can cause maladaptive response in the fetus and increase the risk of insulin resistance and metabolic disease in the offspring. However, the mechanism underlying these fetal adaptations remains obscure. In this way, we aimed to investigate the global expression of proteins in the mitochondrial content of female rats coming from diabetic mothers at weaning and adulthood. Female offspring coming from mothers with diabetes (FOMD) were obtained through pregnancy of *Wistar* rats with streptozotocin neonatally-induced diabetes. At 30 and 110 days of life, FOMD rats were killed, and blood and liver were collected. Blood was used for insulin analysis and liver was processed for obtaining the fresh mitochondrial content. Proteomics analysis were performed in the mitochondrial tissue by nLC-MS/MS mass spectrometry. A total of 399 proteins were identified in the liver mitochondria and 54 and 51 proteins were differentially expressed, respectively at 30 and 110 days of life. In both ages the FOMD rats presented an increase mitochondrial metabolism and biogenesis in addition to increased beta-oxidation and endoplasmic reticulum stress. At 110 days of life the FOMD rats presented also increased response to oxidative stress. Furthermore, prohibitin is upregulated in both ages and appears to have a pivotal role in these adaptive responses found in the FOMD rats.

Keywords: Mitochondria; insulin resistance; prohibitin; proteomics; fetal programming; rat.

INTRODUCTION

The developmental plasticity processes change tissular structure and function by effects on anatomy or microanatomy or on cellular activity in these tissues. Early life can change the physiological configurations of offspring so that the response to an environmental challenge will be different in long-term. This process evolves as an adaptive phenomenon to help the individual to the environment, but that may be maladaptive if the environmental prediction is incorrect because of faulty signal transduction, maternal illness, maternal consumption of a non-repair diet (or even dieting to lose weight) or unpredictable environmental changes [1].

Evidence indicates that children of women with pre-gestational *Diabetes mellitus* (type 1 and type 2 DM) and gestational diabetes had a higher prevalence of glucose intolerance and insulin resistance compared to controls [2]. Pettitt et al. (1991) also confirmed this effect when they verified abnormal glucose tolerance in the offspring of Pima indigenous women during pregnancy [3]. The authors correlated metabolic abnormalities that occur in diabetic pregnancy with states of insulin resistance, hypertension, obesity and diabetes in the offspring. Therefore, the mechanisms by which maternal hyperglycemia increases metabolic risk in offspring have not been still fully elucidated.

Pre-clinical studies are very important to analyze pathophysiological mechanisms and to advance our knowledge regarding the discovery of new treatments for different types of diseases, including *Diabetes mellitus*. In our laboratory, several experimental models of diabetes have been developed in female rats with the aim of associating the diabetic syndrome and pregnancy for a better understanding of the outcomes in the offspring [4–7]. A more recent study about to be published by our research group (Paula et al., 2021 - in press) showed that daughters coming from diabetic rats had impaired glucose tolerance and dyslipidemia in young adulthood. This confirms the harmful repercussions of maternal diabetes on successive generation.

To verify the mechanisms involved in the transgenerational outcomes induced by maternal diabetes, Klöppel et al. (2021 – in press) in our laboratory showed that mature adult female rats from diabetic dams presented mitochondrial alterations; reduced protein expression of transcription factors implicated in the insulin signaling; abnormal relationship between mitochondrial and endoplasmic reticulum (ER) leading to ER stress. Taken together, these findings contributed to insulin resistance in the adult

rats (150 days of life).

Insulin resistance is a major metabolic feature in obesity and a key factor in the pathogenesis of type 2 DM. The underlying molecular mechanisms are complex and still incompletely understood. Insulin signaling is impaired at several levels, but whether these changes are primary or secondary to the metabolic changes remains unclear. In general, insulin resistance is considered “harmful” and is related to Type 2 DM. Therefore, it is necessary to maintain insulin sensitivity as an integral component of normal metabolic physiology [8].

In other hand, liver is a tissue with a high adaptative and metabolic rate and rich in mitochondria. Works as a metabolic sensor and is involved in glycogenesis, glycogenolysis, lipolysis, ketogenesis and in the production of ATP for other tissues, being, therefore, highly responsive to changes related to insulin and increased serum glucose levels [9,10].

Considering the previous results from our laboratory on the evaluation of mature adult offspring (150 days old) from diabetic mothers, there is evidence that the mitochondrial imbalance induced by the intrauterine hyperglycemic insult leads to the adaptive responses present in these animals. Thus, there is a need to develop mechanistic studies to better understand the relationship between insulin resistance, glucose intolerance and plasticity between mitochondria and endoplasmic reticulum in different moments of postnatal life: after weaning (30 days of life) and in young adulthood (110 days of life). Thus, the aim of this study was to investigate how maternal diabetes influences protein expression related to metabolism and mitochondrial function in the offspring's liver at weaning and whether this change remains in adulthood. Furthermore, we intend to verify if this dysfunction altered the expression of specific proteins of mitochondrial energy metabolism induced by adaptive plasticity since intrauterine life.

MATERIALS AND METHOD

All procedures in this study were approved by the Animal Experimentation Ethics Committee of our institution. Wistar female rats at five days after birth were injected with 70 mg/Kg of streptozotocin (beta-cytotoxic drug) by subcutaneous (s.c.) route for composition of future diabetic mothers. The control rats received only citrate buffer (vehicle) in the same conditions as the diabetic group. All the male pups were not

used and anaesthetized (Thiopentax®, 120 mg/kg dosage) and killed. The female rats were kept in polypropylene cages of a maximum of three rats/cage under controlled conditions of light and temperature (7 AM to 7 PM at 22–23°C). Oral glucose tolerance test (OGTT) was used as the inclusion criterion at adulthood (75 days of life). The rats that received STZ and presented glycemic levels equal to or greater than 200 mg/dL in one or more time points were considered as diabetic, while the animals that presented lower glycemia were discarded. In the Control group all female pups with glycemic levels lower than 140 mg/dL during OGTT were used as control mothers [55]. In sequence all included female rats were mated with normal and healthy male rats (3 females: 1 male). The female pups were obtained for vaginal delivery, and composition of two experimental groups: Female offspring from healthy mothers (Control) and female from mothers with diabetes (FOD). All the female pups were maintained with their mother until the weaning (day 30 of postnatal life) in a maximum of 8 pups per mother, equivalent to functional nipple numbers. At 30 days of life each litter of female rats were separated in two: One to compose the group with 30 days old rats, and the other were housed and kept in polypropylene cages of a maximum of three rats/cage under controlled lighting (lights on from 7 am to 7 pm at 22–23°C) until 110 days of life.

At 30 and 110 days of life, according to each experimental group, Control and FOD female rats were killed and blood for serum obtaining and liver for mitochondrial content extraction were collected.

Insulin measurement

Serum insulin levels will still be measured by spectrophotometry using a commercial kit from Cayman® following the manufacturer instructions.

Chemicals

All chemical reagents used in this experiment were purchased from Sigma-Aldrich®.

Mitochondrial isolation

The mitochondrial isolation was performed with fresh liver tissue. The liver

samples were homogenized in 220mM D-Manitol 70mM sucrose 2mM hepes pH 7.2 solution, using a Glass/Teflon Potter Elvehjem homogenizer for 10 seconds. Then, the samples were centrifugated at 600rpm for 10 minutes at 4°C. The supernatant was collected and filtered using a teflon net. The supernatant was again centrifuged at 15.000rpm for 10 minutes at 4°C. The supernatant was then discarded and the pellet with all mitochondrial content was frozen for further analysis.

Mitochondrial protein extration

Around ~30mg of each sample was add to 10-fold volume/weight 6M GuHCl 1mM EDTA 10nM Dithiothreitol (DTT) 0.01% NaN₃ with Protease Inhibitor Cocktail. The samples were then homogenized using a T 10 basic ULTRA-TURRAX® - IKA for 10 seconds. The samples were heated for 5 minutes as 95°C, again homogenized and then and kept during 48 hours in a shaker at 4°C room. After 48 hours, the samples were again heated for 30 minutes at 56°C. After, a 30mM iodoacetamide (IAA) was add and each sample was kept in dark room at 25°C shaking for 45 minutes. Further, the samples were centrifugated at 15.000rpm for 15 min at 4°C. The supernatant was collected and add to a 4-fold volume of cold acetone (-20°C) overnight. The samples were again centrifugated at 15.000rpm for 15 min at 4°C, followed by trypsin (Sigma-Aldrich®) addition in 1/25 volume proportion overnight at 37°C, shaking. By the last, the peptides concentration was measured by commercial kit.

Peptide quantification

The peptides were quantified in order to access the exact amount into to Nano-liquid chromatography equipment. The quantification was done using a commercial kit of quantitative colorimetric peptide assay from Thermo Scientific®

Purification of samples

Purification of samples was performed by extracting tryptic peptides using Stage Tips [11].

Mass spectrometry analysis

The Nano-liquid chromatography procedure, mass spectrometry (MS), and tandem MS (MS/MS) analyses were performed as described in our previous studies [12]. Injection of each sample was 0,2 ug. Database searches were carried out using the Uniprot databases (uniprot.org) as described in [13] with the taxonomy restricted to *Rattus norvegicus*. Only significant hits (MASCOT score ≥ 80 for proteins (at least two peptides for protein) and ≥ 25 for peptides, <http://www.matrixscience.com>) were accepted.

Statistics

The suitable software (Profile Analysis version 2.1, Bruker Daltonik) was used to evaluate differences in the protein composition between groups by means of MS label-free quantification. The peptides under consideration had to be found in at least 40% of all samples, regardless of the group, and had to be found in at least 50% in one group. The p-value was returned by two-sample t-tests (and corrected for multiple-testing by false discovery rate rate (FDR) based on frequency histogram) (adjusted p-value threshold 0.05).

RESULTS AND DISCUSSION

To date, this is the first study to report a proteomic evaluation of the transgenerational effects of maternal diabetes on the hepatic mitochondrial content. No studies have reached what this plasticity period causes to the pattern of protein expression during the weaning period and later in adulthood. From our analysis, 399 proteins were identified in the liver mitochondria of rats (Control and FOMD groups) at 30 days of life (Supplementary Table S1) and 110 days of life (Supplementary Table S2) in which 54 and 51 proteins were differentially expressed, respectively. After analysis of these proteins of the FOMD group at weaning and adulthood periods, respectively, we verified that three and two of them were downregulated, whereas 51 and 49 proteins were upregulated (Tables 1 and 3).

36 KEGG and nine Reactome pathways at 30 days of life, and 31 KEGG and 19 Reactome pathways at 110 days of life were verified. 124 biological processes were

enriched in the protein list of the FOMD group at 30 days of life. Considering the same experimental group at 110 days of life, 162 biological process terms were enriched in the protein list of the FOMD group.

Since of the list of the enriched pathways and processes, we mostly found those related to the lipid metabolism (fatty acid degradation and oxidation), amino acid and mitochondrial metabolism and endoplasmic reticulum stress in both ages while the antioxidant enzymes and mitochondrial biogenesis were increased at 110 days of life (Tables 2 and 4).

Insulin level measurement

The data will be presented and discussed later. This is due to delay in the kit arrival in Brazil, which impaired to conclude this analysis.

Protein Folding and Endoplasmic Reticulum (ER) Stress

The protein enrichment showed the biological process “Response to endoplasmic reticulum stress (ER Stress)” in FOMD at days 30 and 110 of life. On postnatal day 30, the daughters from diabetic rats presented “Positive regulation of protein folding”, and at day 110 “Protein refolding” processes were identified. In these matters, at 30 days of life, the FOMD group presented upregulation of the enriched proteins Calreticulin (*P18418*), Endoplasmic reticulum chaperone (*Q66HD0*), and Protein disulfide-isomerase (*P11598* and *G3V6T7*). At 110 days of life, FOMD rats showed overexpression of mitochondrial heat shock 10-kDa protein (*P26772*) and mitochondrial heat shock 70-kDa protein (*F1M953*) (Table 2), indicating ER stress as well as mitochondrial remodeling and biogenesis [14,15]. Moreover, endoplasmic reticulum chaperone (BiP) was also overexpressed in FOMD groups in both ages (*P06761*) (Tables 2 and 4). BiP is a hallmark with the pivotal role as a chaperone, modulating the unfolded protein response (UPR) under ER accumulation of misfolded protein and stress [16,17], showing the relationship among these proteins involved with ER stress and mitochondrial biogenesis in the female rats from diabetic dams.

In our laboratory team, has been observed that adult female rats (150 days of life) coming from diabetic mothers presented insulin resistance (IR) and glucose intolerance (Klöppel *et al.* 2021– in press). which activates several other stress kinases

that eventually might impair the insulin signaling pathway. According to Klöppel *et al.* (2021 - in press) the mature adult female rats presented lower protein expression of AMPK/ERK1/2 and other transcription factors, leading to damaged insulin signaling pathway. Besides the fact that the accumulation of misfolded or unfolded proteins results in ER stress or UPR, it may be a mechanism related to IR once UPR interacts with AMPK/ERK1/2 [18]. The activation of ER stress-induced kinases impairs the insulin signaling pathway and promotes lipid accumulation through the synthesis of proinflammatory cytokines, which also negatively affect insulin signaling [19]. Moreover, the high glucose concentration in the intrauterine environment might be the cause of excessive mitochondrial respiration as an adaptative response that disturbs mitochondrial homeostasis and dynamics in the young and adult offspring. Then, it is very conceivable that the mechanism behind this ER stress state is a response to an increased mitochondrial metabolism leading to mitochondrial biogenesis.

Lipid Metabolism

“Fatty acid beta-oxidation” biological process and “Fatty acid degradation” and “Mitochondrial Fatty Acid Beta-Oxidation” pathways were strongly enriched in the differentially expressed protein list at the days 30 and 110 of life of the FOMD group. Among these differentially expressed proteins, we highlight the Mitochondrial 3-hydroxy-3-methylglutaryl-CoA synthase (HMG-CoA synthase) (*P22791*), long-chain acyl-CoA dehydrogenase (*P15650*), Electron transfer flavoprotein (*Q68FU3*), Enoyl-CoA hydratase (*P14604*), and mitochondrial long-chain trifunctional enzyme (MTP) (*Q64428*), which were all upregulated at day 30 and 110 of life of the FOMD group (Tables 2 and 4). These proteins are involved in the complete fatty acid β -oxidation process [20–24] and their increased long-life might be related to a ketogenic pathway phenotype in this group, as observed at the 110 days of life. This ketogenic state can be represented by both acetyl-CoA acetyltransferase (*P17764*) and HMG-CoA synthase upregulation, which is one of the enzymes also involved in the last step of the mitochondrial beta-oxidation (thiolases) and first irreversible step of the ketogenesis pathway, respectively, playing a major role in ketogenic liver metabolism [25,26].

Moreover, the MTP complex is recognized as responsible for catalyzing the three last steps of the β -oxidation [27], and its upregulation among the life of the FOMD rats might be explained by an adaptation to the maternal hyperglycemic state, since the

excessive glucose concentrations increase the production of TCA cycle products, such as acetyl-CoA and NADH [28]. In addition, the increase of both acetyl-CoA and NADH levels induces the acetylation of mitochondrial proteins [29], including the MTP complex. There are studies involving MTP overexpression and deacetylation as a protective novel for reducing insulin resistance [20]. This evidence suggests that the MTP might have a pivotal role in the adaptive response to maternal hyperglycemia during the fetal developmental and postnatal periods, which remained present during the adulthood of the FOMD rats related to insulin resistance. It is still pertinent to be elucidated if the increased MTP is just a consequence of an increased mitochondrial β -oxidation or an adaptive mechanism to protect the cell against lipotoxicity during mitochondrial respiration and biogenesis in liver. In this matter, more studies are needed to better understand the MTP's role on glucose metabolism, insulin resistance, and diabetes onset in offspring from diabetic mothers.

Mitochondrial metabolism, biogenesis, and Oxidative stress

The enrichment shows an upregulation in the pathways of “Oxaloacetate metabolic process”, “Citrate cycle (TCA cycle)”, “Pyruvate metabolism” and “Respiratory electron transport” at both studied ages in FOMD group (Tables 2 and 4). The maternal hyperglycemia during pregnancy caused a long-life (30 and 110 days of life) upregulation in the offspring's expression of Electron transfer flavoprotein (*Q68FU3*), Pyruvate carboxylase, mitochondrial (*P52873*), Pyruvate dehydrogenase E1 (*P49432*) and Prohibitin (PHB) (*P67779*). At 30 days of life, the FOMD rats presented upregulation of Alcohol dehydrogenase 1 (*P06757*), Malate dehydrogenase (*O88989*), Pyruvate dehydrogenase (*P49432*) and Cytochrome b-c1 complex (*P32551*), while at 110 days of life the rats presented upregulation of Malate dehydrogenase 2 (*P04636*), and Succinate dehydrogenase (*Q920L2*). In other hand, the Reactome pathways of “Transcriptional activation of mitochondrial biogenesis”, “Mitochondrial biogenesis” and “Cristae formation” was present only in FOMD rats at 110 days of life (Table 4). The increase of these pathways is highlighted by the upregulations in the proteins related to mitochondrial cristae respiration machinery, such as Isocitrate dehydrogenase [NADP] (*P56574*), Mitochondrial membrane ATP synthase F(1)F(0) (*G3V6D3*), ATP synthase subunit O (*Q06647*), ATP synthase subunit f (*D3ZAF6*), ADP/ATP translocase 2 (*Q09073*), Prohibitin (PHB) (*P67779*). The adult FOMD rats presented an increased

expression of the mitochondrial respiratory chain proteins, and this might be related to its driver PHB [30], which was possibly caused by a chronic demand of exacerbated oxidation and degradation of lipid as showed in our results. PHB is considered as a mitochondrial chaperone [31], and has been observed to be related to mitochondrial respiratory chain subunit degradation, association and activity of the oxidative phosphorylation system, mitochondrial biogenesis and networks, and apoptosis, and mitophagy [32]. Then, it is suggested that overexpression of PHB in the weaning and young adult phase of rat daughters coming from diabetic dams might be implicated to plasticity of the offspring involving several mitochondrial alterations, such as mitophagy, found at 150 days of life (Klöppel et al, 2021), confirming an abnormal cellular machinery on the mitochondrial biogenesis, oxidative stress, and PHB expression/levels induced by hyperglycemia since intrauterine life.

The biological process “Reactive oxygen species metabolic process” and the pathways “Glutathione metabolism” and “Detoxification of Reactive Oxygen Species” were enriched in the FOMD groups at 110 days of life (Table 4). The enzymes Glutathione S-transferase (*B6DYP8*), Glutathione peroxidase (*Q6PDW8*), Carbamoyl-phosphate synthase (*P07756*), Superoxide dismutase [Mn], mitochondrial (*P07895*), Catalase (*P04762*) and Isocitrate dehydrogenase [NADP], mitochondrial (*P56574*) were upregulated in this group (Table 3), which represents a complete machinery against reactive oxygen species (ROS) formation. To date, there was not studies reporting transgenerational effects, leading to increased antioxidant defenses of mitochondrial network on the offspring at adulthood. It is recognized that the increased activity in the mitochondrial respiratory chain is the main cause of higher production of ROS in pathologic and adaptive conditions [33,34]. Therefore, it looks to be mostly a consequence of the increased global metabolism in the liver tissue of FOMD rats.

In addition of energetic function, mitochondria are also a major source of reactive oxygen species (ROS) [35]. The energy produced is used by ATP synthetase to transform ADP into ATP. However, mitochondrial respiration also results in the formation of ROS such as superoxide and hydrogen peroxide. For prevention of an excessive ROS production, the lowering the mitochondrial proton gradient (uncoupling substrate oxidation from ATP production) has been performed, resulting in a pronounced lowering in mitochondrial production of ROS [36]. Evidence shows that prohibitin acts against oxidative stress, Muraguchi et al. (2010) verified overexpressed PHB prevented mitochondrial membrane depolarization and cause an inhibition of cell

death induced by hypoxia in H9c2 cardiomyocytes [37]. PHB overexpression protected mitochondria against hydrogen peroxide-induced oxidative stress, reduced apoptosis by mitochondrial pathway, and diminished mitochondrial membrane permeability compared with untransfected cardiomyocytes [31].

Mattox et al. (2021) reported that prohibitin caused an upregulation of glutathione reductase enzyme in the circulation [38]. Besides, PHB is associated to regulation of genes with GSH homeostasis, such as glutamylcysteine synthetase, glutathione peroxidase, and glutathione reductase [39]. Taken together, these evidences indicates that the prohibitin might be a perspective to drible the diabetes or insulin resistance-induced oxidative stress, contributing to decrease the damages from hyperglycemia in dams and successive generation. The upregulated expression of enzymes related to oxidative stress might be implicated to prevent the oxidative stress complications, confirming the protective measurement.

Prohibitin as a metabolism sensor

Prohibitin (PHB) (*P67779*) was strongly upregulated and enriched in both 30 and 110 days of life in FOMD (Tables 1-4), participating in pathways related to mitochondrial metabolism and biogenesis. PHB complex comprises two subunits (1 and 2) in mitochondria. It is an evolutionary conserved, ubiquitously expressed protein with pleiotropic functions [40,41]. For this reason, PHB shows to be able to participate in different biological processes and pathways related to mitochondrial metabolism and biogenesis [41]. PHB is known to control mitochondrial-encoded subunits of the respiratory in cristae formation and function [42,43] as well as prevents ROS by modulating the complexes 1-4 in the mitochondrial respiratory chain [44]. PBH also influence mitochondrial mitophagy when it binds the autophagosomal membrane-associated protein LC3 [45] and activate the mitochondrial fusion trough the dynamin-like GTPase OPA1 [46]. Some studies have reported a controversial role for PHB in cancer [47,48], while PHB appears to be protective against liver diseases [49]. In animal model, PHB1 inhibition caused alteration on the mitochondrial respiratory chain functioning, leading to an enhanced the production of oxidants [50]. Despite the fact that PHB is now also associated to the insulin signaling trough the MAPK/ERK pathway [51], a mystery of the PHB complex mechanisms behind the different protective/adaptative functions still remains. In the present study, the FOMD rats

showed PHB-induced overexpressed pathways on mitochondrial oxidative capacity, which implies that PHB might have also a pivotal role in adaptative responses to maternal hyperglycemia and long-life phenotype. In this sense, although PHB has been studied for decades, there is still a mechanistic gap in the systemic and local effects of PHB on *in vivo* metabolism. Thus, the prohibitin might be the hallmark for better mechanistic knowledge between impaired glycemic control and mitochondrial adaptations, contributing for its understanding in the diabetic syndrome and possible therapies.

Conclusions

In conclusion, we have shown that the mitochondrial content in liver of newly weaned and young adult females rats coming from diabetic mothers undergone to a fetal plasticity that remained for life. These adaptations are associated with different expression of multiple proteins and the abundance of oxidative enzymes in mitochondrial network appears to be part of its phenotype. In addition, our data suggest that this is accompanied by a shift in the glucose oxidative metabolism to a lipid oxidation and degradation. Although of a universal increase of mitochondrial metabolism and ER stress response, the adaptive response to hyperglycemia is largely related to many other mechanisms, such as decreased insulin signaling events that were detected by the current method. The changes in overexpressed protein in these multiple pathways add information on how these interactions happened in the diabetic intrauterine environment. Viewed in this way, the current evidence suggest that PHB can have a key role on adaptative responses related to mitochondrial function through life, although of the minor available knowledge of its biological functions and pathways need more clarification. Therefore, the role and exact pathways in several of the regulated differentially proteins remain to be elucidated.

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Contribution statement

EK, DCD, VH, BK and AE conceived and designed the study. EK, BK and AE performed the experiments. EK, DCD, and AE analyzed and interpreted the data. EK and DCD wrote the manuscript, and all authors will check the manuscript for intellectual content and approved the final version.

Duality of interest

All authors declare that there is no duality of interest associated with this manuscript.

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Table 1. Differentially expressed proteins in the female offspring from rat diabetic mothers at day 30 of life.

Access code	Differentially Expressed Proteins	MW [kDa]	Peptide Number	Expression	FDR <i>p</i> -value
P07756	Carbamoyl-phosphate synthase [ammonia], mitochondrial GN=Cps1	164.5	87	UPREGULATED	p=0.000
G3V6D3	ATP synthase subunit beta GN=Atp5f1b	56.3	22	UPREGULATED	p=0.000
A0A482IDN3	Hsp60 GN=Hspd1	60.9	23	UPREGULATED	p=0.000
G3V9U2	3-ketoacyl-CoA thiolase, mitochondrial GN=Acaa2	41.9	18	UPREGULATED	p=0.000
P10860	Glutamate dehydrogenase 1, mitochondrial GN=Glud1	61.4	20	UPREGULATED	p=0.000
P97852	Peroxisomal multifunctional enzyme type 2 GN=Hsd17b4	79.4	19	UPREGULATED	p=0.000
P52873	Pyruvate carboxylase, mitochondrial GN=Pc	129.7	20	UPREGULATED	p=0.000
Q64428	Trifunctional enzyme subunit alpha, mitochondrial GN=Hadha	82.6	16	UPREGULATED	p=0.000
P06761	Endoplasmic reticulum chaperone BiP GN=Hspa5	72.3	20	UPREGULATED	p=0.000
P18163	Long-chain-fatty-acid--CoA ligase 1 GN=Acs1	78.1	19	UPREGULATED	p=0.009
P22791	Hydroxymethylglutaryl-CoA synthase, mitochondrial GN=Hmgcs2	56.8	18	UPREGULATED	p=0.000
P09118	Uricase GN=Uox	34.9	16	UPREGULATED	p=0.000
P18418	Calreticulin GN=Calr	48.0	15	UPREGULATED	p=0.000
G3V7J0	Aldehyde dehydrogenase family 6, subfamily A1 GN=Aldh6a1	57.7	16	UPREGULATED	p=0.001
Q66HD0	Endoplasmic reticulum chaperone BiP GN=Hsp90b1	92.7	15	UPREGULATED	p=0.000
F1LNW3	Acyl-coenzyme A oxidase GN=Acox2	76.7	15	UPREGULATED	p=0.000
P11598	Protein disulfide-isomerase A3 GN=Pdia3	56.6	15	UPREGULATED	p=0.000
P15650	Long-chain specific acyl-CoA dehydrogenase, mitochondrial GN=Acadl	47.8	11	UPREGULATED	p=0.003
P06757	Alcohol dehydrogenase 1 GN=Adh1	39.6	13	UPREGULATED	p=0.024
A0A0G2JSW3	Globin a4 GN=Hbb	16.0	11	UPREGULATED	p=0.032
P07871	3-ketoacyl-CoA thiolase B, peroxisomal GN=Acaa1b	43.8	10	UPREGULATED	p=0.005
B1H216	Globin c3 GN=Hba-a1	15.3	10	UPREGULATED	p=0.013
Q9WVK7	Hydroxyacyl-coenzyme A dehydrogenase, mitochondrial GN=Hadh	34.4	10	UPREGULATED	p=0.000
P00507	Aspartate aminotransferase, mitochondrial GN=Got2	47.3	11	UPREGULATED	p=0.003
P09811	Glycogen phosphorylase, liver form GN=Pygl	97.4	11	UPREGULATED	p=0.002
F1M1W1	Acyl-coenzyme A synthetase ACSM2, mitochondrial GN=Acsm1	65.3	9	UPREGULATED	p=0.000
F1LZW6	Citrin - Solute carrier family 25 member 13 GN=Slc25a13	54.1	12	UPREGULATED	p=0.017
B6DYP8	Glutathione S-transferase GN=Gsta1	25.3	8	DOWNREGULATED	p=0.025
B4F768	Multifunctional fusion protein (Fragment) GN=Aldh4a1	62.9	11	UPREGULATED	p=0.018
P10634	Cytochrome P450 2D26 GN=Cyp2d26	56.6	9	UPREGULATED	p=0.004
F1LM22	UDP-glucuronosyltransferase GN=Ugt2b	61.0	7	UPREGULATED	p=0.021
Q68FU3	Electron transfer flavoprotein subunit beta GN=Etfb	27.7	7	UPREGULATED	p=0.034

Access code	Differentially Expressed Proteins	MW [kDa]	Peptide Number	Expression	FDR <i>p</i> -value
G3V6T7	Protein disulfide-isomerase A4 GN=Pdia4	72.7	7	UPREGULATED	p=0.002
P67779	Prohibitin GN=Phb	29.8	6	UPREGULATED	p=0.032
Q6LDP3	Rat glutathione S-transferase	25.7	7	DOWNREGULATED	p=0.012
D4A2K1	4-hydroxy-2-oxoglutarate aldolase 1 GN=Hoga1	34.4	5	UPREGULATED	p=0.003
P07824	Arginase-1 GN=Arg1	35.0	6	UPREGULATED	p=0.045
Q6UPE0	Choline dehydrogenase, mitochondrial GN=Chdh	66.3	6	UPREGULATED	p=0.001
M0R6L9	Ferritin	20.7	4	DOWNREGULATED	p=0.046
P16970	ATP-binding cassette sub-family D member 3 GN=Abcd3	75.3	6	UPREGULATED	p=0.014
P11711	Cytochrome P450 2A1 GN=Cyp2a1	56.0	7	UPREGULATED	p=0.006
A0A0G2JY66	Carboxylic ester hydrolase GN=Ces1d	62.0	5	UPREGULATED	p=0.005
P14604	Enoyl-CoA hydratase, mitochondrial GN=Echs1	31.5	5	UPREGULATED	p=0.005
P05197	Elongation factor 2 GN=Eef2	95.2	7	UPREGULATED	p=0.016
Q9WVK3	Peroxisomal trans-2-enoyl-CoA reductase GN=Pecr	32.4	3	UPREGULATED	p=0.031
P32551	Cytochrome b-c1 complex subunit 2, mitochondrial GN=Uqcrc2	48.4	4	UPREGULATED	p=0.008
P35435	ATP synthase subunit gamma, mitochondrial GN=Atp5f1c	30.2	5	UPREGULATED	p=0.001
G3V6S5	C-1-tetrahydrofolate synthase, cytoplasmic GN=Mthfd1	100.9	5	UPREGULATED	p=0.002
P49432	Pyruvate dehydrogenase E1 subunit beta, mitochondrial GN=Pdhb	39.0	2	UPREGULATED	p=0.009
Q6P790	60S ribosomal protein L6 (Fragment) GN=Rpl6	33.5	4	UPREGULATED	p=0.011
O88989	Malate dehydrogenase, cytoplasmic GN=Mdh1	36.5	4	UPREGULATED	p=0.027
D3ZJD3	Ribosomal_L28e domain-containing protein	15.6	3	UPREGULATED	p=0.027
P48500	Triosephosphate isomerase GN=Tpi1	26.8	2	UPREGULATED	p=0.018
P62864	40S ribosomal protein S30 GN=Fau	6.6	1	UPREGULATED	p=0.004

p-value of the *t*-test less than 5% adjusted to false discovery rate (FRD)

GN = Gene name

Table 2. Enriched gene ontology biological process terms, Reactome and KEGG pathways in the list of differentially expressed protein in FOMD group that are involved in Lipid Metabolism, Endoplasmic reticulum stress and Amino acid and mitochondrial metabolism at day 30 of life.

Biological Theme	GO(BP), Reactome or KEGG code	Biological Pathways	Involved proteins (Access code)	<i>p</i> -value
Lipid Metabolism	GO:0006635	Fatty acid beta-oxidation		9.70E-11
	GO:0006637	Acyl-CoA metabolic process	P00507; P22791; P09811; P49432; P14604;	2.86E-09
	GO:0044242	Cellular lipid catabolic process	Q68FU3; G3V7J0; P18163; Q9WVK7;	5.07E-05
	GO:1901568	Fatty acid derivative metabolic process	G3V9U2; Q64428; P15650; P97852;	5.07E-05
	rno00071	Fatty acid degradation	F1LNW3; Q9WVK3; P16970; P10634;	1.87E-09
	RNO-77289	Mitochondrial Fatty Acid Beta-Oxidation	P06757; B6DYP8; P11711	1.43E-05
Endoplasmic reticulum stress	GO:1903334	Positive regulation of protein folding		0.0154
	GO:0006457	Protein folding	P05197; Q66HD0; P11598; G3V6T7;	0.0154
	GO:0034976	Response to endoplasmic reticulum stress	P06761; P18418	0.00037
	rno04141	Protein processing in endoplasmic reticulum		0.001
Amino acid metabolism	GO:1901606	Alpha-amino acid catabolic process		0.0025
	GO:0006575	Cellular modified amino acid metabolic process		0.0154
	GO:0044271	Cellular nitrogen compound metabolic process	Q6UPE0; G3V6S5; D4A2K1; P07824;	7.26E-08
	GO:1901699	Cellular response to nitrogen compound	P07756; P10860; P00507; P49432;	0.0192
	GO:0009065	Glutamine family amino acid catabolic process	G3V7J0; B6DYP8; P22791; Q9WVK7;	0.0032
	rno00280	Valine, leucine and isoleucine degradation	Q64428	1.50E-07
	RNO-71291	Metabolism of amino acids and derivatives		0.00019
Mitochondrial metabolism	GO:0006107	Oxaloacetate metabolic process		0.00038
	rno00020	Citrate cycle (TCA cycle)	P52873; P06757; Q68FU3; O88989;	0.0013
	rno00010	Glycolysis / Gluconeogenesis	P49432; G3V6D3; P48500; P35435;	0.0059
	rno00620	Pyruvate metabolism	P32551; P67779	0.0018
	RNO-1428517	Respiratory electron transport		0.0461

GO(BP) is Gene Ontology (GO) of biological process (BP), RNO is Reactome and RNO is KEGG pathway is Kyoto Encyclopedia of Genes and Genomes biological pathway; *p*-value of <0.05 for the enrichment analyses is significant at Benjamini-Hochberg FDR test.

Table 3. Differentially expressed proteins in the female offspring from rat diabetic mothers at day 110 of life

Access code	Differentially Expressed Proteins	MW [kDa]	Peptide Number	Expression	FDR <i>p</i> -value
P07756	Carbamoyl-phosphate synthase [ammonia], mitochondrial GN=Cps1	164.5	87	UPREGULATED	p=0.000
P04762	Catalase GN=Cat	59.7	26	UPREGULATED	p=0.026
G3V6D3	ATP synthase subunit beta GN=Atp5f1b	56.3	22	UPREGULATED	p=0.002
P10860	Glutamate dehydrogenase 1, mitochondrial GN=Glud1	61.4	20	UPREGULATED	p=0.000
P18163	Long-chain-fatty-acid--CoA ligase 1 GN=Acs11	78.1	19	UPREGULATED	p=0.000
P22791	Hydroxymethylglutaryl-CoA synthase, mitochondrial GN=Hmgcs2	56.8	18	UPREGULATED	p=0.000
P56574	Isocitrate dehydrogenase [NADP], mitochondrial GN=Idh2	42	18	UPREGULATED	p=0.000
G3V9U2	3-ketoacyl-CoA thiolase, mitochondrial GN=Acaa2	41.9	18	UPREGULATED	p=0.001
P52873	Pyruvate carboxylase, mitochondrial GN=Pc	129.7	20	UPREGULATED	p=0.000
P06761	Endoplasmic reticulum chaperone BiP GN=Hspa5	72.3	20	UPREGULATED	p=0.020
P09118	Uricase GN=Uox	34.9	16	UPREGULATED	p=0.001
F1LQC1	Acyl-coenzyme A oxidase GN=Acox1	74.5	11	UPREGULATED	p=0.000
F1LZW6	Solute carrier family 25 member 13 GN=Slc25a13	54.1	12	UPREGULATED	p=0.000
Q64428	Trifunctional enzyme subunit alpha, mitochondrial GN=Hadha	82.6	16	UPREGULATED	p=0.012
Q5M897	Otc protein GN=Otc	39.5	12	UPREGULATED	p=0.000
B4F768	Multifunctional fusion protein (Fragment) GN=Aldh4a1	62.9	11	UPREGULATED	p=0.000
P07871	3-ketoacyl-CoA thiolase B, peroxisomal GN=Acaa1b	43.8	10	UPREGULATED	p=0.002
P15650	Long-chain specific acyl-CoA dehydrogenase, mitochondrial GN=Acadl	47.8	11	UPREGULATED	p=0.021
B6DYP8	Glutathione S-transferase GN=Gsta1	25.3	8	DOWNREGULATED	p=0.001
Q68FU3	Electron transfer flavoprotein subunit beta GN=Etfb	27.7	7	UPREGULATED	p=0.000
P13803	Electron transfer flavoprotein subunit alpha, mitochondrial GN=Etfa	34.9	10	UPREGULATED	p=0.000
F1M953	Stress-70 protein, mitochondrial GN=Hspa9	73.7	10	UPREGULATED	p=0.002
F1M1W1	Acyl-coenzyme A synthetase ACSM2, mitochondrial GN=Acsml	65.3	9	UPREGULATED	p=0.002
D4A2K1	4-hydroxy-2-oxoglutarate aldolase 1 GN=Hoga1	34.4	5	UPREGULATED	p=0.001
P17764	Acetyl-CoA acetyltransferase, mitochondrial GN=Acat1	44.7	5	UPREGULATED	p=0.003
Q920L2	Succinate dehydrogenase flavoprotein subunit, mitochondrial GN=Sdha	71.6	6	UPREGULATED	p=0.032
P04636	Malate dehydrogenase, mitochondrial GN=Mdh2	35.7	10	UPREGULATED	p=0.001
P67779	Prohibitin GN=Phb	29.8	6	UPREGULATED	p=0.001
P00173	Cytochrome b5 GN=Cyb5a	15.3	5	DOWNREGULATED	p=0.010
Q6UPE0	Choline dehydrogenase, mitochondrial GN=Chdh	66.3	6	UPREGULATED	p=0.005
P26772	10 kDa heat shock protein, mitochondrial GN=Hspe1	10.9	6	UPREGULATED	p=0.008
P16970	ATP-binding cassette sub-family D member 3 GN=Abcd3	75.3	6	UPREGULATED	p=0.017

Access code	Differentially Expressed Proteins	MW [kDa]	Peptide Number	Expression	FDR <i>p</i> -value
G3V734	2,4-dienoyl CoA reductase 1, mitochondrial, isoform CRA_a GN=Decr1	36.1	5	UPREGULATED	p=0.005
A0A0G2JY66	Carboxylic ester hydrolase GN=Ces1d	62.0	5	UPREGULATED	p=0.036
P29147	D-beta-hydroxybutyrate dehydrogenase, mitochondrial GN=Bdh1	38.2	5	UPREGULATED	p=0.022
P55159	Serum paraoxonase/arylesterase 1 GN=Pon1	39.3	4	UPREGULATED	p=0.042
O70351	3-hydroxyacyl-CoA dehydrogenase type-2 GN=Hsd17b10	27.2	5	UPREGULATED	p=0.001
Q6PDW8	Glutathione peroxidase GN=Gpx1	16.5	5	UPREGULATED	p=0.022
Q68G40	Cytochrome P450 2C23 GN=Cyp2c23	56.4	3	UPREGULATED	p=0.005
Q06647	ATP synthase subunit O, mitochondrial GN=Atp5po	23.4	4	UPREGULATED	p=0.022
F1LRY5	Sarcosine dehydrogenase, mitochondrial GN=Sardh	101.3	3	UPREGULATED	p=0.008
P07895	Superoxide dismutase [Mn], mitochondrial GN=Sod2	24.7	3	UPREGULATED	p=0.049
P19945	60S acidic ribosomal protein P0 GN=Rplp0	34.2	3	UPREGULATED	p=0.014
D3ZAF6	ATP synthase subunit f, mitochondrial GN=Atp5mf	10.4	3	UPREGULATED	p=0.042
Q09073	ADP/ATP translocase 2 GN=Slc25a5	32.85	30	UPREGULATED	p=0.001
P30839	Aldehyde dehydrogenase family 3 member A2 GN=Aldh3a2	54.0	8	UPREGULATED	p=0.001
Q63342	Dimethylglycine dehydrogenase, mitochondrial GN=Dmgdh	96.0	5	UPREGULATED	p=0.011
P14604	Enoyl-CoA hydratase, mitochondrial GN=Echs1	31.5	5	UPREGULATED	p=0.015
P49432	Pyruvate dehydrogenase subunit beta, mitochondrial GN=Pdhb	39.0	2	UPREGULATED	p=0.006
D3ZCS9	5-hydroxyisourate hydrolase GN=RGD1309350	16.0	3	UPREGULATED	p=0.042

p-value of the *t*-test less than 5% adjusted to false discovery rate (FRD)

GN = Gene name

Table 4. Enriched gene ontology biological process terms, Reactome and KEGG pathways in the list of differentially expressed protein in FOMD group involved in Lipid Metabolism, Endoplasmic reticulum stress and Amino acid, mitochondrial metabolism, oxidative stress, and mitochondrial biogenesis at day 110 of life.

Biological Theme	GO (BP), Reactome or KEGG code	Biological Pathways	Involved proteins (Access code)	<i>p</i> -Value
Lipid Metabolism	GO:0006635	Fatty acid beta-oxidation		8.30E-13
	GO:0006637	Acyl-CoA metabolic process	P18163; P16970; Q68FU3; P29147;	3.42E-05
	rno00072	Synthesis and degradation of ketone bodies	P30839; P22791; P49432; P14604;	7.02E-05
	rno00071	Fatty acid degradation	P17764; P15650; F1LQC1; G3V734;	1.80E-11
	RNO-8978868	Fatty acid metabolism	G3V9U2; P13803; Q64428	3.69E-07
	RNO-77289	Mitochondrial Fatty Acid Beta-Oxidation		0.0024
Endoplasmic reticulum stress	GO:0042026	Protein refolding		0.0258
	GO:0034976	Response to endoplasmic reticulum stress	P06761; P26772; F1M953;	0.00056
	GO:0051085	Chaperone cofactor-dependent protein refolding		0.0012
Amino acid metabolism	GO:0042219	Cellular modified amino acid catabolic process		9.50E-04
	rno00410	Beta-Alanine metabolism	Q63342; F1LRY5; Q6UPE0;	1.81E-05
	rno00280	Valine, leucine and isoleucine degradation	P22791; P30839; O70351;	2.34E-09
	rno00260	Glycine, serine and threonine metabolism		1.50E-03
Mitochondrial metabolism	GO:0006107	Oxaloacetate metabolic process		0.0188
	rno00020	Citrate cycle (TCA cycle)	P13803; Q68FU3; P04636; P56574;	4.61E-07
	rno00620	Pyruvate metabolism	P52873; Q920L2; P49432; G3V6D3;	9.45E-07
	RNO-1428517	Respiratory electron transport	P67779; Q06647; D3ZAF6	3.75E-07
Oxidative stress	GO:0072593	Reactive oxygen species metabolic process		0.0017
	GO:0006749	Glutathione metabolic process	B6DYP8; Q6PDW8; P07756;	0.008
	rno00480	Glutathione metabolism	P07895; P04762; P56574	0.0051
	RNO-3299685	Detoxification of Reactive Oxygen Species		0.0397
Mitochondrial biogenesis (increased content)	GO:0007005	Mitochondrion organization		0.009
	RNO-2151201	Transcriptional activation of mitochondrial biogenesis	O70351; G3V9U2; P56574;	0.0056
	RNO-1592230	Mitochondrial biogenesis	G3V6D3; Q06647; D3ZAF6;	5.48E-07
	RNO-8949613	Cristae formation	Q09073; P67779	0.0125

GO(BP) is Gene Ontology (GO) of biological process (BP), RNO is Reactome and RNO is KEGG pathway is Kyoto Encyclopedia of Genes and Genomes biological pathway; *p*-value of <0.05 for the enrichment analyses is significant at Benjamini-Hochberg FDR test.



UNIVERSIDADE ESTADUAL PAULISTA
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FACULDADE DE MEDICINA



Comissão de Ética no Uso de Animais

Criada através da Portaria DFM nº 511 de 13/12/2012

CERTIFICADO Nº 1326/2019 – CEUA

Certificamos que o projeto intitulado: **"Impacto transgeracional do diabetes materno sobre a morfologia mitocondrial e expressão proteica do músculo esquelético de ratas fêmeas adulta"**, conduzido pelo Pesquisador: **Eduardo Kloppel** – Orientadora: Profa. Dra. Debora Cristina Damasceno M. dos Santos – Colaborador: Yuri Karen Sinzato, registrada com o nº **1326/2019**, que envolve a produção, manutenção ou utilização de animais pertencentes ao filo Chordata, subfilo Vertebrata (exceto humanos), para fins de pesquisa científica – encontra-se de acordo com os preceitos da Lei n. 11.794, de 08 de outubro de 2008, do Decreto n. 6.899, de 15 de julho de 2009, com as normas editadas pelo Conselho Nacional de Controle de Experimentação Animal (CONCEA), e foi APROVADA pela Comissão de Ética no Uso de Animais da Faculdade de Medicina de Botucatu, em reunião ordinária de 30 de outubro de 2019.

Finalidade	() Ensino (X) Pesquisa Científica
Vigência da autorização	28 de janeiro de 2022
Espécie/Linhagem/Raça	Sprague Dawley
Nº de animais	ZERO (Utilizará material do projeto 1218/2017)
Idade/Peso	90 – 120 dias – 250 gramas
Sexo	Fêmeas e Machos
Origem	Biotério

Sara Rosa Stanley Sampaio
Secretária da Comissão de Ética no Uso de Animais
Faculdade de Medicina de Botucatu/UNESP

Profa. Associada Bertha Furlan Polegato
Presidente da Comissão de Ética no Uso de Animais
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