TATIANE APARECIDA RIBEIRO DOMINGOS

DOHaD: METABOLIC PROGRAMMING FOR HEALTH AND DISEASE

Prof. Dr. Paulo Cezar de Freitas Mathias

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Tese apresentada ao Programa de Pós-graduação em Ciências Biológicas (Área de concentração - Biologia Celular e Molecular) da Universidade Estadual de Maringá, para obtenção do grau de Doutor em Ciências Biológicas.

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TATIANE APARECIDA RIBEIRO DOMINGOS

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Tese apresentada à Universidade Estadual de Maringá, como requisito parcial para a obtenção do título de Doutor.

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BIOGRAFIA

Tatiane Aparecida Ribeiro Domingos nasceu em Maringá/PR em 01/03/1985. Possui graduação em Educação Física Licenciatura Plena pela Universidade Estadual de Maringá no ano de 2007 e concluiu em 2012, o Mestrado em Educação Física pela Universidade Estadual de Maringá. Tem experiência na área de Educação Física, Fisiologia, Biologia Celular e Bioquímica, atuando principalmente nos seguintes temas: programação metabólica, vida perinatal, desenvolvimento, diabetes, exerccício físico, síndrome metabólica, secreção de insulina, insultos nutricionais e contaminantes ambientais.

“ Aprender é a única coisa de que a mente nunca se cansa, nunca tem medo e nunca se arrepende. ”
(Leonardo da Vinci)

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APRESENTAÇÃO

Em consonância com as regras do Programa de Pós-graduação em Ciências Biológicas, está tese é composta por 2 artigos científicos, redigidos de acordo com as normas das respectivas revistas. O primeiro artigo, "*Acephate exposure during perinatal life program to type 2 diabetes*" publicado na versão online da revista *Toxicology* (doi 10.1016/j.tox.2016.10.010) em outubro de 2016, apresenta pela primeira vez que a exposição ao pesticida Organofosforado Acefato durante a vida perinatal leva a prole a um quadro de diabetes tipo 2 na vida adulta.

O segundo artigo, "*Maternal low intensity physical exercise prevents obesity in offspring rats exposed to early overnutrition*" está sob revisão na revista *Scientific Reports*. Neste trabalho mostramos pela primeira vez que o exercício físico de baixa intensidade durante a a vida perinatal, impede à instalação de disfunção metabólica na prole de ratos induzidos a obesidade por superalimentação no inicio da vida.

Ambos os trabalhos, confirmam o conceito DOHaD, Developmental Origins of Health and Diseases, o qual propõe que intervenções maléficas ou benéficas à saúde durante a vida perinatal (gestação e lactação), podem determinar as condições de saúde ou doença mais tarde na vida.

Tatiane Aparecida Ribeiro; Kelly Valério Prates; Audrei Pavanello; Ananda Malta; Laize Peron Tófolo; Isabela Peixoto Martins; Júlio Cezar de Oliveira; Rosiane Aparecida Miranda; Rodrigo Mello Gomes, Elaine Vieira; Claudinéia Conationi da Silva Franco, Luiz Felipe Barella; Flávio Andrade Francisco, Vander Silva Alves; Sandra da Silva Silveira, Veridiana Mota Moreira; Gabriel Sergio Fabricio; Kesia Palma-Rigo; Deborah M. Sloboda; Paulo Cezar de Freitas Mathias. **Acephate exposure during perinatal life program to type 2 diabetes,** publicado na versão online da revista toxicology (doi 10.1016/j.tox.2016.10.010) em outubro de 2016.

Tatiane Aparecida Ribeiro; Laize Peron Tófolo; Audrei Pavanello; Júlio Cezar de Oliveira; Kelly Valério Prates; Rosiane Aparecida Miranda; Isabela Peixoto Martins; Claudinéia Conationi da Silva Franco, Flávio Andrade Francisco, Vander Silva Alves; Douglas Lopes de Almeida; Veridiana Mota Moreira; Kesia Palma-Rigo; Elaine Vieira; Gabriel Sergio Fabricio; Marcos Ricardo Rodrigues; Wilson Rinaldi; Ananda Malta; Paulo Cezar de Freitas Mathias. **Maternal low intensity physical exercise prevents obesity in offspring rats exposed to early overnutrition**, sob revisão na revista Scientific Reports.

RESUMO GERAL

INTRODUÇÃO

Estudos epidemiológicos tem demostrado que a vida perinatal desempenha um papel importante em determinar a saúde ou a doença a longo prazo na vida de gerações subsequentes. A hipótese da origem do desenvolvimento da saúde e doença, conceito DOHaD, sugere que doenças não comunicáveis como doenças cardiovasculares e diabetes tipo 2, são originadas durante a vida perinatal e nos primeiros dias de vida, fenômeno conhecido como programação metabólica. Os períodos de gestação e lactação são particularmente sensíveis à composição de dietas materna, individuos cujas mães foram desnutridas ou superalimentadas com dieta rica em gordura durante este período são conhecidos por desenvolverem disfunções metabólicas na vida adulta.

No entanto a programação metabólica não se limita apenas a insultos nutricionais, mas a outros fatores, como poluição ambiental, tabaco, fárrmacos e agrotóxicos, entre outros. Diversas pesquisas tem demonstrado que a exposição materna a contaminantes ambientais como bisfenol e agrotoxicos organofosforados, durante fases criticas de desenvolvimento, está relacionados à disfunção metabólica da prole a longo prazo. Outros estudos tem demostrado que mães com um estilo de vida saudável durante o período perinatal, incluindo dieta balanceada e exercício físico regular, pode levar as gerações subsequantes a efeitos positivos no metabolismo. Assim sendo, insultos durante a vida perinatal pode programar o individuo para mudanças positivas ou negativas no padrão de saúde em fases mais tardias da vida.

OBJETIVOS

Geral

Manuscrito 1: Avaliar os efeitos da exposição materna ao pesticida organofosforado Acefato, durante a vida perinatal na prole de ratos adultos.

Manuscrito *2:* Investigar os efeitos do exercício fisico materno de baixa intensidade durante a gestação e lactação na prole de ratos adultos.

Específicos

Manuscrito 1: Verificar os efeitos da exposição perinatal, ao organofosforado Acefato como programador de disfunções metabólicas na prole, regulação do peso corporal e homeostase glicêmica e insulinêmica.

Manuscrito 2: Analisar os efeitos os efeitos de um programa de exercício físico materno de baixa intensidade durante a vida perinatal na prevenção ou atenuação de disfunção metabólica na prole, regulação do peso corporal e homeostase glicêmica e insulinêmica.

MÉTODOS

Animais Experimentais

Foram utilizados ratos Wistar mantidos sob condições de temperatura (23±2ºC) e fotoperíodo (12h claro/escuro) com água e dieta padrão *ad libitum*. A divisão de todos os grupos está descrita em detalhes em cada artigo.

Coleta de Leite

As amostras de leite foram coletadas manuamente com pipeta Pasteur. Para induzir a secreção de leite foi administrada oxitosina sintética, Oxiton® (5 U.I./ml), e os animais foram anestiados com Thiopental® (0.2 ml, ip).

Homeostase Glicêmica

Os procedimentos cirúrgicos foram realizados com ratos previamente anestesiados. Foi realizada uma cirurgia para implantação de uma cânula de silicone na veia jugular direita, para posterior retirada de sangue durante o teste de tolerância a glicose intravenosa (ivGTT). As mensurações de glicose sanguinea a partir do teste de tolerância a glicose intraperitôneal (ipGTT), teste de tolerancia a insulina intraperitôneal (ipITT) e teste de tolerancia ao piruvato intraperitôneal (ipPTT), foram realizadas por meio de glicosimetro (Accu-Chek Aviva system® - Roche Diagnostics).

Dosagens Bioquímicas

A atividade da enzima Butyrylcholinesterase (BuChE), o perfil lipidico e a glicose foram medidas por espectofotometria usando kits comerciais (Gold Analisa®) especificos. A insulina foi dosada pela técnica de radioimmunoassaio.

Remoção dos Estoques de Gordura

Após eutanásia, os principais estoques de gordura (retroperitôneal, ovariana, uterina, periepididimal and mesenterica) foram removidos e pesados para caracterização da obesidade.

Análises Estatísticas

Os dados foram submetidos ao teste t de Student ou análise de variância (two-way ANOVA), seguido pelo pós-teste de Tukey.

RESULTADOS E DISCUSSÃO

Manuscrito1: O presente estudo mostrou pela primeira vez que a exposição materna ao organofosforado Acefato durante o período perinatal teve efeitos adversos sobre a homeostase da glicose e a sensibilidade à insulina tanto nas mães como na prole. Quando as mães grávidas e lactantes foram expostas ao acefato, desenvolveram intolerância à glicose e aumentaram o peso corporal e a ingestão de alimentos em comparação com mães controle. Curiosamente, a exposição ao acefato durante a gravidez e a lactação programou a prole para ser suscetível ao diabetes tipo 2 durante a idade adulta.

Manuscrito 2: O presente estudo demonstra pela primeira vez que o exercício físico materno de baixa intensidade durante a gestação e a lactação foi capaz de prevenir a obesidade e a disfunção metabólica em descendentes adultos do sexo masculino submetidos a sobrenutrição precoce pós-natal. O exercício físico materno de baixa intensidade melhorou o metabolismo da glicose, a capacidade do VO2max e intensificou a atividade elétrica do nervo simpático nas mães, também promoveu mudanças na composição do leite, incluindo um alto teor de insulina.

CONCLUSÕES

Manuscrito 1: A exposição materna ao Acefato durante a vida perinatal, levou a prole a uma pré-disposição a instalação de dislipidemia e diabetes tipo 2 na vida adulta.

Manuscrito 2: O exercício materno de baixa intensidade, durante a vida perinatal foi capaz de programar os animais para um fenótipo saudavél na vida adulta, protegendo contra as consequências metabólicas da superalimentação em fases inicias do desenvolvimento.

GENERAL ABSTRACT

INTRODUCTION

Epidemiological studies have shown that perinatal life plays an important role in determining long-term health or disease in subsequent generations. The hypothesis of the origin of health and disease development, DOHaD concept, suggests that non communicable diseases, as cardiovascular diseases and type 2 diabetes, are originated during perinatal life and in early life, a phenomenon known as metabolic programming. Pregnancy and lactation periods are particularly sensitive to the composition of maternal diet; individuals whose mothers were malnourished or overfed with a high-fat diet during this period are known to develop metabolic dysfunctions in adult life.

However, metabolic programming is not limited only to nutritional insults, but other factors, such as environmental ar pollution, tobacco, drugs and pesticides, among others. Others studies have shown that maternal exposure to environmental contaminats and organophosphate pesticides during critical phases of development is related to long term offspring metabolic dysfunction. Other studies have shown that mothers with a healthy lifestyle during perinatal life, including a balanced diet and regular physical exercise, can lead subsequent generations to positive effects on metabolism. Thus, insults during perinatal life can program the individual for positive or negative changes in health pattern in adult life.

AIMS

General

Manuscript 1: Evaluate the effects of maternal exposure to organophosphate Acephate pesticide during perinatal life in adult offspring rats.

Manuscript 2: Investigate the effects of maternal low intensity physical exercise during pregnancy and lactation in offspring adult rats.

Specific

Manuscript 1: Verify the effects of perinatal exposure to organophosphate Acephate as a programmer of metabolic dysfunctions in offspring, body weight regulation and glycemic and insulinemic homeostasis.

Manuscript 2: Analyze the effects of maternal low intensity physical exercise during perinatal life in the prevention or attenuation of metabolic dysfunction in offspring, body weight regulation and glycemic and insulinemic homeostasis.

METHODS

Experimental Animals

Wistar rats were kept under conditions of temperature (23±2ºC) and photoperiod cycle (12h light/dark) with water and controlled diet *ad libitum*. The division of all groups is described in detail in each article.

Milk Collection

The milk samples were collected manually with a Pasteur pipette. Oxiton® synthetic oxitosin (5 U.I./ml) was administered to induce milk secretion, and the animals were anesthetized with Thiopental® (0.2 ml, ip).

Glycemic Homeostasis

Surgical procedures were performed with rats previously anesthetized. Surgery was performed to implant a silicone cannula in the right jugular vein for subsequent blood collect during intravenous glucose tolerance test (ivGTT). Measurements of blood glucose from the intraperitoneal glucose tolerance test (ipGTT), intraperitoneal insulin tolerance test (ipITT) and intraperitoneal pyruvate tolerance test (ipPTT) were performed by means of a glycosimetre (Accu-Chek Aviva system® - Roche Diagnostics).

Biochemical measurements

The activity of Butyrylcholinesterase enzyme (BuChE), lipid profile and glucose were measured by spectrophotometry using specific commercial kits (Gold Analisa®). Insulin was measured by the radioimmunoassay technique.

Fat pad stores mesurements

After euthanasia, the fat pad stores (retroperitoneal, ovarian, uterine, periepididimal and mesenterica) were removed and weighed to obesity characterizetion.

Statistical Analyzes

Data were submitted to Student's t-test or analysis of variance (two-way ANOVA), followed by Tukey's post-test.

RESULTS AND DISCUSSION

Manuscript 1: The present study showed for the first time that maternal exposure to organophosphate Acephate during perinatal life had adverse effects on glucose homeostasis and insulin sensitivity in both, mothers and offspring. Mothers exposured to acephate, the offspring during pregnancy and lactation developed glucose intolerance and increased body weight and food intake compared to control mothers. Interestingly, exposure to acephate during pregnancy and lactation programmed the offspring to be susceptible to type 2 diabetes during adulthood.

Manuscript 2: The current study demonstrates for first time that maternal low intensity physical exercise during pregnancy and lactation was able to prevent obesity and metabolic dysfunction in adult male offspring submitted to early postnatal overnutrion. Maternal low physical exercise improved glucose metabolism, VO2max ability and increased sympathetic nerve electrical activity in mothers, also promoted changes in milk composition, including high insulin content.

CONCLUSIONS

Manuscript 1: Maternal Acephate exposure during perinatal life led the offspring to predisposition of dyslipidemia and type 2 diabetes in adulthood.

Manuscript 2: The maternal low intensity physical exercise during perinatal life was able to program offspring to a healthy phenotype in adult life, protecting against the metabolic consequences of overfeeding in early stages of development.

MANUSCRITO 1

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Review

Acephate exposure during a perinatal life program to type 2 diabetes

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ABSTRACT

Acephate has been used extensively as an insecticide in agriculture. Its downstream sequelae are associated with hyperglycemia, lipid metabolism dysfunction, DNA damage, and cancer, which are rapidly growing epidemics and which lead to increased morbidity and mortality rates and soaring health-care costs. Developing interventions will require a comprehensive understanding of which excess insecticides during perinatal life can cause insulin resistance and type 2 diabetes. A Wistar rat animal model suggests that acephate exposure during pregnancy and lactation causes alterations in maternal glucose metabolism and programs the offspring to be susceptible to type 2 diabetes at adulthood. Therapeutic approaches based on preventive actions to food contaminated with insecticides during pregnancy and lactation could prevent new cases of type 2 diabetes.

Contents

Abbreviations: ipGTT, intraperitoneal glucose tolerance test; ipITT, intraperitoneal insulin tolerance test; ivGTT, intravenous glucose tolerance test; ipPTT, intraperitoneal pyruvate tolerance test; BuChE, butyrylcholinesterase; DOHaD, developmental origins of health and disease; OP, organophosphates; COBEA, Brazilian association for animal experimentation; AChE, acetylcholinesterase; Ach, acetylcholine; ACE, acephate; AUC, area under the curve; P0, post, natal day 0; P21, post-natal day 21; P90, post-natal day 90; RIA, radioimmunoassay; K_{itt}, constant for insulin tolerance test; LOAEL, lowest observed adverse effect level; HDL, high density lipoproteins; VLDL, very low density lipoproteins; LDL, low density lipoproteins.

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1. Introduction

Epidemiological studies have resulted in the Developmental Origins of Health and Disease (DOHaD) hypothesis, which suggests that noncommunicable diseases, such as cardiovascular disease and type 2 diabetes, originate in the perinatal period and in early life (Barker, 2004). Intrauterine life plays an important role in determining the long-term health of individuals because maternal factors such as hormones and placental function can affect the developing fetus and lead to metabolic programming of positive or negative health outcomes (Barker and Osmond, 1986; Barker et al., 2005; Hales et al., 1991; Silveira et al., 2007).

The perinatal period is particularly sensitive to changes in maternal diet composition because offspring that experience changes during this period are known to develop metabolic dysfunction in their adult life (Davy and Orr, 2009; de Oliveira et al., 2012; Howie et al., 2009; Morgane et al., 1993; Plagemann et al., 2000; Resnick et al., 1979). Studies have shown that maternal malnutrition is associated with low birth weight and an increased the risk of type 2 diabetes and other chronic diseases later in life among their offspring because of rapid compensatory growth in childhood (Barker, 2004; Barker et al., 2002; Eriksson, 2016; Eriksson et al., 2007).

Metabolic programming is not limited to nutritional insults. Other factors, such as food contaminants like the organophos-phates (OP) that are used in large-scale to improve agricultural production, are also related to long-term metabolic dysfunction (Lassiter and Brimijoin, 2008; Slotkin, 2011; Younes-Rapozo et al., 2015). Consumers are frequently exposed to residual levels of OP pesticides in foods (Omoike et al., 2015). Exposure to different products contaminated with OP pesticides increases the dose and may increase the deleterious effects on long-term health (Du et al., 2014). Many countries have laws that forbid using high doses of these components (Du et al., 2014). The extensive use of acephate in the agricultural fields to improve soya bean culture (Alho and Vieira, 1997) in Brazil, as well as other countries (Liu et al., 2011), increases the possibility that OP residues in food could affect food safety, contributing to the dilemma between public health and crop protection (Du et al., 2014).

OP, which is extensively used in pest control and to kill insects, inhibits acetylcholinesterase (AChE) enzyme involvement in the regulation of neurotransmission by hydrolysis of the neurotrans-mitter acetylcholine (ACh) and leads to cholinergic syndrome in the nervous system (Costa, 2006; Pundir and Chauhan, 2012; Sanghi et al., 2003; Suemizu et al., 2014). Recently, pesticides, such as acephate, have become a public health concern because pesticide exposure leads to harmful effects in human metabolism, such as hyperglycemia, lipid metabolism dysfunction, DNA damage, increased oxidative stress and cancer (Costa, 2006; Du et al., 2014). In the current study, we aimed to investigate whether acephate exposure to dams during pregnancy and lactation could lead to metabolic changes in rat offspring.

2. Material and methods

2.1. Ethical approval

All experiments were conducted according to the guidelines established by the Brazilian Association for Animal Experimenta-tion (COBEA) and were approved by the Ethics Committee in Animal Research of the State University of Maringa (protocol number 9427151014).

2.2. Experimental design and acephate exposure

Adult male and female Wistar rats that were 70 days old (weighing 280–300 g and 200 g, respectively) were housed in the Animal House of the Department of Biotechnology, Genetics and Cellular Biology in polypropylene cages (45 cm/30 cm/15 cm) under light controlled conditions with a 12-h light-dark cycle (07:00 a.m. to 07:00 p.m.) and a temperature of 22.0 2 C. After one week of adaptation, animals were mated at a ratio of three females to each male. Pregnancy was confirmed by the presence of sperm in a vaginal smear, and pregnant dams were individually housed. Dams were randomized into 2 groups, including (1) acephate (ACE)-treated pregnant rats that received OP acephate (2.5 mg/kg/bw) that was diluted in corn oil via gavage from gestational day 7 to lactation day 21 (ACE- Mothers) and (2) control pregnant rats that received gavage with the corn oil vehicle (OIL-Mothers).

The dose of OP acephate, O,S-Dimethyl N-acetylphosphorami-dothioate ($C_4H_{10}NO_3PS$), 2.5 mg/kg was considered no observed adverse effect levels (NOAEL) and in females rats (Pesticides, 2006; Solecki, 2016). Both groups were treated by gavage daily between 9:00 and 10:00 a.m. The acephate was purchased from Chem Service, NORTOX S.A, Parana SEAB/PR no. 466 (purity of 75%). During treatment, dams were constantly observed, and no toxicity characteristics or death were observed. After birth (postnatal day 0, P0), the rats were distributed into 2 groups (offspring of the corn oil mothers, OIL and offspring of the acephate mothers, ACE), and

each lactating dam (4 litters for each experimental group) was housed with 9 pups (preferentially male). However, when the required number of male offspring in the litter was not reached, females newborns were used to adjust the litter size to 9 pups throughout the sucking phase. At weaning (P21), rats were housed with 3 per cage, and only male offspring were used in the experiments. The offspring were placed in an environmentally controlled room and received water and standard chow (Nuvital, Curitiba, Brazil) ad libitum.

Maternal body weight and food intake were measured throughout pregnancy and lactation periods. After birth (P0) and weaning P21, the offspring were weighed, the food intake was determined weekly and the total area under the curve (AUC) for food intake and body weight was calculated.

2.3. Glucose metabolism experiments and glucose measurements

2.3.1. Intraperitoneal glucose tolerance test (ipGTT)

Mothers at pregnancy day 20 and the end of lactation day 21, as well as their offspring (P21), fasted for 6 h and were then intraperitoneally injected with glucose (2 g/kg) for the ipGTT to evaluate glucose tolerance. Blood samples were obtained from the tail vein at 0, 40, 80 and 120 min after injection. Glucose levels were measured using the Accu-Chek Aviva system (Roche Diagnostics) as previously described.

2.3.2. Intraperitoneal insulin tolerance test (ipITT)

Mothers, after weaning and offspring at P90, fasted for 6 h and were then intraperitoneally injected with insulin (1 U/kg) to obtain the ipITT to evaluate insulin sensitivity. Blood samples were obtained from the tail vein at 0, 20, 40, 60 and 80 min after injection. Glucose levels were measured using the Accu-Chek Aviva system (Roche Diagnostics). The constant for the insulin tolerance test (K_{itt}) was calculated using formula K_{itt} (%/min) = $0.693/t^{1/2}$, where $t^{1/2}$ was calculated from the slope of the plasma glucose concentration during ipITT [22].

2.3.3. Intravenous glucose tolerance test (ivGTT)

Adult offspring (P90) underwent a surgical procedure under ketamine and xylazine anesthesia (3 and 0.6 mg/100 g, respective-ly) to implant a silicone cannula into the right jugular vein. All cannula were flushed with heparinized saline solution (50 IU heparin/ml; 0.9% w/v of saline solution) before implantation to avoid blood clots. After a 12 h fast (19:00–07:00 h), the animals were infused with a glucose load (1 g/kg), and blood samples were collected at 0, 5, 15, 30, and 45 min. Blood was collected and centrifuged, and the plasma was collected and stored at 20 C for determination of glucose and insulin concentrations. At the end of the ivGTT, the mothers and offspring were euthanized by an overdose of sodium thiopental (Thiopentax[1], Cristália, Itapira, SP, 120 mg/kg). The adipose tissue stores were removed and weighed as a marker of body fat. Glucose was determined with the glucose oxidase method (Trinder, 1969) using a commercial kit (Gold Analisa, Belo Horizonte, MG, Brazil). Additionally, plasma insulin was measured by a radioimmunoassay (RIA) (Scott et al., 1981) using a gamma counter (Wizard[2] Automatic Gamma Counter, TM-2470, PerkinElmer[1], Shelton, CT, USA). Standard human insulin and anti-rat insulin antibodies (Sigma-Aldrich[1], St. Louis, MO, USA) and recombinant human insulin labeled Iodo125 (PerkinElmer[1], Shelton, CT, USA) were used. The intra-assay coefficients of variation were in the range of 8–10%. The limit of detection was 0.006 ng/ml. The measurements were performed in a single assay.

2.3.4. Intravenous pyruvate tolerance test (ipPTT)

Mothers after weaning and offspring at P90 fasted for 6 h and were then intraperitoneally injected with pyruvate (2 g/kg) for the pyruvate tolerance test in order to evaluate the activity of hepatic gluconeogenesis (Tsuneki et al., 2016). Blood samples were obtained from the tail 0, 15, 30, 45 and 60 min after injection. Glucose levels were measured using the Accu-Chek Aviva system (Roche Diagnostics).

2.4. Butyrylcholinesterase (BuChE) serum and milk activity determination

BuChE activity was used to test the level of toxicity that was eventually caused by the pesticide acephate, as shown in previous studies (Kapka-Skrzypczak et al., 2015). Plasma activity levels of BuChE in mothers at the end of the lactation period and P90 adult offspring were determined using a commercial kit (Gold Analisa, Belo Horizonte, MG, Brazil). Plasma and milk samples (10 μl) were incubated with 500 μl of buffer pyrophosphate (90 mmol/l) and potassium ferricyanide (2 mmol/l) for 3 min at 37 C. Then, 250 μl of butyrylcholine (15 mmol/l) was added, and the progress of the reaction was monitored in a spectrophotometer at 405 nm (BIOPLUS, Bio-200). The final activity was the decreasing absorbance as measured in 3 intervals of 1 min. The results were expressed in units per litter (U/L) (Panteghini and Bonora, 1984).

2.5. Milk collection

Milk samples were collected at 10 and 21 days of age before 2 h pups had been weaned from their dams. To induce milk secretion, a synthetic oxytocin, Oxiton (5 U.I./ml, União Química S/A, Sao Paulo, Brazil), was administered (0.5 ml ip), and the animals were anaesthetized with thiopental (0.2 ml, ip). Milk was collected in a sterile Pasteur pipette by manually massaging the nipple as described previously (DePeters and Hovey, 2009). Milk samples (1 ml/dam) were stored at 80C for subsequent BuChE analysis.

2.6. Lipid profile

Total cholesterol, high density lipoproteins (HDL), very low density lipoproteins (VLDL), low density lipoproteins (LDL) and triglyceride concentrations were measured using the enzymatic colorimetric cholesterol oxidase method with a commercially available kit (Gold Analisa; Belo Horizonte, MG, Brazil).

2.7. Fat pad stores mesurements

After the experimental procedures, the dams and offsprings were euthanized and their fat pad stores (retroperitoneal, ovarian, uterine, periepididymal and mesenteric) were removed and weighted to assess the state of obesity. Each of the fat pad store values were correlated with the bw of each rat and were calculated as g/100 kg of bw (de Oliveira et al., 2016).

2.8. Statistical analyses

Data are presented as the mean standard error of the mean. All data were subjected to Student's t-test and were considered significantly different when the value of $p < 0.05$. Tests and graphics were performed using GraphPad Prism version 6.01 for Windows (GraphPad[1] Software, Inc. San Diego, CA, USA).

Table 1
Adipose tissue stores, fasting glucose and BuChE activity in plasma of rat mothers.

	OIL-Mothers	ACE-Mothers	p value
Retroperitoneal fat pad (g/100 g bw)	1.30 0.19	0.92 0.08	0.38
Ovarian fat pad (g/100 g bw)	1.24 0.24	0.84 0.10	0.05*
Uterine fat pad (g/100 g bw)	1.12 0.06	1.08 0.08	0.24
Mesenteric fat pad (g/100 g bw)	0.87 0.10	0.77 0.09	0.47
Fasting glucose on pregnancy day 20 (mg/dl)	62.5 3.09	60.8 1.74	0.62
Fasting glucose after weaning (mg/dl)	76.79 2.82	85.00 3.57	0.10
K_{itt} (%) min)	1.64 0.12	1.09 0.13	0.03*
BuChE activity after weaning plasma (U/L)	688.33 59.52	591.42 48.37	0.22
Milk BuChE activity on pregnancy day 10 (U/L)	1224 2296.6	1281 66.46	0.89
BuChE activity after weaning milk (U/L)	1591 177.3	2327 227.5	0.02*

*p < 0.05 from comparison of OIL-Mothers with ACE-Mothers with a Student's t test (n = 6–5 rats/group).

3. Results

3.1. Acephate exposure during pregnancy and lactation: consequences for maternal health

We first determined whether 2.5 mg/kg of acephate could be toxic for the dams by measuring the BuChE activity in the plasma after weaning. We could not detect any changes in BuChE activity in the plasma in OIL-Mothers or ACE-Mothers (Table 1). We also observed no signs of cholinergic toxicity, such as tremors, salivation, or diarrhea. However, when we measured BuChE activity in the milk, there was an increase of BuChE activity in ACE-Mothers after weaning compared to in OIL-Mothers ($p< 0.05$, Table 1). To determine whether acephate exposure during pregnancy and lactation was detrimental to the mothers' metabolism after weaning, biometric parameters were measured.

We observed no difference in body weight between the groups during pregnancy. However, during lactation, ACE-Mothers had an increase in body weight compared to OIL-Mothers ($p < 0.05$, Fig. 1A). The increase in food intake was already evident during pregnancy and was exacerbated during lactation in ACE-Mothers ($p < 0.05$, Fig. 1B). There was no difference in maternal body fat weight after pregnancy except in ovarian fat pad stores, which showed a reduction of 32.2% compared to OIL-Mothers ($p< 0.05$, Table 1).

We next tested the effects of acephate on maternal glucose metabolism. The results of the ipGTT at gestational day 20 and after weaning revealed that ACE-Mothers were glucose intolerant in the first 15 min after glucose administration compared to the OIL-Mothers ($p < 0.05$, Fig. 2A and B). Therefore, the ACE-Mothers group displayed a tendency to develop glucose intolerance, and the AUC, which was an index of glucose tolerance, was not significantly different from OIL-Mothers (Fig. 2A and B).

We performed an ipITT to assess insulin sensitivity. In the ACE-Mothers group, insulin caused a modest decrease in plasma glucose levels, which reflects decreased insulin sensitivity, and the

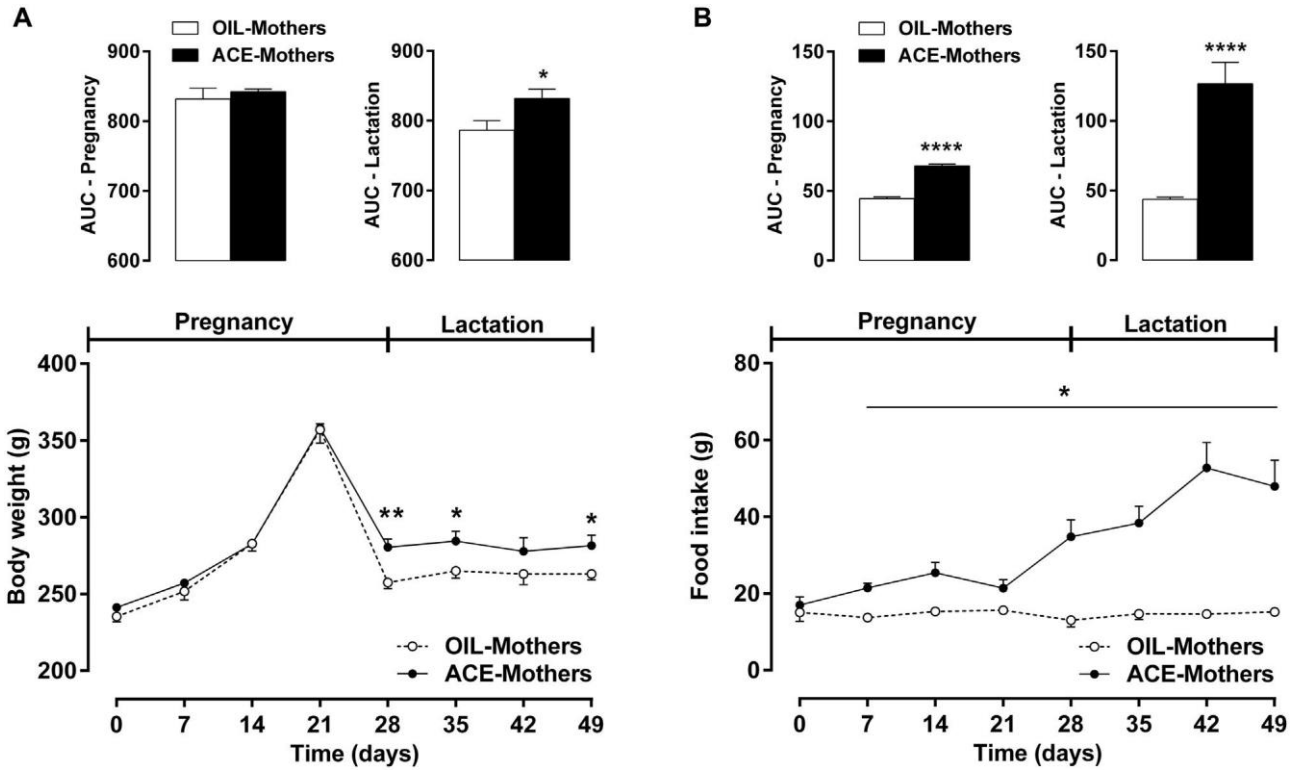

Fig. 1. Effects of acephate exposure on body weight and food intake during pregnancy and lactation in rat mothers. (A) Body weight in OIL-Mothers (n = 6) and ACE-Mothers (n = 5); the inset shows mean total AUC body weight evolution during pregnancy and lactation. (B) Food intake in OIL-Mothers (n = 6), ACE-Mothers (n = 5); the inset shows mean total AUC food intake evolution during pregnancy and lactation. Data are expressed as the mean, SE. *p < 0.05, **p < 0.005 and ****p < 0.0001 by Student's t test.

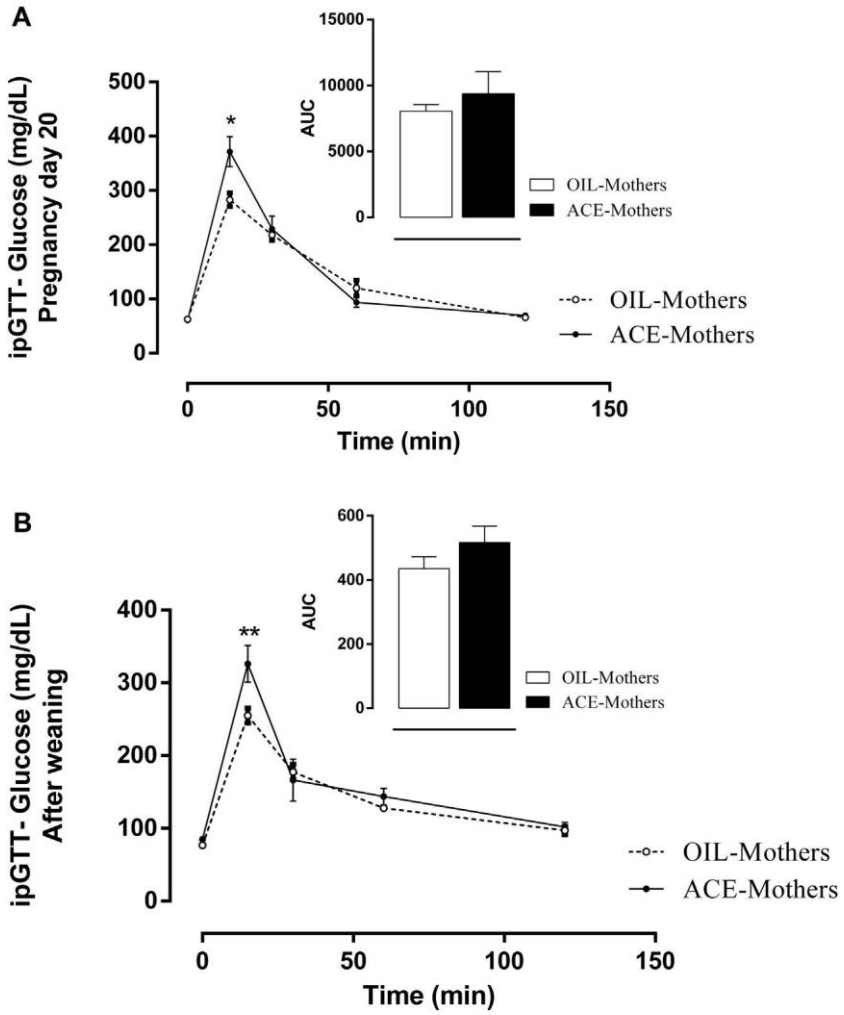

Fig. 2. Effects of acephate exposure on ipGTT in plasma glucose during pregnancy and lactation in rat mothers. (A) ipGTT performed in OIL-Mothers (n = 6) and ACE-Mothers (n = 5); the inset shows mean total AUC in response to a glucose load during pregnancy day 20. (B) ipGTT performed in OIL-Mothers (n = 6), ACE-Mothers (n = 5); the inset shows mean total AUC in response to a glucose load at weaning day. Data are expressed as the mean, SE. *p< 0.05 and **p< 0.005 by Student's t test.

AUC was 30.66% lower than OIL-Mothers (p< 0.05, Fig. 3A). We also estimated the constant for K_{itt} to evaluate the insulin sensitivity, and the K_{itt} index confirmed that ACE-mothers had a decrease of 33.5% in the K_{itt} index compared to OIL-Mothers (p< 0.05, Table 1).

We next studied whether gluconeogenesis was altered in ACE-Mothers by performing an ipPTT after weaning. During ipPTT, plasma glucose levels were increased by 30.1% in ACE-Mothers compared to OIL-Mothers (p< 0.05, Fig. 3B). Thus, acephate exposure during pregnancy and lactation led to alterations in body weight, food intake, insulin sensitivity and gluconeogenesis.

3.2. Acephate exposure during pregnancy and lactation: programming male adult offspring for type 2 diabetes

To test whether acephate exposure in utero predisposes offspring for future development of metabolic abnormalities, we studied two groups of animals: ACE offspring and OIL offspring. Note that these offspring received no direct treatment with acephate but that their mothers were exposed to acephate during gestation and lactation. We first measured BuChE activity in the plasma of the offspring. We could not detect any changes in BuChE activity in plasma in OIL-offspring or ACE offspring (Table 2).

The ACE offspring weighed 7.8% less than the OIL offspring at birth, P0, and the relative difference persisted until weaning when they weighed 8.5% less at P21 (p< 0.05, Fig. 4A). In contrast, body weights at P90 were not significantly different between the groups (p< 0.05–Table 2).

When we observed the AUC body weight curve of the animals during the period from P0 to P21, ACE offspring group showed an 13,12% decreased in AUC bw compared to OIL offspring group (p < 0.0001, Fig. 4A). However, at P21 to P90 the AUC bw increased 13,86% in the ACE groups compared to the OIL group (p < 0.0001, Fig. 4B).

The ACE offspring also showed a 21.22% increase of AUC food intake compared to the OIL offspring (p< 0.05, Fig. 4C). The increase in body weight was not followed by changes in fat pad stores because the ACE and OIL offspring did not exhibit any significant difference in fat pad stores (Table 2). Interestingly, the ACE offspring showed alterations in their plasma lipid profile. These animals had an increase in total cholesterol, HDL, VLDL, LDL and triglyceride plasma levels compared to the OIL offspring (p< 0.05, Table 2).

Glucose intolerance was detected in the ACE offspring as early as P21. These animals showed an increase of glucose plasma levels during ipGTT with an increase of 47.7% in the AUC compared to the

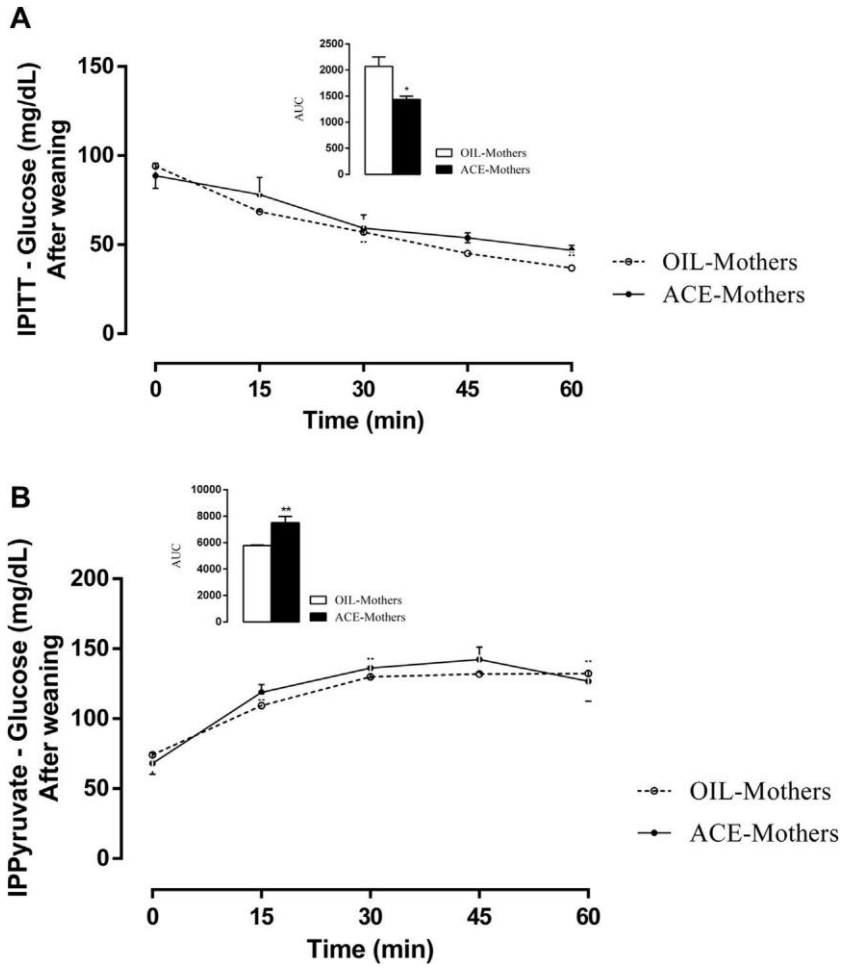

Fig. 3. Effects of acephate exposure on ipITT and ipPTT in plasma glucose during pregnancy and lactation in rat mothers. (A) ipITT performed in OIL-Mothers (n = 6) and ACE-Mothers (n = 5); the inset shows mean total AUC in response to an insulin load after weaning. (B) ipPTT performed in OIL-Mothers (n = 6), ACE-Mothers (n = 5); the inset shows mean total AUC in response to a pyruvate load after weaning. Data are expressed as the mean, SE. *p< 0.05 and **p< 0.005 by Student's t test.

Table 2
Body weight, adipose tissue stores, fasting plasma glucose, insulin, lipid profile and BuChE activity in rat offspring.

	OIL Offspring	ACE Offspring	p value
Body Weight P0 (g)	6.64 0.06	6.12 0.66	0.0001****
Body Weight P21(g)	52.10 0.88	47.63 1.22	0.009**
Fasting glucose P21 (mg/dl)	86.91 1.84	89.83 1.66	0.29
Body Weight P90 (g)	377.5 2.01	385.83 6.04	0.21
Retroperitoneal fat pad (g/100 g bw)	1.19 0.051	1.23 0.054	0.58
Periepididymal fat pad (g/100 g bw)	1.13 0.050	1.14 0.048	0.9
Mesenteric fat pad P90 (g/100 g bw)	0.72 0.022	0.65 0.049	0.16
Fasting Glucose P90 (mg/dl)	86.220 1.70	109.6 17.81	0.03*
Fasting insulin P90 (ng/ml)	0.34 0.01	0.54 0.06	0.001***
Total cholesterol (mg/dl)	75.57 3.66	95.42 4.14	0.005**
Total HDL (mg/dl)	7.82 0.96	14.30 0.90	0.0008****
Total VLDL (mg/dl)	16.61 2.32	65.80 8.95	0.0001****
Total LDL (mg/dl)	52.90 57	67.96 4.13	0.021*
Triglycerides (mg/dl)	44.29 2.46	65.80 8.95	0.022*
BuChE activity P90 (U/L)	458.88 24.12	475.55 32.75	0.68

*p < 0.05, **p < 0.005, ***p < 0.001 and ****p < 0.0001 when compared to OIL with ACE offspring with a Student's t test. (18–20 rats at least 3 different litters).

OIL offspring (p< 0.05, Fig. 5). At P90, fasting plasma glucose and insulin were 21.3% and 37% higher in ACE offspring compared to OIL offspring, respectively (p< 0.05, Table 2).

We also performed an ivGTT and ipITT to evaluate glucose tolerance and insulin sensitivity, respectively, of the offspring at P90. At this age, the ACE offspring were highly glucose intolerant and exhibited alterations in plasma glucose levels at all time points during the ivGTT compared to the OIL offspring (Fig. 6A). The insulin levels during the ivGTT were also increased at all time points in the ACE offspring (Fig. 6B). As expected, insulin sensitivity

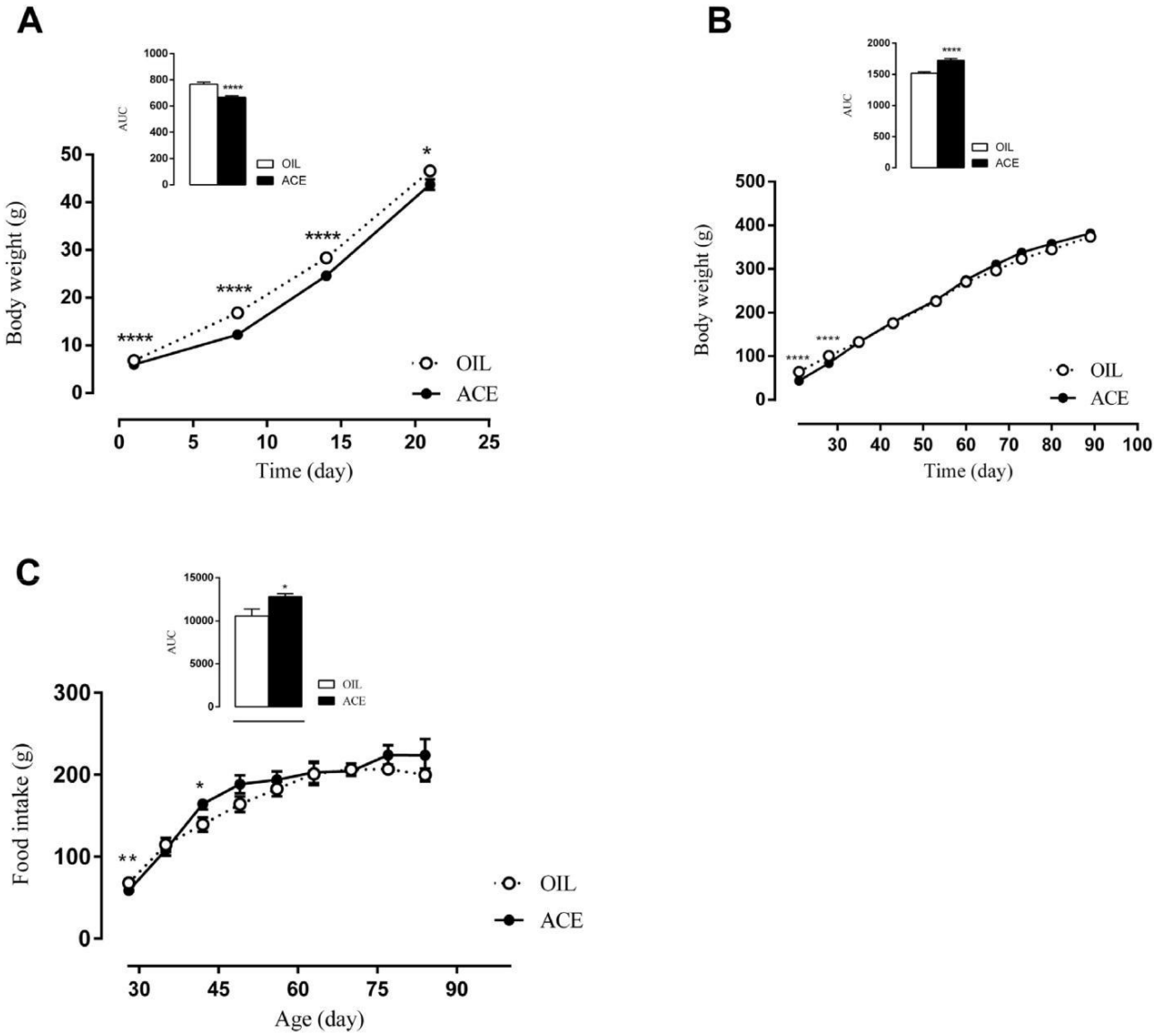

Fig. 4. Effects of exposing pregnant and lactating rat mothers to acephate on body weight and food intake of their offspring. (A) Body weight in OIL offspring (n = 18) and ACE offspring (n = 20); the inset shows the mean total AUC body weight evolution from the P0 to the P21 nursing periods. (B) Body weight in OIL offspring (n = 18) and ACE offspring (n = 20); the inset shows the mean total AUC body weight evolution from P21 to P90. (C) Food intake in OIL offspring (n = 18) and ACE offspring (n = 20); the inset shows the mean total food intake evolution from P21 to P90. Data are expressed as the mean, SE. *p< 0.05 and ****p< 0.0001 by Student's t test.

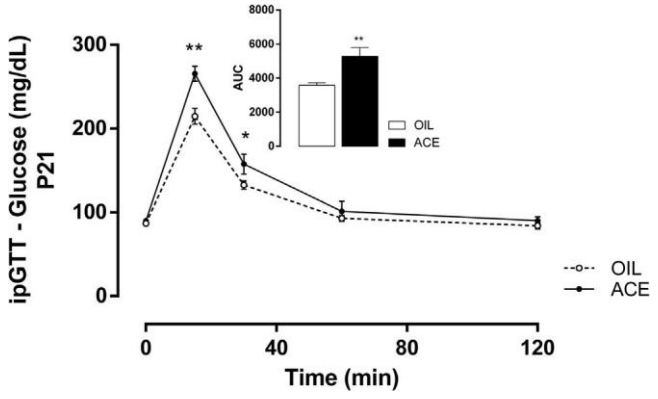

Fig. 5. Effects of exposing pregnant and lactating rat mothers to acephate on ipGTT plasma glucose of their offspring. ipGTT performed in OIL offspring (n = 9) and ACE offspring (n = 9); the inset shows the mean total AUC in response to a glucose load at P21. Data are expressed as the mean, SE. *p< 0.05 and **p< 0.005 by Student's t test.

detected by IPITT was significantly decreased in ACE offspring compared to the OIL offspring (p< 0.05 Fig. 6C). Our results demonstrated that acephate exposure during pregnancy and lactation disrupt body weight, food intake, glucose metabolism and lipid metabolism in the offspring.

4. Discussion

In the present study, we showed for the first time that exposure to low doses of acephate during critical periods of life had adverse effects on glucose homeostasis and insulin sensitivity both in the mothers and the offspring. When pregnant and lactating mothers were exposed to acephate, they developed glucose intolerance and increased body weight and food intake compared to control mothers. Interestingly, exposure to acephate during pregnancy and lactation programmed the offspring to be susceptible to type 2 diabetes during adulthood.

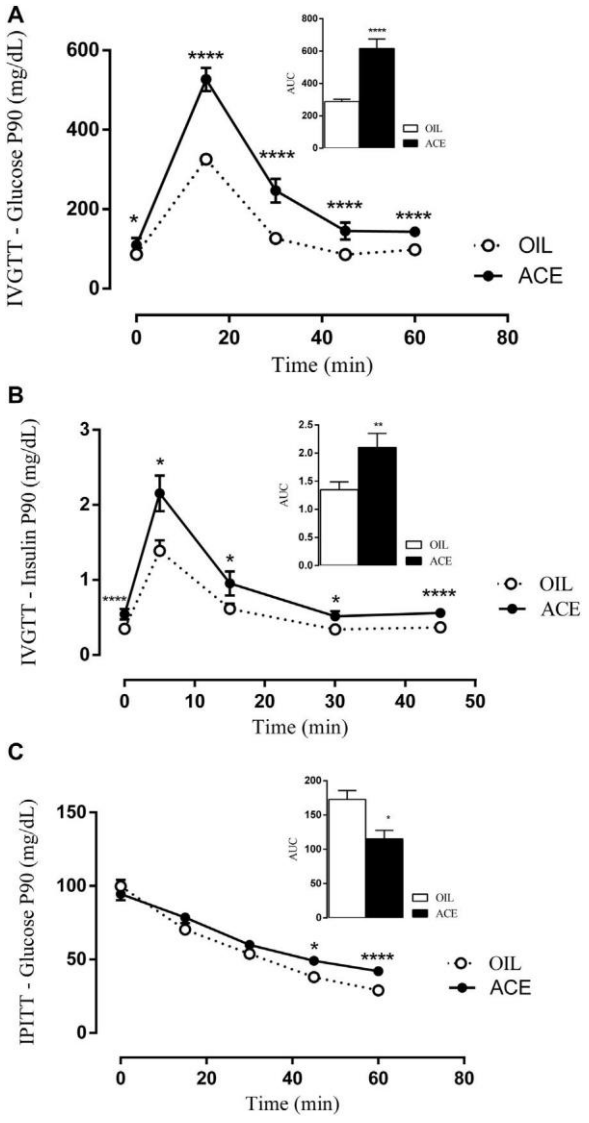

Fig. 6. Effects of exposing pregnant and lactating rat mothers to acephate on glucose and insulin plasma during ivGTT and glucose plasma in ipITT in their offspring. (A) ivGTT performed in OIL offspring (n = 9) and ACE offspring (n = 12); the inset shows the mean total AUC in response to a glucose load at P90. (B) ipGTT performed in OIL offspring (n = 9) and ACE offspring (n = 12); the inset shows the mean total AUC in response to a glucose load at P90. (C) ipITT performed in OIL offspring (n = 9) and ACE offspring (n = 12); the inset shows the mean total AUC in response to an insulin load at P90. Data are expressed as the mean, SE. *p< 0.05, **p< 0.005 and ****p< 0.0001 by Student's t test.

Dangerous OP compounds, such as acephate, have been used as insecticides in agriculture and in chemical warfare. These compounds are very toxic when absorbed by humans because of the acetylcholinesterase deactivation. Recently, acephate residues were found to be present in 22.3% of the samples, especially in soybean meals at a Brazilian university restaurant (Caldas et al., 2011). The dose of acephate that we used in our study (2.5 mg/kg/day) was considered the lowest dose to cause an observeable adverse effect according to the US Environmental Protection AgencyUnited States Environmental Protection Agency (US-EPA: IRIS 0354) (Storm, 2001).

At a dose of 2.5 mg/kg, the rats did not present clinical toxic symptoms, such as exophthalmos, tremors, salivation, and diarrhea (Bhadaniya et al., 2015; Lassiter and Brimijoin, 2008).

In the present study, we observed no changes in BuChE activity in the plasma of mothers and offspring, which suggested that there were no toxic effects in mothers and in the offspring. For the first time, we showed the presence of BuChE activity in milk. However, we detected high levels of BuChE activity in the milk of ACE-Mothers. The increase in BuChE activity in the milk of ACE-Mothers could be a mechanism of protection for the acephate exposure because this enzyme was shown to prevent intoxication of animals exposed to OP compounds. (Cerasoli et al., 2005; Lenz et al., 2007). On the other hand, a decrease of plasma BuChE activity in patients is associated with acephate intoxication (Kapka-Skrzypczak et al., 2015).

Experimental studies in humans and animals suggest that gestational diabetes is characterized by high plasma glucose and insulin levels, which increase the risk of obesity and type 2 diabetes in offspring later in life (Kahn et al., 2006; Reece et al., 2009). Our data showed that ACE-Mothers had high plasma glucose levels in the first 15 min after a glucose load in the ipGTT as well as high glucose levels and insulin resistance during ipPTT. OP induced glycogenolysis, gluconeogenesis and insulin resistance for maintaining normal glycemic blood glucose levels (Joshi and Rajini, 2009; Rahimi and Abdollahi, 2007). Hepatic gluconeogene-sis is the main source of hepatic glucose production during prolonged fasting and contributes to the development of type 2 diabetes (Pilkis and Granner, 1992).

In addition, ACE-Mothers had an increase in body weight and food intake. All these metabolic abnormalities that were caused by acephate exposure during pregnancy and lactation could explain the low birth weight in early life in the ACE offspring. Indeed, experimental and epidemiological studies in rodents and humans have shown that low birth weight and small body size in infancy were strongly associated with risk factors for type 2 diabetes later in life (Barker, 2004; Barker et al., 2005; Eriksson, 2016; Eriksson et al., 2007).

Several studies have shown an association between low birth weight and coronary heart disease and type 2 diabetes because low birth weight can led to alterations in lipid profile, high triglyceride and insulin levels (Kajantie et al., 2008; Perala and Eriksson, 2012; Perala et al., 2011). In the present study, we found that the ACE offspring had reduced glucose tolerance, insulin resistance, and altered levels of plasma insulin and total cholesterol, HDL, VLDL, LDL and triglycerides, which suggested a high predisposition to metabolic diseases.

The observed metabolic effects of acephate exposure during pregnancy and lactation may be due to altered glucose metabolism in the mothers. Whether the effects that we observed in the offspring were due to a direct effect of exposing the fetus to acephate during pregnancy or because the pups were exposed to an altered maternal glucose metabolism during pregnancy and lactation or the combination of both factors remains unknown.

Nevertheless, both situations are plausible. First, acephate could affect the placenta during pregnancy (Farag et al., 2000) or might affect the milk during breast feeding (Boobis et al., 2008; Sanghi et al., 2003), and then acephate could impair glucose metabolism during gestation and lactation, which could alter fetal growth (Hales et al., 1991; Kajantie et al., 2008; Perala and Eriksson, 2012; Perala et al., 2011). Indeed, maternal metabolism is important, and the differences observed in glucose tolerance and plasmatic parameters in ACE-Mothers may explain, at least in part, the metabolic abnormalities that were displayed subsequent-ly in their ACE offspring. In any case, the results of the present study suggest that the endocrine disruptor acephate should be evaluated as a possible risk factor for gestational diabetes and type 2 diabetes associated with metabolic dysfunction. Moreover, our findings demonstrate that fetal exposure to acephate may

predispose offspring to type 2 diabetes and dyslipidemia during adulthood.

Conflicts of interest

All of the authors have no potential conflict of interests, including any financial, personal or other relationships that could influence the study.

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Author contributions

TAR, AP and PCFM designed the study. TAR, AP, KVP, AP, AM, LPT and IPM collected the data. TAR, AP and AM analyzed the data. TAR, PCFM and EV wrote the manuscript. All authors (TAR, KVP, AP, AM, LPT, IPM, JCO, RAM, RMG, CCSF, LFB, FFA, VSA, SSS, VMM, GSF, KPR, EV, DMS and PCFM) contributed intellectually as well as reviewed, edited and approved the final version of the manuscript that was submitted for publication.

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MANUSCRITO 2

Maternal low intensity physical exercise prevents obesity in offspring rats exposed to early overnutrition.

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Abstract

Low intensity exercise during pregnancy and lactation may create a protective effect against the development of obesity in offspring exposed to overnutrition in early life. To test these hypotheses, pregnant rats were randomly assigned into 2 groups: Sedentary and Exercised, low intensity, on a rodent treadmill at 30% VO_{2Max} /30-minute/session/3x/week throughout pregnancy and the lactation. Male offspring were raised in small litters (SL, 3 pups/dam) and normal litters (NL, 9 pups/dam) as models of early overnutrition and normal feed, respectively. Exercised mothers showed low mesenteric fat pad stores and fasting glucose and improved glucose-insulin tolerance, VO_{2max} during lactation and sympathetic activity. Moreover, the breast milk contained elevated levels of insulin. In addition, SL of sedentary mothers presented metabolic dysfunction and glucose and insulin intolerance and were hyperglycemic and hyperinsulinemic in adulthood. SL of exercised mothers showed lower fat tissue accretion and improvements in glucose tolerance, insulin sensitivity, insulinemia and glycemia. The results suggest that maternal exercise during the perinatal period can have a possible reprogramming effect to prevent metabolic dysfunction in adult rat offspring exposed to early overnutrition, which may be associated with the improvement in maternal health caused by exercise.

Introduction

Nutritional, hormonal and metabolic insults during early critical periods of life can predispose individuals to long-lasting deleterious effects later in life [1]. This phenomenon has been known as metabolic programming [2]. Studies have shown that poor or overnutrition during perinatal life is associated with an increased risk of type 2 diabetes and other chronic diseases later in life [3].

A healthy lifestyle including a balanced diet and regular physical exercise during perinatal life can have positive effects on maternal metabolism and that of the subsequent generation [4,5]. Physical exercise during pregnancy is known to have beneficial effects on maternal health, decreasing the risk of preeclampsia and gestational diabetes [6]. In addition, aerobic physical exercise in lactating woman improves maternal maximal oxygen consumption (VO_{2max}) and plasma high-density lipoprotein (HDL) cholesterol concentrations [7].

On the other hand, high intensity physical exercise during pregnancy in women can affect fetal health, inducing maternal hyperthermia [8], increased uterine contractility by hormone stimulation [9], fetal hypoglycemia [10], and reduction in visceral and placental blood flow due to diverted blood to the working muscles mass and skin [11]. Higher intensity exercise over a long duration during pregnancy can induce negative outcomes in human and rodent offspring[12,13].

The current study highlights the scarcity of a clear recommendation regarding the type, timing, intensity, frequency and duration and benefits of exercise; therefore, we aim to evaluate whether maternal low intensity exercise during pregnancy and lactation can attenuate the adult metabolic dysfunction induced by early postnatal overnutrition in offspring rats.

Results

Effects of low intensity physical exercise during pregnancy and lactation on theVO_{2max} parameters of the dams.

Table 1 shows the VO_{2max}values in the dams. There was no difference in theVO_{2max} values before physical exercise between groups (Table 1). However, after physical exercise, at lactational day 3 (LD3), exercised mothers (EM) showed an increase of 22.56% in the VO_{2max} compared with that of SM ($p<0.05$ - Table 1).

Effects of low intensity physical exercise during pregnancy and lactation on the body composition of the dams.

No difference was found in the AUC for body weight during pregnancy and lactation between the two groups (Fig. 2). There were differences in maternal mesenteric fat pad stores in LD21, with EM showing 29% less maternal mesenteric fat pad stores than SM ($p<0.001$ – Table 1).

Effects of low intensity physical exercise during pregnancy and lactation on the milk and plasma biochemical parameters of the dams.

Low intensity exercise in EM resulted in a lower fasting plasma glucose than in SM by 18.69% ($p<0.004$ – Table 1), while plasma insulin levels were not different between the groups (Table 1). During the ivGTT, EM plasma glucose and insulin increments, showed significant difference at 5 and 15 minutes peak time points of the plasma glucose, as well as in the 15min time point of the plasma insulin, compared to SM ($p<0.0001$ - Fig. 3a and 3b). The AUC, which is shown in the inset of Fig. 3a, showed that the glucose presented an reduction of 54% compared with the SM mothers ($p<0.0001$). And the inset of Fig. 3b, in the same test, the plasma insulin concentration was 23% lower in the EM animals than SM mothers ($p<0.05$). The HOMA-IR values were 36.14% lower in EM (p=0.05 – Table 1). There was no difference in the glucose and lipid composition of the milk between the groups. However, total cholesterol content at day 21 was 20.61% lower in EM compared to SM ($p<0.03$). Interestingly, EM exhibited an increase in insulin levels in the milk on the 10^{th} and 21^{st}day of lactation by 87.21% and 145.63%, respectively, compared to that of SM ($p<0.005$ – Table 2).

Effects of low intensity physical exercise during pregnancy and lactation on the autonomic nervous system of the dams.

We observed no difference in parasympathetic nervous system activity between the groups (Table 1). Nevertheless, the EM presented a 39.6% increase in sympathetic nervous system activity compared to that of the SM ($p<0.05$ – Table 1).

Long-term effects of low intensity physical exercise in pregnant and lactating dams on the body composition of the adult offspring.

As observed in Table 3, there was no difference in birth weight between the offspring groups ($p=0.6$). Early overnutrition induced an increase in the bw of the rats in the SL-SM group at P21 and P90 of 44.6% ($p_l<0.001$) and 10% ($p_l<0.05$), respectively, compared to that of rats in the NL-SM group. The SL-SM group showed higher body weigh at P21 through P90, compared to that of the NL-SM, as well as, the SL-EM group showed low body weigh at P21 through P90, compared to that of the SL-SM ($p<0.05$ - Fig. 4). In Fig. 4, the evolution of the body weight, as indicated by the AUC, in the SL-SM group was 22.1% higher than in the NL-SM rats ($p_l<0.001$). In contrast, the NL-EM group showed no difference in the bw curve compared to that of the NL-SM group, and SL-EM rats had a lower body weight than the SL-SM rats, resulting in a significant interaction between litter and maternal exercise ($p_{lxe}<0.001$).

Low intensity physical exercise during pregnancy and lactation mediated changes in the offspring's fat pad stores in adulthood. The SL-SM rats exhibited higher weights of the retroperitoneal, periepididymal and mesenteric fat pad stores than the NL-SM rats ($p_l<0.05$, Table 3). Although the NL-EM rats only showed a 15% decrease in the periepididymal fat pad stores compared to that of the NL-SM rats, the SL-EM rats exhibited a lower weight in all evaluated fat pad stores when compared to that of the SL-SM rats, which demonstrated a significant effect of maternal exercise on the offspring fat depots ($p_e<0.0001$, Table 3).

Long-term effects of low intensity physical exercise in pregnant and lactating dams on the glucose-insulin homeostasis of the adult offspring.

At P90, SL-SM rats showed a higher fasting plasma glucose than NL-SM rats ($p_{lxe}<0.001$, Table 3), while SL-EM rats exhibited a 21.5% decrease compared to that of the SL-SM rats ($p_e<0.0001$; $p_{lxe}<0.0001$, Table 3). Glucose intolerance was detected in the SL-SM group, where rats exhibited alterations in plasma glucose levels during the ivGTT compared to that of the NL-SM rats, as shown by the 16.9% increase in the AUC ($p_{lxe}<0.005$, Fig. 5a). There was no significant difference in the glucose levels between adult rat offspring from exercised mothers in relation to their counterpart control groups (Fig. 5a).

During the ivGTT, the insulin plasma levels were reduced in both the NL-EM (28.8%) and SL-EM (45.5%) groups compared to the levels of their counterpart groups, the NL-SM and SL-SM rats, respectively ($p_e<0.01$- Table 3). During the ivGTT, SL-SM plasma glucose and insulin increments, showed significant difference at 5, 15, 30 and 45 minutes peak time points of the plasma glucose, as well as in the 5, 15 and 45 minutes time point of the plasma insulin, compared to NL-SM ($p<0.05$ - Fig. 5a and 5b). SL-SM plasma glucose and insulin increments, also showed significant difference at 5, 30 and 45 minutes peak time points of the plasma glucose, as well as in the 5 and 15 minutes time point of the plasma insulin, compared to SL-EM ($p<0.05$ - Fig. 5a and 5b). The glucose levels were increased during ivGTT in the SL-SM compared to that of the NL-SM group. These animals showed a 15.9% increase in the AUC of the glucose plasma levels compared to that of the NL-SM group ($p_l<0.005$, Figure 5a). As well as, the insulin levels were increased during ivGTT in the SL-SM compared to that of the SL-EM group. These animals showed a 50% increase in the AUC of the insulin plasma levels compared to that of the SL-EM group ($p_l<0.001$, Fig. 5b).

At P90, the SL-SM rats presented a 54.2% increase in fasting insulinemia compared with that of the NL-SM rats ($p_l<0.001$, Table 3). In relation to their counterparts, the NL-EM and SL-EM animals showed decreases in fasting insulin of 28.5% and 33.3%, respectively, indicating a not able effect of maternal exercise on insulin levels ($p_e<0.01$, Table 3). The HOMA–IR values of the SL-SM rats were increased by 102.0% when compared to that of the NL-SM rats ($p_l<0.0001$). In contrast, the NL-EM rats exhibited a 26.3% decrease in HOMA-IR values compared to that of the NL-SM rats ($p_{lxe}<0.01$, Table 3), and the values of the SL-EM rats were 46.8% lower than the values observed in their counterpart rats ($p_{lxe}<0.01$, Table 3). Altogether, the results showed a significant interaction between maternal exercise and small litter size.

Discussion

The current study demonstrates for first time that maternal low intensity physical exercise during pregnancy and lactation was able to prevent obesity and metabolic dysfunction in adult male offspring exposed to early postnatal overnutrition. Small-litter offspring from exercised dams presented low depots of adipose tissue and low fasting insulin and glucose plasma levels, as well as normal glucose tolerance and insulin sensitivity. Interestingly, maternal low physical exercise improved maternal glucose metabolism and VO_{2max} capacity and enhanced sympathetic nerve electrical activity and increase insulin milk levels. Our results highlight the beneficial effects of low intensity maternal physical exercise on the health status of the offspring and mother.

Early overnutrition is an established model for the study of its long-term consequences in an animal model. Studies have shown that small litter size during the suckling period leads to overnutrition because of the reduced competition for milk and increase caloric intake [14]. Early-overfeeding has been shown to malprogram hypothalamic leptin resistance [15], and reduce the thermogenic activity of brown adipose tissue [16]. Combined, these changes may well predispose individuals to exhibit hyperphagic behavior and adipose tissue accumulation due to lack of sympathetic-induced energy wastage. Interestingly, in the current study, mothers that performed low intensity physical exercise throughout pregnancy and lactation displayed reduced sympathetic nervous tone, and normoinsulinemia but elevated concentrations of insulin in their milk. We hypothesised that it may be the altered levels of insulin in the milk that contributed to attenuation of early-overfeeding induced obesity in their rat offspring.

Beyond the well-characterised action of insulin on food intake, body weight and energy balance in the hypothalamus [17], insulin also regulates the function of several hypothalamic areas by modifying neuronal plasticity, especially during early life by promoting metabolic derangement and neuronal dysfunction associated with impaired synaptic plasticity [18]. Accordingly, it is possible that the offspring of physical exercise dams ingested more insulin via milk during the first 21 days of life and that this attenuated or modulated the early overfeeding effects in neuronal pathways involved in energy regulation and thermogenesis function in rat immature brain.

Postnatal early nutrition, especially breast-feeding, is essential to infant development, protecting against obesity and metabolic dysfunction in later life [19]. Studies have shown that human milk contains high concentrations of bioactive substances such as proteins, peptides, steroids, growth factors and hormones, including insulin [20-23].

Oral insulin from the mother may function in the regulation of the growth and development of the neuroendocrine system, newborn immune system and gastrointestinal tract [24]. Additionally, insulin levels in milk appear to have a beneficial effect on gut maturation and prevent later diseases such as Crohn's disease, celiac disease and type 1 diabetes [25-28]. Studies have documented the presence of insulin receptors in the mammalian intestine, in the jejunal and ileal brush border and intestinal crypt, in the fetal period, during the suckling period, at weaning, and in adults [20,29-32].

The macromolecule insulin is digested in the lumen of the gut to avoid absorption into the blood stream; however, there is some evidence that oral insulin treatment decreases bw, cholesterol and the triglyceride blood level in different animal models [22,33]. In insulin-resistant states, the intestine significantly enhances the production of lipoproteins [34] and glucose [35]. The mechanism by which

luminal insulin influences intestinal metabolism even without being absorbed is not completely understood, but its capacity to downregulate gut insulin receptor expression [33] might be the cornerstone factor.

Interestingly our study showed that exercised dams have a modified milk composition on the10th and 21st days of the lactation period with significantly higher levels of insulin. Studies have shown that exercise during lactation does not affect the quality of breast milk composition, but improves the maternal health condition [36-38]. Interestingly milk insulin levels during lactation was different in exercised mothers, where dams displayed high milk insulin levels, on the other hand, maternal low intensity physical exercise reduced plasma insulin. This result suggests a possible high skeletal muscle adaptation on uptake nutrients without insulin action. It is known that physical exercise training increases the peripheral insulin-sensitivity as a compensatory response to better uptake glucose for the physiological energy demand [39].

Regular physical exercise during pregnancy improves maternal health conditions [40,41] that are important to fetal growth and development, which primarily depend on maternal placental transport for adequate fetal hormones, nutrients and oxygen supply to the fetus [42]. Low to moderate maternal exercise, approximately 40–65% of VO_{2max}, during pregnancy has beneficial effects on offspring metabolism development in exercised mothers exposed to undernutrition [43]. The suggested mechanisms of these effects may be related to metabolic changes, promoted through blood flow and changes in the production of fetal and placental hormones that control development [44].

Low intensity exercise (30% of the VO_{2max}) during pregnancy and lactation was beneficial for maternal and offspring health. Mothers that were exposed to low intensity exercise, 30% VO_{2max}, in the current study maintained a VO_{2max} after pregnancy similar to theVO_{2max} before pregnancy; on the other hand, the sedentary mothers showed a reduction in theVO_{2max} after pregnancy compared to their VO_{2max}before pregnancy. The maintenance of theVO_{2max} may have resulted in improvements in placental growth and functional capacity. Aerobic exercise increases blood flow and provides better delivery of nutrients and oxygen, allowing for a better overall growth rate of the fetus in later pregnancy [45]. The improvement of the VO_{2max} may induce an increase in blood supply to tissues leading to high diffusion of oxygen, improving the ability to extract oxygen from the blood into all tissues [46].

Physical exercise during pregnancy and lactation also contributed to a reduction in the fat pad stores and an improvement in glucose/insulin metabolism in dams. Exercised mothers exhibited a

reduction in mesenteric adipose tissue stores and improved glucose metabolism associated with an increase in sympathetic electrical activity. Maternal exercise during pregnancy promotes autonomic nervous system balance and, consequently, has beneficial effects on brain function and structure in both the mother and her offspring in an animal model [47]. Studies have shown that the increase in parasympathetic nervous system activity and reduction in sympathetic nervous system activity contributes to obesity onset and insulin resistance in obese humans and animal models [48]. However, moderate physical exercise in adult male rats is able to improve the sympathetic nerve tone, enhance energy expenditure, and decrease fat stores and body weight [49].

Interestingly, the improvement in the autonomic nervous system (ANS) activity is related to the VO_{2max} balance [50]. A sedentary lifestyle decreases the VO_{2max} and leads to the development of an increase in fat deposition and body weight gain in humans [51]. Studies have shown that the beneficial effect of physical exercise on metabolism in pregnant humans and animals is dependent on the type, intensity and frequency of exercise [13,52]. The American College of Sports Medicine guidelines recommend 30 minutes or more of moderate exercise daily for pregnant women in the absence of medical or obstetric complications[53]. According to most protocols, the exercise is considered moderate when theVO_{2max} is between 50-70% [5]. High intensity exercise promotes a deleterious effects in pregnant mothers [11] and subsequent generations [8].

The ANS is involved in the fatty acid mobilization induced by physical exercise [54]. The activity of the heart is also stimulated by the SNS during exercise, which functions to increase blood flow, particularly to the muscles, improving nutrition [55,56], and adipose tissue, stimulating the lipolysis pathway [57,58]. Previous studies from our group have shown that moderate physical exercise promotes a beneficial effect on glucose metabolism by improvement of pancreatic islet function and ANS activity in an adult obese animal model [59] and induces a decrease in fat pad stores, related to activation of the sympathoadrenal axis [60,61]. Furthermore, we found that mothers submitted to low intensity exercise show an improvement in ANS activity, suggesting a balance in glucose metabolism, and a decrease in mesenteric fat pad stores and increase in VO_{2max} compared to that of sedentary mothers after pregnancy. This may contribute to the improvement of maternal health, leading to the prevention of metabolic programming associated with overnutrition.

In conclusion, the current study suggests that maternal low intensity physical exercise during the perinatal period improves the health of the mother and prevents metabolic dysfunction in offspring later in life. These protections can be associated with changes in the maternal milk composition, including high insulin content, suggesting a potential reprogramming effect.

Materials and Methods

Ethical approval

The handling of animals and the experimental procedures were in accordance to the rules of the National Council of Animal Experiment Control (CONCEA) and the Brazilian Society of Science in Laboratory Animals (SBCAL) and approved by the Ethics Committee on Animal Use of Universidade Estadual de Maringa – CEUA/UEM (protocol number 9427151014).

Animals

At 70days of age, female *Wistar* rats were mated with 80-day-old male rats insets of 3:1, respectively. Pregnancy was confirmed by the presence of sperm cells in the vaginal plug (pregnancy day 0.5). The pregnant rats were maintained in individual cages and distributed into two groups: Exercised mothers (EM) and sedentary mothers (SM). The exercise was performed throughout pregnancy and lactation. The maternal body weight (bw) was measured throughout the pregnancy and lactation periods.

After birth on postnatal day 0 (P0), the rats were distributed into 4 groups: Normal litter of sedentary mothers (NL-SM), small litter of sedentary mothers (SL-SM), normal litter of exercised mothers (NL-EM) and small litter of exercised mothers (SL-EM), and each lactating dam (4 litters for each experimental group) was housed with 9 pups (preferentially male). Considering the issue of sexual dimorphism and that litter size manipulation has been shown to have significant effects on male rats compared to females [62,63],we used only males in this study. However, when the required number of male offspring in the litter was not reached, females newborns were used to adjust the litter size to 9 pups throughout the sucking phase. To induce early overnutrition, on the third day after birth, the litter size was adjusted to 3 male pups per dam. The offspring were placed in an environmentally controlled room and received water and standard chow (Nuvital, Curitiba, Brazil) *ad libitum*.

Exercise protocol

Adaptationand protocol for the maximal efforttest

During the mating period (approximately 1 to 2 weeks), female rats were acclimated, using a modified protocol, to a treadmill for rats (Panlab, Harvard Apparatus®, Cornellà- Barcelona – Spain) 10 minutes per day, 3 times a week at 10 cm/s[64]. After detection of pregnancy, the animals were submitted to an effort test to determine the velocity of the training throughout pregnancy. On the third day of lactation, the second test was performed to determine the intensity of the exercise protocol (30% VO_{2max}). The test was performed twice: on pregnancy day 0.5 and lactation day 3 (the period of parturition), using a treadmill for rodents with an indirect calorimetry analyzer *(Panlab technology for bioresearch, Harvard Apparatus®- Le405, gas analyzer)* for the determination of the O_2/CO_2 gas concentrations.

The test began with a warm up (5 minutes, 10 cm/s, 0° of inclination), after which the velocity was increased by 5 cm/s every 3 minutes until the exhaustion of the animal [65]. The VO_{2max}determination was used to calculate the intensity of the training (30%) for each phase of training, based on the maximal velocity (100% maximal effort) of the VO_{2max} test. Exercised and sedentary dams were submitted to the effort test at the same time to compare the physical performance.

Physical training protocol

The physical exercise training began 24 h after the effort test. On lactation day 3, the animals performed an other effort test to adjust the speed training for the lactational period (30%). The training was performed three times a week, 30 minutes/day during pregnancy and the lactation period, using 30% of the maximal velocity obtained in the effort test (Fig. 1). We did not use an electrical stimulus to keep the animals running.

Body weight

Maternal bw (n=10-12 per group) was measured throughout pregnancy and the lactation period. The bw of the offspring (n=6-18 per group) was determined once weekly throughout the experimental protocol. The total area under the curve (AUC) for body weight was calculated[66].

Milk sample collection

Milk samples were collected at P10 and P21 (n=8-10 per group);lactating mothers were separated from their pups for 2 h before collection. The fed dams were anesthetized with sodium thiopental

(45 mg/kg of BW, i.p., Thiopentax®, Cristália, Itapira, São Paulo, Brazil) and received an injection (2.5 UI/kg of BW, i.p.) of synthetic oxytocin (Oxytocin®, Chemical Union, Embu, São Paulo, Brazil) to induce milk secretion [67,68]. Breast milk samples were collected by manually massaging the nipple (0.5 ml/dam) and stored at -20^0 C for subsequent analysis. Milk samples were diluted (1:20 v/v) in saline solution (0.9% NaCl) for glucose measurement using the enzymatic colorimetric glucose oxidase method with a commercial kit (Gold Analisa; Belo Horizonte, Minas Gerais, Brazil) [69].

Lipid profile

Milk samples (n=8-10 per group)were diluted (1:20 v/v) in saline solution (0.9% NaCl) for total cholesterol measurement using the enzymatic colorimetric cholesterol oxidase method with a commercial kit (Gold Analisa; Belo Horizonte, Minas Gerais, Brazil)[70] and triglyceride concentration using the enzymatic colorimetric glycerol-3-phosphate oxidase method with a commercial kit (Gold Analisa; Belo Horizonte, Minas Gerais, Brazil) [71].

Intravenous glucose tolerance test (ivGTT)

Offspring at P90 (n=6-18 per group) anda batch of dams(n=5-6 per group), after weaning on LD21 underwent a surgical procedure under ketamine and xylazine anesthesia (3 and 0.6 mg/100 g of bw) to implant a silicone cannula into the right jugular vein for the ivGTT, as previously described [72]. Animals were allowed to recover 24 hours after surgery. Rats fasted for 12 hand were then infused with a glucose load (1 g/kg bw). Blood samples were obtained from the silicone canula 0, 5, 15, 30 and 45 minutes after glucose injection. Glucose and insulin levels were measured using biochemical analyses. The delta peak glucose in the ivGTT was calculated by the subtraction of the fasting plasma glucose and insulin concentration was used to obtain the glucose (Δglucose) and insulin changes (Δinsulin) for each time point of the ivGTT. Increases in total Δglucose and Δinsulin were calculated with the glucose and/or insulin AUC for the 45 minutes of the ivGTT [73].

Radioimmunoassay and biochemical analyses

Plasma and milk insulin were measured by radioimmunoassay (RIA) in a gamma counter (Wizard2 Automatic Gamma Counter, TM-2470, PerkinElmer®, Shelton, CT, USA). Standard human insulin, and anti-rat insulin antibody (Sigma-Aldrich®, St. Louis, MO, USA), and ^{125}I-labeled recombinant human insulin (PerkinElmer®, Shelton, CT, USA) were used. The intra-assay coefficients of

variation were in the range of 8-10%. The limit of detection was 0.006 ng/ml. The plasma glucose, milk glucose, and lipid profile was determined by using a commercial kit (Gold Analisa®, Belo Horizonte, MG, Brazil)[74].

Sympathetic and parasympathetic electrical activity assessment

For the autonomic nerve activity assessment at LD21, another batch of dams (n=5-6 per group) fasted for 12 h and was subsequently anesthetized with thiopental (45 mg/kg bw); longitudinal incisions were made on the anterior cervical region under a dissection microscope to isolate the nerve bundle of the left superior branch of the vagus nerve from the carotid artery. The nerve was covered with silicone oil to prevent dehydration and placed on a pair of curved silver recording electrodes (0.6 mm diameter) connected to an electronic device (Bio-Amplificator, Insight®; Riberão Preto/SP, Brazil) that amplified the electrical signals up to 10,000 times, and the low and high frequencies, 1-80 kHz, were filtered. The neural signal output was acquired by an Insight interface (Insight®, Riberão Preto, Brazil), viewed online and stored by a personal computer running software (Bio-Amplificator, Insight®; Riberão Preto/SP, Brazil). For data acquisition, recordings took place in a Faraday cage to avoid any electromagnetic interference. Nerve activity was analyzed as the number of spikes/ 5 s (after a 2-minute period of signal stabilization), and 20 record frames of 15s from each animal were randomly chosen for spike counting. The average number of spikes was used as the nerve firing rate for each rat. Also, the branch of the sympathetic nerve from the lumbar plexus (retroperitoneal white adipose tissue innervation - greater splanchnic nerve) was dissected. The electrode was placed under the greater splanchnic nerve, close to the retroperitoneal area. Firing rates from the nerve were obtained as described for the vagus nerve[64].

Removal of fat pad stores for measurement

After the experimental procedures, dams (n=10-12) and offspring (n=6-18) were euthanized, and fat pad stores (mesenteric, retroperitoneal and periepididymal) were removed and weighed to assess the state of obesity. Fat pad store values were correlated with the rat bw and calculated as g/100 kg of bw [73].

Statistical analysis

Results were reported as means SEM. Statistical analysis and graphics were performed using GraphPad Prism® version 6.01 for Windows (GraphPad Software, Inc. San Diego, CA, USA). Data

sets with only two groups (mothers) were analyzed for statistical significance using Student's*t* test. The data sets with more than two groups (offspring) were analyzed using Two-way analysis of variance (ANOVA) followed by Tukey's *post hoc* test. $p<0.05$ was considered significantly different when considering the main effect of exercise (E), litter size (L), their interaction (LxE; litter size vs exercise) and the differences between groups. Changes in body weight and ivGTT were analyzed using repeated measure ANOVA.

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Author contribution statement

TAR, AM and PCFM contributed to the design and conduct of the study; TAR, AM, LPT and AP contributed to the acquisition, analysis and interpretation of the data and reviewed and approved the manuscript. All authors (TAR, KVP, AP, AM,LPT, IPM, JCO, RAM, RMG, CCSF, DLA, FAF, VSA, VMM, GSF, KPR, EV, MRSR, WR and PCFM) contributed intellectually as well as reviewed, edited and approved the final version of this manuscript

Additional Information

Competing financial interests: The authors declare that they have no competing interests.

Figure legends

Figure 1. Low intensity physical training program in female rats during pregnancy and lactation periods, according to velocity, duration and intensity of sessions.

Figure 2. Body weight in mothers. Effect of low intensity physical exercise training on the body weight of the mothers. The upper panel represents the area under the curve (AUC) of bw during pregnancy and lactation of the SM (n=10) compared with that of the EM (n=12), *$*p<0.05$* by Student's *t* test. And *$*p<0.05$*, each time points of the bw curve, was calculated by repeated measures ANOVA.

Figure 3. Effect of low intensity physical exercise training on glucose and insulin increments (Δ) in mothers during the ivGTT. The upper panel represents the area under the curve (AUC) of the plasma glucose **(a)** and plasma insulin levels **(b)** after weaning until LD21 in the SM (n=5) compared with that in the EM (n=6), *$*p<0.05$* and *$***p<0.001$* by Student's *t* test. And *$****p<0.0001$*, each time points of the plasma glucose and insulin during ivGTT, was calculated by repeated measures ANOVA.

Figure 4. Effect of low intensity physical exercise training on adult offspring body weight. Area under the curve of the body weight from P0-P90. NL-SM, SL-SM, NL-EM and SL-EM groups. p_{LxE}, interaction between the exercise factor and the litter factor; $p_{e,}$ exercise factor and p_l, litter factor; *$*p<0.05$ and *$***p<0.0001$* by two-way ANOVA and Tukey's test. (n=6–18). The $^{\#}$ or * represents $p<0.05$ in each time points of the bw curve, was calculated by repeated measures ANOVA. Over the lines and bars, (*) represents NL-SM compared with SL-SM and ($^{\#}$) represents SL-SM compared to SL-EM.

Figure 5. Effect of low intensity physical exercise training in adult offspring plasma glucose and insulin levels during the ivGTT. Area under the curve of plasma glucose **(a)** and insulin levels **(b)** evaluated during the ivGTT at P90. NL-SM, SL-SM, NL-EM and SL-EM groups. p_{LxE}, interaction between sedentary mother and exercised mother factors; p_e, exercise factor and p_l, litter factor;*$*p<0.05$ **$p<0.005$ and *$***p<0.001$, *$****p<0.0001$* by two-way ANOVA and Tukey's test. (n=6–18). The $^{\#}$ or * represents $p<0.05$ in each time points of the bw curve, was calculated by repeated measures ANOVA. Over the lines and bars, (*) represents NL-SM compared with SL-SM and ($^{\#}$) represents SL-SM compared to SL-EM.

604 **Table 1.**

Parameters	SM	EM	p value
Fasting Insulin LD21 (ng/mL)	0.53 ± 0.05	0.43 ± 0.069	0.27
Fasting Glucose LD21(mg/dL)	98.1 ± 2.2	79.5 ± 3.75**	0.004
HOMA-IR LD21	3.68 ± 0.25	2.35 ± 0.35	0.05
Mesenteric fat pad LD21(g/100 g bw)	0.72 ± 0.05	0.51 ± 0.07***	0.001
VO_{2max} Pregnancy day 0.5 (mL/kg/min)	25.13 ± 0.94	22.46 ± 0.19	0.11
VO_{2max} LD3 (mL/kg/min)	18.70 ± 1.05	22.51 ± 0.36*	0.006
Parasympathetic electrical activity LD21(spike/s)	16.04 ± 1.94	16.79 ± 1.88	0.70
Sympathetic electrical activity LD21(spike/s)	17.14 ± 1.94	23.79 ± 2.84*	0.04

605 **Effect of low intensity physical exercise training on metabolism and fat pad stores in mothers.**
606 Data are expressed as the mean±SEM. SM, sedentary mothers (n=5-10); EM, exercised mothers
607 (n=6-10). The analyses were performed by Student's *t* test.
608

609 **Table 2.**

Milk	SM	EM	p value	SM	EM	p value
Lactation	**Day 10**			**Day 21**		
Insulin (ng/mL)	1.33 ± 0.08	2.49 ± 0.24****	0.0001	1.03 ± 0.10	2.53 ± 0.34****	0.0001
Glucose (mg/dL)	140.4 ± 12.4	145 ± 12.7	0.80	190 ± 40.72	206.7 ± 27.5	0.75
Triglycerides (mg/dL)	2119 ± 284.2	1579 ± 204.0	0.27	4124 ± 1053	3089 ± 405.9	0.41
Total Cholesterol (mg/dL)	84.07 ± 9.96	79.16 ± 9.61	0.73	163.5 ± 6.29	129.8 ± 11.51*	0.03

610 **Effect of low intensity physical exercise training on milk composition.** Data are expressed as the
611 mean±SEM. SM, sedentary mothers (n=5); EM, exercised mothers (n=6). The analyses were
612 performed by Student's *t* test.

613

614

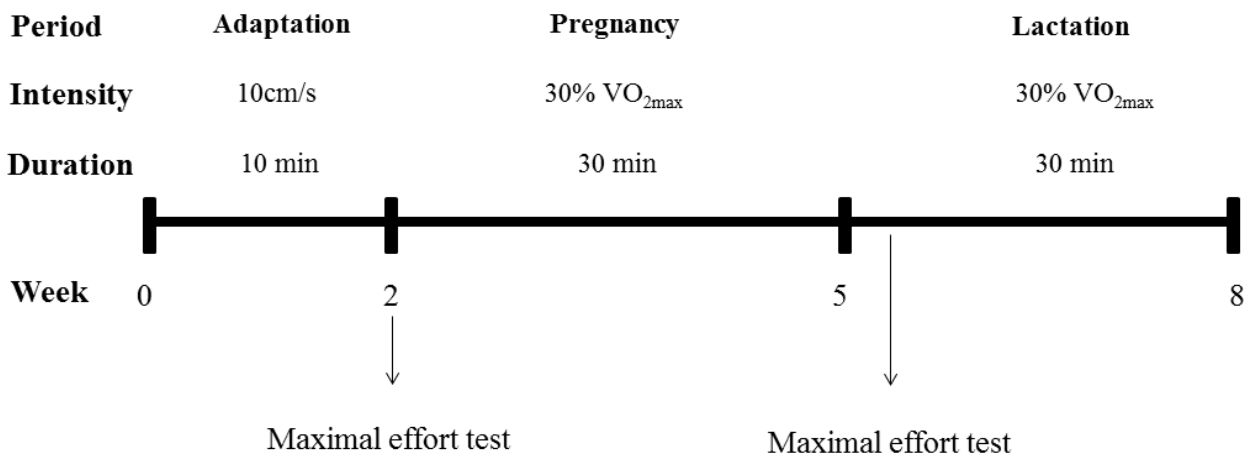
615 **Table 3.**

Parameters	**SM Pups**		**EM Pups**		***p* value**
Birth weight (g)	6.06 ± 0.01		6.05 ± 0.01		0.6
	NL-SM	**SL-SM**	**NL-EM**	**SL-EM**	**Source of variation**
Body weight (g) P21	47.0 ± 2.0	68.0 ± 1.7	46.9 ± 0.7	49.2 ± 1.3	I^{*} / E^{**} / L^{****}
Body weight (g) P90	370.4 ± 6.6	406.6 ± 8.5	366.3 ± 3.9	374.2 ± 10.7	I^{ns} / E^{**} /L^{*}
Retroperitoneal fat pad (g/100 g) P90	1.248 ± 0.042	1.603 ± 0.099	1.09 ± 0.03	1.19 ± 0.113	I^{ns} / E^{****}/ L^{*}
Periepididymal fat pad (g/100 g) P90	1.14 ± 0.040	1.39 ± 0.091	0.97 ± 0.02	1.03 ± 0.091	I^{ns} / E^{***}/ L^{*}
Mesenteric fat pad (g/100 g) P90	0.69 ± 0.030	1.06 ± 0.060	0.70 ± 0.03	0.73 ± 0.033	I^{****} / E^{****}/ L^{****}
Fasting Glucose (mg/dL) P90	83.57± 1.59	112.02 ± 1.68	97.08 ± 2.11	87.9 ± 2.46	I^{****} / E^{****}/ L^{****}
Fasting Insulin (ng/mL) P90	0.35 ± 0 01	0.54 ± 0.04	0. 25 ± 0.03	0.36 ± 0 05	I^{ns} / E^{**}/ L^{***}
HOMA-IR P90	1.86 ± 0.07	3.76 ± 0.30	1.37 ± 0.17	2.00 ± 0.29	I^{**} / E^{****}/ L^{****}

616 **Effect of low intensity physical exercise training on metabolism and fat pad stores in adult rat**
617 **offspring.** Data are expressed as the mean±SEM (n=6–18). NL-SM, normal litter of sedentary
618 mothers; SL-SM, small litter of exercised mothers; NL-EM, normal litter of exercised mothers; SL-
619 EM, small litter of exercised mothers, P90, postnatal day 90; P21, postnatal day 21; L, litter size
620 factor; E, exercise factor; and LxE, interaction between L and E factors. *$p<0.05$, ** $p<0.01$, ***
621 $p<0.001$, ****$p<0.0001$ and ns, no significant difference, based on a two-way analysis of variance
622 or Student's *t* test.

623

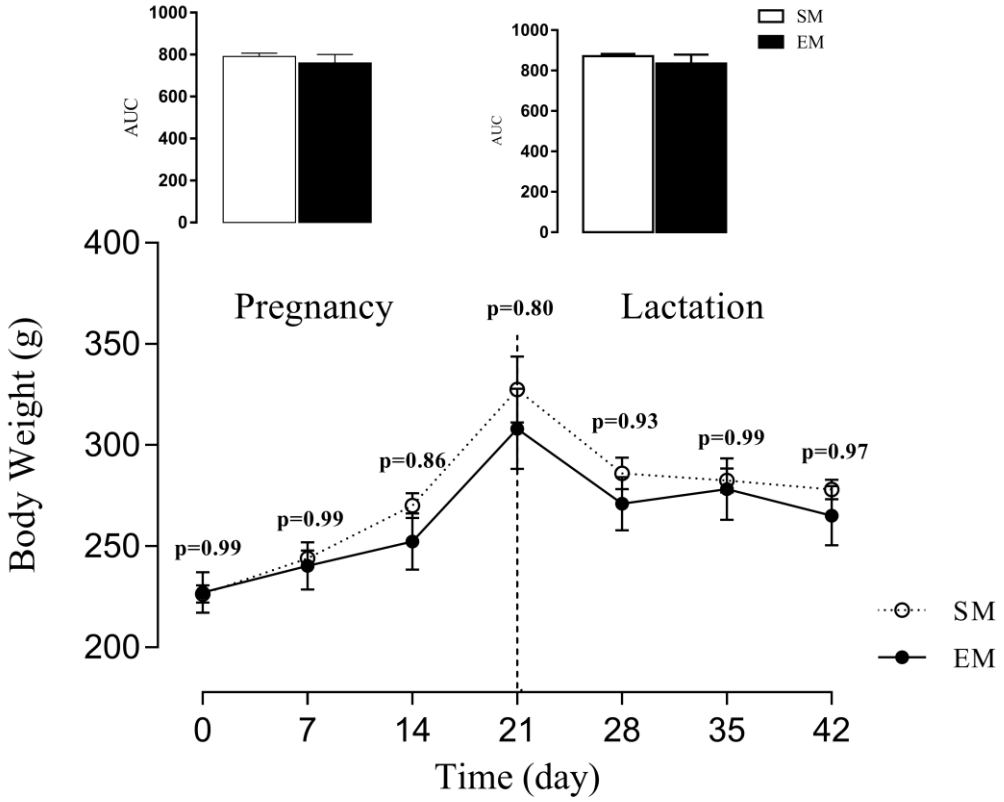
624 **Figure 1**



625

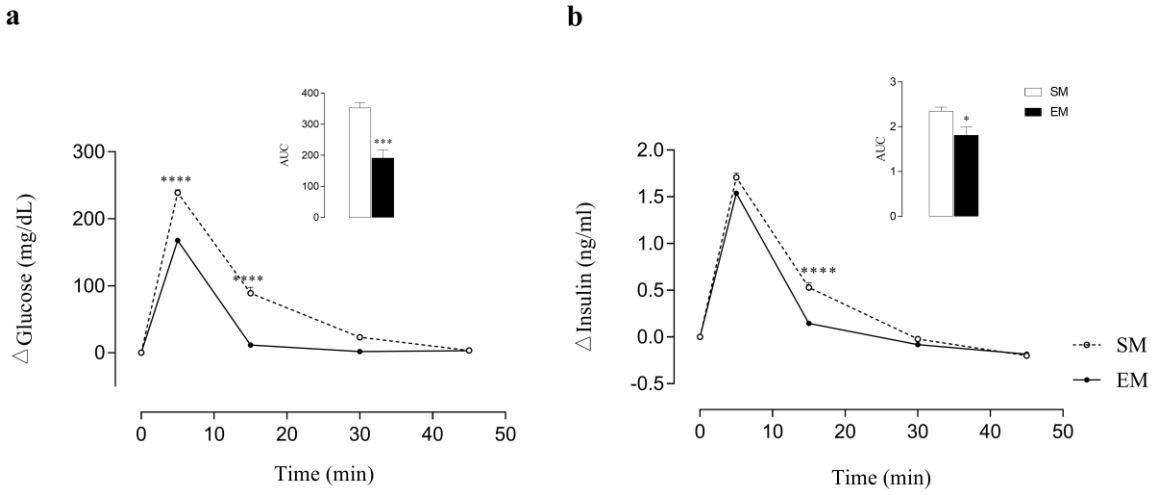
626

627 **Figure 2**



628

629 **Figure 3**



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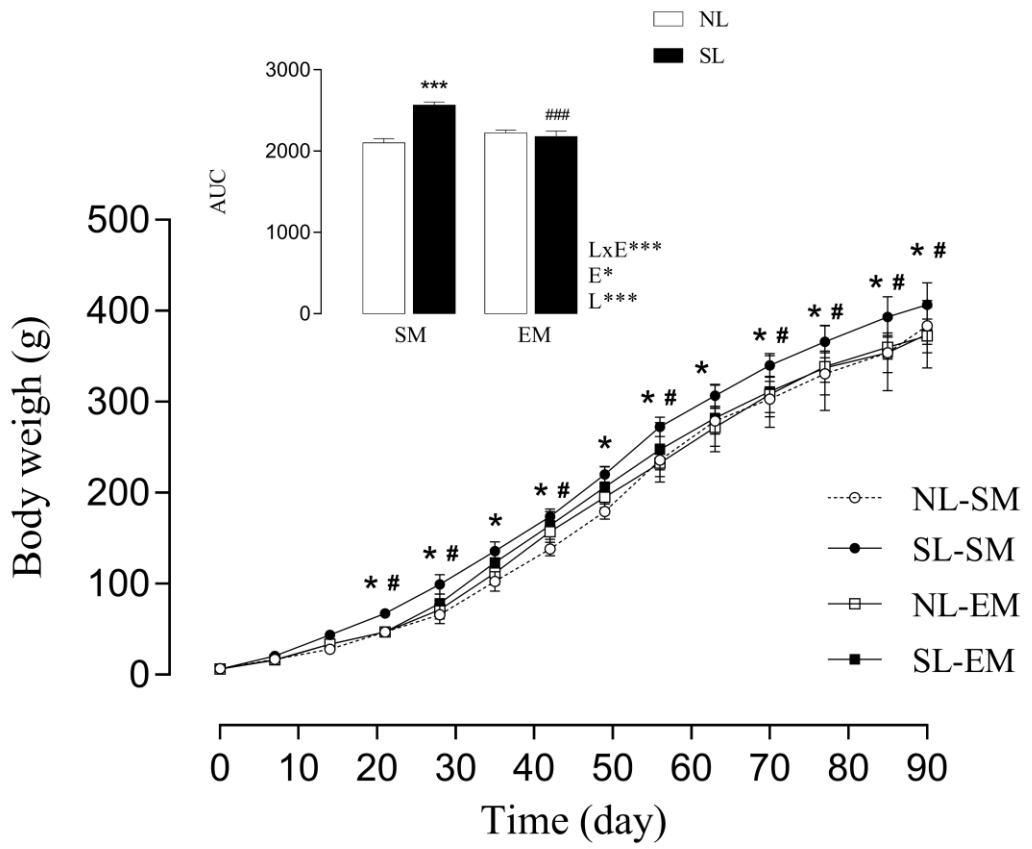
631

632 **Figure 4**



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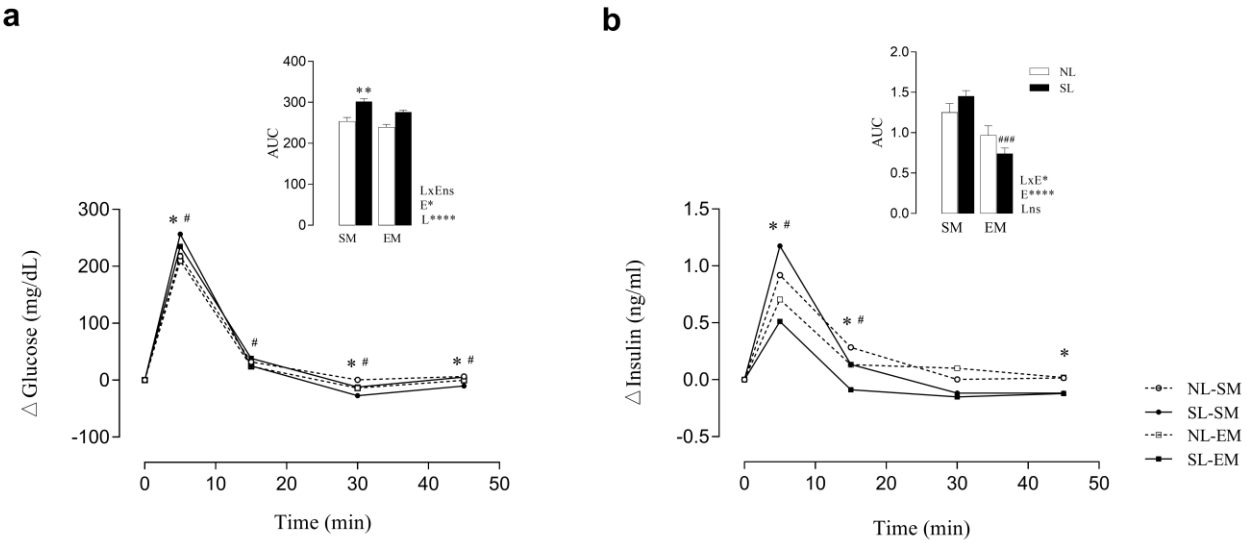
634 **Figure 5**



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