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MILENE APARECIDA BOBATO

"Metformin during critical stages of development: A promising approach to prevent metabolic dysfunction in adulthood."

Maringá
2023

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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.

Orientador: Prof. Dr. Paulo Cezar de Freitas Mathias

Coorientador(a): Dra. Ananda Malta

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BANCA EXAMINADORA

Prof. Dr. Paulo Cezar de Freitas Mathias Universidade Estadual de Maringá	Prof. Dra. Anacharis Babeto de Sá Nakanishi Universidade Estadual de Maringá
Prof. Dr. Rodrigo Mello Gomes Universidade Federal de Goiás	Prof. Dra. Veridiana Mota Moreira Universidade Estadual de Maringá
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Biografia

Milene Aparecida Bobato nasceu em 01/01/1978 em Curitiba/PR. Possui graduação em Biomedicina pela Universidade Cesumar - UNICESUMAR (2015). Concluiu o mestrado em ciências Fisiológicas no ano de 2018, na Universidade Estadual de Maringá, com a dissertação intitulada “Ftalocianina alumínio-hidróxido altera o metabolismo hepático e a sensibilidade à insulina em camundongos Swiss diabéticos tipo 1.” Atualmente é doutoranda no Programa de Pós-graduação em Ciências Biológicas da Universidade Estadual de Maringá. Tem experiência na área de biologia celular e fisiologia, atuando principalmente nos seguintes temas: obesidade, diabetes tipo 1 e tipo 2, secreção de insulina e homeostase da glicose.

Agradecimentos

Hoje, diante desta banca acadêmica, repleta de expectativas e desafios, gostaria de expressar minha profunda gratidão por ter chegado a este momento crucial da minha jornada: a defesa do meu doutorado. Este feito só foi possível graças ao apoio incondicional de pessoas especiais em minha vida, que compartilharam comigo cada passo dessa trajetória desafiadora.

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Por fim, O PRESENTE TRABALHO FOI REALIZADO COM APOIO DA COORDENAÇÃO DE APERFEIÇOAMENTO DE PESSOAL DE NÍVEL SUPERIOR - BRASIL (CAPES) - CÓDIGO DE FINANCIAMENTO 001. Desta forma eu gostaria de expressar minha gratidão às agências de fomento, em especial à CAPES, pela concessão de bolsa de estudos financiando minha pesquisa. Sem seu apoio financeiro, este projeto de doutorado não teria sido possível.

Dedicatória

Dedico esta tese de doutorado ao meu amado pai que, mesmo não estando mais fisicamente presente, permanece eternamente vivo em minha inspiração e em meu coração. É com profunda gratidão que escolho este dia, o dia do seu aniversário, para homenagear sua memória e sua influência duradoura em minha vida. Seu exemplo de dedicação, determinação e amor pela busca do conhecimento continuam a ser a luz que ilumina meu caminho acadêmico. Pai, você vive em mim, em cada conquista e em cada passo que dou nesta jornada. Esta tese é dedicada ao senhor, com amor e saudade, em celebração ao seu legado inspirador.

Apresentação

Esta dissertação é composta de dois artigos científicos, sendo o primeiro manuscrito uma revisão intitulada: **"Metformin and the developmental origin of health and disease (DOHaD): A comprehensive review of implications for metabolic programming and health across the lifespan"**, e o segundo manuscrito, um trabalho experimental intitulado: **“Metformin Administration During Lactation Mitigates Hyperphagia and Preserves Beta Cell Structure in Early Overfed Rats.”** Os estudos em questão investigam o efeito da administração da metformina em fases críticas do desenvolvimento, um tópico de pesquisa alinhado com o conceito DOHaD (Developmental Origins of Health and Disease). A presente revisão de literatura examina os efeitos da administração da metformina durante diversas fases do desenvolvimento, nomeadamente, gestação, lactação e adolescência, tanto em humanos quanto em modelos animais e esses efeitos perduram na fase adulta inferindo em programação metabólica. Além disso, o estudo experimental apresenta os benefícios associados à administração da metformina durante o período de lactação em um modelo animal de superalimentação na infância, com foco nas ilhotas pancreáticas.

Em consonância com as regras do programa de pós-graduação em ciências biológicas, o artigo de revisão foi redigido de acordo com as normas da revista **DIABETES RESEARCH AND CLINICAL PRACTICE** com atual fator de impacto **5.1**. Enquanto o trabalho experimental foi redigido de acordo com as normas da revista **JOURNAL OF ENDOCRINOLOGY**, com atual fator de impacto **4.0**.

Resumo geral:

Introdução:

Primeiro manuscrito: A revisão aborda a utilização da metformina com o propósito de promover a programação metabólica. O conceito DOHaD, que representa "Origens do Desenvolvimento da Saúde e da Doença", enfatiza a importância das condições ambientais e nutricionais durante momentos cruciais do processo de desenvolvimento na determinação da saúde ao longo da vida. A metformina, comumente empregada no tratamento da diabetes tipo 2, tem atraído considerável atenção devido ao seu potencial para mitigar disfunções metabólicas, tais como resistência à insulina e disfunção das células beta pancreáticas. Este artigo explora a interação entre os princípios DOHaD e o uso da metformina, com foco em sua administração durante fases críticas do desenvolvimento, tais como a gestação, lactação e adolescência.

Segundo manuscrito: Realiza-se um estudo experimental que se concentra nos efeitos da metformina durante o período de lactação em animais superalimentados durante a infância e programados para desenvolver obesidade na infância perdurando à fase adulta. A obesidade infantil suscita preocupações devido à sua associação com o surgimento de condições como diabetes mellitus tipo 2 e doenças cardiometabólicas na idade adulta. Nossa hipótese é que a metformina, quando administrada durante a lactação em animais superalimentados na infância, melhora a função das células β prevenindo a formação de hipertrofia celular.

Metodologia:

Primeiro manuscrito: A revisão de literatura abrangeu uma busca ampla na base de dados PubMed para identificar artigos relacionados à administração de metformina durante os períodos de gestação, lactação e adolescência, com ênfase nos resultados relacionados à regulação do metabolismo.

Segundo manuscrito: Na análise experimental, ratos da linhagem Wistar foram usados como modelo animal. Fêmeas virgens com 70 dias de idade e machos com 80 dias de idade foram acasalados. O dia do nascimento das ninhadas foi considerado o dia zero (0), e as ninhadas foram padronizadas com 9 filhotes (4 fêmeas e 5 machos) no primeiro dia após o parto. Para induzir a superalimentação precoce, algumas ninhadas foram reduzidas para 3 ratos machos no terceiro dia. A administração de metformina (100 mg/kg de peso corporal por dia) ou solução salina foi realizada por injeção intraperitoneal (I.P.) do

primeiro ao 12º dia de lactação. Os filhotes foram distribuídos em quatro grupos: ratos de ninhadas reduzidas que receberam solução salina (SLS), ratos de ninhadas reduzidas que receberam metformina (SLM), ratos de ninhadas normais que receberam solução salina, (NLS) e ratos de ninhadas normais que receberam metformina, (NLM). O desmame dos filhotes ocorreu no 21º dia. As condições ambientais, como temperatura e iluminação, foram mantidas sob controle durante todo o período do experimento, e água e ração padrão para roedores foram fornecidas à vontade. Peso corporal e consumo foram avaliados semanalmente durante a vida do animal até os 90 dias de vida, foram feitas análises *in vivo e ex vivo*. Este estudo optou por utilizar apenas ratos machos devido às diferenças de gênero nas respostas de longo prazo à superalimentação, sendo os machos mais suscetíveis aos efeitos do tamanho reduzido da ninhada.

Resultados:

Primeiro manuscrito: Uma parcela significativa dos estudos avaliados tanto em humanos quanto em roedores, revela que a administração de metformina durante a gravidez, lactação e ambos os períodos sequencialmente, pode ter um impacto positivo na regulação da glicemia, na tolerância à glicose e na resistência à insulina. Entretanto, alguns estudos relataram efeitos adversos, como baixo peso ao nascer, acúmulo de gordura abdominal e subcutânea em crianças e ganho de peso na fase adulta. Estudos sobre os efeitos da metformina quando administrada na adolescência com efeito de programação metabólica não foram encontrados.

Segundo manuscrito: Filhotes superalimentados na infância e tratados com metformina, SLM, apresentam redução da área da ilhota pancreática, sugerindo redução de hipertrofia celular, em comparação com animais não tratados, SLS. Ao analisar consumo, ganho de peso, tolerância à glicose e resistência à insulina em animais SLS, esses parâmetros estão aumentados desde a infância até a idade adulta em comparação aos animais NLS. Porém, esses parâmetros são normalizados através do tratamento lactacional com metformina, SLM.

Conclusão:

Primeiro manuscrito: Em suma, foi possível verificar que a metformina quando administrada em fases críticas do desenvolvimento pode atenuar ou impedir o desenvolvimento de disfunções metabólicas como o diabetes mellitus tipo 2 e doenças metabólicas na idade adulta. Contudo, sugerimos que os efeitos adversos podem estar relacionados ao delineamento experimental como o modelo de estudo, a dosagem e a via de administração da metformina em contextos específicos.

Segundo manuscrito: Mostra-se aqui, pela primeira vez, que a metformina administrada durante a lactação em animais superalimentados na infância tem a capacidade de impedir a hipertrofia nas células beta bem como a hiperfagia, evitando disfunções metabólicas associadas à obesidade como o diabetes mellitus tipo 2**.**

General abstract

Introduction:

First Manuscript: The review approaches the use of metformin for the purpose of promoting metabolic programming. The concept of DOHaD, which stands for "Developmental Origins of Health and Disease," underscores the importance of environmental and nutritional conditions during critical developmental periods in determining lifelong health. metformin, commonly employed in the treatment of type 2 diabetes, has garnered significant attention due to its potential to mitigate metabolic dysfunctions such as insulin resistance and pancreatic beta-cell dysfunction. This article explores the interaction between DOHaD principles and the use of metformin, with a focus on its administration during critical developmental phases, including pregnancy, lactation and adolescence.

Second Manuscript: We conducted an experimental study that focuses on the effects of metformin during the lactation period in animals that were overfed during childhood and programmed to develop obesity persisting into adulthood. Childhood obesity raises concerns due to its association with the onset of conditions such as type 2 diabetes and cardiometabolic diseases in adulthood. Our hypothesis is that metformin, when administered during lactation in overfed animals during childhood, improves β-cell function by preventing cellular hypertrophy.

Methods:

First Manuscript: The literature review encompassed a comprehensive search in the PubMed database to identify articles related to the administration of metformin during pregnancy, lactation and adolescence, with an emphasis on outcomes related to metabolism regulation.

Second Manuscript: In the experimental analysis, Wistar strain rats were used as the animal model. Virgin females at 70 days of age and males at 80 days of age were mated. The day of litter birth was considered day zero (0), and litters were standardized with 9 pups (4 females and 5 males) on the first day postpartum. To induce early overfeeding, some litters were reduced to 3 male pups on the third day. Metformin administration (100

mg/kg of body weight per day) or saline solution was performed via intraperitoneal injection (I.P.) from the first to the 12th day of lactation. The pups were divided into four groups: reduced litter rats receiving saline (SLS), reduced litter rats receiving metformin (SLM), normal litter rats receiving saline (NLS), and normal litter rats receiving metformin (NLM). Weaning of the pups occurred on the 21st day. Environmental conditions, such as temperature and lighting, were kept under control throughout the experiment, and water and standard rodent chow were provided ad libitum. Body weight and consumption were assessed weekly throughout the animal's life until 90 days of age, and both *in vivo and ex vivo* analyses were conducted. This study chose to use only male rats due to gender differences in long term responses to overfeeding, with males being more susceptible to the effects of reduced litter size.

Results:

First Manuscript: A significant portion of the assessed studies reveals that the administration of metformin during pregnancy, lactation, and both periods sequentially can have a positive impact on glycemic regulation, glucose tolerance, and insulin resistance. These effects are even more pronounced when metformin is administered during pregnancy and lactation simultaneously. However, some studies have reported adverse effects, such as low birth weight, accumulation of abdominal and subcutaneous fat in children, and weight gain in adulthood. Studies on the effects of metformin when administered during adolescence with a metabolic programming effect were not found.

Second Manuscript: Overfed pups treated with metformin, SLM, show a reduction in pancreatic islet area, suggesting reduced cellular hypertrophy, compared to untreated animals, SLS. When analyzing intake, weight gain, glucose tolerance and insulin resistance in SLS animals, these parameters are increased from childhood to adulthood compared to NLS animals. However, these parameters are normalized through lactational treatment with metformin, SLM.

Conclusion:

First Manuscript: In summary, it was possible to observe that metformin, when administered during critical phases of development, can mitigate or prevent the development of metabolic dysfunctions such as type 2 diabetes mellitus and metabolic diseases in adulthood. However, we suggest that adverse effects may be related to experimental design factors such as the study model, dosage, and route of metformin administration in specific contexts.

Second Manuscript: We show for the first time that metformin administered during lactation in animals overfed in infancy has the ability to prevent hypertrophy in beta cells as well as hyperphagia, preventing metabolic dysfunctions associated with obesity such as type 2 diabetes mellitus.

Sumário

First Manuscript:

Manuscript 1 – Review Article

"Metformin and the developmental origin of health and disease (DOHaD): A comprehensive review of implications for metabolic programming and health across the lifespan"

Authors

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Abstract

Background

Metformin is a medication commonly used to treat type 2 diabetes mellitus, gestational diabetes and polycystic ovary syndrome. The drug, when administered at critical stages of development, can program the organism in accordance with the DOHad concept.

Objective

Elucidate the available literature findings concerning the impacts of metformin administration during crucial developmental stages like pregnancy, lactation, and adolescence, with the goal of understanding its role in metabolic programming during adulthood.

Methods and materials

We performed a comprehensive narrative review in PubMed, evaluating the lasting effects of metformin during critical developmental stages. All studies involving metabolic programming effects were included.

Results

Metformin during pregnancy and lactation benefits glycemic control, but some adverse effects exist. Research on metformin's effects during adolescence is lacking.

Conclusion

In summary, it is evident that metformin, when administered during critical developmental phases, predominantly exhibits beneficial effects in mitigating or preventing metabolic dysfunctions such as type 2 diabetes mellitus and metabolic diseases in adults. Although few studies have reported adverse effects, it is important to recognize that the drug's positive impacts remain undeniable. These occasional adverse effects can be attributed to various experimental factors, such as study designs, dosages, and routes of administration in specific contexts.

Keywords: Metformin, Metabolic Programmig, DOHaD concept

Abbreviations

T1D, Type 1 Diabetes Mellitus; T2D, Type 2 Diabetes Mellitus; GD, Gestational Diabetes; PCOS, Polycystic Ovary Syndrome; DOHaD, Developmental Origins of Health and Disease; FDA, Food and Drug Administration; WHO, World Health Organization; OCT, Organic Cation Transporters; MATE, Multiple Drug and Toxin Extrusions; AMP, Adenosine Monophosphate; AMPK, Adenosine Monophosphate Kinase; ATP, Adenosine Triphosphate; LDL, Low Density Lipoprotein Cholesterol; GLP-1, Hormone Glucagon-Like Peptide-1; NPY, Orexigenic Peptide Neuropeptide Y; STAT3, Signal Transducer and Activator of Transcription 3; IL-6, Interleukin 6; CRP, C-Reactive Protein; mTORC, Inhibiting Mammalian Target of Rapamycin Complex; TSC, Tuberous Sclerosis Complex; BMI, Body Mass Index; PDX1, Duodenal Homeobox 1; NG3+, Neurogenin 3; HFD, High-Fat Diet; IFN-γ, Interferon-Gamma; PVAT, Perivascular Adipose Tissue; GLUT, Glucose Transporter; ITT, Insulin Tolerance Test; NEFA, Non-Esterified Fatty Acid; PPAR-gamma, Proliferator-Activated Receptor; METGL, Metformin during Pregnancy and Lactation; METG, Metformin during Pregnancy; HPG, Hypothalamic-Pituitary-Gonad; IGF, Insulin-like Growth Factors; HPS, Hypothalamic-Pituitary-Somatic; HPA, Hypothalamic-Pituitary-Adrenal, ACTH, Adrenocorticotropic Hormone

1. Background

Metformin is a medication derived from the plant *Gallega Officinalis* also known as French lilac. Dimethyl biguanide, a specific compound of metformin, was synthesized for the first time in 1922, but only in 1950 did the Frenchman Jean Sterne publish about its anti-hyperglycemic properties. In 1994, it was approved by a Food and Drug Administration (FDA) in the United States, increasing its popularity [1].

Studies related to the effects of metformin infer that the drug initially demonstrates its effectiveness in the treatment of T2D and that it later shows effectiveness in the treatment of T1D concomitant with insulin and the progression of pre-diabetes, in PCOS, and possibly dementia-like neurodegenerative disorders [2-5]. Other studies also show promising effects on the development of certain types of cancer and on the intestinal microbiome, as well as its anti-aging ability [6, 7]. In addition to the effects mentioned, experimental data have shown that metformin has an effect on controlling food intake and reducing body weight, which makes this drug even more attractive, taking into account its effects on obesity control [8, 9].

According to the World Health Organization (WHO), it is estimated that by 2025, 667 million people, including adults and children, will be overweight or obese [10]. The obesity can develop in infancy and adolescents and may last into adulthood. Regardless of the stage, if obesity cannot be contained it can trigger a series of consequences such as T2D, cardiovascular disease, chronic kidney disease and cancer, in addition to an increased risk of premature death [11-14].

Observing the development of metabolic diseases from the beginning of life, the Developmental Origins of Health and Disease (DOHaD) concept determines that the metabolic programming infers that the interferences that occur in periods of vulnerability of the organism (pre-conception, pregnancy, lactation, infancy and adolescence), whether due to nutritional deficiencies, exposure to endocrine disruptors or even toxic substances, not only interrupt or delay the growth process, but can also manifest metabolic abnormalities that challenge health in the short term and can extend into adulthood [15, 16].

These periods of greater vulnerability of the organism are defined as “critical windows of development”, characterized by intense cell proliferation, differentiation and rapid tissue expansion [15, 16].Therefor, considering the effects of metformin and understanding the

importance of the DOHaD concept in the development of health and disease, this review seeks to elucidate the results obtained in scientific research published when the drug is administered in critical periods of development.

2. Metformin: mechanism of action

Metformin, a positively charged biguanide, requires transporters to cross cell membranes. It accumulates in organs such as the intestine, liver and kidneys, where absorption, metabolism and excretion occur, with minimal concentration in the periphery. Organic cation transporters (OCT3) and multiple drug and toxin extrusions (MATE1) are also important for their absorption [17, 18].

The mechanism of action of metformin involves the activation of 5′-adenosine monophosphate (AMP) kinase (AMPK), which regulates several metabolic pathways, including glucose and lipid metabolism [19]. Metformin acts on mitochondria, inhibiting complex I of the electron transport chain, reducing adenosine triphosphate (ATP) synthesis and affecting the activation of AMPK [20]. In the liver, through the activation of AMPK, it inhibits gluconeogenesis, increases the oxidation of fatty acids, and reduces triglyceride and low-density lipoprotein cholesterol (LDL) levels [21]. It also increases glucose uptake in muscle [22].

In the intestine, metformin can increase the release of the hormone glucagon-like peptide-1(GLP-1), which is secreted by L cells, mainly in the ileum. It increases the expression of precursor proteins such as pre-proglucagon and proglucagon in the intestine, potentially increasing GLP-1 [23]. Already in the kidneys, metformin has a protective role in diabetic kidney disease, reducing glycolipid metabolism disorders, oxidative stress and promoting autophagy. It also attenuates albuminuria and extracellular matrix production [24, 25].

Metformin is promising in weight control and dietary intake, being beneficial in patients undergoing restrictive diets for weight control and in patients with type 2 diabetes on insulin therapy [26]. It can affect food intake by reducing the expression of the orexigenic peptide neuropeptide Y (NPY) in the hypothalamus and inhibiting the role of ghrelin [27]. Furthermore, it affects the leptin - Signal transducer and activator of transcription 3 (STAT3) system (60) and reduces leptin resistance in the hypothalamus [28, 29]. Therefore, metformin has varied effects on the body, including metabolic regulation, glucose reduction, weight control and effects on the central nervous system.

3. Metformin exposure during pregnancy

Metformin is a safe and effective option for insulin resistance in pregnant women with T2D, GD, PCOS, and non-diabetic obese women during pregnancy [30] Its effects on fetal and neonatal metabolism are scrutinized due to placental transfer, with cord blood concentrations matching maternal levels [31]. Metformin enters the enterohepatic circulation, reaching embryofetal cells via cationic transporters present in the placenta, including OCTN1, OCTN2, and significant OCTN3 [32]. OCT3 expression increases with gestational age, elevating drug transfer to fetal tissues [33].

GD treatment with metformin, compared to insulin, improves postprandial glycemia, minimizes hypoglycemia, curbs early pregnancy weight gain, and reduces premature births [34]. Obese non-diabetic pregnant women on metformin had reduced body weight, a 50% lower incidence of large-for-gestational-age newborns, lower fasting blood glucose, HOMA-IR score, interleukin 6 (IL-6), CRP levels, and fewer cases of pre-eclampsia [35, 36].

Complications like pre-eclampsia, GD, low birth weight, miscarriage, and premature birth are common in women with PCOS [37]. Metformin use in PCOS women is associated with full-term deliveries, possibly by inhibiting mammalian target of rapamycin complex 1 (mTORC) through TSC 1 and TCS2 gene activation [38]. mTORC activation induces preterm labor, while inhibition prevents or delays labor onset [39]. Metformin's effects on children exposed in utero showed no impact on birth weight or long-term risks like obesity, hypoglycemia, hyperglycemia, diabetes, or social motor development [40].

Some studies contradict the positive effects of metformin. Babies born to mothers with gestational diabetes on metformin were smaller than those on insulin. However, metformin-exposed children exhibited accelerated postnatal growth, leading to higher body mass index (BMI) in mid-childhood. Low birth weight and postnatal catch-up growth may have long-term cardiometabolic effects, warranting further perinatal and longitudinal studies [41].

Studies on children aged 12 months to 9 years exposed to metformin during embryonic and fetal development showed increased size in weight, arm, waist circumference, waist-to-height ratio, BMI, abdominal fat mass, and subcutaneous fat content. Metformin's specific action, as well as fetal nutritional status, sex, and postpartum environment, may contribute to these phenotypic characteristics [41, 42].

Animal research on the impact of metformin on embryonic and fetal development is also performed, most often in rodents. In a diabetic embryopathy model in mice, metformin reduced neural tube defect formation by normalizing metabolic defects, with hyperglycemia as the primary pathogenic feature [43]. Offspring of rats exposed to metformin during pregnancy and later fed a high-fat diet gained less weight and adipose tissue, with improved glucose tolerance and lipid profiles. Metformin down-regulated genes associated with electron transport chain complexes I, III, and ATP synthase in the liver, affecting gluconeogenesis [44, 45].

Greeg et al., demonstrated in their experiments the effects of metformin when administered to mothers during pregnancy *in vitro* and *in vivo* in the embryonic pancreas of mice. *In vitro* studies showed increased pancreatic germ cell size and mesenchymal cell number with elevated pancreatic and duodenal homeobox 1 (PDX1) expression and a reduction in Neurogenin 3 expression (NGN3+ cells). *In vivo*, metformin exposure increased pancreatic progenitor cell number and size, along with elevated PDX1 expression. NGN3+ fraction increased, but it didn't lead to cell proliferation. Notably, metformin in vivo led to an expansion of pancreatic β-cell numbers, potentially influencing adulthood outcomes[46].

When evaluating the effects of metabolic programming in young mice aged 20 weeks and in females between the first and second year of life exposed to metformin in utero, Gregg et al., show that both male and female offspring of metformin-exposed mothers displayed improved glucose tolerance and insulin secretion, without changes in β-cell mass. Elderly females (1-2 years) exposed to metformin in utero also exhibited improved glucose tolerance. These findings suggest a pattern of metabolic programming with lasting effects on glucose metabolism [47].

In another study, female Wistar rats were divided into those fed a standard diet and those on a high-fat diet (HFD) for 5 weeks. Half of the HFD-fed mothers received daily metformin until the 19th day of pregnancy. This hypercaloric diet significantly affected maternal and fetal fatty acid metabolism. Metformin treatment during pregnancy reduced Interferon-gamma (IFN-γ) levels in fetal livers, potentially mitigating hepatic steatosis and endoplasmic reticulum stress. While the study didn't explore long-term effects on offspring, it hints at the possibility of fetal reprogramming due to maternal obesity [48]. With that in mind, our investigation found studies on the effects of metformin administered to mothers during pregnancy on offspring in adulthood.

Puppies on a high-fructose and fat diet developed hypertension-related gene expression, but those exposed to metformin in utero had normalized parameters. The diet also reduced mTORC expression, which was restored with metformin [49]. In a study with Wistar rats aged 75-80 days, intrauterine metformin exposure didn't affect vascular reactivity in the abdominal aorta, regardless of vasodilators, vasoconstrictors, or the presence of endothelium and/or perivascular adipose tissue (PVAT). Biometric data, including maternal weights during metformin treatment, pups' birth weights, and pup numbers, remained unchanged [50]. We can highlight that these data suggest that metformin administered during pregnancy, a phase considered of high plasticity and a critical period of development, was able to restore the changes in metabolic patterns generated by a maternal diet rich in fructose and fat.

Shoonejans et al., used obese mothers receiving metformin from one week before mating until E18.5. Male pups from obese mothers showed increased adiposity, adipocyte hypertrophy, pro-inflammatory gene expression, hyperleptinemia, and hepatic lipid accumulation at 12 months of age. Offspring exposed to metformin in utero exhibited an adiposity phenotype with hyperinsulinemia, hyperleptinemia, adipose tissue inflammation, and ectopic lipid deposition, with a stronger effect in older female offspring [51].

As in humans, animal studies show adverse effects of metformin when administered to dams during pregnancy in their offspring. Salomaki et al. (2013), administered metformin orally to mothers from E0.5 to E17.5 with a standard diet. Offspring exposed to metformin exhibited programming effects during a later high-fat diet (HFD) phase, including increased body weight, mesenteric fat, impaired glucose tolerance in males, and reduced glucose transporter isoform (GLUT4) mRNA expression in epididymal fat. These effects were observed at the end of the HFD phase [52].

Studies in humans demonstrate that metformin administration during pregnancy to mothers with Type 2 diabetes, gestational diabetes, polycystic ovary syndrome, and obesity exerts beneficial adjustments in maternal metabolism. It reduces glucose levels, enhances glucose tolerance, improves insulin resistance, and mitigates inflammatory factors. Nonetheless, adverse effects such as low birth weight and increased abdominal and subcutaneous fat deposition in children have been observed. Furthermore, animal studies also indicate positive effects, including the reduction of neural tube defects and

influence on pancreatic development, but suggest metabolic challenges in offspring, including insulin resistance and glucose intolerance.

Among the studies reviewed, the majority, both in humans and animals, underscore the beneficial effects of metformin administration during this developmental phase. We suggest that adverse effects may be related to dosage and exposure duration, which appear to vary between study models, compromising result reproducibility.

3.1 Metformin exposure during lactation

This discussion revolves around the utilization of metformin during lactation and its potential metabolic effects on offspring, with most studies conducted in rodents. In human research, only one article on metformin use during lactation and its metabolic impact was identified. Breast milk is recognized as the optimal source of postnatal nutrition, with the WHO recommending exclusive breastfeeding for the first six months of life [53]. Breast milk contains various bioactive agents that influence the gastrointestinal tract, immune system, and neurological development. Recent studies suggest that breast milk may mitigate the risk of late metabolic diseases, particularly obesity and type 2 diabetes, in childhood [54, 55].

The critical phase of development during lactation is highlighted, particularly in rodents where processes like proliferation, differentiation, and maturation occur during this period [56]. This vulnerability in newborns underscores the importance of addressing the development of key organs involved in metabolic development. While the gastrointestinal tract is fully mature in humans at birth, in rodents, both anatomical and functional features, along with immune maturity of the gut, develop gradually during lactation [57]. Similarly, liver development, hepatocytic differentiation, hepatic glycogen accumulation, and hematopoiesis in both humans and rodents occur during gestation.

However, in rodents, hepatocyte differentiation continues into the first week of life, contrasting with humans where it completes at the end of gestation [56]. Renal development, although initiated during fetal life in both humans and rodents, concludes during lactation in rodents [56]. Pancreas formation begins earlier in mice than in humans, but at birth, the pancreas remains immature in both. Rodent pancreas experiences significant growth during the first postnatal month, while in humans, islet size increases without a corresponding increase in number from birth to adulthood [58, 59].

Metformin has been identified in both maternal blood and milk, however, its concentration in milk is nearly negligible [60]. Human research reveals that metformin levels in breast milk are minimal, constituting less than 0.5% of the maternal ingested dose. While detectable in low levels in infant serum, a significant study determined no adverse effects on breastfed infants [61]. Despite consistent presence in milk, the estimated infant dose remains around 1% or less of the mother's weight-adjusted dose, and there have been no observed glycemic alterations in infants. Consequently, metformin is considered compatible with breastfeeding [60].

Recent animal research aims to uncover the metabolic effects of administering metformin during pregnancy and its long-term impacts on postnatal health, yielding varied results. In one study, Wistar rat pups received metformin injections during early lactation, preventing adult obesity, glucose intolerance, and insulin resistance caused by early overnutrition [62]. However, adult rats receiving early metformin and a high-fat diet showed no changes in body weight or fat stores [63]. Nonetheless, metformin protected these rats from reproductive organ damage, improved sperm morphology, and enhanced antioxidant defenses while reducing inflammatory markers [63].

In another study with C57BL/6J mice dams exposed to metformin during lactation, their offspring exhibited a lean phenotype, reduced glucose and insulin levels, and improved glucose/insulin metabolism, particularly in males. Females did not show significant alterations. Intriguingly, the offspring of metformin-exposed dams demonstrated protection from metabolic diseases when challenged with an adult high-fat diet, with varying responses observed in males and females [64].

Hafner et al., study in female mice explored the effects of metformin administered during lactation alongside a high-fat diet (HFD). Their findings showed that 21-day-old pups exposed to metformin during lactation from HFD mothers had similar weights to controls. At 2 months, insulin levels were consistent across groups, and at 6 months, there was a reduction in visceral adiposity. While there were no significant changes in the Insulin Tolerance Test (ITT), non-esterified fatty acid (NEFA) levels, or lipolysis suppression, a trend toward normality was observed [65].

The metformin appears to be a well-tolerated drug during lactation, with a lack of reported adverse effects in both observational human and experimental animal studies. However, the limited amount of metformin passing into breast milk suggests it may not exert

significant effects on breastfeeding infants. While observational studies in humans do not provide evidence of long-term effects, rodent experiments, whether involving metformin exposure through breast milk or direct administration to offspring, show programming effects in adulthood, including improved metabolic parameters and reversal of obesity-related changes in the reproductive system.

We believe that the adverse effects are not related to the organism's exposure to metformin during this developmental phase but are directly associated with variables such as animal models, dosages, and routes of exposure. In this regard, we dare to consider that metformin administration programs the organism by enhancing metabolism and preventing the development of obesity and related comorbidities in adulthood.

3.2 Metformin exposure during pregnancy and lactation

A study involving mice offspring exposed to metformin during pregnancy and lactation after being induced into obesity through an HFD diet showed that at weaning, maternal body weights in the HFD plus metformin group were higher than the control group, with no differences in serum insulin and triglyceride levels [66]. In contrast, in another study using Sprague-Dowley rats, mothers in the HFD plus metformin group had lower body weight during late pregnancy and weaning, along with reduced plasma triglycerides, cholesterol, and insulin levels [67].

Regarding the birth weight of the mice offspring, they had a higher birth weight compared to the control group. However, throughout the experiment, the pups of mothers in the HD plus metformin group showed a decrease in weight gain [66] In the study with the offspring of Sprague-Dowley rats, metformin effectively restored AMP-activated protein kinase (AMPK) and reduced peroxisome proliferator-activated receptor (PPAR-gamma) protein expression in offspring from high-fat diet (HFD) mothers. Maternal metformin lowered leptin levels in 60-day-old male offspring and reduced triglycerides and cholesterol in both genders at 21 days but not in adulthood. Metformin also positively influenced myogenic regulatory factors (MRFs) affected by HFD in adult male and female offspring.

Maternal metformin decreased mitochondrial biogenesis genes (*Ppargc1a* and *Tfam*) without affecting *Nrf1* expression. Maternal HFD increased mitochondrial dynamics genes (*Drp1* in adult males, *Drp1* and *Mfn2* in both genders), but metformin restored

balance [67]. AMPK controls skeletal muscle metabolism and energy development by improving insulin signaling, inhibiting adipogenesis, and preventing insulin resistance [19]. PPAR-gamma is vital for adipogenesis, thus elevated PPAR-gamma in the muscles of HFD offspring signals increased adipogenesis [68].

Novi et al., investigated the effects of exposure to metformin during pregnancy and lactation in rats, comparing the METGL group (metformin during pregnancy and lactation) with the METG group (metformin only during pregnancy) and their respective controls. The results of this study indicate that METG group and METGL group did not affect the birth weight, body weight, or blood pressure of the offspring. However, METG group reduced litter size, and removal of the endothelium increased the contractile response to phenylephrine in isolated aortic rings, regardless of the period of exposure. [69, 70]

Song et al., study focused on maternal HFD diet and concurrent metformin exposure during pregnancy and lactation. Results showed reduced body weight, food intake, and visceral fat in metformin-treated HFD-fed mothers. Their offspring had lower body weight and retroperitoneal fat at weaning, with normalized body fat at 16 weeks. Metformin restored intestinal epithelial cell integrity and mitigated inflammatory gene expression. It also impacted the offspring's gut microbiota composition, reducing species diversity but increasing beneficial microbes. These findings suggest long-term effects of maternal metformin treatment on offspring metabolism and microbiota [71].

The continuous metformin use during pregnancy and lactation, especially in individuals at risk of obesity, exerts notable impacts on metabolic programming. Evidence from human observational studies and experimental rodent research underscores enhancements in glucose and lipid metabolism, alterations in the gut microbiome, and regulation of inflammatory cytokines linked to adult-onset obesity induced by high-fat diets. These findings confirm that metformin has a protective effect in metabolic programming, preventing the development of dysfunctions related to glucose metabolism and the onset of obesity and its complications.

3.3 Metformin use during adolescence

Adolescence involves significant changes in body composition, metabolism, cognition, and behavior, driven by the reactivation of the hypothalamic-pituitary-gonad (HPG) axis

[72]. Hormonal shifts, including increased Growth Hormone (GH) and insulin-like growth factors I and II (IGF I/II) secretion, lead to insulin resistance, altered glucose metabolism, and enhanced fatty acid oxidation. Puberty also activates the hypothalamic-pituitary-somatic (HPS) and hypothalamic-pituitary-adrenal (HPA) axes, influencing the release of GH, adrenocorticotropic hormone (ACTH), testosterone, and estradiol [72]. Additionally, puberty prompts neural pathway maturation, impacting energy balance regulation via processes like neurogenesis and epigenetic events [73].

The research aims to uncover the potential impact of administering metformin during adolescence on long-term health outcomes. Adolescence is a crucial period, as it involves significant physiological changes and is susceptible to environmental and chemical influences that can shape future health. Considering metformin's effects on controlling food intake, improving glucose tolerance, and reducing insulin resistance, along with the metabolic challenges associated with childhood obesity and the increased risk of developing type 2 diabetes, it is essential to investigate the use of metformin during adolescence and its potential long-term consequences in adulthood, yet such studies remain elusive.

For instance, a study involving obese adolescents treated with metformin for 48 weeks, the initial reduction in body mass index was eventually reversed after treatment cessation, and there were no observed effects on HOMA-IR, fat mass, lean mass, or cholesterol levels [74]. However, the effects of metformin were more enduring in girls with low birth weight and precocious pubarche, persisting for up to 5 years [75]. During this extended period, metformin continued to reduce fat mass, increase lean mass, and modify endocrine-metabolic profiles in these individuals [75]. No experimental investigations were identified pertaining to adolescence as a pivotal developmental stage and the potential programming effects of metformin.

The short-term effects of metformin on glycemic control, insulin resistance, glucose tolerance, and obesity stimulate research into long-term programming. The DOHaD concept and prior research on pregnancy and lactation windows have demonstrated long-term benefits of metformin. Childhood obesity, a programmed condition, leads to hypertension, type 2 diabetes, and cardiometabolic diseases. Adolescence is a crucial phase for potential metformin treatment to reverse programmed childhood metabolic disorders. Therefore, it is of paramount importance that investigations in this context be conducted.

4. Strength and weakness

One of the strengths of our narrative review lies in its comprehensiveness, surpassing the constraints of a systematic review by not being confined to predetermined inclusion criteria. Consequently, this narrative review stands as a valuable resource for elucidating the impacts of metformin within the context of metabolic programming, particularly considering the drug's chronic usage. Nonetheless, potential biases may exist, such as those associated with the author's discretion in selecting and interpreting literature. It is important to note that we did not identify any apparent limitations or conflicts of interest in this current narrative review.

5. Conclusion

In summary, metformin is not only a well-established tool in the treatment of type 2 diabetes and obesity-related disorders, it also proves to be a powerful ally in metabolic programming when administered during crucial stages of development. This scientific review, by combining evidence from human studies and animal models, illustrates that metformin acts as a guardian, shielding the body from metabolic dysfunctions that could arise in adulthood. This discovery broadens the scope of metformin as a therapeutic intervention that transcends conventional applications. It presents us with the exciting prospect of an early intervention capable of generating long-term positive impacts, sculpting a healthier metabolism and mitigating metabolic complications that might otherwise afflict individuals in adulthood. Ultimately, this scientific review underscores metformin as an invaluable ally in promoting metabolic health throughout life, highlighting its potential not only as a treatment but also as an essential prevention strategy.

Author agremment

The final version of the submitted manuscript was reviewed and approved by all authors. They confirm that the article is an original work and has not been previously published. They also state that it is not being submitted for publication in another journal.

Author contributions

Milene Aparecida Bobato carried out the bibliographic search and wrote the text. Paulo Cezar de Freitas Mathias made the final adjustments. Each of the authors carefully read the journal's authorship agreement, and the manuscript was reviewed and received unanimous approval from all authors.

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Second Manuscript:

Manuscript 2 – Experimental Article

Metformin Administration During Lactation Mitigates Hyperphagia and Preserves Beta Cell Structure in Early Overfed Rats.

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Metformin prevents hypertrophy in β-cells

Keywords: metformin, lactation, overfeeding, β-cells

ABSTRACT

Overfeeding in childhood increases the risk of chronic non-communicable diseases in adults, such as diabetes, obesity and cardiovascular disease. The DOHaD, or metabolic programming, concept suggests that interventions during key developmental stages (pregnancy, lactation, childhood, and adolescence) can alter metabolic pathways, potentially leading to chronic disease or lifelong health. Metformin plays a crucial role in mitigating these metabolic changes resulting from obesity. Wistar rats reared in small litters (SLS, 3 pups/dam) and normal litters (NLS, 9 pups/dam) were employed as models of early overfeeding and normal feeding, respectively. Over the initial 12 days of lactation, animals in the SL and NL groups received metformin (M) therapy, whereas control subjects were administered saline (S) injections. Metformin treatment yielded reductions in food intake, attenuated body weight gain, and lowered triglyceride and cholesterol levels in both juvenile and adult rats. These animals displayed enhancements in fasting blood glucose, improved glucose tolerance, and heightened insulin sensitivity at both the weaning and adult stages. Furthermore, a marked decrease in visceral fat deposits was evident in adult rats. Notably, the treated SL group exhibited reduced pancreatic islets area in both infant and adult rats. The results indicate that metformin administered during lactation prevented hyperphagia and β-cells hypertrophy in early overfed animals, suggesting a metabolic programming effect that inhibits the development of obesity-related comorbidities, such as type 2 diabetes mellitus.

INTRODUCTION

The trajectory of childhood growth and development is critical to metabolic health later in life. In particular, accelerated childhood growth is associated with an increased risk of developing non-communicable chronic diseases in adulthood, such as diabetes, obesity and cardiovascular disease (Baird, et al. 2017; Barker 1995; Gluckman, et al. 2008; Korner, et al. 2019). This rapid growth is primarily mediated by high caloric intake and overeating (Singhal, et al. 2010).

These observations in humans can be reproduced in various experimental animal models. In rodents, accelerated neonatal growth can be induced by reducing litter size at birth (Habbout, et al. 2013; Plagemann, et al. 1999a). Rats reared in small litters (2-4 pups per female) during lactation exhibit neonatal overfeeding, due to less competition for food, leading to rapid weight gain and later development of metabolic diseases such as impaired glucose tolerance, insulin resistance, obesity and cardiometabolic dysfunctions (Parra-Vargas, et al. 2020). Understanding the biological mechanisms involved, as well as the means to prevent the harmful effects of neonatal overfeeding on health, are important challenges for scientific research.

The DOHaD concept "Developmental Origins of Health and Disease"; also known as metabolic programming or metabolic imprinting, argues that interference occurring in specific stages of development, such as pregnancy, lactation, childhood and adolescence, are capable of promoting changes in metabolic pathways, favoring the emergence of chronic diseases or preventing these changes promote health throughout life (Charles, et al. 2016).

Organ development in humans and rodents largely occurs during the gestational period. However, in rodents, the processes of proliferation, differentiation and maturation can only be concluded during lactation, which predisposes the newborn to a situation of

vulnerability, which makes this phase a critical phase of development (Rodríguez-González, et al. 2020; Zambrano and Nathanielsz 2013).

Pharmacological interventions can produce important responses in the health of a developing organism. Metformin, an antidiabetic drug from the biguanide class, is the most prescribed drug for type 2 diabetes mellitus (DM2) and gestational diabetes (GD) commonly used by mothers during the lactation period (Briggs, et al. 2005). Its main pharmacological hypoglycemic effect is mediated by the decrease in hepatic gluconeogenesis, but improvement in insulin sensitivity, lipid profile, body weight control and inflammation has been observed (He 2020).

Previously, our group shows that metformin when administered during lactation in overfed rats caused improvement in insulin sensitivity and glucose tolerance as well as attenuated obesity, a preponderant factor for the development of metabolic alterations. Regarding insulin secretion, the results demonstrate that rats from litter reduction and metformin treated have a decrease in insulin secretion at postprandial levels compared to their control (Previate, et al. 2020).

During the progression of glucose intolerance and insulin resistance, β-cells exhibit an adaptive capacity by increasing cell proliferation and insulin secretion, therefore, when the demand for insulin can no longer be met by the adaptive process of β-cells, the diabetes develops (Prentki and Nolan 2006; Weir, et al. 2001). Pancreatic β-cells in an obese animal model are hyperplastic and secrete up to 3 times more insulin in the basal state (Paulsen, et al. 2010). On the other hand, studies show that metformin is capable of inhibiting apoptosis in β-cells when they are under lipotoxicity, exerting a protective condition (Dai, et al. 2015).

Considering these studies, we hypothesize that metformin improves β-cell function when administered during lactation to early overfed animals, preventing the

formation of hypertrophied β-cells. Therefore, we investigated the effects of metformin treatment on metabolic parameters and immunohistochemistry in both childhood and adulthood animals subjected to early-life overfeeding.

METHODS

Ethical approval

All experimental protocols were performed in accordance with the standards of the Brazilian College of Animal Experimentation (COBEA) and approved by the Ethics Committee of the State University of Maringa (CEUA), protocol no. 1923021018. We understand the ethical principles under which the journal operates, and our work complies with the animal ethics checklist. We have taken all steps to minimize the pain and suffering of the animals.

Animal model and experimental design

Wistar rats were used in this research. The animals were acquired at the central vivarium of the State University of Maringá/Paraná/Brazil. 70-day-old female virgin rats and 80-day-old male rats were housed in the sectoral vivarium for 5 days for adaptation and placed for mating in a ratio of (3:1) remaining together for 2 weeks. On day 1 after birth, day 0 being considered the day of birth, the litters were standardized in 9 pups (4 females and 5 males) preferably. On day 03, with the aim of inducing early overfeeding, some litters were reduced to 3 male rats, giving rise to small litters (SL). The litters composed of 9 animals originated the groups of normal litters (NL). At day 1, the pups received either an intraperitoneal (I.P.) injection of metformin (Pharmakon - compounding pharmacy, Maringá/Paraná, Brazil) [M; 100 mg (kg bw) −1 day−1] until

12th day of lactation or saline solution (S; NaCl, 0.9%, I.P.) for the same period. The administered dose of metformin was determined based on a dose-response evaluation, where the adopted drug concentration did not induce mortality or observable toxic effects. All puppies in a litter received the same treatment Thus, the offspring were distributed into four groups: reduced litter rats that received saline solution (SLS), reduced litter rats that received metformin (SLM), normal litter rats that received saline solution (NLS) and normal litters that received metformin (NLM). The pups were weaned on the 21st. After weaning, with the aim of standardizing the animals' growth environment, 3 animals from both the SL and NL groups were kept in the box.

Studies show that male and female rats have some different long-term responses to overnutrition, with males being more susceptible to the effects of reduced litter size (Argente-Arizón, et al. 2016), we used only males in this study.

Throughout the protocol period, all animals were housed under controlled conditions on a 12-hour light-dark cycle (lights on from 7:00 am to 7:00 pm) at a temperature of 21 ± 2 °C. Water and standard rodent chow (NuVital®, Curitiba, PR, Brazil) were provided *ad libitum.*

Metabolic and biometric parameters

Body weight was assessed at 07, 14, 21 and 90 days of age (n = 8-12 rats from three to four different litters). Food consumption was evaluated once a week from weaning to 90 days of age. Absolute food intake was calculated as the difference between the total food provided 1 week before (Dinitial) and the amount of food remaining (Dfinal), divided by the number of rats per cage: [food intake (in grams) = (Dinitial − Dfinal)/3] (de Oliveira, et al. 2018; Vicente, et al. 2004). Relative food intake was calculated as the value of absolute food intake divided by the mean body weight of the

three rats in each cage. The area under the curve (AUC) of the entire observation period was also calculated for food intake measurement. Under general anaesthesia [thiopental, Thiopentax, Cristalia, Sao Paulo, Brazil, 45 mg (kg bw) −1, I.P.], rats from both groups were killed by decapitation at 90 days of age. Subsequently, blood samples were collected and centrifuged (5040 g for 5 min) to obtain the plasma, which was stored at −20◦C for further biochemical analysis. The retroperitoneal, perigonadal and mesenteric fat pad stores were harvested (n = 12 rats from four different litters), and the weight of the fat pads was expressed in relationship to the body weight of each animal (in grams per 100 g bw).

Milk consumption

Assessment of milk ingestion by rat-offspring was adapted of previous study (Bautista et al., 2019). At 6th, 11th and 16th days of lactation, rat offspring were fasted by 4 hours, by separation from their respective mothers. After that, rats were weighed and returned to the cages to suckling per a period of 1 hour. The difference between rat's weight after- and before-suckling time-point was considered as the amount of milk ingested (per grams) by each on of rats. The values of milk ingested was related with the percentage of the rats' body weight, considering the body weight obtained after the suckling period, as follow: milk ingestion = [(rat's body weight (after-suckling)) – (rat's body weight (before-suckling))/rat's body weight (after-suckling) x 100].

Intravenous glucose tolerance test

At 21 days old, the group of pups (n=12 from four different litters) underwent an intraperitoneal glucose tolerance test (ipGTT). For this, after 6 hours of fasting, the

animals were weighed and then blood was collected from the tail to obtain fasting blood glucose (time 0) with the aid of a glucometer (FreeStyle OptimumH®, Abbott Laboratories). Afterwards, the animals received an intraperitoneal injection of glucose at a concentration of 2g/kg of body weight. Glycemia will be checked at times 15, 30, 60 and 120 min after glucose administration.

At 90 days, all animals (n=12 from four different litters) underwent the intravenous glucose tolerance test (ivGTT). For this, they were weighed and anesthetized with a Ketamine/Xylazine mixture (75mg + 15mg/kg bw) for the implantation of a silicone cannula in the right jugular vein (Previate et al. 2020). After a 12-hour overnight fast, the animals received an intravenous glucose load (1 g/kg bw). Blood samples (300-350 μL) were taken through the cannula at the following times: 0 (before the glucose injection), 5, 15, 30 and 45 minutes after the infusion of the glucose load. After each blood collection, an equivalent volume of saline solution (NaCl, 0.9%) was administered intravenously. Plasma glucose was measured using the enzymatic-colorimetric method (GoldAnalisa®; Belo Horizonte, Brazil). The color reagent was added to plasma samples and incubated for 10 min at 37°C in a water bath for plasma glucose determination. The absorbance readings of the samples were performed in a spectrophotometer (Semi-automatic biochemical analyzer, BIO 200FL, Bio Plus®, São Paulo/SP, Brazil). Test AUCs were calculated.

Insulin tolerance test (ITT)

At 21 days and 90 days of age, part of the animals in each group underwent an insulin tolerance test (1 U/kg of body weight, i.p.) with human recombinant insulin (Eli Lilly®, São Paulo, Brazil), 2% v/v (2mL/100mL), diluted in saline solution (0.9% NaCl). Blood samples were collected through a small cut at the tip of the tail and blood glucose

measured using a glucometer (FreeStyle OptimumH®, Abbott Laboratories), at times 0 (baseline); 5, 15, 30, 45, and 60 minutes after insulin injection.

Blood glucose decay rate constant (kITT)

The kITT was calculated using the formula 0.693/(t1/2). Blood glucose t1/2 was calculated from analysis of the slope angle of the straight line of plasma glucose concentrations during the linear phase of glycemic decline during the ITT (Lundbaek 1962). The result is given as kITT (%)/min.

Total cholesterol and triglycerides

Total cholesterol and triglyceride levels were measured in plasma samples using a colorimetric method and commercial kits (GoldAnalisa; Belo Horizonte, MG, Brazil).

Immunostaining and morphometric analysis of the pancreas

Pancreatic tissue samples obtained from rats at the ages of 07, 14, 21, and 90 days were initially fixed in 10% formalin solution, dehydrated, and subsequently embedded in paraffin for histological analysis. The fixed samples were then sliced into 5-µm-thick semi-serial sections using a microtome. Following deparaffinization, the sections underwent rehydration and were subjected to blocking to inhibit endogenous peroxidase activity, accomplished with a 3% H2O2 solution. Subsequently, the sections were rinsed in 0.01 M phosphate-buffered saline (PBS, pH 7.4) and incubated in 10% non-immune goat serum (Histostain-Plus®, Invitrogen) for 10 minutes. After the blocking step, the sections were exposed to an anti-insulin monoclonal antibody, diluted at a ratio of 1:500 (Sigma®, St. Louis, MO, USA), for 1 hour at room temperature. Following a PBS wash

(0.01 M), specific biotinylated secondary antibodies (Histostain-Plus®, Invitrogen) were applied to the sections for 10 minutes. Subsequently, the sections were incubated with streptavidin-peroxidase enzyme conjugate (Histostain-Plus®, Invitrogen) for 10 minutes and then subjected to two 5-minute washes. The detection of streptavidin-biotin complexes was achieved using a chromogenic diaminobenzidine solution (Histostain-Plus® Invitrogen), and counterstaining with hematoxylin was performed for 15 seconds.

Statistical analysis

The results are expressed as the mean ± SEM and were subjected to a D'Agostino Pearson normality test to assess the Gaussian distribution. Data were subjected to a variance analysis by two-way ANOVA to determine the effect of different factors: litter size, treatment or their interaction (litter size and treatment) followed by Tukey's post hoc test. A P-value <0.05 was considered statistically significant. Analyses were performed using GraphPad Prism v.7.0 for Windows (GraphPad Software, Inc., San Diego, CA, USA).

Results

Effects of metformin on milk consumption.

The Figure 1 show that the litters that underwent a reduction in the number of pups, SLS, consume more milk on the 6, 11 and 16 days of life compared to the control litter. However, the treatment with metformin in this period reduces milk consumption in SLM animals. An interaction between litter size and treatment is observed on milk consumption at all observed ages.

Effects of metformin related to body weight, fasting glucose, triglycerides and cholesterol in puppies.

In the Table 1, at 07, 14 and 21 days of life, the SLS group has higher body weight compared to their control ($p=0.0049$; $p<0.0001$; $p=0.0007$), but the treatment had no effect in the SLM group compared to the untreated group in these periods. About glycemia, it is possible to observe an interaction effect between the litter and the treatment factors. The SLS group had higher fasting glucose levels compared to the NLS group ($p<0.0001$), however, treatment with metformin during lactation resulted in a glucose decrease in the SLM group compared to their control in all ages ($p< 0.0001$). Triglycerides and total cholesterol were also analyzed. Triglycerides and total cholesterol are increased in the SLS group compared to their control ($p<0.0001$). When analyzing the treatment with metformin, the treated group, SLM, presented a decrease triglyceride at 07,14, and 21 days ($p<0.0001$) and on 14 and 21 days of life regarding total cholesterol ($p<0.0001$). At 07 days of life, total cholesterol analyzes show no effect of metformin treatment in the treated group, SLM compared to its control. The analyzes of triglycerides and total cholesterol show an interaction effect between the litter and treatment factors only with exception for total cholesterol at 07 days of life.

Effects of metformin on glucose tolerance and insulin sensitivity in puppies.

During the IpGTT at 21 days of life, (Figure 2A; AUC), the SLS animals show lower glucose tolerance compared to their control ($p<0.01$; AUC). An interaction between litter size and treatment on glucose concentration during ipGTT is observed. SLM

animals that were treated with metformin during lactation showed better glucose tolerance compared to their controls ($p<0.01$; AUC). These results were accompanied by a change in insulin sensitivity. The kiTT (Figure 2C) was affected by litter size, treatment and the interaction between these two factors. As expected, animals reared on reduced litters, SLS, exhibit insulin resistance compared to the normal litter group, NLS ($p<0.0001$). On the other hand, the SLM group submitted to intraperitoneal treatment with metformin during lactation showed greater insulin sensitivity ($p<0.0001$) compared to the SLS.

Effects of metformin on weight gain, relative food intake, visceral adipose, fast glucose, triglycerides and total cholesterol

Regarding weight gain over the 90 days of life, (Figure 3A; $p<0.0001$) as expected, we observed that the SLS animals showed greater body weight gain compared to their control, however the treatment with metformin in the SLM animals reduces this parameter compared to untreated animals ($p<0.05$). This result is confirmed by the analysis of the final weight at 90 days of age (Figure 3C). When checking the relative consumption of these animals (Figure 3D, we found that SL animals have a higher consumption in (g/100g) compared to their control ($p<0.05$). However, it is possible to observe that treatment with metformin in SLM animals reduced the relative consumption compared to animals that received salina, SLS ($p<0.01$).

The weight of mesenteric fat (Figure 3F), perigonadal fat (Figure 3G) and retroperitoneal fat (Figure 3H) were affected by litter size and treatment determined by an interaction effect. SLS animals showed greater weight of mesenteric, perigonadal and retroperitoneal fat compared to their NLS control ($p<0.0001$). Metformin treatment

during lactation in SLM group reduced all three fat stores when compared to untreated SLS animals ($p<0.0001$).

Investigations regarding glucose dosage (Table 2) showed that the SLS group had higher fasting glycemia when compared to the control group ($p<0.0001$). It is possible to verify an interaction between the bed and the treatment factors in this context. However, the group indicated to treatment with metformin during lactation, SLM, presents a reduction in these values when compared to the untreated group ($p<0.0001$). Also in Table 2, the results referring to triglycerides and total cholesterol can be observed. Both parameters show an interaction between bedding and treatment factors. Both triglycerides and total cholesterol are elevated in animals from the SLS group when compared to the NLS group ($p<0.0001$). However, when observing the animals in the group treated with metformin during lactation, SLM, both parameters are reduced when compared to the control group. ($p<0.0001$).

Effects of metformin on glucose tolerance and insulin sensitivity in adult rats.

An interaction between litter size and treatment is observed on glucose concentration during ivGTT. (Figure 4A; 4B-AUC). The SLS animals showed lower glucose tolerance compared to their NLS control ($p<0.0001$). However, SLM animals that were treated with metformin during lactation improved this parameter considerably when compared to their control ($p<0.0001$; AUC). These results accompany a clear improvement insulin sensitivity as shown in Figure 4C and 4D ($p=0.0594$). The kiTT was affected by the interaction between litter and treatment factors. As expected, animals reared on reduced litters, SLS, exhibit insulin resistance compared to the normal litter group, NLS ($p<0.01$) whose litters received saline. On the other hand, the SLM group

submitted to intraperitoneal treatment with metformin during lactation showed a tendency of insulin sensitivity (p = 0.0594) compared to untreated animals.

Pancreatic morphology

The analysis of islet area revealed interesting results in the current study. During the first 7 and 14 days of life, no significant changes were observed between groups. However, a significant increase in this parameter was observed at 21 and 90 days of age in animals subjected to early overfeeding, SLS group (Figure 5C and 5D). On the other hand, when examining the group treated with metformin during lactation, SLM group, a reduction in the size of the islets compared to the untreated group, SLS, was observed over the same time period (Figure 5C, p= 0.0019 and Figure 5D, p=0.0003). An interaction between litter and treatment factors was also observed (Figure 5C, p= 0.0171 and Figure 5D, p= 0.0043).

When evaluating the imunodensity of islets, it was observed an interaction between factors litter size and treatment at days 7 and 21 (Figure 5E and 5G, p=0.0160; p= 0.0275) and litter size effect on results at 07 and 14 days of life (Figure 5E and 5F, p= 0.0416; p=0.0076), however, no differences was observed between the groups (Tukey's post hoc test). At 90 days of life, no changes were observed between the groups (Figure 5H). Representative images can be found in supplementary figure 1 and 2.

Figures

Figure 1

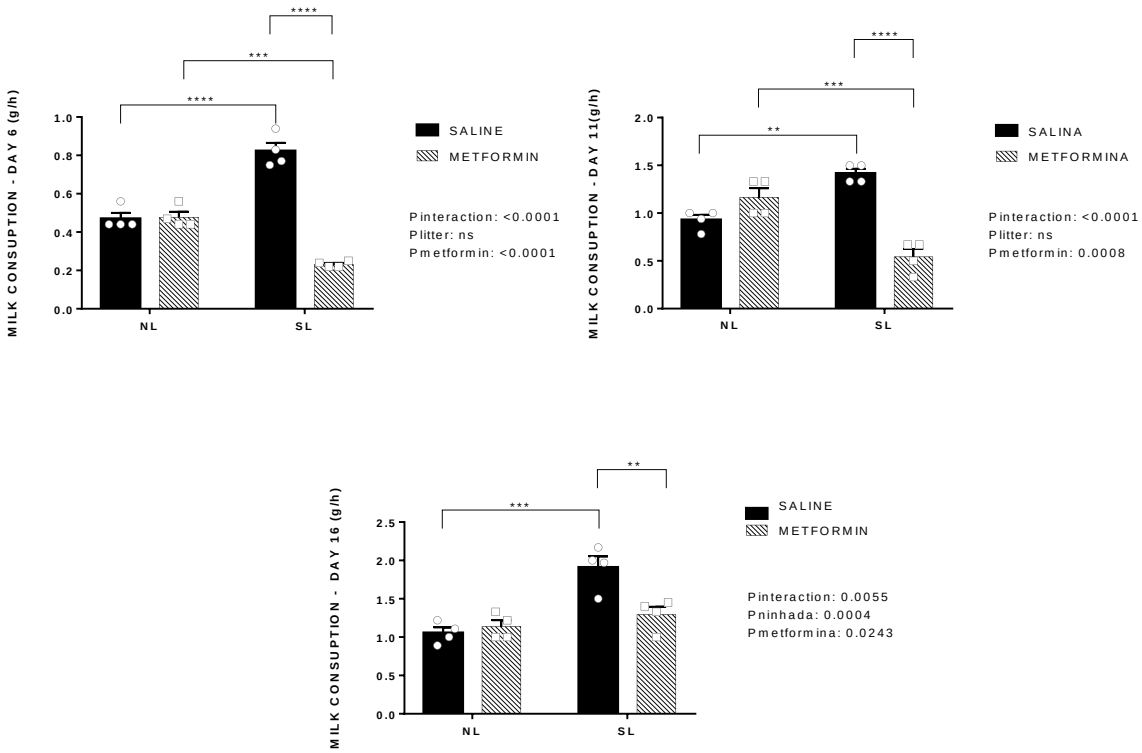


Figure 1 – Milk consumption on the 6sixth day of life, day of life (B), and sixteenth day of life (C). Data are presented as mean ± standard error of the mean of four litters per group. Analyzes performed by two-way Anova based on factors treatment, litter and interaction between factors. $p<0.05$ was considered statistically significant. **$p<0.01$, ***$p<0.001$, ****$p<0.0001$.

Figure 2

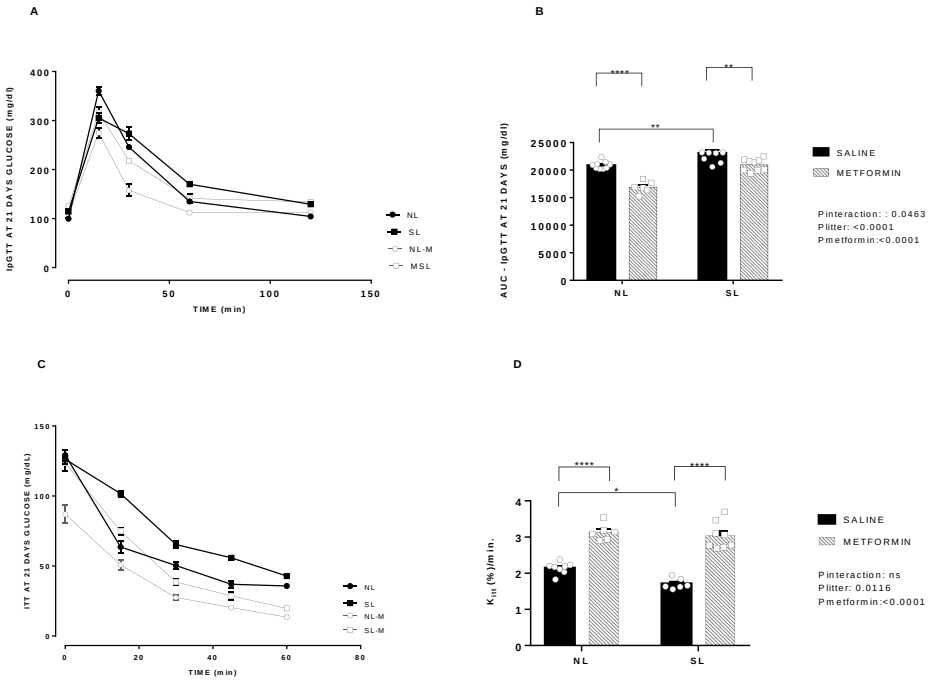
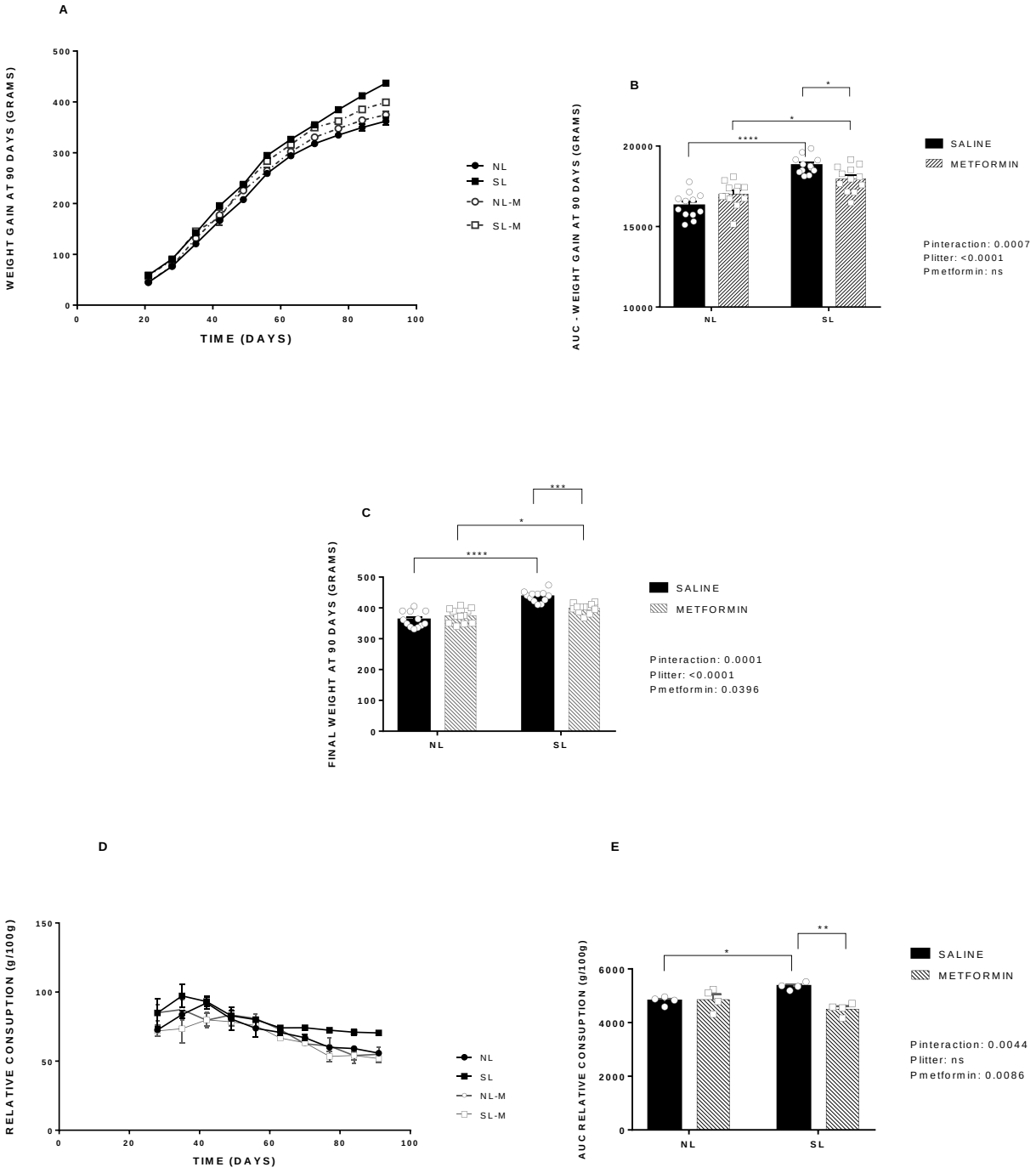


Figure 2 – Intraperitoneal Glucose Tolerance Test, ipGTT (A), Intraperitoneal Insulin Tolerance Test, ipITT (C) and Blood Glucose Decay Rate Constant, -kITT (D) of pups at 21 days. Data are presented as mean ± standard error of the mean of 12 pups from at least 4 litters per group. Analyzes performed by two-way Anova based on factors treatment, litter and interaction between factors. $p<0.05$ was considered statistically significant. In (B) the bar graph represents the ipGTT AUC. *$p<0.05$, **$p<0.01$, ****$p<0.0001$.

Figure 3


A
WEIGHT GAIN AT 90 DAYS (GRAMS)
TIME (DAYS)
NL
SL
NL-M
SL-M
B
AUC - WEIGHT GAIN AT 90 DAYS (GRAMS)
SALINE
METFORMIN
Pinteraction: 0.0007
Plitter: <0.0001
Pmetformin: ns
C
FINAL WEIGHT AT 90 DAYS (GRAMS)
SALINE
METFORMIN
Pinteraction: 0.0001
Plitter: <0.0001
Pmetformin: 0.0396
D
RELATIVE CONSUPTION (g/100g)
TIME (DAYS)
NL
SL
NL-M
SL-M
E
AUC RELATIVE CONSUPTION (g/100g)
SALINE
METFORMIN
Pinteraction: 0.0044
Plitter: ns
Pmetformin: 0.0086

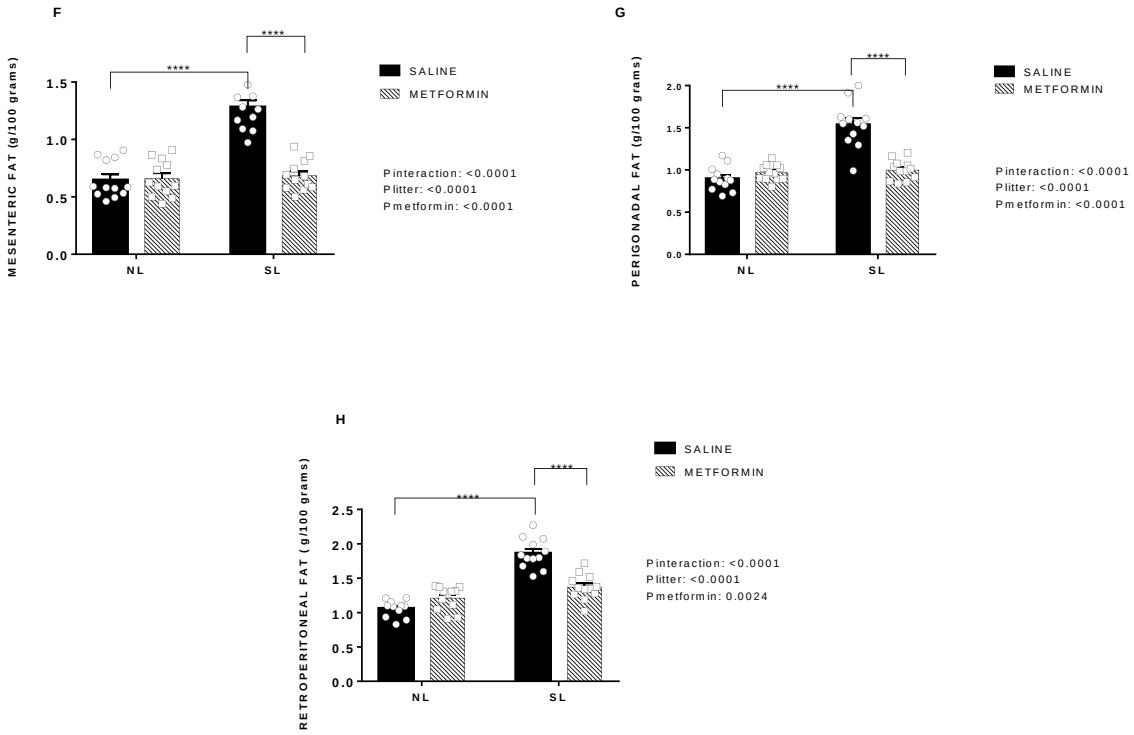


Figure 3 - Evolution of body weight gain (A), Final weight at 90 days (C), Relative consumption (D), mesenteric fat (F), perigonadal fat (G) and retroperitoneal fat (H) of rats at 90 days. Data are presented as mean ± standard error of the mean of 12 pups from at least 4 litters per group. Analyzes performed by or two-way Anova based on factors, treatment, litter and interaction between factors. $p<0.05$ was considered statistically significant. In (B and E) the bar graph represents the AUC. *$p<0.05$, **$p<0.01$, ***$p<0.001$, ****$p<0.0001$.

Figure 4

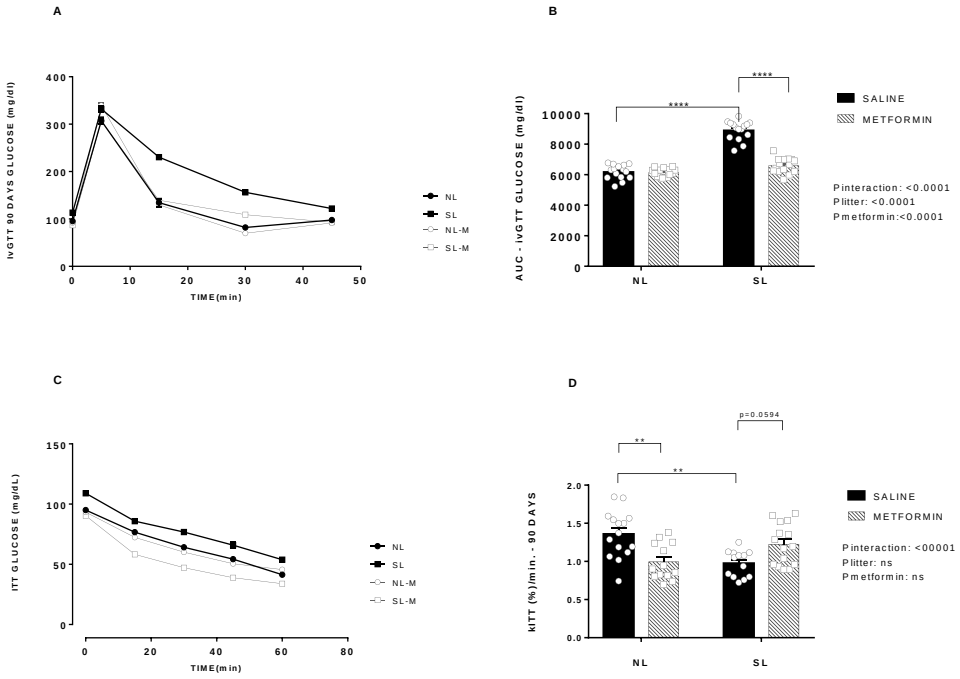
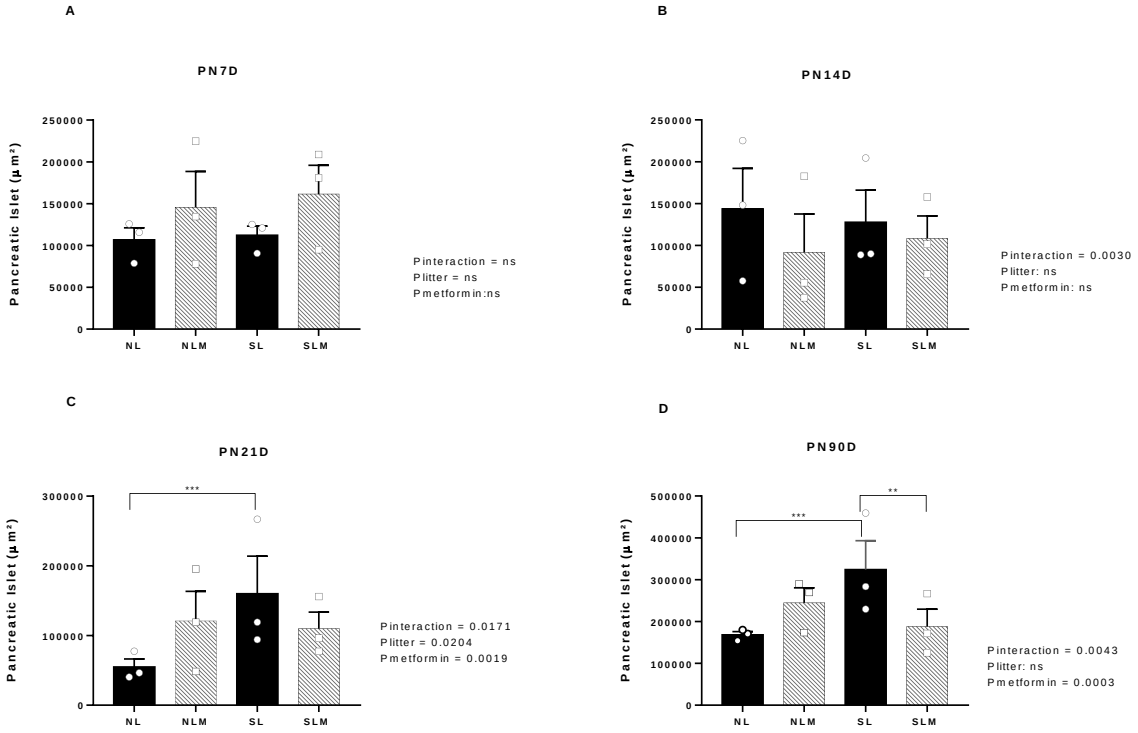


Figure 4 - Intraperitoneal Glucose Tolerance Test, ipGTT (A), Intraperitoneal Insulin Tolerance Test, ipITT (C) and Blood Glucose Decay Rate Constant, -kITT (D) of rats at 90 days. Data are presented as mean ± standard error of the mean of 12 rats from at least 4 litters per group. Analyzes performed by two-way Anova based on factors treatment, litter and interaction between factors. $p<0.05$ was considered statistically significant. In (B) the bar graph represents the ipGTT AUC. **$p<0.01$, ****$p<0.0001$.

Figure 5


A
PN7D
Pancreatic Islet (µm²)
250000
200000
150000
100000
50000
0
NL
NLM
SL
SLM
Pinteraction = ns
Plitter = ns
Pmetformin:ns
B
PN14D
Pancreatic Islet (µm²)
250000
200000
150000
100000
50000
0
NL
NLM
SL
SLM
Pinteraction = 0.0030
Plitter: ns
Pmetformin: ns
C
PN21D
***
Pancreatic Islet (µm²)
300000
200000
100000
0
NL
NLM
SL
SLM
Pinteraction = 0.0171
Plitter = 0.0204
Pmetformin = 0.0019
D
PN90D
***
**
Pancreatic Islet (µm²)
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400000
300000
200000
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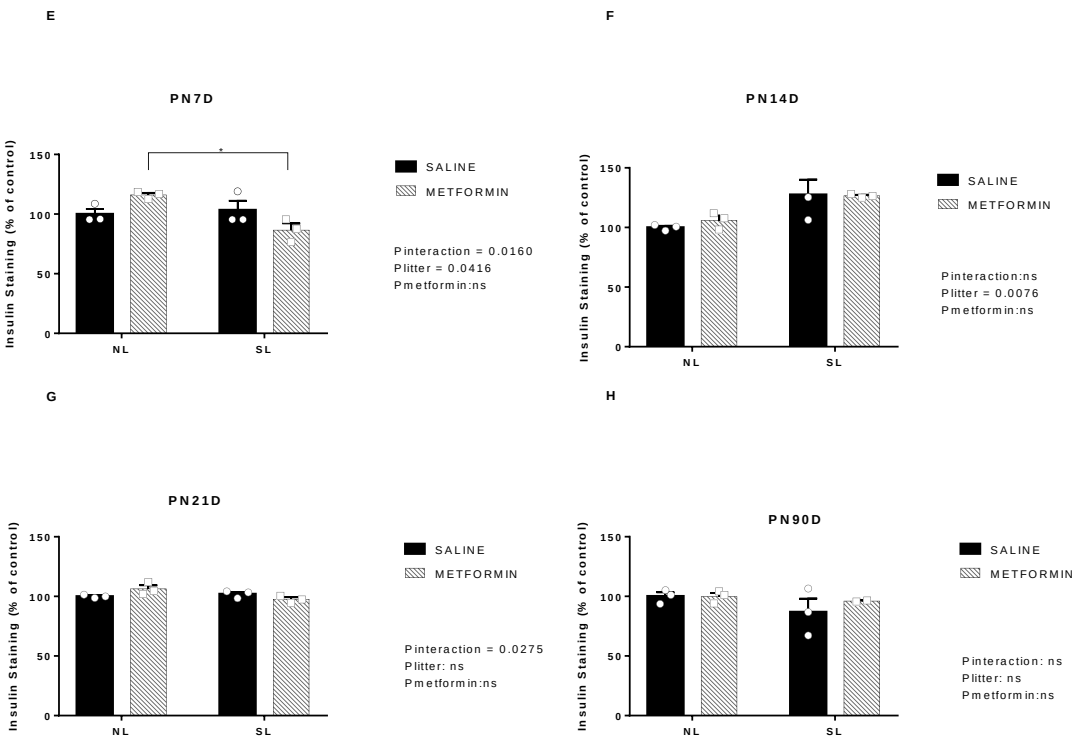

Figure 5 – Pancreatic islet area at 07 (A), 14 (B), 21 (C) and at 90 days of life (D). Pancreatic islet insulin immunodensity at 07 (E), 14 (F), 21 (G), and at 90 days of life (H). Data are presented as mean ± standard error of the means of 3 to 4 pups from at least 4 litters per group. Analyzes performed by two-way Anova based on factors, treatment, litter and interaction between factors. $p<0.05$ was considered statistically significant. *$p<0.05$, **$p<0.01$, ***$p<0.001$, ****$p<0.0001$. Representative images in supplementary Figure 1.

Table 1 - Puppies metabolic parameters

	GROUPS				TWO-WAY ANOVA		
PARAMETERS	NLS	NLM	SLS	SLM	I	L	T
07 DAYS OLD							
GLUCOSE (mg/dl)	99.67±1.06	86.37±4,47	165.90±3.09*	108.87±6.78#$	<0.0001	<0.0001	<0.0001
BODY WEIGHT (grams)	16.93±0.68	16.47±0.62	18.31±0.23	18.46±0,21	ns	0.0049	ns
TRYGLYCERIDES (mg/dl)	57.39±2.20	45.29±3.69*	106.54±1.97*	62.36±1.05#$	<0.0001	<0.0001	<0.0001
TOTAL CHOLESTEROL (mg/dl)	68.45±3.28	68.61±3.44*	94.60±3.00	84.05±2.31#	ns	<0.0001	ns
14 DAYS OLD							
GLUCOSE (mg/dl)	78.50±1.56	78.21±2.59	127.94±2.03*	108.26±2.40#$	<0.0001	<0.0001	<0.0001
BODY WEIGHT (grams)	30.39±0.75	30.35±0.54	40.50±0.93*	38.96±0.48#	ns	<0.0001	ns
TRYGLYCERIDES (mg/dl)	132.60±3.09	113.32±3.30*	187.36±6.09*	120.28±5.20$	<0.0001	<0.0001	<0.0001
TOTAL CHOLESTEROL (mg/dl)	85.88±2.48	82.33±1.42	106.29±1.08*	86.36±2.91$	0.0004	<0.0001	<0.0001
21 DAYS OLD							
GLUCOSE (mg/dl)	91.72±1.45	103.86±2.34*	141.44±4.00*	124.44±2.94$	<0.0001	<0.0001	ns
BODY WEIGHT (grams)	45.15±3.31	45.94±2.15	58.44±0.82*	54.41±0.72	ns	0.0007	ns
TRYGLYCERIDES (mg/dl)	34.86±2.21	37.72±1.59	94.64±1.99*	94.64±1.99$	<0.0001	<0.0001	<0.0001
TOTAL CHOLESTEROL (mg/dl)	52.60±2.31	54.30±2.16	93.30±0.86*	67.47±2.48#$	<0.0001	<0.0001	<0.0001

The data represent the mean ± SEM, n = 12 from four distinct litters of each experimental group at 07, 14 and 21 days old. Small litter animals that received metformin (SLM). The symbols (*) means statistical difference between NLM vs NLS, (#) means statistical difference between SLS vs NLS and ($) means statistical difference between SLM vs SLS based on Tukey's post hoc test. P values are expressed for interaction (I), for litter (L) and for treatment (T). The abbreviation (ns), means no significant difference. This data reflects the parameters based on the two-way Anova analysis.

Table 2 - Adult metabolic parameters

	GROUPS				TWO-WAY ANOVA		
PARAMETERS	NLS	NLM	SLS	SLM	I	L	T
90 DAYS OLD							
GLUCOSE (mg/dl)	92.40±1.98	93.70±2.79	109.50±1.45*	90.30±1.72$	<0.0001	0.0019	<0.0001
TRYGLYCERIDES (mg/dl)	84.07±1.44	84.46±1.69	159.07±2.04*	99.86±2.10#$	<0.0001	<0.0001	<0.0001
TOTAL CHOLESTEROL (mg/dl)	63.07±0.69	67.60±0.77*	76.88±0.92*	53.95±1.42#$	<0.0001	ns	<0.0001

The data represent the mean ± SEM, n = 12 from four distinct litters of each experimental group at 90 days old. The symbols (*) means statistical difference between NLM vs NLS, (#) means statistical difference between SLS vs NLS and ($) means statistical difference between SLM vs SLS based on Tukey's post hoc test. P values are expressed for interaction (I), for litter (L) and for treatment (T). The abbreviation (ns), means no significant difference. This data reflects the parameters based on the two-way Anova analysis.

DISCUSSION

Our studies demonstrate for the first time, that intraperitoneal treatment with metformin during the lactation period had a significant impact on preventing β-cells hypertrophy as well as reducing hyperphagia, in adult rats that were programmed to develop obesity and metabolic dysfunctions since infancy. This set of results suggests that metformin intervention may play a crucial role in preventing the development of DM2 induced malnutrition during early life.

Childhood obesity has been an issue of global concern, as there is a strong correlation between childhood body weight, adult obesity, and metabolic syndrome (Zheng, et al. 2018). Studies indicate that rapid weight gain during the first two years of life can increase the chance, by almost 4 times, of developing overweight and/or obesity in adulthood (Zheng et al. 2018). The experimental rodent model of litter size reduction is capable of mimicking the condition of early postnatal overfeeding, promoting greater susceptibility to obesity and its metabolic disorders throughout life (Plagemann, et al. 1992; You, et al. 1990). In this study, we observed that milk consumption in small litter (SLS) puppies is increased at different times throughout the lactation period. It is known that a normal rodent litter consists of an average of 8-12 puppies. The mother has a smaller number of nipples than the number of puppies in the litter and the amount of milk per nipple also differs and may have nipples that secrete less milk (Parra-Vargas et al. 2020). When reducing the litter, as the animal does not need to compete for food and the number of nipples is greater than the number of puppies, the food supply increases (Peckham, et al. 1979). A puppy can drink about 1.5 times more milk than a puppy from a standard litter (Cunha, et al. 2009). Another preponderant factor is characterized by the change in the composition of the milk offered to puppies. Mothers from small litters have milk with increased lipid content and reduced protein content (Cunha et al. 2009). Thus, puppies

from litters overfed at the beginning of life show accelerated body weight gain and increased adiposity before weaning, with body weight being up to 30% greater than that of puppies from control litters at the time of weaning (Stefanidis and Spencer 2012). During the lactation period, the hypothalamic circuits that regulate energy homeostasis are still developing (Debarba, et al. 2020). Early overfeeding contributes to hormonal changes involving altered responses in the expression of orexigenic and anorexigenic neuropeptides resulting in hyperphagia (Davidowa, et al. 2003; Parra-Vargas et al. 2020). Interestingly, our studies demonstrate that the intervention carried out with the administration of metformin during lactation prevented exacerbated milk consumption in animals from litter reduction, SLM. This effect directly implicates the development of the obese phenotype that results in the metabolic syndrome observed in this model.

Metformin is capable of crossing the blood-brain barrier, which implies modulating the activities of the central nervous system (Łabuzek, et al. 2010). In a study using rat hypothalamic cell culture, metformin inhibited the activation of AMP-activated protein kinase (AMPK), which decreased the expression of neuropeptide Y (NPY) and consequently its orexigenic activity (Chau-Van, et al. 2007). Another study using intracerebroventricular administration of metformin demonstrated an anorexigenic effect through the negative regulation of AMPK induced by the ghrelin signaling pathway (Stevanovic, et al. 2012). Lee et al. (2012), when analyzing the effect of metformin on food intake, propose that metformin appears to be in part related to the phosphorylation of Signal transducer and activator of transcription 3 (STAT3) (Lee, et al. 2012). STAT3 is activated by phosphorylation through tyrosine kinase activity associated with autophosphorylation of the leptin receptor (Shek and Scarpace 2000). Increased phosphorylated STAT3 leads to increased Pro-opiomelanocortin (POMC) and allows appetite suppression (Scarpace, et al. 2001). These analyzes allow us to verify that studies

that aim to understand the mechanism by which metformin decreases food intake, show that the inhibition of AMPK in hypothalamic centers can produce this effect, but that there are also AMPK-independent pathways showing the direct action of metformin in molecular mediators involved with satiety. Our result infers that treatment with metformin decreases food intake at the beginning of treatment. These effects persist throughout development until adulthood. We can state that there is a programming effect, which prevents these animals from developing hyperphagia, a preponderant factor in the development of obesity, but the possible molecular pathways involved in this effect have not been analyzed.

Peripheral and central insulin resistance, central leptin resistance, glucose intolerance, increased food intake, increased visceral fat stores as well as high serum triglyceride and cholesterol levels are characteristics of animals programmed for obesity through overfeeding during lactation that they can be observed early in life and persist into adulthood (Argente-Arizón et al. 2016; Bei, et al. 2015; Conceição, et al. 2017; Davidowa and Plagemann 2007; Moreira, et al. 2009; Plagemann, et al. 1999b; Sominsky, et al. 2017).

Studies investigating the effects of metformin treatment on metabolic parameters at the end of treatment are widely documented in the literature (de Oliveira Santana, et al. 2016; Geerling, et al. 2014; Huang, et al. 2017; Lee and Morley 1998; Portela, et al. 2015). In particular, studies looking at the effects in puppies weaned from dams given metformin during lactation in conjunction with a high-fat diet, or in adult offspring of control dams on a high-fat diet, show resistance to weight gain, improvements in glucose tolerance and insulin sensitivity (48, 49). Malta et al. (2016) revealed that SL rats, susceptible to metabolic dysfunction, exhibited increased islet count and size, along with increased β-cells density. However, subsequent investigations by Previate et al. (2020), highlighted

the notable influence of metformin administration on lactation in SL animals. the intervention alleviated the hyperinsulinemia observed at weaning, and this effect lasted into adulthood, emphasizing its lasting role in metabolic reprogramming (Malta, et al. 2016; Previate et al. 2020)

In the early stages of type 2 diabetes (T2D), a condition favored by our study model, pancreatic beta cells respond to insulin resistance by increasing their mass (β-cell hypertrophy) and insulin secretion (hyperinsulinemia) (Yoon, et al. 2003). Our research demonstrated that administration of metformin during the lactation period had a significant impact on preventing β-cell hypertrophy.

At birth, in both humans and rodents, the pancreas is in a stage of immaturity. In the case of rodents, pancreatic growth is particularly notable during the first month after birth, reaching its peak between weeks 2 and 4 of the lactation period (Bonner-Weir, et al. 2016). Before weaning, neogenesis from undifferentiated cells contributes substantially to the increase in β-cells mass. As weaning occurs, there is a delicate balance between apoptosis and β-cells proliferation. After weaning, cellular plasticity, pancreatic islet renewal, and a reduction in the β-cells population are observed, emphasizing the critical importance of adequate prenatal and postnatal development of the pancreas (Moullé, et al. 2017; Rankin and Kushner 2009).

When considering the conditions to which the pancreatic β cells of SL animals are exposed during lactation, where they are still in the development phase, it is plausible to suggest that the observed dysfunctions originate from toxicity caused by hyperlipidemia and hyperglycemia.

Hyperlipidemia accompanied by constant hyperglycemia are toxic to pancreatic β cells (Robertson, et al. 2004) . Prentki & Corkey., proposed that at physiological glucose concentrations, excess fatty acids are easily eliminated through mitochondrial oxidation.

Diverging from this point, when glucose and fatty acids are elevated, there is an accumulation of metabolites derived from the acid esterification of fat, which impairs the function of β cells (Prentki and Corkey 1996). Other studies support this hypothesis. When exposing isolated rat islets to palmitate in the presence of high glucose, insulin gene expression is inhibited (Jacqueminet, et al. 2000). Furthermore, when triglyceride synthesis is stimulated in cell culture with a high concentration of glucose, insulin secretion is inhibited (Kelpe, et al. 2002). *In vivo*, when using a hypoglycemic agent in hyperglycemic rats, the triglyceride content of islets decreases while maintaining insulin mRNA levels, a result not observed with the use of a lipid-lowering agent (Harmon, et al. 2001). Hyperglycemic rats on a high-fat diet have impaired insulin secretion compared to normoglycemic rats (Briaud, et al. 2002). Our results show that animals overfed during lactation have increased glucose concentration, insulin resistance and high cholesterol and triglyceride levels from weaning until 90 days of life. When performing immunohistochemistry on the pancreas of this animal using insulin markers, cellular hypertrophy was demonstrated at weaning and in adulthood, thus confirming cellular dysfunction.

Metformin causes apoptosis in rat insulinoma under normal conditions, however when cells are incubated in the presence of palmitic acid, metformin, AICAR (AMPK activator) and compound C (AMPK inhibitor) it is possible to observe that metformin increases autophagy factors and inhibits apoptotic factors demonstrating an involvement of the AMPK signaling pathway in the positive regulation of autophagy and negative regulation of apoptosis (Jiang, et al. 2014).

The β-cell apoptosis can be induced by mediators of endoplasmic reticulum stress generated by lipotoxicity (Akerfeldt, et al. 2008). There is evidence that proteins involved in the incorrect folding of the endoplasmic reticulum (ER) as well as the increase in

reactive oxygen species (ROS) in pancreatic β-cells induce cell death (Back, et al. 2009; Malhotra, et al. 2008; Song, et al. 2008).

Other studies reveal that metformin has different effects depending on the concentration used. At low concentrations, metformin demonstrates the ability to restore insulin secretion, reduce oxidative stress, and improve pancreatic β-cells functions, independently of AMPK activation (Kim, et al. 2019). At higher concentrations, metformin mitigates lipotoxicity, but does not modulate oxidative stress through the AMPK pathway (Simon-Szabó, et al. 2014). Furthermore, also at higher concentrations, metformin demonstrates the ability to attenuate β-cells dysfunctions, but does not influence stress markers in the endoplasmic reticulum (ER) (Jung, et al. 2012).

These findings highlight the complexity of metformin's action and suggest that it may offer protection to pancreatic β-cells at different concentrations and in varying contexts of metabolic stress.

In summary, the results obtained in our investigation are intriguing when considering that animals subjected to overfeeding during infancy exhibit β-cell hypertrophy. However, the administration of metformin during lactation played a highly effective role in preventing the development of pancreatic β-cell hypertrophy during infancy and this effect persisted into adulthood, which suggest that metformin is acting on parameters of protection and metabolic programming. Furthermore, lactational treatment with metformin has demonstrated its efficacy in controlling food consumption, inhibiting hyperphagia, preventing obesity development, and combating insulin resistance. While we cannot precisely define whether metformin acts on central or peripheral metabolic pathways, or both, due to the lack of detailed studies on intrinsic molecular mechanisms, we can conclusively state that metformin attenuated the adverse metabolic programming induced by overfeeding during infancy. These results have

significant implications for the understanding and treatment of metabolic diseases, highlighting metformin as a promising tool in the prevention and treatment of type 2 diabetes mellitus and other conditions associated with childhood obesity.

Supplementary data

Figure 1

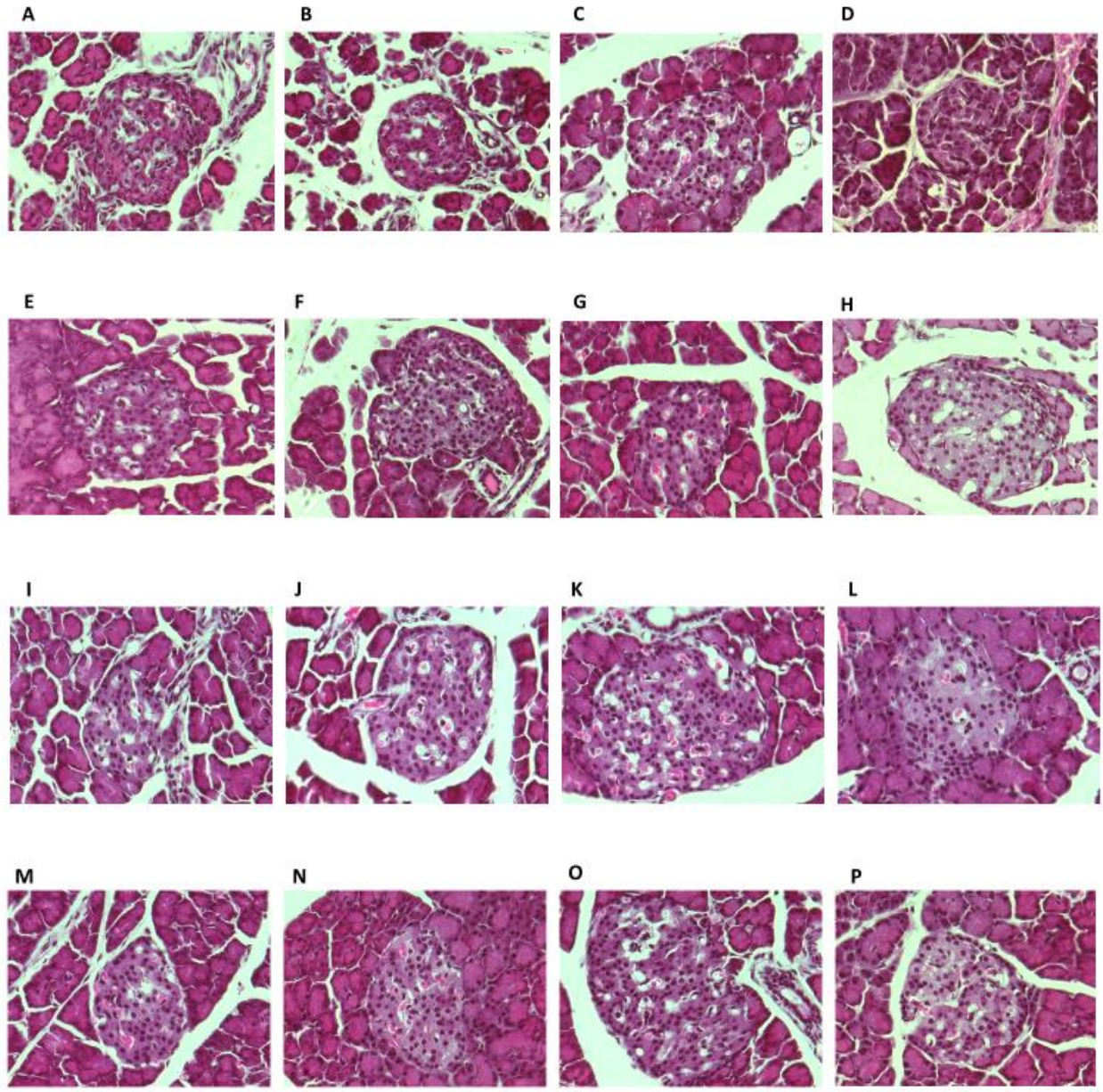

Figure 1 – Representative images of pancreatic islet area (A-P) × 400 magnification, scale bars = 50 μm. NLS group at 07 days (A), at 14 days (E), at 21 days (I) and at 90 days of life (M). NLM group at 07 days (B), at 14 days (F), at 21 days (J) and at 90 days of life (N). SLS group at 07 days (C), at 14 days (G), at 21 days (K) and at 90 days of life (O). SLM group at 07 days (D), at 14 days (H), at 21 days (K) and at 90 days of life (O).

Figure 2

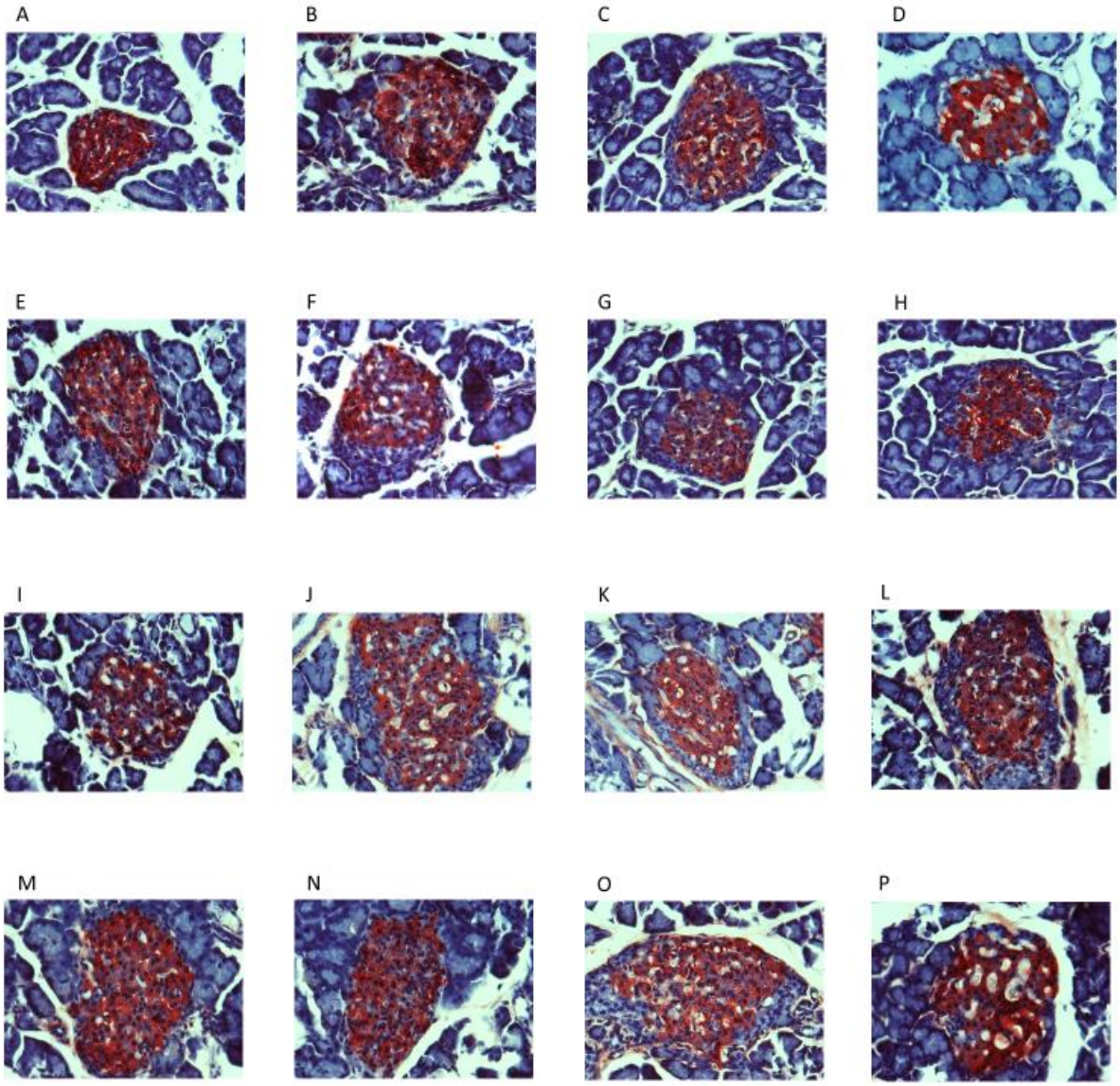

Figure 2 – Representative images of pancreatic sections immunostained with an anti-insulin antibody (A-P) × 400 magnification, scale bars = 50 µm. NLS group at 07 days (A), at 14 days (E), at 21 days (I) and at 90 days of life (M). NLM group at 07 days (B), at 14 days (F), at 21 days (J) and at 90 days of life (N). SLS group at 07 days (C), at 14 days (G), at 21 days (K) and at 90 days of life (O). SLM group at 07 days (D), at 14 days (H), at 21 days (K) and at 90 days of life (O).

Declaration of interest

The final version of the submitted manuscript was reviewed and approved by all authors. They confirm that the article is an original work and has not been previously published. They also state that it is not being submitted for publication in another journal. The authors maintained the legitimacy and integrity of their work and declared no conflicts of interest.

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