



UNIVERSIDADE FEDERAL DE UBERLÂNDIA
FACULDADE DE MEDICINA
PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS DA SAÚDE

**Estudos em Diabetes *mellitus* gestacional: da busca de um
protocolo eficiente à investigação sobre o desenvolvimento neural
da prole**

FERNANDA NAVES A. P. MASCARENHAS

Uberlândia, MG

2023

FERNANDA NAVES A. P. MASCARENHAS

**Estudos em Diabetes *mellitus* gestacional: da busca de um
protocolo eficiente à investigação sobre o desenvolvimento neural
da prole**

Tese apresentada ao Programa de Pós-Graduação
em Ciências da Saúde da Faculdade de Medicina
da Universidade Federal de Uberlândia, como
requisito parcial para a obtenção do título de
Doutor em Ciências da Saúde.

Área de concentração: Ciências da Saúde

Orientador: Prof. Dr. Carlos Ueira Vieira

Coorientadora: Profa. Dra. Renata Graciele Zanon

Uberlândia, MG

2023

Ficha Catalográfica Online do Sistema de Bibliotecas da UFU
com dados informados pelo(a) próprio(a) autor(a).

M395 2023	Mascarenhas, Fernanda Naves Araújo do Prado, 1994- Estudos em Diabetes mellitus gestacional: da busca de um protocolo eficiente à investigação sobre o desenvolvimento neural da prole [recurso eletrônico] / Fernanda Naves Araújo do Prado Mascarenhas. - 2023. Orientador: Carlos Ueira Vieira. Coorientadora: Renata Graciele Zanon. Tese (Doutorado) - Universidade Federal de Uberlândia, Pós-graduação em Ciências da Saúde. Modo de acesso: Internet. Disponível em: http://doi.org/10.14393/ufu.te.2023.626 Inclui bibliografia. Inclui ilustrações. 1. Ciências médicas. I. Vieira, Carlos Ueira, 1981-, (Orient.). II. Zanon, Renata Graciele, 1980-, (Coorient.). III. Universidade Federal de Uberlândia. Pós-graduação em Ciências da Saúde. IV. Título. CDU: 61
--------------	---

Bibliotecários responsáveis pela estrutura de acordo com o AACR2:

Gizele Cristine Nunes do Couto - CRB6/2091
Nelson Marcos Ferreira - CRB6/3074



UNIVERSIDADE FEDERAL DE UBERLÂNDIA
 Coordenação do Programa de Pós-Graduação em Ciências da
 Saúde - Acadêmico
 Av. Pará, 1720, Bloco 2H, Sala 11 - Bairro Umuarama, Uberlândia-MG, CEP 38400-902
 Telefone: (34) 3225-8628 - www.ppcsafamed.ufu.br - ppcsafamed@ufu.br



ATA DE DEFESA - PÓS-GRADUAÇÃO

Programa de Pós-Graduação em:	Ciências da Saúde				
Defesa de:	Tese de Doutorado Nº 15/PPCSA				
Data:	01.12.2023	Hora de início:	10:00h	Hora de encerramento:	13:06h
Matrícula do Discente:	12013CSD007				
Nome do Discente:	Fernanda Naves Araújo do Prado Mascarenhas				
Título do Trabalho:	Estudos em Diabetes Gestacional: a busca de um protocolo eficiente à investigação sobre o desenvolvimento neural das proles				
Área de concentração:	Ciências da Saúde				
Linha de pesquisa:	3: Fisiopatologia das Doenças e Agravos à Saúde				
Projeto de Pesquisa de vinculação:	Modelos Biológicos Alternativos Para Triagem De Novos Fármacos Neuromoduladores				

Reuniu-se em web conferência pela plataforma Google Meet, em conformidade com a PORTARIA Nº 36, DE 19 DE MARÇO DE 2020 da COORDENAÇÃO DE APERFEIÇOAMENTO DE PESSOAL DE NÍVEL SUPERIOR - CAPES, pela Universidade Federal de Uberlândia, a Banca Examinadora, designada pelo Colegiado do Programa de Pós-graduação em Ciências da Saúde, assim composta: Profs. Drs. Pedro Augusto Silva Nogueira (UTSouthwestern), Alex Dias Assis (UNICAMP), Vivian Alonso Goulart (UFU), Robson José de Oliveira Júnior (UFU) e Carlos Ueira Vieira (UFU), orientador da candidata.

Iniciando os trabalhos, a presidente da mesa, Prof. Dr. Carlos Ueira Vieira, apresentou a Comissão Examinadora e a candidata, agradeceu a presença do público, e concedeu a Discente a palavra para a exposição do seu trabalho. A duração da apresentação da Discente e o tempo de arguição e resposta foram conforme as normas do Programa.

A seguir o senhor(a) presidente concedeu a palavra, pela ordem sucessivamente, aos(às) examinadores(as), que passaram a arguir o(a) candidato(a). Ultimada a arguição, que se desenvolveu dentro dos termos regimentais, a Banca, em sessão secreta, atribuiu o resultado final, considerando o(a) candidato(a):

Aprovada.

Esta defesa faz parte dos requisitos necessários à obtenção do título de Doutor.

O competente diploma será expedido após cumprimento dos demais requisitos, conforme as normas do Programa, a legislação pertinente e a regulamentação interna da UFU.

Nada mais havendo a tratar foram encerrados os trabalhos. Foi lavrada a presente ata que após lida e achada conforme foi assinada pela Banca Examinadora.



Documento assinado eletronicamente por **Carlos Ueira Vieira, Professor(a) do Magistério Superior**, em 01/12/2023, às 13:07, conforme horário oficial de Brasília, com fundamento no art. 6º, § 1º, do [Decreto nº 8.539, de 8 de outubro de 2015](#).



Documento assinado eletronicamente por **Robson José de Oliveira Junior, Professor(a) do Magistério Superior**, em 01/12/2023, às 13:07, conforme horário oficial de Brasília, com fundamento no art. 6º, § 1º, do [Decreto nº 8.539, de 8 de outubro de 2015](#).



Documento assinado eletronicamente por **Vivian Alonso Goulart, Professor(a) do Magistério Superior**, em 01/12/2023, às 13:10, conforme horário oficial de Brasília, com fundamento no art. 6º, § 1º, do [Decreto nº 8.539, de 8 de outubro de 2015](#).



Documento assinado eletronicamente por **Pedro Augusto Silva Nogueira, Usuário Externo**, em 01/12/2023, às 16:32, conforme horário oficial de Brasília, com fundamento no art. 6º, § 1º, do [Decreto nº 8.539, de 8 de outubro de 2015](#).



Documento assinado eletronicamente por **Alex Dias Assis, Usuário Externo**, em 01/12/2023, às 22:58, conforme horário oficial de Brasília, com fundamento no art. 6º, § 1º, do [Decreto nº 8.539, de 8 de outubro de 2015](#).



A autenticidade deste documento pode ser conferida no site https://www.sei.ufu.br/sei/controlador_externo.php?acao=documento_conferir&id_orgao_acesso_externo=0, informando o código verificador **5011503** e o código CRC **EB49E543**.

Dedico esse presente estudo aos meus pais Saulo (“In memoriam”) e Marilúcia, meu irmão e meu marido Matheus, que foram os meus maiores incentivadores e apoiadores em toda minha formação acadêmica.

AGRADECIMENTOS

Primeiramente, quero agradecer aos meus pais, Saulo (“*In memoriam*”) e Marilúcia, pelo exemplo de pessoas que são para mim, por cada ensinamento, apoio e dedicação em cada jornada, e pelo amor depositado, além de sempre me oferecerem as melhores oportunidades para eu alcançar meus objetivos. E ao meu irmão Saulo, que esteve comigo em cada passo dado, ofertando sua amizade, carinho e apoio;

À meu marido, Matheus, por todo companheirismo, cuidado, paciência e amor, que esteve ao meu lado nas melhores horas e também nas mais difíceis, você é essencial na minha vida;

Aos meus orientadores, Prof. Dr. Carlos Ueira Vieira, por todo empenho em conseguir as melhores oportunidades e soluções aos problemas encontrados, sou-lhe eternamente grata, e Prof^a. Dra. Renata Graciele Zanon, por toda orientação desde a graduação até aqui, me motivando e apoiando sempre, um exemplo de profissionalismo e dedicação, compartilhando seus ricos conhecimentos, e pela amizade adquirida;

À minha coorientanda de IC, Maria Júlia Neves Ribeiro, por todo auxílio e dedicação para com o desenvolvimento do projeto, uma pessoa dedicada e brilhante. E a todos os meus companheiros de laboratório, LAMOC, principalmente a Msc. Natália Ferreira, por toda parceria construída, por toda disponibilidade e pelos conhecimentos compartilhados, além da amizade adquirida;

À Rede de Biotérios (REBIR) da UFU, pela oferta e manutenção do nosso modelo experimental. E a todos os laboratórios e professores parceiros, que contribuíram e se propuseram a contribuir no enriquecimento do projeto, Profa. Dra. Danielle Lisboa, Prof. Dr. Foued Salmen Espindola (LABIBI) e Profa. Dra. Renata Rouland Teixeira. E aos seus alunos que estiveram ali sempre auxiliando nos experimentos, Msc. Rener Mateus e Msc. Luiz Felipe.

À todos os professores do Programa de pós-graduação em Ciências de Saúde (PPCSA), que contribuírem de diversas maneiras para enriquecimento, pessoal, profissional e científico. E à coordenação e secretaria, Profa. Dra. Yara Cristina de Paiva Maia, Gisele de Melo e Viviane Pires, que contribuíram na organização de todas as burocracias necessárias;

À todos os meus amigos e familiares que sempre me apoiaram, se orgulharam a cada conquista e ajudaram sempre que necessário;

À banca pelo aceite do convite, para enriquecer ainda mais nossa pesquisa;

E à CAPES, pelo apoio financeiro.

Muito obrigada!

RESUMO

Introdução: O diabetes *mellitus* (DM) é uma síndrome metabólica caracterizado por uma disfunção na produção, secreção e/ou atuação da insulina, causando hiperglicemia. As alterações nessa doença são metabólicas e estruturais atingindo diversos tecidos. Quando a doença ocorre durante a gestação, denomina-se diabetes *mellitus* gestacional (DMG), e o estado hiperglicêmico atinge o feto em desenvolvimento podendo causar diversos danos. O excesso de glicose pode comprometer as células neurais que reagem através de mudanças neurofisiológicas e estruturais, com prejuízos na condução do impulso nervoso, além de alterações em neurotransmissores, em vesículas sinápticas e na atividade glial. **Objetivos:** Desenvolver e padronizar um modelo experimental de indução ao DMG que seja eficiente em ratos Wistar, utilizando a estreptozotocina (STZ), além de entender se filhotes de ratas diabéticas apresentam comprometimento estrutural e/ou funcional no cerebelo. **Metodologia:** Os animais obtidos foram colocados para acasalamento e verificação de presença de espermatozoides no lavado vaginal das fêmeas, indicativo de primeiro gestacional (G1), e após cinco dias (G5) foram induzidas ao diabetes em jejum, por via intraperitoneal, utilizando STZ, 50 mg/kg. Foram avaliados o peso e a glicemia, pré-acasalamento, no sétimo e décimo sétimo dia de gestação, sendo consideradas diabéticas quando glicemia ≥ 200 mg/dL, e marcadores de danos oxidativos no soro plasmático. Após o nascimento, os filhotes passaram por avaliação de qualidade de ninhada (número de filhotes, sexo e natimortos). Posteriormente, os filhotes seguiram para avaliação física, índice de massa corporal e glicemia (mg/dL), e, foram eutanasiados nas idades de sete, 30 e 90 dias, com extração e avaliação física dos encéfalos (g) e dissecação do cerebelo, estes foram preparados para análises histomorfométrica, imunofluorescentes, e expressão de proteínas alvo, dados indicativos de inflamação, gliose, desbalanço oxidativo e morte celular. Além disso, avaliar os impactos funcionais, os filhotes foram submetidos a testes comportamentais motores. **Resultados:** Obtivemos sucesso de 55,6% na obtenção do modelo experimental de GDM, pois conseguimos implementar uma sequência de cuidados essenciais, com comprovação do estabelecimento da patologia caracterizada por evolução de ganho de peso, hiperglicemia e dano oxidativo no sangue materno, além de alteração na qualidade da ninhada. Os filhotes de mães diabéticas apresentaram menores índices de massa corporal aliado ao baixo peso encefálico, a histomorfometria cerebelar mostrou presença de camadas corticais mais delgadas, com presença de gliose, além de um desbalanço oxidativo, e, apoptose. Além de demonstrarem menos comportamentos exploratórios. **Conclusão:** Obtivemos um protocolo eficiente de indução do DMG e o estudo do cerebelo da prole mostrou que a hiperglicemia durante o período

gestacional pode interferir no desenvolvimento cerebelar da prole com impacto funcional na vida adulta dos ratos.

Palavras-chave: diabetes gestacional, hiperglicemia, sistema nervoso motor, cerebelo, desenvolvimento.

ABSTRACT

Introduction: Diabetes mellitus (DM) is a metabolic syndrome characterized by a dysfunction in the production, secretion and/or action of insulin, causing hyperglycemia. The changes in this disease are metabolic and structural, affecting various tissues. When the disease occurs during pregnancy, it is called gestational diabetes mellitus (GDM), and the hyperglycemic state affects the developing fetus and can cause various damages. Excess glucose can compromise neural cells that react through neurophysiological and structural changes, with impairments in the conduction of nerve impulses and changes in neurotransmitters, synaptic vesicles, and glial activity. **Objectives:** To develop and standardize an experimental model of induction of GDM that is efficient in Wistar rats, using streptozotocin (STZ), in addition to understanding whether offspring of diabetic rats present structural and/or functional impairment in the cerebellum. **Methodology:** The animals obtained were placed for mating and checking for the presence of sperm in the females' vaginal wash, indicative of the first pregnancy (G1), and after five days (G5) they were induced to diabetes while fasting, intraperitoneally, using STZ, 50 mg/kg. Weight and blood glucose levels were evaluated, pre-mating, on the seventh and seventeenth day of pregnancy, being considered diabetic when blood glucose levels ≥ 200 mg/dL, and markers of oxidative damage in plasma serum. After birth, the puppies underwent litter quality assessment (number of puppies, sex and stillbirths). Subsequently, the puppies underwent physical evaluation, body mass index and blood glucose (mg/dL), and were euthanized at the ages of seven, 30 and 90 days, with extraction and physical evaluation of the brains (g) and dissection of the cerebellum, these were prepared for histomorphometric, immunofluorescent analysis, and expression of target proteins, data indicative of inflammation, gliosis, oxidative imbalance and cell death. Furthermore, evaluate the functional impacts, the puppies were subjected to motor behavioral tests. **Results:** We achieved 55.6% success in obtaining the experimental model of GDM, because we were able to implement a sequence of essential care, with proof of the establishment of the pathology characterized by the evolution of weight gain, hyperglycemia, and oxidative damage in maternal blood, in addition to change in litter quality. The puppies of diabetic mothers had lower body mass indexes combined with low brain weight, cerebellar histomorphometry showed the presence of thinner cortical layers, with the presence of gliosis, in addition to an oxidative imbalance, and apoptosis. In addition to demonstrating less exploratory behavior. **Conclusion:** We obtained an efficient GDM induction protocol and the study of the offspring's cerebellum showed that hyperglycemia during the gestational period can interfere with the offspring's cerebellar development with a functional impact on the rats' adult life.

Keywords: gestational diabetes, hyperglycemia, motor nervous system, cerebellum, development.

LISTA DE ABREVIATURAS

AGEs	Advanced glycation end products
ALX	Alloxan
AOPP	Advanced Oxidation Protein Products
BMI	Body mass index
BSA	Albumin bovine
CAPES	Coordination of Superior Level Staff Improvement
CEUA	Comitê de ética de uso animal
CGE	Camada granular externa
CGI	Camada granular interna
CNS	Central Nervous System
CT	Controle
D	Diabetic
DAPI	4',6-diamidino-2-phenylindole
DM	Diabetes <i>Mellitus</i>
DM-1	Diabetes <i>Mellitus</i> tipo 1
DM-2	Diabetes <i>Mellitus</i> tipo 2
DMG	Diabetes <i>Mellitus</i> Gestacional
DNA	Ácido desoxirribonucleico
DPN	Neuropatia Diabética Periférica
ECM	Elevated Cross Maze
EGL	External granular layer
FRAP	Ferric reducing antioxidant power
G1	Primeiro dia de gestação
G4	Quarto dia de gestação
G5	Quinto dia de gestação
G6	Sexto dia de gestação
G7	Sétimo dia de gestação
G10	Décimo dia de gestação
G17	Décimo sétimo dia de gestação
GDM	Gestacional Diabetes <i>Mellitus</i>
GFAP	Glial fibrillary acidic protein

GH	Growth hormone
GLUT-2	Transportador de glicose tipo 2
GPx	Glutathione peroxidase
GR	Glutathione reductase
GSH	Reduced glutathione
GSSG	Oxidized glutathione
IGL	Internal granular layer
IMC	Índice de massa corporal
IP	Intraperitoneal
IV	Intravenoso
MGO	Methylglyoxal
MPA	Metaphosphoric acid
NADPH	Nicotinamide adenine dinucleotide phosphate
OFT	Open Field Test
P7	Sete dias pós natal
P30	Trinta dias pós natal
P90	Noventa dias pós natal
PBS	Phosphate buffer saline
REBIR	Rede de Biotérios
ROS	Reactive oxygen species
SAP BA	Stretched out in open arms
SAP BF	Stretched out in closed arms
SNC	Sistema Nervoso Central
SOD	Superoxide dismutase
SPF	Specific pathogenic germs
STZ	streptozotocin
TPTZ	2,4,6-tri (2 pyridyl)-s-triazine
UFU	Universidade Federal de Uberlândia

SUMÁRIO

1. INTRODUÇÃO.....	1
2. FUNDAMENTAÇÃO TEÓRICA	2
2.1. Diabetes mellitus.....	2
2.2. Diabetes mellitus gestacional (DMG)	3
2.3. O estudo do DMG em modelo experimental animal	3
2.4. DMG e seu impacto no Sistema Nervoso Central dos fetos	4
2.5. DMG no desenvolvimento cerebelar da prole	6
3. OBJETIVOS.....	7
3.1. Gerais	7
3.2. Específicos	7
4. ARTIGO 1	8
4.1. Development of a successful protocol for experimental gestational diabetes	9
5. ARTIGO 2	27
5.1. Gestational Diabetes influences the development of the cerebellum in rats	28
REFERÊNCIAS.....	56
ANEXO 1 - Certificado de aprovação do CEUA/UFU	60

1. INTRODUÇÃO

Sabe-se que o Diabetes *mellitus* (DM) é uma síndrome metabólica causado pela alteração insulínica, que leva ao aumento do nível de glicose no sangue. Sendo classificada em 4 tipos, e um deles é o Diabetes Mellitus Gestacional (DMG), que é desenvolvido e diagnosticado durante a gestação, onde os próprios hormônios deste período acarretam em uma resistência insulínica, colocando esse grupo em maior susceptibilidade de desenvolvimento da doença. Existem fatores de risco que aumentam a prevalência dessa doença, como idade > 35 anos, excesso de peso, histórico de DM na família, hipertensão arterial, entre outros. O DMG possui os mesmos parâmetros de diagnóstico e tratamento do DM comum.

Quando descontrolado e não tratado o DM pode levar a consequências perinatais, sendo o responsável por altos índices de mortalidade perinatal e complicações fetais, como macrosomia, hipoglicemia, hiperinsulinemia, e anomalias congênitas. Além de afetar o desenvolvimento do Sistema Nervoso Central (SNC) do feto, pois esse sistema se mostrou em pesquisas anteriores, sensível a hiperglicemia, podendo levar a distúrbios do neurodesenvolvimento, incluindo deficiências no funcionamento motor, capacidade de atenção e aprendizagem. Essas alterações são devido a uma neuroinflamação e oxidação ocasionada pelo excesso de glicose nos tecidos.

Pensando em sistema nervoso motor, o cerebelo é reconhecido por ser uma importante área motora, com 80% de todas os neurônios do SNC, além disso estudos atuais mostraram o cerebelo com grande importância funcional em outras áreas de atuação, como aprendizagem motora, discriminação sensorial e realização de tarefas cognitivas complexas. Devido tais complexidades o torna um alvo para novas pesquisas, que busca as alterações causadas pela hiperglicemia materna nos cerebelos das proles.

Para esse tipo de pesquisa ser desenvolvida há a necessidade de utilização de um modelo experimental, no caso, de indução ao DMG. Nesse contexto, pesquisas mostram sobre a dificuldade em obtenção de um modelo eficiente, os protocolos atuais apontam gastos de dinheiro e tempo excessivos, e o uso de quantidade excessiva de animais, o que justificou padronizarmos um protocolo que fosse eficiente de indução ao DMG, com o passo a passo de todos os cuidados necessários para a obtenção de ratas com DMG, reprodutível e que levasse a uma redução no uso de animais. Com os filhotes obtidos dessa metodologia, utilizamos os cerebelos para uso na pesquisa que busca respostas a essas alterações fetais causadas pela hiperglicemia materna.

2. FUNDAMENTAÇÃO TEÓRICA

2.1. Diabetes *mellitus*

Diabetes *mellitus* (DM) é uma síndrome metabólica caracterizado pela hiperglicemia, causado pela alteração da produção, secreção e/ou ação da insulina. Pode ser classificado em diabetes *mellitus* tipo 1 (DM-1), ocorre pela deficiência absoluta na secreção de insulina, decorrente da destruição autoimune das células pancreáticas, e em tipo-2 (DM-2), ocorre pela combinação de resistência à ação da insulina e um processo compensatório inadequado de resposta secretora da insulina, ocorre mais em adultos e idosos. Existe também os tipos específicos de diabetes devido diferentes causas, como as síndromes de diabetes monogênicas, doenças de pâncreas exócrino, e diabetes induzido quimicamente por medicamentos. E por último o diabetes mellitus gestacional (DMG), que é diagnosticado no segundo ou terceiro trimestre de gravidez, em mulheres antes não diabéticas (AMERICAN DIABETES ASSOCIATION, 2021).

À longo prazo, as altas concentrações plasmáticas de glicose levam ao desenvolvimento de degenerações crônicas com falência de diversos tecidos, podendo afetar a retina, os glomérulos renais, a micro vascularização e os nervos periféricos (FERREIRA et al., 2011; REMOR et al., 2011). Mas o DM pode também desencadear encefalopatia caracterizada pela diminuição cognitiva, dificuldade de aprendizagem e aumento do risco de demência (PITITTO, ALMADA FILHO & CENDOROGLO, 2008).

Periféricamente o DM pode levar ao desenvolvimento de neuropatia periférica diabética (DPN), uma doença difusa dos nervos periféricos que causa perda de sensibilidade e dificuldades motoras. A maioria dos pacientes com essa doença apresentam um padrão misto: sensorial, autonômico e com perturbação motora que pode levar à fraqueza e atrofia muscular das pernas e dos pés, o que contribui para o aumento de quedas (REMOR et al., 2011).

O trabalho de FRANCIS e colaboradores (2011) analisou possíveis perdas motoras em ratos diabéticos no decorrer do tempo e a eficiência do uso de insulina na prevenção dessas possíveis alterações. E observaram que neurônios motores apresentam maior resistência a hiperglicemia à longo prazo quando comparados aos sensitivos. Mas as áreas motoras também sofrem neurodegeneração, mas tardiamente. Os danos iniciam a nível das placas motoras, com perda de mielina e da junção neuromuscular, posteriormente alterações no corpo neuronal, com comprometimentos estruturais e moleculares, levando a redução da quantidade e da velocidade de condução da informação motora.

2.2. Diabetes *mellitus* gestacional (DMG)

O DMG é caracterizado por intolerância à glicose, de magnitude variável, com início e diagnóstico durante a gestação. Sua prevalência mundial é de 15,2% entre as gestantes (SUN et al., 2022), geralmente se resolve no pós-parto, mas retorna anos depois na maioria dos casos. Recomenda-se detectá-lo com os mesmos procedimentos e parâmetros diagnósticos empregados fora da gravidez (GOLBERT & CAMPOS, 2008).

Os fatores de risco para desenvolvimento de diabetes gestacional são: maternidade com idade avançada, 35 anos ou mais, sobrepeso, obesidade ou ganho excessivo de peso na gravidez atual, deposição excessiva de gordura abdominal, histórico familiar de diabetes em parentes de primeiro grau, crescimento fetal excessivo e polidrâmnio, hipertensão ou pré-eclâmpsia na gravidez atual, antecedentes obstétricos de abortamentos de repetição, malformações, e, síndrome dos ovários policísticos (MILECH et al., 2006).

O DMG é uma das intercorrências mais frequentes durante a gestação e quando não diagnosticado e tratado adequadamente, é determinante para diversos riscos maternos e perinatais. É o responsável por índices elevados de mortalidade perinatal e complicações fetais, como a macrosomia, traumatismos no parto, hipoglicemia, hiperbilirrubinemia neonatal, síndrome do desconforto respiratório do recém-nascido, hipocalcemia. Tal incidência de anomalias congênitas está relacionada com os níveis de glicose aumentados durante a gestação. Além dos riscos futuros, há maiores chances de síndrome metabólica (obesidade, hipertensão e diabetes) na vida adulta, por herança epigenética (FALEIROS et al., 2021)

O controle da glicemia no período pré-concepcional e nas primeiras semanas de gestação é crucial para reduzir os riscos de aborto e de malformações congênitas. O tratamento com insulina permite normalizar os níveis de glicemia, protegendo o cérebro e outros tecidos dos efeitos adversos da hiperglicemia (MAGANHA et al., 2003; SILVA et al., 2007).

2.3. O estudo do DMG em modelo experimental animal

O estudo do diabetes e de tentativas terapêuticas é feito em modelos animais que utilizam principalmente roedores destacando-se ratos e camundongos. Diferenciam-se animais que desenvolvem espontaneamente a doença, devido a uma predisposição genética, e, animais que são submetidos a diferentes técnicas para a indução da doença, como a pancreatectomia, exposição a vírus específicos ou administração intravenosa (IV)

ou intraperitoneal (IP) de agentes citotóxicos, como a estreptozotocina (STZ) ou aloxana (ALX) (GISPEN & BIESSELS, 2000). Os roedores têm sido amplamente utilizados devido suas vantagens: fácil manuseio (alimentação, limpeza e alocação), elevada resistência à infecção, facilidade para dissecação e extração de tecidos, além de desenvolverem semelhanças fisiopatológicas com o diabetes humano (LERCO et al., 2003).

A droga STZ possui uma maior especificidade sobre as células beta pancreáticas (LERCO et al., 2003). E sua finalidade é causar danos no DNA (ácido desoxirribonucleico), depletando a Nicotina Adenina Dinucleotídeo (NAD⁺), inibindo a síntese e secreção de insulina, culminando no processo de morte das células beta pancreáticas devido a depleção energética, resultando nos sintomas diabetogênicos no animal (KIRSTEN, SESTERHEIM & SAITOVITCH, 2010). Sua ação pode ser influenciada pela concentração da droga, velocidade de infusão, dose, via de administração, dieta, tempo de jejum e peso do animal (SANTOS, 2018). A escolha da via de administração da droga é influenciada diretamente pelo tipo de ação desejada (local ou geral), da velocidade de ação da droga e pela facilidade de acesso à via. Sendo que as vias mais utilizadas para a indução ao diabetes são: intravenosa (IV), intraperitoneal (IP) e a subcutânea (MONTANHA & AZEVEDO, 2013).

De acordo com a revisão sistemática conduzida por FERREIRA & NICOLAU (2011) o STZ mostrou ser a droga mais utilizada nos estudos (70% dos estudos), porém, com administração de doses em diferentes concentrações e vias de acesso. Assim, faz necessário a padronização de um protocolo consistente e eficiente de indução do DMG em modelos animais.

2.4. DMG e seu impacto no Sistema Nervoso Central dos fetos

Diante de todas as anormalidades congênitas existentes, as do Sistema Nervoso Central (SNC) são as mais comuns, pois esse sistema tem seu desenvolvimento iniciado antes dos outros sistemas no período embrionário, e dessa forma suas estruturas estão mais vulneráveis a sofrerem alterações por diversos agentes durante seu desenvolvimento (KOSTOVIĆ, SEDMAK & JUDAŠ, 2019). Estudos mostraram que filhos de mães diabéticas possuem de três a quatro vezes mais chances de desenvolverem malformações fetais quando comparados aos filhos de mães normoglicêmicas (DE MORAIS RIBEIRO, 2008).

No SNC as malformações congênitas mais frequentes são falhas de fechamento do tubo neural, anencefalia, microcefalia, hidrocefalia, catarata congênita e hipoplasia do

nervo óptico. Adicionalmente, anomalias do coração, do esqueleto, do trato gastrointestinal e do trato urinário também estão presentes (PANTE et al., 2011).

A fisiopatologia do DMG no feto durante a gestação está relacionada à elevada oferta de glicose pela circulação materna gerando hiperglicemia no feto (NOMURA et al., 2002). Esta hiperglicemia leva a hiperinsulinemia fetal, com aumento do depósito de glicogênio e de lipídeos (CHANDNA et al., 2015). O aumento do metabolismo celular tem como consequência um maior consumo de oxigênio pelos tecidos e, ao mesmo tempo, a glicosilação da hemoglobina promove um aumento da afinidade pelo oxigênio facilitando a ocorrência de estresse oxidativo e hipóxia tecidual (HAMI et al. 2015).

Outra anormalidade desencadeada pela hiperinsulinemia fetal é a eritropoiese extra medular em resposta às alterações dos padrões de crescimento do feto, levando a poliglobulia e aumento da viscosidade sanguínea. Estas alterações podem influenciar os padrões de velocidade de fluxo sanguíneo na circulação do feto (AMARAL, et al., 2012). Fetos que possuem maior predisposição a hiperinsulinemia tendem a desenvolver macrossomia, que é uma doença caracterizada pelo excesso de peso do recém-nascido. Estes terão como consequências maiores riscos de complicações no parto e morbidade neonatal elevada (RUDGE et al., 2005; SILVA et al., 2009).

Tais fatores alteram a trajetória normal do desenvolvimento do sistema nervoso fetal, o predispondo a distúrbios do neurodesenvolvimento, incluindo deficiências no funcionamento motor, capacidade de atenção e aprendizagem, possivelmente devido uma neuroinflamação e oxidação ocasionada pelo excesso de glicose. Logo, o controle glicêmico materno durante a gravidez favorece a manutenção do estado metabólico fetal em condições adequadas para preservar sua vitalidade (VILELA, 2021).

A glicose e o oxigênio exercem papéis fundamentais na função do SNC, especialmente sobre a homeostase de neurônios e astrócitos (MILLIGAN & WATKINS, 2009). Sabe-se que os astrócitos possuem um sistema de comunicação, que regula a excreção de neurotransmissores, ativação neuronal e sinapse (FELLIN & CARMIGNOTO, 2004). Prejuízos na regulação metabólica, atinge diretamente o aporte energético e morfologia dessas células, contribuindo para a formação de um microambiente inflamatório, disfunção sináptica e mitocondrial, levando à morte celular, com consequentes alterações fenotípicas cognitivas (GARCIA-SERRANO & DUARTE, 2020).

A astrogliose ocorre em resposta a um dano tecidual neural, e é caracterizada por hiperplasia e/ou hipertrofia das células gliais (ANCIUTI, 2016). O estudo de DE SOUZA MELLO, SANTOS & FUNCHAL (2012) mostrou que os astrócitos quando expostos a

uma alta oferta de glicose, eles sofrem uma gama de alterações, como: redução no conteúdo de glutathione, redução de proteínas marcadoras da reatividade dessas células, proteína ácida fibrilar glial (GFAP), liberação de quimiocinas, alteração no metabolismo de glutamato e produção de espécies reativas de oxigênio. Esse conjunto de respostas dos astrócitos num ambiente hiperglicêmico traduz alterações da atividade celular e, portanto, da atividade neuronal (NARDIN, 2006). ANCIUTI (2016) mostrou que a hiperglicemia intrauterina e pós-natal pode estar relacionada à predisposição ao desenvolvimento de desordens durante a vida adulta, pois verificou danos no hipocampo de ratos jovens, alterações neuronais e gliais, e, aumento de espécies reativas de oxigênio (ROS).

2.5.DMG no desenvolvimento cerebelar da prole

Atualmente sabe-se que o cerebelo além de área motora e de equilíbrio, está relacionado com a aprendizagem motora, discriminação sensorial e tarefas cognitivas complexas (ARLEO et al., 2023; MANTO et al., 2012). Sendo dividido filogeneticamente em arquicerebelo (relacionado ao sistema vestibular), paleocerebelo (relacionado a funções motoras e propriocepção), e, neocerebelo (responsável pela programação motora e atualmente relacionada à cognição). A substância cinzenta do cerebelo maduro apresenta três camadas celulares identificadas pela histologia clássica: camada molecular, camada de células de Purkinje, e camada de células granulares (DAMIANI et al., 2016).

Em ratos o desenvolvimento cerebelar continua nas primeiras três semanas pós nascimento, o que corresponde ao terceiro trimestre embrionário em humanos (GOLDOWITZ & HAMRE, 1998). Caracterizado por ser um período ativo de proliferação neuronal, migração e diferenciação de células granulares na região de córtex cerebelar desses animais. Iniciando pela proliferação de neurônios imaturos na camada granular externa (CGE), e então iniciam a migração ao longo dos processos radiais das células gliais pela camada molecular em direção a camada granular interna (CGI), e então começam sua diferenciação em neurônios maduros (YONG et al., 2015). Nesta fase, o córtex cerebelar possui quatro camadas: CGE, a camada molecular, a camada de células de Purkinje e CGI, sendo CGE temporária, pois desaparece após a conclusão da diferenciação cerebelar total, o denominado cerebelo maduro, agora com as mesmas camadas de um cerebelo humano maduro (MASCARENHAS et al., 2022).

Nesse contexto, o cerebelo se mostrou uma área de grande interesse de estudo, durante o desenvolvimento embrionário e pós-natal, pelo fato de suas várias funções de grande importância, e por apresentarem grande volume de células neurais, e estas serem

sensíveis ao excesso de glicose. Alguns estudos mostram que o desenvolvimento fetal em um ambiente hiperglicêmico está diretamente relacionado com diversas patologias que tem como manifestações alterações motoras, do comportamento e cognição, uma das funções do cerebelo, como por exemplo o autismo e a esquizofrenia (XIANG et al., 2018).

3. OBJETIVOS

3.1 Geral

Padronizar um protocolo eficiente de indução ao DMG, desde o momento do acasalamento, comprovação da patologia durante a gestação e nascimento da prole. E avaliar o impacto dessa doença no desenvolvimento das proles, com enfoque nos aspectos morfofuncionais cerebelares.

3.2 Específicos

- ✓ Padronizar a metodologia de um modelo experimental de indução ao DMG utilizando ratos *Wistar*;
- ✓ Avaliar a evolução de peso corporal, glicemia e qualidade de ninhadas das mães, dos grupos controle (CT) e DMG;
- ✓ Analisar marcadores de danos oxidativos no soro das mães induzidas ao DMG;
- ✓ Avaliar o Índice de Massa Corporal (IMC), glicemia e peso encefálico dos filhotes, dos grupos CT e DMG;
- ✓ Avaliar o cerebelo dos filhotes, quanto a morfologia tecidual, a reatividade glial, a presença de danos oxidativos e apoptose, dos grupos CT e DMG;
- ✓ Além de analisar alterações comportamentais dos filhotes, utilizando testes de caráter motor, dos grupos CT e DMG.

ARTIGO 1

Development of a successful protocol for experimental gestational diabetes

Submitted in *Journal of Pharmacological and Toxicological Methods*, Qualis B1.

Development of a successful protocol for experimental gestational diabetes

Fernanda Naves Araújo do Prado Mascarenhas^{a*}; Maria Júlia Neves Ribeiro^a; Natália Ferreira Silva^a; Rener Mateus Francisco Duarte^b; Foued Salmen Espindola^b; Renata Graciele Zanon^a

^aInstitute of Biomedical Sciences, Federal University of Uberlandia (UFU), Uberlandia, MG, Brazil

^bInstitute of Biotechnology, Federal University of Uberlandia (UFU), Uberlandia, MG, Brazil.

**Corresponding author:* Msc. Fernanda N. A. P. Mascarenhas, Instituto de Ciências Biomédicas, Universidade Federal de Uberlândia (UFU), CP 592, CEP 38400-902, Uberlândia, MG, Brasil. e-mail: mascarenhas.nutri@hotmail.com

ABSTRACT

Diabetes mellitus (DM) is characterized by a dysfunction in insulin secretion and/or action which causes hyperglycemia. When the disease occurs during pregnancy, it is called gestational diabetes mellitus (GDM), and hyperglycemia affects the fetus's development. The present study aimed to develop an efficient model of GDM induction in Wistar rats using streptozotocin (STZ). Therefore, the animals are placed for mating overnight, and the next day the presence of sperm is verified in the vaginal wash, indicating the first day of gestation (G1), and after five days, the pregnant rats were induced to diabetes, intraperitoneally, using STZ, at a dose of 50 mg/kg. The animals were weighed, and their blood glucose levels were measured (pre-mating, G7, and G17), being considered diabetics when blood glucose ≥ 200 mg/dL. After birth, the puppies were evaluated for the number of individuals, gender, and stillbirths. The blood of females was collected to assess oxidative damage caused by diabetes. The results showed efficacy in the use of the alternative dye gentian violet for sperm and cell identification. Weight gain, hyperglycemia, and the presence of oxidative damage showed an efficient GDM induction for 55.6% of total animals, considered a successful protocol superior to those commonly observed by those researchers in the GDM study area. Also, we observed a higher number of stillbirths in the GDM group, confirming data from the literature that characterize the hyperglycemic environment as hostile to the development of the fetus.

Keywords: diabetes, gestation, methods, streptozotocin

1. Introduction

Gestational diabetes mellitus (GDM) is characterized by glucose intolerance that begins during pregnancy. Its worldwide prevalence is 15.2% among pregnant women (SUN et al., 2022), it generally resolves postpartum but returns years later in most cases. The recommendation for treatment and diagnosis is the same parameters used outside of pregnancy (MILECH et al., 2007; GOLBERT & CAMPOS, 2008; RUDGE et al., 2005). GDM is responsible for high rates of perinatal mortality, especially due to very large fetuses, the presence of malformations, and congenital anomalies, related to the presence of increased glucose levels at the beginning of pregnancy (GARCÍA, 2008; SILVA et al., 2009).

The study of diabetes and therapeutic attempts is carried out in animal models, mainly in rats and mice (SILVA & PRESRAVE, 2008). A distinction is made between animals that spontaneously develop the disease, due to a genetic predisposition, and animals that are subjected to different techniques for inducing the disease, such as pancreatectomy, exposure to specific viruses, or intravenous (IV) or intraperitoneal (IP) administration of cytotoxic agents, such as streptozotocin (STZ) and alloxan (ALX) (GISPEN & BIESSELS, 2000). The agent ALX presents high mortality after induction and ketosis. STZ has greater stability and cellular selectivity, as it is absorbed via the glucose transporter GLUT-2, and pancreatic beta cells express this transporter, making them more sensitive to STZ. At the same time, the extrapancreatic parenchyma remains intact (LENZEN, 2008).

Rodents have been widely used due to their advantages of easy handling (feeding, cleaning, and allocation), high resistance to infection, ease of dissection, and tissue extraction, in addition to developing pathophysiological similarities with human diabetes (LERCO et al., 2003). However, the lack of standardization in mating protocols combined with diabetogenic induction leads to large losses of animals, unnecessary expenses, and inexhaustible repetitions, which culminate in a major delay in the development of study in this area. In this context, the present study aimed to develop and standardize an experimental model for inducing GDM in rats that is highly efficient.

2. Methods

2.1. Animals

All procedures performed with the animals were approved by the Animal Use Ethics Committee of the Federal University of Uberlandia (UFU), protocol 056/18. We

used 51 Wistar rats (*Rattus norvegicus*), 39 females and 12 males, that were kept in polysulfone boxes, with adequate air circulation, controlled temperature between 18-22°C, a humidity of 45 to 55%, with an autoclaved wood shavings bed, and a 12/12-hour light-dark cycle. For feeding, commercial feed and drinking water were used in drinking fountains, *ad libitum*.

2.2. Mating

Rats reach puberty at 30 days of age, but it is suggested that, for the ability to maintain pregnancy to be adequate, they wait longer. Therefore, the animals used were 60 days old, and during this period, the males weighed around 200-250g and females 150-180g.

Nulliparous females were previously evaluated in relation to weight (g), using an electronic scale for weighing with a precision of 0.1 gram (KERN®), and fasting blood glucose (mg/dL) by obtaining a drop of blood from the animal's tail, with the aid of a scalpel, and the reading was taken using a glucometer (Descarpack Plus®). Therefore, females were transferred to the male boxes in the late afternoon only for mating, in a ratio of two females to one male (polygamous system). On the morning of the following day, the females were returned to their original boxes, and the vaginal fluid was collected to control copulation.

In the vaginal washing technique, the rats were restrained manually, then a Pasteur pipette containing saline solution (0.9%) was introduced superficially into the vaginal canal, and the collected material was deposited on the identified glass slides. Subsequently, the slides were dried in an oven for histological staining, using gentian violet, washing in 70% alcohol and water, mounting the slide, using mounting medium and a coverslip. Then, the reading and capture were carried out under an optical microscope, with a 20x objective. Thus, it was possible to identify with greater precision the presence of sperm in the vaginal wash (**Figure 1**), indicating the first day of gestation (G1), or its absence, and in this case, it was used to characterize which phase of the reproductive cycle the rat was in what is known as the estrous cycle, which lasts 4 to 5 days, estimating the female's fertile day, to determine the best day for re-mating (**Figure 2**).

2.3. Induction to GDM

On the fourth day of gestation (G4), the rats were fasted at the end of the day for 24 hours. In G5, the diabetes induction procedure was performed. Induction occurred through an intraperitoneal (IP) injection of streptozotocin (STZ) (Santa Cruz Biotechnologies®).

The drug was diluted at the time of induction, at a dose of 50 mg of STZ per kg of animal weight, diluted in citrate buffer (0.01M and pH 4.5), as STZ is only active in an acidic environment. After dilution, the animals were mobilized manually, and with a sterilized syringe with a fixed 6mm needle, the drug was applied to the animals intraperitoneally, in the third abdominal quadrant (**Figure 3**). After application, the animals remained fasting for one hour, as the mechanism of action used by STZ includes its binding to GLUT-2 receptors in the intestine, the same ones used by dietary glucose, so fasting guarantees absorption exclusive to the drug.

After this time, commercial food and drinking fountains containing water with 1.5% glucose were offered, and this remained for 24 hours, with the aim of avoiding death from hypoglycemia. Which is common to occur in the first 24 hours post-induction.

A parallel group of pregnant rats, determined as the control group (CT), were induced under the same conditions, using only the citrate buffer diluent (without the drug) for comparison.

After 48 hours of induction, exactly on the seventh day of gestation (G7), the females were fasted for eight hours. At the end of the day, body weight and blood glucose assessments were carried out, aiming to verify weight gain resulting from pregnancy and the success of GDM induction, with rats that presented fasting blood glucose ≥ 200 mg/dL being considered diabetic. In G17, the same assessments were repeated.

After approximately 21 days, the puppies began to be born. At this time, the following were measured: total number of puppies per mother, sex, and number of live and dead births. After this time, mothers who did not go into labor were counted as a failed pregnancy.

2.4.Biochemical analysis

To check the damage caused by GDM in the mothers, they were anesthetized using Ketamine and Xylazine (1:1, 0,2g/100ml), and then 1.5 ml of blood was collected via intracardiac puncture. Subsequently, the blood was centrifuged at 1500 rpm for 10 minutes at 25 °C to obtain serum. This is stored in an ultrafreezer at -80 °C, to wait for biochemical tests: Fluorescent AGE assay and Advanced Oxidation Protein Products Assay (AOPP).

Measurement of fluorescent AGEs (F-AGEs) concentrations was based on the spectrofluorimetric detection (KALOUSOVA, SKRHA & ZIMA, 2002). The serum (250 μ L) was transferred into black 96-well plates and phosphate buffer saline (PBS) solution was used as blank. The excitation and emission wavelengths were 320 nm and 440 nm, respectively (Molecular Devices, Menlo Park, CA, USA). The fluorescence intensity was expressed in arbitrary units per milliliter of serum (AU/mL) and in AU/g of total protein. The Bradford method measured the total protein (BRADFORD, 1976). Absorbance was measured at 570 nm in a 96-well plate reader spectrophotometer (brand®).

The AOPPs levels as a marker of protein oxidative damage were measured by the action of ROS (chlorinated compounds) on proteins, leading to the formation of dityrosine residues and protein cross-linking. AOPP was determined according to the method of WITKO-SARSAT and collaborators (1996) using a spectrophotometer (brand®) and calibrated with a chloramine-T solution that absorbs at 340 nm in the presence of potassium iodide. The 10 μ L of serum sample was mixed with 170 μ L of PBS and 20 μ L of citric acid. The curve was made using chloramine and potassium iodide (1.16M), 200 μ L of the standards were mixed with 10 μ L of potassium iodide and 20 μ L of citric acid, and the plate was shaken for six minutes before reading. The absorbance of the reaction mixture was read at 340 nm. AOPP concentrations were expressed as μ mol $\cdot$ L⁻¹ of chloramine-T equivalents.

3. Results

3.1. Copulation control

To verify successful copulation, we used gentian violet staining of vaginal washings, as this dye is easily accessible and cheap, it brought better visibility of all the cells present in the slide, being essential for identifying sperm (**Figure 1B**) and characterization of the estrous cycle, which allowed predicting an ideal moment for a new mating attempt (**Figure 2**). After the end of the gestational period, we achieved 100% pregnancy success in the CT group, and 55.6% success in the GDM group (**Figure 4C**, $p = 0.005$).

3.2. Evolution of body weight and blood glucose

With the data on weight and blood glucose evolution, in the pre-gestational, seventh, and seventeenth gestational days, we evaluated the success of induction of GDM (**Figure 4**).

After administration of STZ, we obtained a group of non-pregnant and diabetic rats, characterized by weight maintenance (**Figure 4A**) and an increase in glycemic rate (**Figure 4B**, G7 and G17 $p < 0.00001$). We had a GDM group, of pregnant and diabetic rats, all of which showed progress in weight gain (**Figure 4A**, G7 $p = 0.0026$, G 17 $p = 0.00001$), and hyperglycemia (**Figure 4B**, G7 and G17 $p < 0.00001$).

3.3. Offspring assessment

Regarding the number of puppies born alive (**Figure 5A**, $p = 0.41$) and the ratio of males ($p = 0.32$) and females ($p = 0.32$) per mother, there were no statistically significant differences between the CT and GDM groups (**Figure 5B**). However, in relation to the rate of stillborn and dead puppies after birth, the GDM group presented 38% of stillborns ($p < 0.0001$), 16.3% of puppies that died between 1 and 7 days ($p < 0.0001$), and 6.5% of puppies that died from 8 to 30 days ($p = 0.02$), while the CT group had a rate of 1.8% of stillbirths and 0.9% of dead pups between 1 and 7 days.

3.4. Maternal blood analysis

When evaluating oxidative stress through the determination of advanced glycation end products (AGEs) and advanced oxidation protein products (AOPP), we saw that both components were increased in diabetic animals (**Figure 6**).

4. Discussion

The gestational diabetes (GDM) induction protocol must be initiated by mating the animals, in this way, the mating was carried out in the proportion of two females for each male, a method called temporary polygamous, and after mating the rats are separated from the males for evaluation of successful pregnancies. In the study by MATTARAI & MOURA (2012), the authors verified the reproductivity of Wistar rats in different mating systems and concluded that the temporary polygamous system was the most efficient, guaranteeing animal welfare, as it respects the biology of the females, and, the reproductive capacity of males is maximized, which represents a saving in physical space and resources used.

To accurately determine the beginning of pregnancy, which was important for inducing diabetes on the correct days of pregnancy, copulation was performed at night, and in the morning the vaginal washes obtained were stained for better identification of cells (BARRIL et al., 2016). The slides were stained with an alternative dye, gentian violet,

facilitating the identification of sperm and the determination of the phase of the female's estrous cycle, an essential method for correctly determining the first day of pregnancy (G1). The use of alternative dyes demonstrates effectiveness for staining animal and plant cells and can be easily acquired for histological preparations and visualization under a microscope (DA SILVA, DE ARAÚJO & BEZERRA, 2019; DA ROCHA et al., 2021). Gentian violet, used in the present study, is an alternative dye with antiseptic activity, bacteriostatic and bactericidal power, low cost, and ease of use and access.

Many authors who study GDM report the difficulty of obtaining pregnant and diabetic animals, and the consequent use of a large number of animals to obtain a significant sample (RUDGE et al., 2013). In most experimental studies, a lot of variation in the methodology for inducing GDM is observed, which consequently creates difficulty in replicating the procedures by other researchers. In the studies seen, the authors carry out vaginal washings at non-standard times, they do not stain the slides, only a few studies monitor the estrous cycle, not all of them specify the use of temporary or permanent polygamous methods and the proportions of females per male, other studies induce diabetes prior to pregnancy, among other divergences, or do not clearly describe this in their methodologies (CALUWAERTS et al., 2003; DAMASCENO et al., 2004; LIRA et al., 2016; FRANÇA, OLIVEIRA & BALBI, 2016). All these details were studied and, in this work, we bring the most efficient procedures from the moment of copulation.

The gestational period of Wistar rats lasts from 19 to 22 days, and after the tenth day, an increase in abdominal volume begins to be noticed. Cases of dystocia are rare, and birth lasts around 1 to 2 hours, with an average of 8 puppies per birth (ANDRADE, PINTO & OLIVEIRA, 2006). However, diabetes during pregnancy can impact gestation time, weight gain, and the number of stillborn and live-born puppies.

The drug of choice for inducing diabetes was streptozotocin (STZ). It causes DNA damage by inhibiting insulin synthesis and secretion, culminating in the process of death of pancreatic beta cells (LERCO et al., 2003; KIRSTEN, SESTERHEIM & SAITOVITCH, 2010). Its action can be influenced by concentration, infusion speed, dose, route of administration, diet, fasting time, and animal weight (SANTOS, 2017). STZ has a glucose molecule in its chemical structure and is therefore able to use GLUT-2 transporters to enter the cell. As pancreatic beta cells have many GLUT-2 type glucose transporters, they become more affected by the drug, with preservation of the extra-pancreatic parenchyma (ELSNER et al., 2007; LENZEN, 2008). In a systematic review, FERREIRA & NICOLAU (2011) carried out a survey of articles on diabetes induction and concluded that STZ is the

most used drug (70% of studies), but with variable doses and routes, of 35 at 65 mg/kg and intraperitoneal (IP) and intravenous (IV), respectively.

To begin the induction of GDM, on the fourth day of gestation, the females were kept fasting for 24 hours, to be induced to diabetes on the fifth gestational day, using the drug STZ, via IP, considered easier to manage than the IV (DEEDS et al., 2011), and uses smaller doses than the subcutaneous via. Fasting prior to induction aims to inhibit the competition of glucose arising from food with the glucose molecule present in the drug. The half-life of STZ is short, ranging from 5 to 15 minutes, therefore, it must be diluted before induction. An acidic buffer must be used for the best activity, such as citrate buffer with pH 4.4 - 4.5 (ELEAZU et al., 2013). The dose will depend on several factors such as animal species, administration, age, weight, and nutritional and hydration status (ETUK & MUHAMMED, 2010). The time of day in which STZ is administered also affects the success of the induction, the study by CANDELA, HERNANDEZ & GAGLIARDINO (1979) showed that inductions carried out in the late afternoon were more successful when compared to those carried out in the morning. Therefore, in our work, the animals were induced at 5 p.m., and we achieved 100% success in inducing diabetes. The study of ELEAZU, IROAGANACHI & ELEAZU (2013) concluded that effective doses for inducing diabetes in rats range from 55 to 75 mg/kg, using the via IP, in a single dose. When very low doses are applied, the tendency is to lead to unstable and reversible diabetes. In our study, we used a dose of 50 mg/kg, a slightly lower dose, but which proved to be effective, leading to stable and irreversible diabetes.

One of the concerns of the GDM protocol is the possibility of the STZ drug crossing the placental barrier, and acting directly on embryonic development and organogenesis, or even on the fetal pancreas. In this context, it is known that induction before conception or in the first gestational days can affect early development and cause miscarriages (FRASER et al., 2007). Our choice of induction in G5 has based on the information that the period of organogenesis in rats begins from G6 and extends to G15 (MEDEIROS et al., 2014), and the development of beta-pancreatic cells in rats occurs during the last week of gestation (BLONDEAU et al., 2011), thus, the endocrine pancreas of fetuses would not be affected by STZ. In the literature, we find inductions at the most different gestational ages, from pre-conception to G10 (BLONDEAU et al., 2011; CALUWAERTS et al., 2003; RAMOS, et al., 2012; NATIF et al., 2003), also, induction in G5 (MERZOUK et al., 2002).

Our success rate for inducing GDM was 55,6%, considered very efficient compared to reports from researchers who study diabetes. Some studies report on how unfavorable

the maternal environment becomes when mothers are in hyperglycemia. In severe GDM, severe hyperglycemia alters placental maturation, making it insufficient in its fetal nutrition function, causing developmental harm and leading to a higher incidence of newborns with restricted body weight. On the other hand, a mildly hyperglycemic environment results in fetuses with macrosomia and hypoinsulinemia (CALDERON et al., 1999). One of the factors involved in these complications caused by GDM is the oxidative damage caused by excess glucose in maternal-fetal blood (JAKUS et al., 2014).

The formation and accumulation of advanced glycation end products (AGEs) and AOPP in groups of mothers with hyperglycemia reflect the damage caused by the induction of GDM. AGEs are generated by the non-enzymatic reaction of sugar with the free amino groups of proteins and amino acids under conditions of hyperglycemia, altering their structure and making them dysfunctional (BARBOSA, OLIVEIRA & SEARA, 2008; VLASSARA & URIBARRI, 2014; LOBO et al., 2017). A variety of toxic α -oxoaldehydes are produced under hyperglycemia in diabetes mellitus, with methylglyoxal (MGO) being one of the most potent glycating agents, playing a key role in endothelial dysfunction that leads to insulin resistance, hypertension, and nephropathy in diabetic patients (REYAZ et al., 2020). The plasma level of MGO, among others, was significantly increased in diabetic rats compared to healthy controls, demonstrating that the protocol applied was capable of generating hyperglycemia during pregnancy by increasing the presence of glycoxided products in plasma serum.

The increase in the oxidation of proteins, lipids, and carbohydrates occurs when the production of reactive oxygen species exceeds the local antioxidant capacity, playing an essential role in the pathogenesis of many diseases (KALOUSOVA, SKRHA & ZIMA, 2002). AOPPs are cross-linked protein products containing di-tyrosine that result in the oxidation of proteins and are generated as a secondary response to oxidative stress, which occurs due to the reaction of chlorinated oxidants with plasma proteins. These AOPPs are elevated in diabetes, they have a structure similar to AGEs and have the ability to induce the production of pro-inflammatory cytokines and adhesive molecules (TSUKAHARA et al., 2003; CONTI et al., 2019; ZHOU et al., 2021).

The puppies from the group of diabetic mothers had higher mortality rates. Many studies report how unfavorable the maternal environment becomes when mothers are in hyperglycemia, which can alter placental maturation, making it insufficient in its function of fetal nutrition, causing harm to the development of the offspring (Ejdesjö et al.; Da Cruz

et al., 2023). One of the factors involved in these complications caused by GDM is the oxidative damage caused by excess glucose in maternal-fetal blood (Jakuš et al., 2014).

The objective of this study was to develop an efficient protocol for inducing GDM, from copulation to proving the disease in mothers during pregnancy and childbirth. In this sense, we were able to show a sequence of essential care to better obtain positive results in the experimental model, as well as prove the disease established not only by measuring blood glucose but also by studying oxidative damage commonly linked to diabetes mellitus.

Declaration of Competing Interest

I wish to confirm that there are no known conflicts of interest associated with this publication.

Acknowledgments

Rodent Bioterium Network (REBIR) of the Federal University of Uberlandia (UFU) and Coordination of Superior Level Staff Improvement - Brazil (CAPES) - Finance Code 001.

Data availability

Data will be made available on request.

References

- Andrade, A., Pinto, S. C., & Oliveira, R. S. D. (2006). Animais de laboratório: criação e experimentação. *Editora Fiocruz*, 118-121. <https://doi.org/10.7476/9788575413869>
- Barbosa, J. H. P., Oliveira, S. L., & Seara, L. T. (2008). O papel dos produtos finais da glicação avançada (AGEs) no desencadeamento das complicações vasculares do diabetes. *Arquivos Brasileiros de Endocrinologia & Metabologia*, 52, 940-950. <https://doi.org/10.1590/S0004-27302008000600005>
- Barril, N., Grisotto, A. C. D., Coelho, A. C. T. E. R., Pereira, G. H., & Garcia, J. C. (2016). Implantação da técnica de citologia vaginal para a identificação e monitorização das fases do ciclo estral em ratas Wistar. *CuidArte Enfermagem*, 162-164.
- Blondeau, B., Joly, B., Perret, C., Prince, S., Bruneval, P., Lelièvre-Pégorier, M., ... & Van Huyen, J. P. D. (2011). Exposure in utero to maternal diabetes leads to glucose intolerance and high blood pressure with no major effects on lipid metabolism. *Diabetes & Metabolism*, 37(3), 245-251. <https://doi.org/10.1016/j.diabet.2010.10.008>
- Bradford, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical biochemistry*, 72(1-2), 248-254. [https://doi.org/10.1016/0003-2697\(76\)90527-3](https://doi.org/10.1016/0003-2697(76)90527-3)

Candela, S., Hernandez, R. E., & Gagliardino, J. J. (1979). Circadian variation of the streptozotocin-diabetogenic effect in mice. *Experientia*, 35, 1256-1257. <https://doi.org/10.1007/BF01963323>

Calderon, I. D. M. P., Rudge, M. V. C., Ramos, M. D., & Peraçoli, J. C. (1999). Estudo longitudinal, bioquímico e histoquímico, de placentas de ratas diabéticas: relação com a macrosomia e o retardo de crescimento intra-uterino. *Revista Brasileira de Ginecologia e Obstetrícia*, 21, 91-98. <https://doi.org/10.1590/S0100-72031999000200006>

Caluwaerts, S., Holemans, K., van Bree, R., Verhaeghe, J., & Van Assche, F. A. (2003). Is low-dose streptozotocin in rats an adequate model for gestational diabetes mellitus?. *The Journal of the Society for Gynecologic Investigation: JSGI*, 10(4), 216-221. [https://doi.org/10.1016/S1071-5576\(03\)00044-3](https://doi.org/10.1016/S1071-5576(03)00044-3)

Conti, G., Caccamo, D., Siligato, R., Gembillo, G., Satta, E., Pazzano, D., ... & Santoro, D. (2019). Association of higher advanced oxidation protein products (AOPPs) levels in patients with diabetic and hypertensive nephropathy. *Medicina*, 55(10), 675. <https://doi.org/10.3390/medicina55100675>

da Cruz, L. L., Barco, V. S., Paula, V. G., Gallego, F. Q., Souza, M. R., Corrente, J. E., ... and Damasceno, D. C. (2023). Severe Diabetes Induction as a Generational Model for Growth Restriction of Rat. *Reproductive Sciences*, 1-13. <https://doi.org/10.1007/s43032-023-01198-9>

da Rocha, R. D. C. (2021). Estratégias Didáticas para o Ensino de Botânica: Microscópio Artesanal Simplificado e Práticas Laboratoriais com Corantes Alternativos. <https://repositorio.ufsc.br/handle/123456789/233968>

da Silva, J. J., de Araújo, S. L. S. M., & Bezerra, M. L. D. M. B. (2019). Experimentação em ciências com o uso de um microscópio artesanal e corante alternativo. *Experiências em Ensino de Ciências*, 14(1), 344-352.

Damasceno, D. C., Volpato, G. T., Calderon, I. D. M. P., Aguilar, R., & Rudge, M. C. (2004). Effect of Bauhinia forficata extract in diabetic pregnant rats: maternal repercussions. *Phytomedicine*, 11(2-3), 196-201. <https://doi.org/10.1078/0944-7113-00348>

Deeds, M. C., Anderson, J. M., Armstrong, A. S., Gastineau, D. A., Hiddinga, H. J., Jahangir, A., ... & Kudva, Y. C. (2011). Single dose streptozotocin-induced diabetes: considerations for study design in islet transplantation models. *Laboratory animals*, 45(3), 131-140. <https://doi.org/10.1258/la.2010.010090>

Ejdesjö, A., Wentzel, P., and Eriksson, U. J. (2012). Influence of maternal metabolism and parental genetics on fetal maldevelopment in diabetic rat pregnancy. *American Journal of Physiology-Endocrinology and Metabolism*, 302(10), E1198-E1209. <https://doi.org/10.1152/ajpendo.00661.2011>

- Eleazu, C. O., Eleazu, K. C., Chukwuma, S., & Essien, U. N. (2013). Review of the mechanism of cell death resulting from streptozotocin challenge in experimental animals, its practical use and potential risk to humans. *Journal of diabetes & metabolic disorders*, 12, 1-7. <https://doi.org/10.1186/2251-6581-12-60>
- Eleazu, C. O., Iroaganachi, M., & Eleazu, K. C. (2013). Ameliorative potentials of cocoyam (*Colocasia esculenta* L.) and unripe plantain (*Musa paradisiaca* L.) on the relative tissue weights of streptozotocin-induced diabetic rats. *Journal of diabetes research*, 2013. <https://doi.org/10.1155/2013/160964>
- Elsner, M., Guldbakke, B., Tiedge, M., Munday, R., & Lenzen, S. (2000). Relative importance of transport and alkylation for pancreatic beta-cell toxicity of streptozotocin. *Diabetologia*, 43, 1528-1533. <https://doi.org/10.1007/s001250051564>
- Etuk, E. U., & Muhammed, B. J. (2010). Evidence based analysis of chemical method of induction of diabetes mellitus in experimental rats. *Int J Res Pharm Sci*, 1(2), 139-142.
- Ferreira, C. L. R., & Nicolau, R. A. (2011). *Diabete experimental em ratos-revisão sistemática*. Encontro Latino Americano de Iniciação Científica, Encontro Latino Americano de Pós-Graduação: Anais de trabalhos completos XV Encontro Latino Americano de Iniciação Científica, XI Encontro Latino Americano de Pós-Graduação, 1-5.
- França-Silva, N., Oliveira, N. D. G., & Balbi, A. P. C. (2016). Morphofunctional renal alterations in rats induced by intrauterine hyperglycemic environment. *Archives of Medical Science*, 12(2), 243-251. <https://doi.org/10.5114/aoms.2015.48220>
- Fraser, R. B., Waite, S. L., Wood, K. A., & Martin, K. L. (2007). Impact of hyperglycemia on early embryo development and embryopathy: in vitro experiments using a mouse model. *Human reproduction*, 22(12), 3059-3068. <https://doi.org/10.1093/humrep/dem318>
- García, C. G. (2008). Diabetes mellitus gestacional. *Medicina interna de México*, 24(2), 148-156.
- Gispen, W. H., & Biessels, G. J. (2000). Cognition and synaptic plasticity in diabetes mellitus. *Trends in neurosciences*, 23(11), 542-549. [https://doi.org/10.1016/S0166-2236\(00\)01656-8](https://doi.org/10.1016/S0166-2236(00)01656-8)
- Golbert, A., & Campos, M. A. A. (2008). Diabetes melito tipo 1 e gestação. *Arquivos Brasileiros de Endocrinologia & Metabologia*, 52, 307-314. <https://doi.org/10.1590/S0004-27302008000200018>
- Jakuš, V., Sándorová, E., Kalninová, J., & Krahulec, B. (2014). Monitoring of glycation, oxidative stress and inflammation in relation to the occurrence of vascular complications in patients with type 2 diabetes mellitus. *Physiological Research*, 63(3). <https://doi.org/10.33549/physiolres.932672>

- Kalousova, M., Skrha, J., & Zima, T. (2002). Advanced glycation end-products and advanced oxidation protein products in patients with diabetes mellitus. *Physiological research*, 51(6), 597-604. <https://doi.org/10.1159/000066956>
- Kirsten, V. R., Sesterheim, P., & Saitovitch, D. (2010). Modelos experimentais para o estudo do diabetes tipo 1. *Medicina*, 43(1), 3-10. <https://doi.org/10.11606/issn.2176-7262.v43i1p3-10>
- Lenzen, S. (2008). The mechanisms of alloxan-and streptozotocin-induced diabetes. *Diabetologia*, 51(2), 216-226. <https://doi.org/10.1007/s00125-007-0886-7>
- Lerco, M. M., Spadella, C. T., Machado, J. L. M., Schellini, S. A., & Padovani, C. R. (2003). Caracterização de um modelo experimental de Diabetes Mellitus, induzido pela aloxana em ratos: estudo clínico e laboratorial. *Acta Cirúrgica Brasileira*, 18, 132-142. <https://doi.org/10.1590/S0102-86502003000200010>
- Lira, E. C., dos Santos, G. M. R. F., da Silva, J. J. T., Duarte, G. I. B. P., de Aguiar Júnior, F. C. A., & de Oliveira Silva, C. (2016). Exposição ao diabetes gestacional: alterações sobre os parâmetros de estresse oxidativo e acúmulo de ferro esplênico em ratos. *Infarma-Ciências Farmacêuticas*, 27(4), 205-213. <http://dx.doi.org/10.14450/2318-9312.v27.e4.a2015.pp205-213>
- Lobo, J. P., Brescansin, C. P., Santos-Weiss, I. C., Welter, M., Souza, E. M. D., Rego, F. G. D. M., ... & Alberton, D. (2017). Serum Fluorescent Advanced Glycation End (F-AGE) products in gestational diabetes patients. *Archives of endocrinology and metabolism*, 61, 233-237. <https://doi.org/10.1590/2359-3997000000238>
- Mattaraia, V. G. D. M., & Moura, A. S. A. M. T. (2012). Produtividade de ratos Wistar em diferentes sistemas de acasalamento. *Ciência Rural*, 42, 1490-1496. <https://doi.org/10.1590/S0103-84782012000800026>
- Medeiros, M. A., Riet-Correa, F., Dantas, F. P., Santos, J. R., & Medeiros, R. M. (2014). Efeitos teratogênicos de *Prosopis juliflora* em ratos e análise da toxicidade das vagens. *Pesquisa Veterinária Brasileira*, 34, 1089-1093. <https://doi.org/10.1590/S0100-736X2014001100010>
- Merzouk, H., Madani, S., Hichami, A., Prost, J., Moutairou, K., Belleville, J., & Khan, N. A. (2002). Impaired lipoprotein metabolism in obese offspring of streptozotocin-induced diabetic rats. *Lipids*, 37, 773-781. <https://doi.org/10.1007/s11745-002-0960-9>
- Milech, A., Forti, A. C., Golbert, A., Gomes, M. B., & Dib, S. A. (2007). Tratamento e acompanhamento do Diabetes mellitus. São Paulo: *Diretrizes da Sociedade Brasileira de Diabetes*.
- Natif, N., Sclarovsky-Benjaminov, F., Van Dijk, D. J., Sulkes, J., Gafter, U., Boner, G., & Erman, A. (2003). Insulin and losartan reduce proteinuria and renal hypertrophy in the

pregnant diabetic rat. *Journal of Laboratory and Clinical Medicine*, 142(3), 166-171. [https://doi.org/10.1016/S0022-2143\(03\)00113-6](https://doi.org/10.1016/S0022-2143(03)00113-6)

Ramos-Alves, F. E., de Queiroz, D. B., Santos-Rocha, J., Duarte, G. P., & Xavier, F. E. (2012). Increased cyclooxygenase-2-derived prostanoids contributes to the hyperreactivity to noradrenaline in mesenteric resistance arteries from offspring of diabetic rats. *PLoS One*, 7(11), e50593. <https://doi.org/10.1371/journal.pone.0050593>

Reyaz, A., Alam, S., Chandra, K., Kohli, S., & Agarwal, S. (2020). Methylglyoxal and soluble RAGE in type 2 diabetes mellitus: Association with oxidative stress. *Journal of Diabetes & Metabolic Disorders*, 19, 515-521. <https://doi.org/10.1007/s40200-020-00543-y>

Rudge, M. V. C., Calderon, I. D. M. P., Ramos, M. D., Brasil, M. A. M., Rugolo, L. M. S. S., Bossolan, G., & Odland, J. O. (2005). Hiperglicemia materna diária diagnosticada pelo perfil glicêmico: um problema de saúde pública materno e perinatal. *Revista Brasileira de Ginecologia e Obstetrícia*, 27, 691-697. <https://doi.org/10.1590/S0100-72032005001100010>

Rudge, M. V. C., Piculo, F., Marini, G., Damasceno, D. C., Calderon, I. M. P., & Barbosa, A. P. (2013). Pesquisa translacional em diabetes melito gestacional e hiperglicemia gestacional leve: conhecimento atual e nossa experiência. *Arquivos Brasileiros de Endocrinologia & Metabologia*, 57, 497-508. <https://doi.org/10.1590/S0004-27302013000700001>

Santos, G. M. R. F. D. (2017). Diabetes gestacional induzido por estreptozotocina: contribuição do estresse oxidativo e da enzima NADPH oxidase no desenvolvimento de lesões cardiovasculares na prole. <https://repositorio.ufpe.br/handle/123456789/30718>

Silva, F. C., Presgrave, O. A. F. (2008). *Manual de utilização de animais/FIOCRUZ* (1th ed.).

Silva, J. C., Bertini, A. M., Ribeiro, T. E., Carvalho, L. S. D., Melo, M. M., & Barreto Neto, L. (2009). Fatores relacionados à presença de recém-nascidos grandes para a idade gestacional em gestantes com diabetes mellitus gestacional. *Revista Brasileira de Ginecologia e Obstetrícia*, 31, 5-9. <https://doi.org/10.1590/S0100-72032009000100002>

Sun, H., Saeedi, P., Karuranga, S., Pinkepank, M., Ogurtsova, K., Duncan, B. B., ... & Magliano, D. J. (2022). IDF Diabetes Atlas: Global, regional and country-level diabetes prevalence estimates for 2021 and projections for 2045. *Diabetes research and clinical practice*, 183, 109119. <https://doi.org/10.1016/j.diabres.2021.109119>

Tsukahara, H., Sekine, K., Uchiyama, M., Kawakami, H., Hata, I., Todoroki, Y., ... & Mayumi, M. (2003). Formation of advanced glycosylation end products and oxidative stress in young patients with type 1 diabetes. *Pediatric research*, 54(3), 419-424. <https://doi.org/10.1203/01.PDR.0000076662.72100.74>

Vlassara, H., & Uribarri, J. (2014). Advanced glycation end products (AGE) and diabetes: cause, effect, or both?. *Current diabetes reports*, 14, 1-10. <https://doi.org/10.1007/s11892-013-0453-1>

Witko-Sarsat, V., Friedlander, M., Capeillère-Blandin, C., Nguyen-Khoa, T., Nguyen, A. T., Zingraff, J., ... & Descamps-Latscha, B. (1996). Advanced oxidation protein products as a novel marker of oxidative stress in uremia. *Kidney international*, 49(5), 1304-1313. <https://doi.org/10.1038/ki.1996.186>

Zhou, C., Zhang, Y., Chen, J., Mei, C., Xiong, F., Shi, W., ... & Hou, F. F. (2021). Association between serum advanced oxidation protein products and mortality risk in maintenance hemodialysis patients. *Journal of Translational Medicine*, 19(1), 1-8. <https://doi.org/10.1186/s12967-021-02960-w>

Figure captions

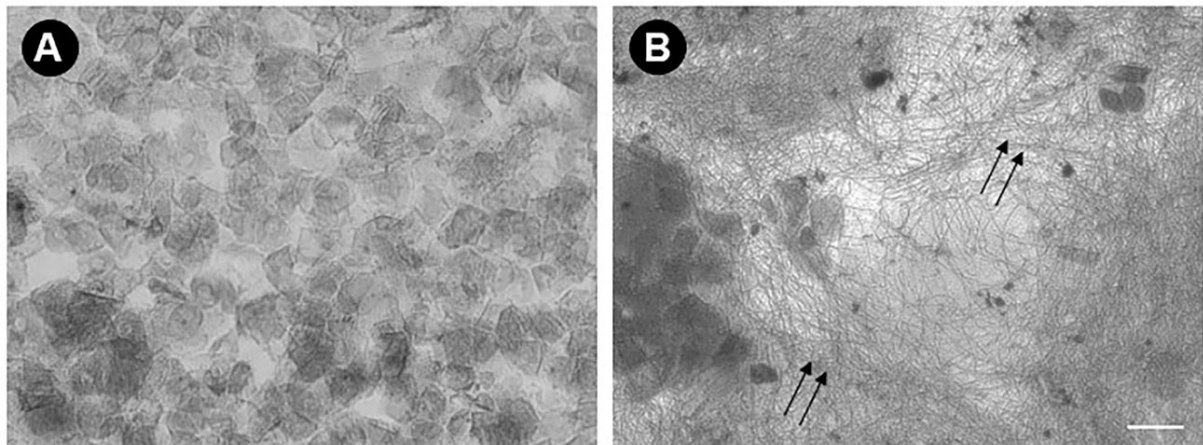


Figure 1 – Vaginal washes from rats stained with gentian violet. **A:** Fertile phase absent of sperm (estrus, see figure 2); **B:** Fertile phase combined with the presence of sperm (arrows), indicating G1. Scale = 10 μ m.

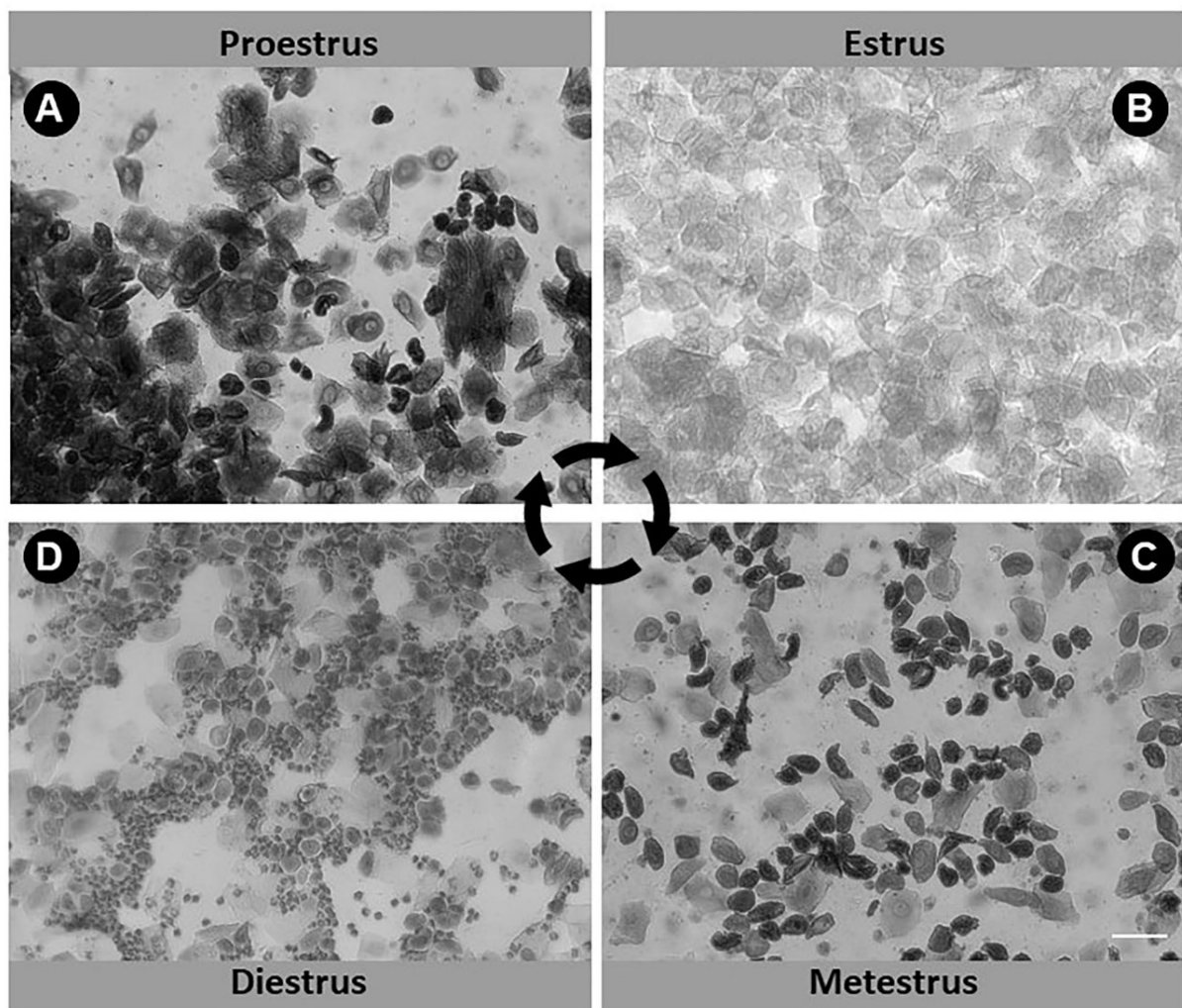


Figure 2 – Phases of the estrous cycle observed in vaginal washes from Wistar rats stained with gentian violet. **A: Proestrus**, characterized by a large number of round and polynucleated cells, found scattered or grouped, and keratinized cells; **B: Estrus**, presents

only keratinized and anucleated cells; **C: Metestrus**, presence of few leukocytes, little mucus, and some keratinized cells; **D: Diestrus**, numerous leukocytes (very small) and mucus filaments are visualized. Scale = 10 μ m.

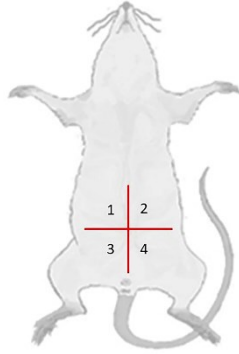


Figure 3 – Abdominal quadrants for intraperitoneal application. STZ was applied in the third quadrant.

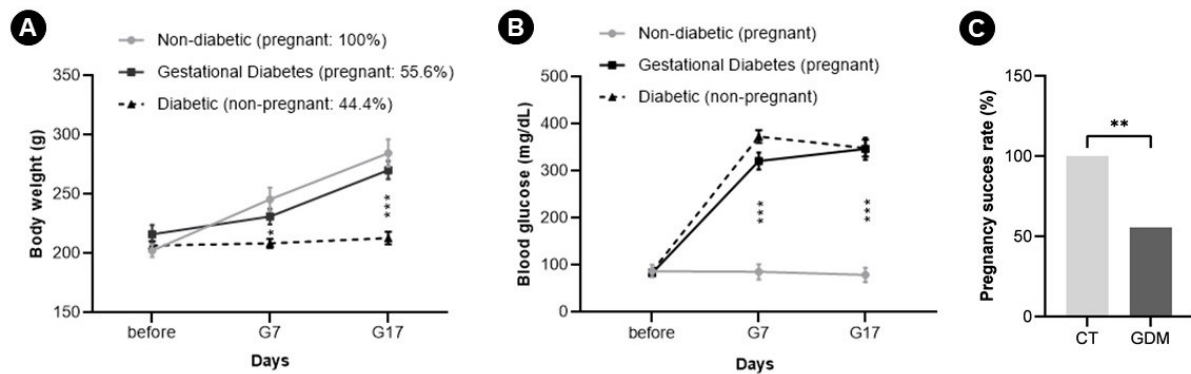


Figure 4 – Assessment of rats, before pregnancy, in G7 and G17, in the different comparison groups, non-diabetic (CT), gestational diabetes (GDM), and diabetic (D). **A:** Evolution of body weight in grams: the dotted line represents animals that did not gain weight in G7 and G17. **B:** Evolution of blood glucose in mg/dL: Before ($p > 0.9999$), G7 and G17 ($***p < 0.0001$). **C:** Percentage of pregnancy success: shows 100% gestational success in the CT group, and 55.6% for the GDM group ($**p = 0.005$). Holm-Sidak test (A and B) and Binomial test (C).

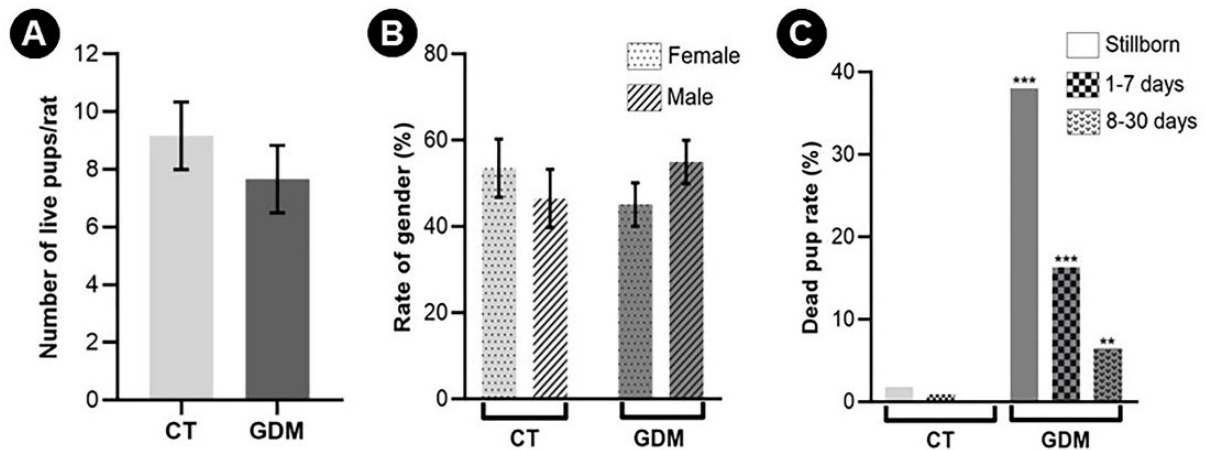


Figure 5 – Assessment of gestational quality. **A:** Number of pups born alive per rat ($p = 0.41$); **B:** Sex ratio of offspring (male and female $p = 0.32$); **C:** Rate of stillborn puppies, dead from 1 to 7 days, and from 8 to 30 days. A higher percentage of stillborn and dead puppies after birth ($***p < 0.0001$ and $**p = 0.02$) was noted in the gestational diabetes group. Mann Whitney U test (A), t-test (B), and Binomial test (C).

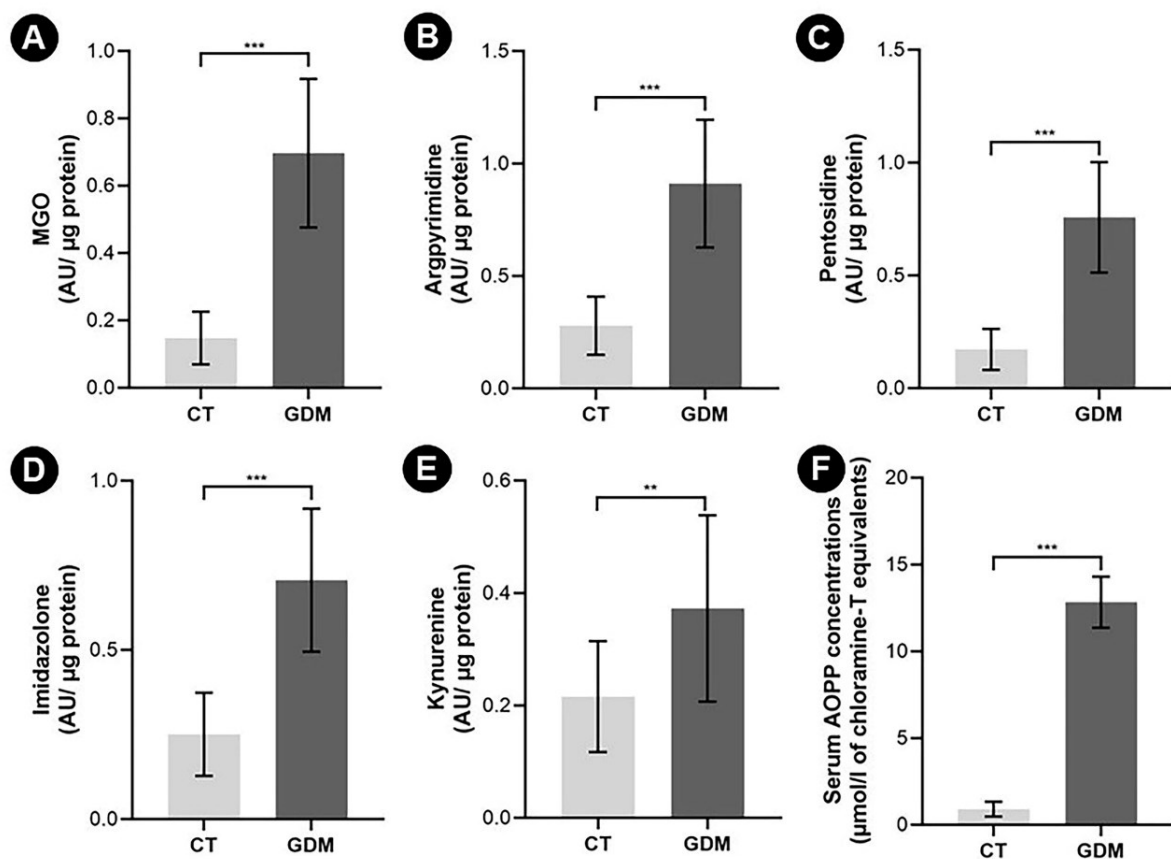


Figure 6 – Assessment of oxidative stress in the serum of mothers in the control (CT) and gestational diabetes (GDM) groups. **A, B, C, D, and E:** quantification of advanced glycation products (AGEs). A higher concentration of AGEs was noted in the GDM group when compared to the CT, (A, B, C, and D: $p < 0.0001$; E: $p = 0.0085$). **F:** advanced protein oxidation products (AOPP), showing a higher concentration of AOPPs in the GDM group ($p < 0.0001$). Mann Whitney U test.

ARTIGO 2

Gestational Diabetes influences the development of the cerebellum in rats

Submitted in *Metabolic Brain Disease*, Qualis A3

Gestational Diabetes influences the development of the cerebellum in rats

Fernanda Naves Araújo do Prado Mascarenhas¹; Maria Júlia Neves Ribeiro¹; Natália Ferreira Silva¹; Rener Mateus Francisco Duarte²; Renata Rouland Teixeira²; Foued Salmen Espindola²; Daniele Lisboa Ribeiro¹; Carlos Ueira-Vieira²; Renata Graciele Zanon^{1,*}

¹Institute of Biomedical Sciences, Federal University of Uberlandia (UFU), Uberlandia, MG, Brazil

²Institute of Biotechnology, Federal University of Uberlandia (UFU), Uberlandia, MG, Brazil.

Orc id: Fernanda N. A. do Prado: 0000-0002-1499-9850; Maria Júlia N. Ribeiro: 0009-0000-0303-5733; Natália F. Silva: 0000-0001-6277-0367, Rener M. F. Duarte: 0009-0005-4719-1494; Renata R. Teixeira: 0000-0002-5081-1384, Foued S. Espindola: 0000-0002-6937-1411, Daniele L. Ribeiro: 0000-0002-2800-4084, Carlos Ueira-Vieira: 0000-0002-8369-9069, Renata G Zanon: 0000-0001-5930-3821

Corresponding author: Dr. Renata Graciele Zanon, Instituto de Ciências Biomédicas, Universidade Federal de Uberlândia (UFU), CP 592, CEP 38400-902, Uberlândia, MG, Brasil. e-mail: rezanon@ufu.br

ABSTRACT

Diabetes mellitus (DM) is characterized by a dysfunction in the secretion and/or action of insulin, causing hyperglycemia. When the disease occurs during pregnancy, it is called gestational diabetes mellitus (GDM), and the hyperglycemic state affects the developing fetus, potentially causing various damages. Excess of glucose can compromise neural cells, leading to neurophysiological and structural changes. This study aims to understand whether offspring of diabetic rats show structural and/or functional impairment of the cerebellum, due to its range of functions in body control and having approximately 80% of the brain's neurons. For this purpose, the offspring were physically evaluated, and after euthanasia, their brains were collected, followed by cerebellum dissection. These were prepared for histomorphometric, immunofluorescent analyses, and target protein expression, indicating inflammation, gliosis, oxidative imbalance, and cell death. To assess functional impacts, the offspring underwent motor behavioral tests. The results showed that the GDM group offspring had lower body mass indices, along with lower brain weight. Cerebellar histomorphometry revealed thinner cortical layers with gliosis, as well as oxidative imbalance and apoptosis. They also exhibited less exploratory behaviors, indicative of reduced cognition. Thus, we conclude that maternal hyperglycemia interferes with the cerebellar development of their offspring, potentially impacting adult life.

Keywords: gestational diabetes, hyperglycemia, motor nervous system, cerebellum, development.

1. Introduction

Gestational diabetes mellitus (GDM) is considered a public health problem, characterized by glucose intolerance that begins during pregnancy (Batista et al., 2021) its worldwide prevalence is 15.2% among pregnant women (Sun et al., 2022), and that generally resolves postpartum but returns years later in most cases. The pathophysiology of the disease is similar to type I diabetes mellitus, involving either deficiency in insulin secretion or resistance to its action in different organs, and it is known that the gestational phase itself contributes to these diabetogenic factors, as insulin and carbohydrate metabolism, they occur to ensure an adequate supply of glucose to the fetus (Montenegro and Rezende Filho, 2008). This insulin resistance is caused in part by the elevation of placental hormones, such as growth hormone (GH), estrogen, human chorionic somatotropin, and prolactin (Mançú and Almeida, 2016).

Maternal hyperglycemia during pregnancy can cause hyperinsulinemia in the fetus, oxidative stress, tissue hypoxia, and a decrease in iron levels (Hami et al. 2015), altering the normal trajectory of the development of the fetal Nervous System, predisposing it to neurodevelopmental disorders, including deficiencies in motor functioning, attention, and learning capacity, possibly due to neuroinflammation and oxidation caused by excess glucose (Chandna et al., 2015; Van Lieshout and Voruganti, 2008). Our work contributes information about the development of the cerebellum, a tissue with around 80% of the neurons present in the entire Central Nervous System (CNS), in the intrauterine environment of diabetic females, and in the postnatal life until adulthood of the offspring. Recent studies provide evidence that the cerebellum, in addition, to function in balance and motor control, is central to motor learning, sensory discrimination, and complex cognitive tasks (Arleo et al., 2023; Manto et al., 2012).

The cerebellum can be phylogenetically divided into archicerebellum (composed of the flocculonodular lobe and related to the vestibular system), paleocerebellum (composed of the vermis and medial region of the cerebellar hemisphere with motor functions and proprioception), and neocerebellum (lateral region of the cerebellar hemisphere, responsible for motor programming and currently related to cognition). The gray matter of the mature cerebellum has three cellular layers identified by classical histology: the molecular layer, the Purkinje cell layer, and the granular cell layer (Damiani et al., 2016).

In rats, cerebellar maturation occurs during the first three postnatal weeks (Goldowitz and Hamre, 1998), which corresponds to the third trimester of embryonic brain development in humans. In relation to the cerebellum, this is the active period of neuronal

proliferation, migration, and differentiation of granule cells in the cortex region. Starting with the rapid proliferation of thousands of immature neurons in the external granular layer (EGL) of the cerebellum, covering the entire external surface of the cerebellar cortex, and these migrate along the radial processes of glial cells through the molecular layer towards the internal granular layer (IGL) and begin their differentiation into mature neurons (Yong et al., 2015). At this stage, the cerebellar cortex has four layers: the external granular layer (EGL), the molecular layer, the Purkinje cell layer, and the internal granular layer (IGL), with the EGL being temporary, as it disappears after the completion of total cerebellar differentiation, the so-called mature cerebellum (Mascarenhas et al., 2022).

Some studies show that fetal development in a hyperglycemic environment is directly related to several pathologies that manifest as motor, behavioral, and cognition changes, one of the functions of the cerebellum, such as autism and schizophrenia (Xiang et al., 2018; Van Lieshout and Voruganti, 2008). In this sense, our work seeks to understand how the cerebellum develops in a potentially injured environment, and whether there is an impact on behaviors involving the functions of the cerebellum in adulthood in rats.

2. Methods

2.1. Animals and mating

All procedures performed with the animals were duly approved by the Animal Use Ethics Committee (CEUA) of the Federal University of Uberlandia (UFU), protocol 056/18. The model used was heterozygous rats of the Wistar lineage (*Rattus norvegicus*), free of specific pathogenic germs (SPF), obtained and maintained in the Rodent Animal Facility Network (REBIR) at the Federal University of Uberlandia (UFU).

39 animals were used, 12 males and 27 females (12 CT e 15 GDM) and they were kept in polysulfone boxes, with adequate air circulation, controlled temperature between 18-22°C, humidity of 45 to 55%, with an autoclaved wood shavings bed, and a 12/12-hour light-dark light cycle. For feeding, commercial feed and drinking water were used in dedicated drinking fountains, ad libitum.

After the animals reached eight weeks of age, the age of sexual maturity, and during this period, males weighed around 200-250g and females weighed around 150-180g. Nulliparous females were previously evaluated in relation to weight (g), using an electronic scale with a precision of 0.1 gram (KERN 440-49n®), and blood glucose (mg/dL) after an 8-hour fast, to obtain a drop of blood from the animal's tail with the aid of a scalpel, and the reading taken on a glucometer (Descarpac Plus®).

Subsequently, the females were transferred to the male boxes in the late afternoon for mating, in a ratio of two females to one male (temporary polygamous system). And in the morning of the following day, the females were returned to their original boxes, to collect vaginal fluid to control copulation.

The rats were restrained manually, then a Pasteur pipette containing saline solution (0.9%) was introduced superficially into the vaginal canal, and the collected material was deposited on the identified glass slides and stained with gentian violet. Then, the reading and capture were carried out under an optical microscope, with a 20x objective. Thus, it was possible to identify with greater precision the presence of sperm in the wash, indicating the first day of gestation (G1), or its absence, and in this case, it was used to characterize which phase of pregnancy reproductive cycle the rat was in, called the estrous cycle, which lasts 4 to 5 days, with this it was possible to estimate the female's fertile day, this being the determinant of the best day to put her back into mating.

2.2 Induction to GDM

On the fourth day of gestation (G4), the rats were fasted at the end of the day and remained fasting for 24 hours. And on the fifth day (G5), the diabetes induction procedure was carried out, through an intraperitoneal (IP) injection of streptozotocin (STZ) (Santa Cruz Biotechnologies®). The drug was diluted at the time of induction, at a dose of 50 mg of STZ per kilogram of weight, in citrate buffer (0.01M and pH 4.5), as STZ is only active in an acidic environment. After dilution, the animals were manually mobilized, and with a sterilized syringe with a fixed needle, the drug was applied to the animals via IP. A parallel group of pregnant rats, determined as the control group (CT), were induced under the same conditions, but without the drug, using only the citrate buffer diluent (0.01M and pH 4.5) for subsequent comparison.

After 48 hours of induction, exactly on the seventh day of gestation (G7), the females were fasted in the morning and kept for eight hours, and at the end of the day, they were weighed (g) and blood glucose was measured (mg/ dL), rats with fasting blood glucose ≥ 200 mg/dL is considered diabetic.

2.3 Offspring and groups

The puppies were divided according to Table 1. They underwent assessments of body weight (g), nasoanal length (cm), blood glucose (mg/dL), total brain weight (g) and relative (%), at 7, 30, and 90 days of age.

2.4 Cerebellum analysis

2.4.1 Tissue morphology

After euthanasia, half of the animals per group and age underwent thoracotomy for transcardiac perfusion with 5-20 ml of buffered saline (PBS) (0.01 M and pH 7.4) and, subsequently, with 5-20 ml of 3.4% formaldehyde fixative solution in PBS. The brains were then extracted, first weighed (g), and then followed by histological preparation.

The tissue remained for 48 hours in a fixative solution at 4°C, was submerged in sucrose solutions in increasing concentrations (10, 20, and 30%) for dehydration and cryoprotection and, after 72 hours, they were included in Tissue-tek and frozen in chilled isopentane (-40°C) with the aid of liquid nitrogen (-195.8°C). These were stored in a freezer (-20°C) until the histological sections were obtained using a cryostat (Leica®), serial frontal sections 10 µm thick. To locate the region of interest for the study, the cerebellum, the brain coordinates map of rats Paxinos and Watson (2006) was used. The sections were transferred to previously gelatinized glass slides and stored in a freezer (-20°C) until histochemical and immunofluorescence markings were performed.

Part of the slides containing frontal sections of the cerebellum were stained with Nissl corpuscles, that is, rough endoplasmic reticulum with basophilic characteristics, which stain purple and are found in neural cells. First, they were submerged in a cresyl violet solution (1g of cresyl violet, 100 ml of distilled water, and 10 ml of 10% acetic acid) for 10 minutes, washed in distilled water, dehydration process in an increasing series of ethanol (70, 80, 90 and 100%1, 100%2 and 100%3) for 1 minute each, for dehydration, diaphanization process in xylene (1, 2 and 3) for 5 minutes each. Then mount the slides using a mounting medium (Entellan®) and a properly cleaned coverslip. Subsequently, the slides were digitized in a slide scanner (ScanScope AT®), at 20x magnification, to be analyzed, with the ImageJ software, in relation to the measurement of the external granular layer (EGL) at P7, measurement of the cerebellar layers (molecular, Purkinje and granular cells) and quantification of Purkinje cells at P30 and P90.

Another part of the slides containing frontal sections of the cerebellum were sent for specific immunostaining for astrocytes. First, they were washed three times in buffered saline (PBS) (0.01 M and pH 7.4) for five minutes each, treated with ice-cold methanol for antigen retrieval for five minutes, then non-specific binding was blocked using albumin. bovine diluted in PBS (BSA 5%) for one hour and 30 minutes, and incubated with the primary antibody, mouse anti-GFAP (Santa Cruz Biotechnologies® 1:100, astrocyte marker), overnight at 4°C. The next day, the slides were washed three times in PBS for five

minutes each, and then incubation began with the secondary antibody, mouse conjugated with Alexa-Fluor 488 nm (Jackson Lab® 1:200), for two hours, and washed in PBS. Afterward, the slides were mounted using glycerol containing DAPI (4',6-diamidino-2-phenylindole) (Santa Cruz Biotechnologies® 1:1000, nuclear marker) and a properly cleaned coverslip, stored at 4°C and protected from light. Subsequently, the slides were photographed under a fluorescence microscope (TSi NIKON®), at 20x magnification, to be qualitatively analyzed in relation to the presence of astrogliosis at P7, P30, and P90.

2.2.1 Tissue protein extract

After euthanasia, half of the animals per group and age did not undergo perfusion for washing and fixation. The brains were then extracted, first weighed (g), and subsequently, the cerebellum was separated for protein extraction. The extraction of total proteins was started with the addition of homogenization buffer (0.01 M and pH 7.4), in amounts calculated per sample weight (g), with the aid of a sample homogenizer, followed by refrigerated centrifugation (800xg for 15 min at 4°C), the supernatants were separated, and using the Bradford method (Bradford, 1976) the total protein was quantified by reading the absorbance in an Elisa reader (Perkin-Elmer LS 55®) at 595nm. Such data are plotted in spreadsheets (Excel®) for normalization of the other tests.

Aliquots containing supernatant were stored in an ultra-freezer (-80°C) after oxidative stress tests, to perform apoptosis analyses.

- **Oxidative stress**

The aliquots containing cerebellum protein extract underwent redox balance evaluations, and for this purpose, the formation of reactive oxygen species (ROS), indicators of protein damage (AOPP), and antioxidant activity were measured, using the iron reduction method (FRAP), enzymatic antioxidant system, including the enzymes superoxide dismutase (SOD) and glutathione levels (GSH, GR and GPx).

a. ROS: The determination of ROS levels was performed following the method described by Sommerfeld et al. (2022). In brief, the homogenates were incubated with dichloro-dihydro-fluorescein diacetate (10 µM) and Tris-HCl buffer (5 mM, pH 7.4) for 3 minutes. Subsequently, the fluorescence was measured in 96-well microplates in an Elisa reader (Perkin-Elmer LS 55®) at 530 nm.

b. AOPP: AOPP (Advanced Oxidation Protein Products) is a method for quantifying advanced protein oxidation products. The determination of AOPP levels was carried out by spectrophotometric measurements using chloramine-T as a standard reference. Briefly, the sample was diluted in PBS, or standard chloramine-T solutions (0–500 $\mu\text{mol/L}$), placed in a 96-well plate, followed by acetic acid and potassium iodide (KI) (1.16 M). Absorbance was immediately read at 340 nm. AOPP concentrations were expressed in $\mu\text{mol/liter}$ of chloramine-T equivalents (Conti et al., 2009)

c. FRAP: The antioxidant capacity is determined through the ferric reducing antioxidant potential. In this assay, the antioxidants present in the sample reduce Fe^{3+} to Fe^{2+} , which is chelated by 2,4,6-tri (2 pyridyl)-s-triazine (TPTZ) to form the Fe^{2+} - TPTZ complex, with an intense blue color. This colored complex is read on the spectrophotometer (brand®) at 593 nm. The antioxidant capacity of the sample is determined using an analytical curve, constructed with Trolox as a standard. Trolox (6-hydroxy-2,5,7,8-tetramethachroman-2-carboxylic acid), is a hydrophilic analogue of vitamin E and is used as a standard antioxidant agent (Benzie and Strain, 1996).

d. SOD: SOD catalyzes the dismutation reaction of two superoxide radicals to hydrogen peroxide and oxygen and is called the primary defense against oxidative stress, as the superoxide radical is a strong initiator of chain reactions. The hydrogen peroxide formed is less reactive than the superoxide radical and is further degraded by other systems, such as catalase and glutathione peroxidase. The method is based on the inhibition of the superoxide radical reaction with pyrogallol. Superoxide is generated by the autoxidation of pyrogallol in a basic medium. The SOD present in the sample competes for the superoxide radical with the detection system. The oxidation of pyrogallol leads to the formation of a colored compound, detected on the spectrophotometer (brand®) at 420 nm. SOD activity is determined by measuring the rate of formation of oxidized pyrogallol. Results are expressed in units of SOD/mg protein (U/mg). One unit of SOD is defined as 50% of the autoxidation of pyrogallol (Marklund and Marklund, 1974).

e. Measurement of glutathione (GSH), oxidized (GPx), and reduced (GR) equivalents: To reduced glutathione (GSH), the protein content of the samples was initially precipitated by metaphosphoric acid (MPA) (1:1). The samples were centrifuged (7000xg for 10 minutes), and the supernatant was collected and mixed with sodium phosphate buffer (100

mM, pH 8.0), containing EDTA (5mM) and ortho-phthaldialdehyde (1 mg/mL in methanol). The mixture was incubated in the dark at room temperature for 15 min and fluorescence was measured at 350 nm (excitation) and 420 nm (emission). A standard curve of GSH (0.001–0.1 mM) was used for linear regression. To measure the glutathione peroxidase (GPx) activity, the homogenate was incubated with GPx buffer (100 mM potassium phosphate containing 1 mM EDTA, pH7.7), sodium azide (40 mM), GSH (diluted in 5% metaphosphoric acid), GR (diluted in GPx buffer), NADPH (diluted with sodium bicarbonate 5%) and tert-butyl (0.5 mM). The reduction in NADPH concentration was evaluated for 10 minutes in a spectrophotometer (brand®), at 340 nm. To glutathione reductase (GR) activity, was evaluated using oxidized glutathione (GSSG) and nicotinamide adenine dinucleotide phosphate (NADPH) as substrates. The activity of the enzyme was determined using sodium phosphate buffer (200 mM, pH 7.5), EDTA (6.3 mM), GSSG (1 mM), NADPH (1 mM) and the samples. The consumption of NADPH was measured at 340 nm for 10 minutes. A GR unit is defined as one μ mol of reduced GSSG per minute. The specific activity was calculated as U/mg of protein (Diniz et al., 2016).

• Detection of caspase-3 activation

Caspase-3 proteolytic activity was measured by fluorimetry, using the EnzChek® Caspase-3 Assay kit, according to the manufacturer's specifications (Thermo Fisher Scientific®). The basis of the technique consists of the property that caspase-3 exhibits in cleaving the c-terminal site of the aspartate residue of the DEVD sequence (Asp-Glu-Asp). In this way, the kit provides the substrate for caspase 3 activity linked to fluorescein rhodamine 110 (Z-DEVD.AMC Substrate) which, in contact with the samples containing the enzymes, displays fluorescence, which is read in a microplate reader on the wavelength of 441nm.

2.2.1 Behavioral analyzes

The puppies in the 90-day group (CT and GDM) were subjected to behavioral assessment tests before euthanasia. The Open Field Test (OFT) is a behavioral test used to study locomotion and anxiety. The animal's activity is evaluated for approximately five minutes in an apparatus suitable for the test, made of wood, circular in shape, containing a base divided into peripheral and central quadrants, and a wall. The animals are filmed for five minutes and exposed to OFT. In it, locomotor activity is analyzed (time spent in the center and on the periphery, number of exploratory events carried out, and distance

covered), with the help of Smart Video Tracking Software (Panlab®). The Elevated Cross Maze (ECM) is a behavioral test used to observe movement, exploration of the environment, and anxiety levels. The animal's activity is evaluated for approximately five minutes in an apparatus suitable for the test, elevated 90 centimeters from the ground and formed by two open arms that form a cross with two closed arms. The animals are filmed for five minutes and exposed to the ECM. In it, locomotor activity is evaluated (time spent in the closed and open arms, number of exploratory events performed, and distance covered) with the help of Smart Video Tracking Software (Panlab®). It is based on the animal's "avoidance" behavior in an unknown environment combined with the natural aversion to open, potentially dangerous regions. Thus, the percentage of preference (time spent) for the open and closed arms is considered a reliable index of anxiety: the higher the level of anxiety, the lower the percentage of staying in the open arms.

3. Results

3.1. Body and brain weights and blood glucose of offspring

Assessment data on body mass index (BMI), brain weight, and blood glucose levels of puppies, at seven, 30, and 90 days of age (**Figure 1**). Pups from sick mothers during pregnancy had lower body mass indexes (**Figure 1A**, P7 $p < 0.0001$, P30 $p = 0.0042$, and P90 $p = 0.0255$) when compared to pups from healthy mothers during pregnancy. In relation to the glycemic assessment (**Figure 1B**), the puppies of diabetic mothers at thirty days old had lower blood glucose levels ($p = 0.0114$), and at ninety days old they had higher blood glucose levels ($p = 0.0092$), both when compared to the group control. However, all puppies were normoglycemic in the evaluations.

We measured the absolute (g) and relative weight (%) of the puppies' brains in the periods of seven, 30, and 90 days of age (**Figure 1C**). Pups from sick mothers during pregnancy had lower brain mass weights at both ages ($p < 0.0001$) when compared to pups from control mothers. In relation to brain weight relative to body weight (**Figure 1D**), pups in the GDM group had larger brains for their body weight at both ages (P7 $p < 0.0001$, P30 $p = 0.0006$, and P90 $p = 0.0368$), when compared to control puppies. In other words, the puppies of diabetic rats are born with smaller brains, and these are even larger than their body proportion.

3.2. Morphological assessments of the developing cerebellum

Figures 2 and 3 present data from histological analyses of the cerebellum at 10x and 20x magnification, specifically in the neocerebellum region, located with the aid of the Paxinos and Watson map (2006), at the ages of seven, 30, and 90 days of age. Seven-day-old pups showed no differences in terms of the length of the external granular layer (EGL) between the study groups (CT and GDM) (**Figure 2**, $p > 0.9999$). The pups in the GDM group had thinner cerebellar layers (**Figure 3**), when compared to the CT group, at the ages of 30 ($p < 0.0001$ and $p = 0.0005$) and 90 days ($p = 0.0150$ and $p = 0.0020$). But there were no differences in relation to the quantity of Purkinje cells between the CT and GDM groups, at the ages of 30 ($p = 0.3299$) and 90 ($p = 0.2043$) days of age.

3.3. Assessment of neocerebellar astrogliosis

To verify the presence of reactive astrocytes (or precursors cells) in the neocerebellum region, in both groups (CT and GDM) and ages (P7, P30, and P90), immunostainings were performed for a specific protein in the cerebellum, using the mouse anti-GFAP antibody (glial fibrillary acidic protein, an intermediate filament protein that is expressed by astrocytes). The images were qualitatively compared between the groups (CT and GDM) by age (**Figure 4**), and it was noted that the GDM group at ages 30 and 90 days presented more reactive astrocytes, with hypertrophic characteristics, which is called astrogliosis.

3.4. Redox balance assessment and cerebellar cell death

Figure 5 presents the quantifications of the redox balance analysis in the cerebellum of puppies from CT and GDM mothers, at the ages of seven, 30, and 90 days. Oxidant activity, antioxidants, and their enzymes are measured. The GDM group showed higher rates of oxidative stress at P30 ($p = 0.0226$) and P90 (0.0002) (**Figure 5A**) but without signs of protein damage (**Figure 5B**). In relation to the antioxidant system, at P7 the FRAP levels were increased ($p = 0.0219$), while at P30 the antioxidant activity was decreased ($p = 0.0171$) (**Figure 5C**), in the evaluation of antioxidant enzymes, SOD presented was increased at the ages of P7 ($p = 0.0078$) and P30 ($p = 0.0049$) (**Figure 5D**) and glutathione was increased only at P90 ($p = 0.0260$) (**Figure 5G**). It is noted that the cerebellum of puppies in the GDM group presents oxidative stress when compared to puppies in the control group.

The data obtained after the caspase-3 activation assay (**Figure 5H**) showed that it was more activated in the GDM group of seven-day-old pups ($p = 0.0139$) but decreased at ages P30 ($p = 0.0065$) and P90 ($p = 0.0061$) when compared with the control group.

3.5. Motor behavior assessments

For these results, animals aged 90 days (P90) were subjected to two behavioral tests, the elevated plus maze (ECM) and the open field test (OFT), evaluating parameters of time spent in different test regions, behaviors exploratory surveys and total distance covered (**Figure 6**). In the ECM, the animals in the GDM group explored less the apparatus, stretched out in closed arms (SAP BF $p < 0.0001$), stretched out in open arms (SAP BA $p = 0.0083$), and traveled a greater distance ($p = 0.0282$), when compared to the CT group. While in OFT, no differences were observed between the groups in both parameters evaluated.

4. Discussion

Maternal hyperglycemia interfered with the direct development of the puppies, as they had lower body mass indexes, lower blood glucose levels, lower brain weights, and sizes, they had cerebellum with thinner layers, and astrocytes present in the molecular layers were more reactive, that is, hypertrophied in response to hyperglycemia. This damage is found in the increase in reactive oxygen species, indicative of oxidative damage, where the antioxidant system is not able to contain oxidation. When these animals reached adulthood, we saw in behavioral tests that they tended to have anxieties and were less exploratory indicating impact on cerebellar function even after its maturity. Next, we will discuss each result.

The puppies from the group of diabetic mothers had lower body mass indexes, and birth weight is an important indicator of postnatal and adult health and survival. Many studies report how unfavorable the maternal environment becomes when mothers are in hyperglycemia. In severe GDM, severe hyperglycemia alters placental maturation, making it insufficient in its fetal nutrition function, causing harm to development and leading to a higher incidence of newborns with restricted body weight. On the other hand, a mildly hyperglycemic environment results in fetuses with macrosomia and hypoinsulinemia (Ejdesjö et al.; Da Cruz et al., 2023). One of the factors involved in these complications caused by GDM is the oxidative damage caused by excess glucose in maternal-fetal blood (Jakuš et al., 2014).

In the glycemic assessment, the animals in the GDM group showed lower blood glucose levels as they aged but were still all considered normoglycemic. Studies in humans have shown that maternal hyperglycemia can lead to fetal hyperinsulinemia, with postnatal hypoglycemia, which recovers spontaneously over time, but this hypoglycemia may be responsible for deficient neurological development (Pereira et al., 2021). The work of Blondeau et al. (2011), with GDM models in Sprague Dawley, showed that over time the offspring developed insulin resistance, with hyperglycemic evolution, but without histomorphometric changes in the pancreatic tissue. It is known that maternal diabetes can alter several tissues that may be related to insulin dysfunction in addition to the pancreas, such as the liver, skeletal muscles, and adipose tissue (Petropoulos et al., 2015).

The puppies of diabetic mothers had smaller brains but were still considered large in proportion to the size of the individual. Such results may be related to the low BMI and lower blood glucose levels presented by these animals. It is known that GDM can lead to growth restriction, with consequent damage to the development of various tissues (Lazo-De-LaVega et al., 2020; Pereira et al., 2021). Studies show how GDM and the mother's immune activation can affect fetal brain development, leading to increased risks of psychiatric disorders in several areas of adult life. The work of Money et al. (2018) showed that the combination of insults linked to hyperglycemia and immune activations alter factors circulating in the mother's serum, which alter the embryo's gene expression associated with inflammation and neurodevelopment, pathways implicated in the differentiation of dopaminergic neurons and innate immune responses. Studies show that uncontrolled diabetes during pregnancy induces adverse effects on the offspring's brain nerve cells, and these effects remain during the postnatal period (Ghafari et al., 2015), which can directly impact the size and development brain.

Knowing the restriction in the brain development of the puppies, the focus was on the histomorphometric study of the cerebellum, since the puppies of diabetic mothers had thinner cerebellar cortex layers, compared to the healthy group, which may imply a lower brain weight. In the study by Hami et al. (2016), the effect of maternal diabetes on the volume and morphogenesis of the cerebellar cortex of neonatal rats during the first two weeks of these animals' lives was investigated, and they concluded that maternal hyperglycemia-induced neurotoxic effects on the granules and Purkinje cells, with a significant reduction in the thickness of the cerebellar cortical layers, which can result in delay in normal cerebellar development and lead to structural, motor, behavioral and cognitive abnormalities in these offspring of diabetic mothers. The study by Razi et al.

(2015) concluded that GDM led to a reduction in cell density and in the various layers of the cerebellum, in Wistar rats at the ages of 21 and 28 days.

We evaluated these changes in the puppies by age, and at seven days, we measured the external granular layer (EGL), which contains precursor cells for the formation of the other layers, and it showed no differences in terms of thickness. But caspase-3 levels were high at this age, as this phase is marked by intense cell proliferation and apoptosis, due to it being a precursor layer (Hami et al., 2016). No increase in oxidative damage was observed, and the total antioxidant system (FRAP) of these animals was elevated, as were their enzymes (SOD). Cell death, via caspase-3, is essential for normal brain development, in ways specific to region, cell types, and death stimuli (Porter and Jänicke, 1999), which is why it is seen in the control group. However, when exacerbated by injurious or non-physiological stimuli, it can cause functional impairment.

The reduction in the thickness of the cerebellar layers may be linked to the high cell death seen initially at seven days of age, or to neurogenesis blockade pathways caused directly by maternal hyperglycemia (Hami et al., 2016). Previous studies have shown that exposure to high levels of glucose leads to the inhibition of the proliferation of neural precursor cells in areas of the brain undergoing neurogenesis (Jackson-Guilford et al., 2000). Furthermore, evidence indicates that prolonged maternal hyperglycemia during critical periods of development deregulates apoptosis levels and affects hippocampal neurogenesis, decreasing cell proliferation and survival (Piazza et al., 2019).

In this same tissue, we observed the presence of astrogliosis in the group of GDM puppies at all ages, through immunostaining of the astrocytic cytoskeleton, demonstrating hypertrophy of these cells, indicative of a response to possible damage caused by maternal hyperglycemia. Studies with gestational diabetes focusing on other brain regions found a high level of GFAP expression in the hippocampus in groups of puppies from diabetic mothers during pregnancy, found to be active even in the group treated with insulin (Sadeghi et al., 2016), showing a relationship of hyperglycemia and astrogliosis.

Brain function requires a constant supply of glucose and oxygen and homeostasis between astrocytes and neurons. Impairments in this metabolic regulation alter energy supply and neuronal morphology, with the accumulation of neurotoxins, which contributes to the maintenance of an inflammatory microenvironment, dysfunction synaptic and mitochondrial, leading to neuronal death and consequent cognitive phenotypic changes (Garcia-Serrano and Duarte, 2020). In the oxidative stress test, animals aged 30 and 90 days showed high concentrations of ROS, indicative of oxidative damage, and their

antioxidant systems, even when active, are insufficient to combat the damage (FRAP, SOD, and Glutathione). The increase in the oxidation of proteins, lipids, and carbohydrates occurs when the production of reactive oxygen species exceeds the local antioxidant capacity, playing an essential role in the pathogenesis of many diseases (Kalousova et al., 2002). High and prolonged exposure to glucose decreases the activity of glutathione in various tissues, such as vascular endothelium, kidney cells, and brain mitochondria, that is, hyperglycemia weakens the nervous antioxidant system (Ballatori et al., 2009).

After all the morphological changes were found, we went in search of what the impacts would be on the adult life of these animals. For this we use behavioral tests, ECM and OFT, to evaluate the locomotor and exploratory activities (cognitive tasks) of the animals, as it is known that the natural tendency of rodents in a new environment is to explore it, despite the stress and fear caused by it (Arruda et al., 2012). The animals in the GDM group covered greater distances, within closed arms, their locomotor capacity was not altered, and they showed less exploratory behavior in a new environment, indicating behavioral impairment. Cognitive impairment appears because of several cellular events intensified by hyperglycemia and hyperinsulinemia, which can culminate in neuronal loss, activation of inflammatory cells, and impairment of the blood-brain barrier function (Calvo-Ochoa et al., 2014; Vilela, 2021). Research by Schneider (2020) showed data on the progression of diabetes in rodents, with changes observed in areas of cerebral glucose metabolism related to locomotion and cognition, and saw a direct induction of cognitive impairment, but without changes in locomotor activity, using the same behavioral tests.

In summary of our results, hyperglycemia during the gestational period impacts the postnatal development of the cerebellum, and possibly of other brain areas, these changes are metabolic, morphological, and cellular, probably other morphophysiological aspects are also impacted and which were not investigated in the present study. It was confirmed that such changes, seen and not seen here, can impact the function in the animal's adult life, despite focusing on the cerebellum, motor skills, and cognition are tasks controlled by several regions of the nervous system, in this sense, the impact of diabetes gestational age is probably greater than what we were able to show in this work.

Acknowledgments

Rodent Bioterium Network (REBIR) of the Federal University of Uberlandia (UFU), Postgraduate Program in Health Sciences (PPCSA) for financial assistance, and

Coordination of Superior Level Staff Improvement - Brazil (CAPES) - Finance Code 001.

This manuscript is a contraction of Mascarenhas doctoral thesis.

Conflict of interest

I wish to confirm that there are no known conflicts of interest associated with this publication.

Data availability

Data will be made available on request.

Authors contributions

All authors listed in the manuscript have contributed to the work presented in the paper. Fernanda N.A.P. Mascarenhas and Maria Júlia N. Ribeiro carried out the experiments. Fernanda N.A.P. Mascarenhas and Natália F. Silva performed the interpretation of the results. Rener M.F. Duarte, Renata R. Teixeira, and Foued S. Espindola performed the oxidative stress experiments. Daniele L. Ribeiro performed the caspase assay. Carlos Ueira-Vieira and Renata Graciele Zanon were involved in all stages of this work.

References

- Arleo, A., Bareš, M., Bernard, J. A., Bogoian, H. R., Bruchhage, M. M., Bryant, P., and Manto, M. (2023). Consensus Paper: Cerebellum and Ageing. *The Cerebellum*, 1-31. <https://doi.org/10.1007/s12311-023-01577-7>
- Arruda, E. J. D., Kitamura, J. H., Chaves, T. P., Silva, C. A. D., and Mascarim, A. L. (2012). Comportamento exploratório e ansiolítico de ratos e ratas submetidos à estimulação somatossensorial. *Revista Brasileira de Terapias e Saúde*, 2(1), 7-12.
- Ballatori, N., Krance, S. M., Notenboom, S., Shi, S., Tieu, K., and Hammond, C. L. (2009). Glutathione dysregulation and the etiology and progression of human diseases. *Biol. Chem*, 390, 191-214. <https://doi.org/10.1515/BC.2009.033>

Batista, M. H. J., de Sousa, L. P., de Souza, D. M. D., Silva, R. O., dos Santos Lima, E., Nunes, T. S., ... and Rocha, M. A. (2021). Diabetes Gestacional: origem, prevenção e riscos. *Brazilian Journal of Development*, 7(1), 1981-1995. <https://doi.org/10.34117/bjdv7n1-135>

Benzie, I. F., and Strain, J. J. (1996). The ferric reducing ability of plasma (FRAP) as a measure of “antioxidant power”: the FRAP assay. *Analytical biochemistry*, 239(1), 70-76. <https://doi.org/10.1006/abio.1996.0292>

Blondeau, B., Joly, B., Perret, C., Prince, S., Bruneval, P., Lelièvre-Pégorier, M., ... and Van Huyen, J. P. D. (2011). Exposure in utero to maternal diabetes leads to glucose intolerance and high blood pressure with no major effects on lipid metabolism. *Diabetes and Metabolism*, 37(3), 245-251. <https://doi.org/10.1016/j.diabet.2010.10.008>

Bradford, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical biochemistry*, 72(1-2), 248-254. [https://doi.org/10.1016/0003-2697\(76\)90527-3](https://doi.org/10.1016/0003-2697(76)90527-3)

Calvo-Ochoa, E., Hernández-Ortega, K., Ferrera, P., Morimoto, S., and Arias, C. (2014). Short-term high-fat-and-fructose feeding produces insulin signaling alterations accompanied by neurite and synaptic reduction and astroglial activation in the rat hippocampus. *Journal of Cerebral Blood Flow and Metabolism*, 34(6), 1001-1008. <https://doi.org/10.1038/jcbfm.2014.4>

Chandna, A. R., Kuhlmann, N., Bryce, C. A., Greba, Q., Campanucci, V. A., and Howland, J. G. (2015). Chronic maternal hyperglycemia induced during mid-pregnancy in rats increases RAGE expression, augments hippocampal excitability, and alters behavior of the offspring. *Neuroscience*, 303, 241-260. <https://doi.org/10.1016/j.neuroscience.2015.06.063>

Conti, G., Caccamo, D., Siligato, R., Gembillo, G., Satta, E., Pazzano, D., ... and Santoro, D. (2019). Association of higher advanced oxidation protein products (AOPPs) levels in patients with diabetic and hypertensive nephropathy. *Medicina*, 55(10), 675. <https://doi.org/10.3390/medicina55100675>

da Cruz, L. L., Barco, V. S., Paula, V. G., Gallego, F. Q., Souza, M. R., Corrente, J. E., ... and Damasceno, D. C. (2023). Severe Diabetes Induction as a Generational Model for Growth Restriction of Rat. *Reproductive Sciences*, 1-13. <https://doi.org/10.1007/s43032-023-01198-9>

Damiani, D., Gonçalves, V. P., Kuhl, L., Aloï, P. H., and Nascimento, A. M. (2016). Aspectos neurofuncionais do cerebelo: o fim de um dogma. *Arquivos Brasileiros de Neurocirurgia: Brazilian Neurosurgery*, 039-044. <http://dx.doi.org/10.1055/s-0035-1570498>

Diniz Vilela, D., Gomes Peixoto, L., Teixeira, R. R., Belele Baptista, N., Carvalho Caixeta, D., Vieira de Souza, A., ... and Espindola, F. S. (2016). The role of metformin in controlling oxidative stress in muscle of diabetic rats. *Oxidative medicine and cellular longevity*, 2016. <https://doi.org/10.1155/2016/6978625>

Ejdesjö, A., Wentzel, P., and Eriksson, U. J. (2012). Influence of maternal metabolism and parental genetics on fetal maldevelopment in diabetic rat pregnancy. *American Journal of Physiology-Endocrinology and Metabolism*, 302(10), E1198-E1209. <https://doi.org/10.1152/ajpendo.00661.2011>

Garcia-Serrano, A. M., and Duarte, J. M. (2020). Brain metabolism alterations in type 2 diabetes: what did we learn from diet-induced diabetes models?. *Frontiers in neuroscience*, 14, 229. <https://doi.org/10.3389/fnins.2020.00229>

Ghafari, S., Golalipour, E., and Golalipour, M. J. (2015). Alterations of the giant pyramidal neurons (Betz cells) in brain cortex of rat offspring born from gestational diabetic dams: A morphometric study. *International Journal of Morphology*, 33(3), 1120-1125. <http://dx.doi.org/10.4067/S0717-95022015000300049>

Goldowitz, D., and Hamre, K. (1998). The cells and molecules that make a cerebellum. *Trends in neurosciences*, 21(9), 375-382. [https://doi.org/10.1016/S0166-2236\(98\)01313-7](https://doi.org/10.1016/S0166-2236(98)01313-7)

Hami, J., Shojae, F., Vafae-Nezhad, S., Lotfi, N., Kheradmand, H., and Haghir, H. (2015). Some of the experimental and clinical aspects of the effects of the maternal diabetes on developing hippocampus. *World journal of diabetes*, 6(3), 412. <https://doi.org/10.4239%2Fwjcd.v6.i3.412>

Hami, J., Vafaei-Nezhad, S., Ghaemi, K., Sadeghi, A., Ivar, G., Shojae, F., and Hosseini, M. (2016). Stereological study of the effects of maternal diabetes on cerebellar cortex development in rat. *Metabolic brain disease*, 31, 643-652. <https://doi.org/10.1007/s11011-016-9802-5>

Jackson-Guilford, J., Leander, J. D., and Nisenbaum, L. K. (2000). The effect of streptozotocin-induced diabetes on cell proliferation in the rat dentate gyrus. *Neuroscience letters*, 293(2), 91-94. [https://doi.org/10.1016/S0304-3940\(00\)01502-0](https://doi.org/10.1016/S0304-3940(00)01502-0)

Jakuš, V., Sándorová, E., Kalninová, J., and Krahulec, B. (2014). Monitoring of glycation, oxidative stress and inflammation in relation to the occurrence of vascular complications in patients with type 2 diabetes mellitus. *Physiological Research*, 63(3). <https://doi.org/10.33549/physiolres.932672>

Kalousova, M., Skrha, J., and Zima, T. (2002). Advanced glycation end-products and advanced oxidation protein products in patients with diabetes mellitus. *Physiological research*, 51(6), 597-604. <https://doi.org/10.1159/000066956>

Lazo-de-la-Vega, M. L., Mata-Tapia, K. A., Garcia-Santillan, J. A., Corona-Figueroa, M. A., Gonzalez-Dominguez, M. I., Gomez-Zapata, H. M., ... and Barbosa-Sabanero, G. (2020). Association of placental nutrient sensing pathways with birth weight. *Reproduction*, 160(3), 455-468. <https://doi.org/10.1530/REP-20-0186>

Mançú, T. D. S., and Castro Almeida, O. S. (2016). Knowledge and feelings of diabetic pregnant women about gestational diabetes mellitus and treatment. *Journal of Nursing UFPE*. 10(3), 1474-1482. <https://doi.org/10.5205/reuol.7057-60979-3-SM-1.1003sup201616>

Manto, M., Bower, J. M., Conforto, A. B., Delgado-García, J. M., Da Guarda, S. N. F., Gerwig, M., ... and Timmann, D. (2012). Consensus paper: roles of the cerebellum in motor control—the diversity of ideas on cerebellar involvement in movement. *The Cerebellum*, 11, 457-487. <https://doi.org/10.1007/s12311-011-0331-9>

Marklund, S., and Marklund, G. (1974). Involvement of the superoxide anion radical in the autoxidation of pyrogallol and a convenient assay for superoxide dismutase. *European journal of biochemistry*, 47(3), 469-474. <https://doi.org/10.1111/j.1432-1033.1974.tb03714.x>

Mascarenhas, F. N. A. D. P., Silva, N. F., Menezes-Reis, L. T., Vieira, L. G., Hirano, L. Q. L., Botelho, F. V., ... and Zanon, R. G. (2022). Prenatal effects of alprazolam treatment on the immature cerebellum of rats. *International Journal of Developmental Neuroscience*, 82(8), 726-734. <https://doi.org/10.1002/jdn.10222>

Money, K. M., Barke, T. L., Serezani, A., Gannon, M., Garbett, K. A., Aronoff, D. M., and Mirnics, K. (2018). Gestational diabetes exacerbates maternal immune activation effects in the developing brain. *Molecular Psychiatry*, 23(9), 1920-1928. <https://doi.org/10.1038/mp.2017.191>

Montenegro, C. A. B., and Rezende Filho, J. (2011). Obstetrícia fundamental. In *Obstetrícia Fundamental* (pp. 724-724).

Paxinos, G., and Watson, C. (2006). *The rat brain in stereotaxic coordinates: hard cover edition*. Elsevier.

Pereira, L. F. M., Menezes, R. V. D., Correa, T. R., Braga, V. C., Mesquita, G. A., Pires, V. C. L., ... and Coelho, L. B. P. (2021). Fisiopatologia e prevenção da hipoglicemia neonatal: revisão de literatura. *Brazilian Journal of Health Review*, 4(2), 5852-5865. <https://doi.org/10.34119/bjhrv4n2-148>

Petropoulos, S., Guillemin, C., Ergaz, Z., Dimov, S., Suderman, M., Weinstein-Fudim, L., ... and Szyf, M. (2015). Gestational diabetes alters offspring DNA methylation profiles in human and rat: identification of key pathways involved in endocrine system disorders, insulin signaling, diabetes signaling, and ILK signaling. *Endocrinology*, 156(6), 2222-2238. <https://doi.org/10.1210/en.2014-1643>

Piazza, F. V., Segabinazi, E., de Meireles, A. L. F., Mega, F., Spindler, C. D. F., Augustin, O. A., ... and Marcuzzo, S. (2019). Severe uncontrolled maternal hyperglycemia induces microsomnia and neurodevelopment delay accompanied by apoptosis, cellular survival, and

neuroinflammatory deregulation in rat offspring hippocampus. *Cellular and molecular neurobiology*, 39, 401-414. <https://doi.org/10.1007/s10571-019-00658-8>

Porter, A. G., and Jänicke, R. U. (1999). Emerging roles of caspase-3 in apoptosis. *Cell death and differentiation*, 6(2), 99-104. <https://doi.org/10.1038/sj.cdd.4400476>

Razi, E. M., Ghafari, S., and Golalipour, M. J. (2015). Effect of gestational diabetes on purkinje and granule cells distribution of the rat cerebellum in 21 and 28 days of postnatal life. *Basic and clinical neuroscience*, 6(1), 6.

Sadeghi, A., Esfandiary, E., Hami, J., Khanahmad, H., Hejazi, Z., and Razavi, S. (2016). Effect of maternal diabetes on gliogenesis in neonatal rat hippocampus. *Advanced Biomedical Research*, 5, 142-142. <https://doi.org/10.4103/2277-9175.187376>

Schneider, S. I. D. R. (2020). Avaliação da modulação cognitiva, locomotora, neurometabólica e da lateralidade encefálica em ratos wistar com diabetes mellitus induzidos por estreptozotocina.

Sommerfeld, S., Mundim, A. V., Silva, R. R., Queiroz, J. S., Rios, M. P., Notário, F. O., ... and Fonseca, B. B. (2022). Physiological changes in chicken embryos inoculated with drugs and viruses highlight the need for more standardization of this animal model. *Animals*, 12(9), 1156. <https://doi.org/10.3390/ani12091156>

Sun, H., Saeedi, P., Karuranga, S., Pinkepank, M., Ogurtsova, K., Duncan, B. B., ... and Magliano, D. J. (2022). IDF Diabetes Atlas: Global, regional and country-level diabetes prevalence estimates for 2021 and projections for 2045. *Diabetes research and clinical practice*, 183, 109119. <https://doi.org/10.1016/j.diabres.2021.109119>

Van Lieshout, R. J., and Voruganti, L. P. (2008). Diabetes mellitus during pregnancy and increased risk of schizophrenia in offspring: a review of the evidence and putative mechanisms. *Journal of Psychiatry and Neuroscience*, 33(5), 395-404.

Vilela, W. R. (2021). Disfunção metabólica central e periférica associada ao diabetes Mellitus: impacto sobre a cognição e função mitocondrial.

Xiang, A. H., Wang, X., Martinez, M. P., Page, K., Buchanan, T. A., and Feldman, R. K. (2018). Maternal type 1 diabetes and risk of autism in offspring. *Jama*, 320(1), 89-91. <https://doi.org/10.1001/jama.2018.7614>

Yong, Y., Meng, Y., Ding, H., Fan, Z., Tang, Y., Zhou, C., and Ke, Z. J. (2015). PACT/RAX regulates the migration of cerebellar granule neurons in the developing cerebellum. *Scientific reports*, 5(1), 7961. <https://doi.org/10.1038/srep07961>

Table and caption

Table 1 - Division of puppies by groups referring to their mothers, control (CT), and gestational diabetes (GDM), and by euthanasia age group, seven (P7), thirty (P30) and ninety (P90) days.

	CT	GDM
P7	n = 27	n = 26
P30	n = 28	n = 22
P90	n = 28	n = 11

Figures and captions

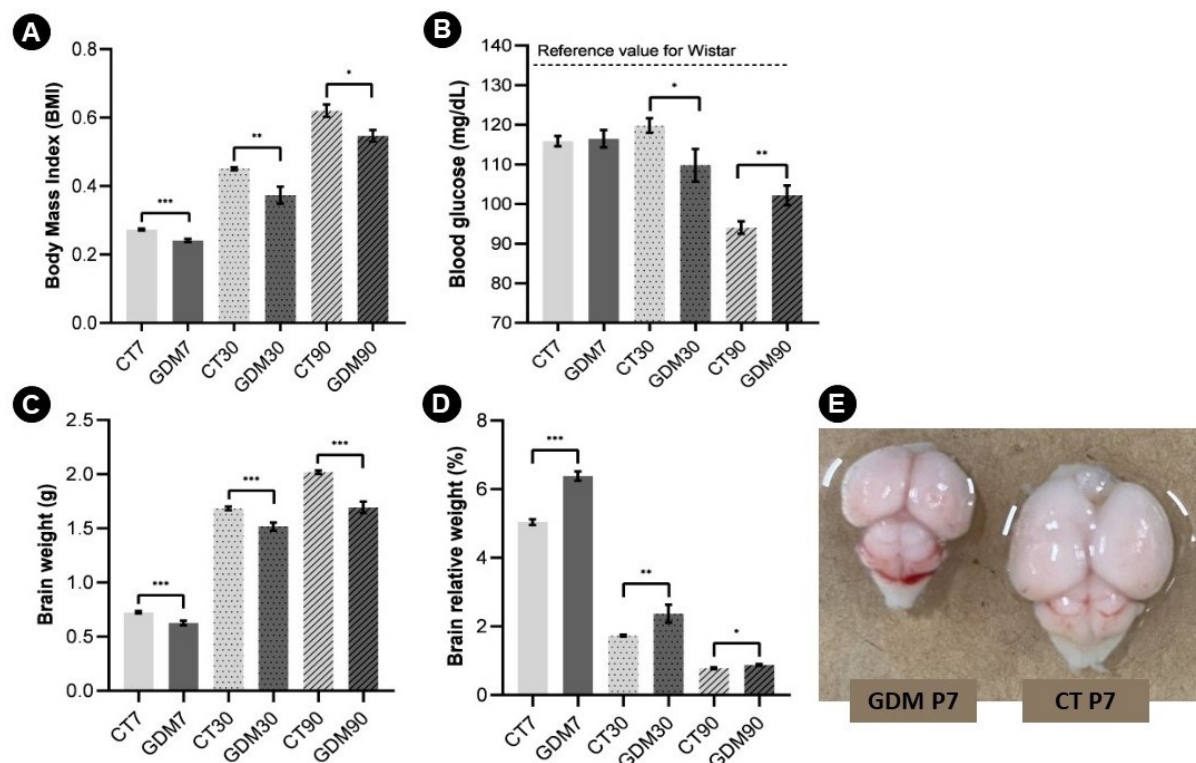


Figure 1 – Assessment of BMI, brain weight, and blood glucose (mg/dL) of puppies, seven (P7), thirty (P30) and ninety (P90) days old, in the different comparison, control (CT) and gestational diabetic (GDM) groups. **A:** Body mass index (BMI) graph, pups from the GDM group had lower BMIs at both ages, P7, P30 and P90, when compared to the CT group. **B:** Blood glucose graph in mg/dL, at P30 the puppies in the GDM group had lower blood glucose levels and at P90 higher blood

glucose levels, when compared to the control group, but still considered normoglycemic. **C:** Graph of brain weight in grams (g), pups in the GDM group had smaller brain masses at both ages, when compared to the CT group; **D:** Graph of brain mass relative to the pups' body weight, pups in the GDM group had larger brains in relation to their weight at both ages, when compared to the control group; **E:** Photos of the brains of seven-day-old pups, comparing GDM and CT groups, showing the difference in brain sizes. Unpaired t-test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.0001$.

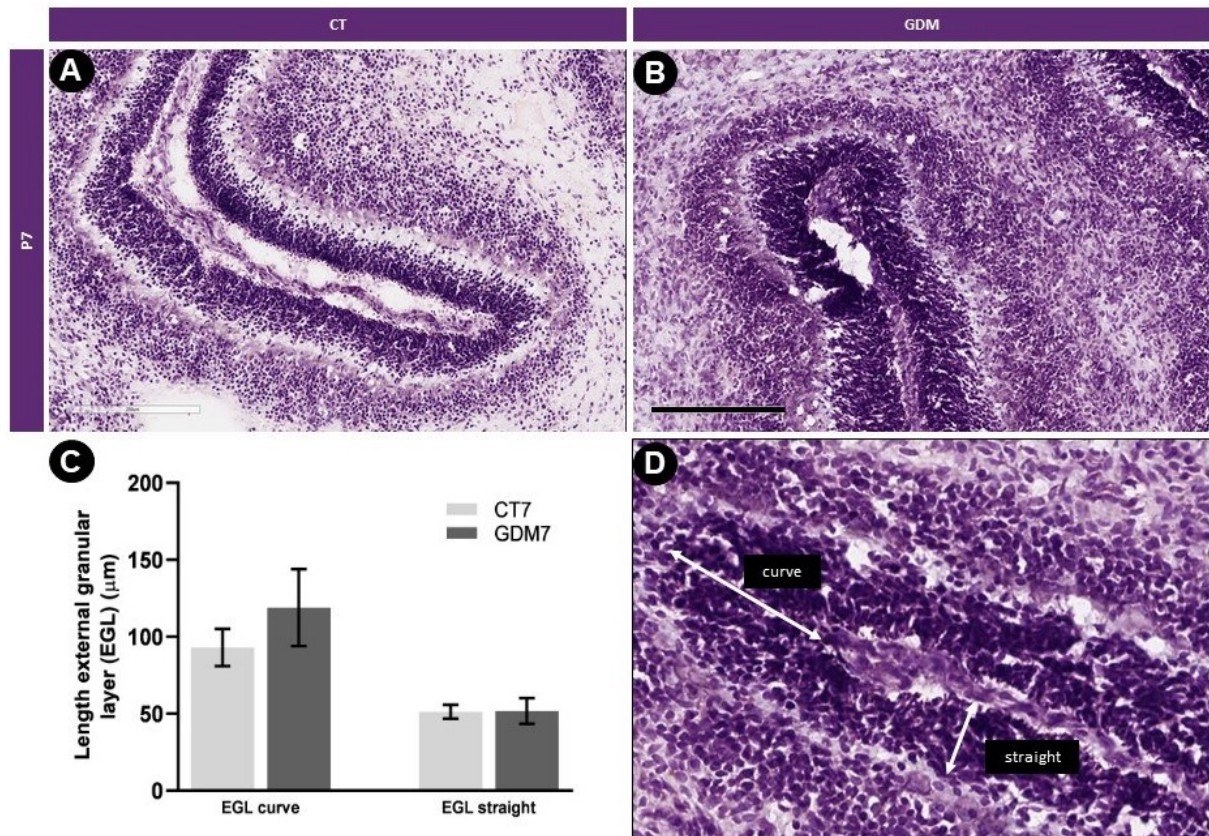


Figure 2 – Morphological evaluation of the cerebellum of seven-day-old pups. **A** and **B:** Photomicrographs at 20x magnification of the CT (**A**) and GDM (**B**) groups, of the cerebellum of seven-day-old Wistar rat pups (P7). **C:** Average graph comparing the length of the external granular layer (EGL) between groups, but without differences between groups. **D:** Photomicrograph showing in detail the regions that were measured. Unpaired t-test. Scale = 200 μm.

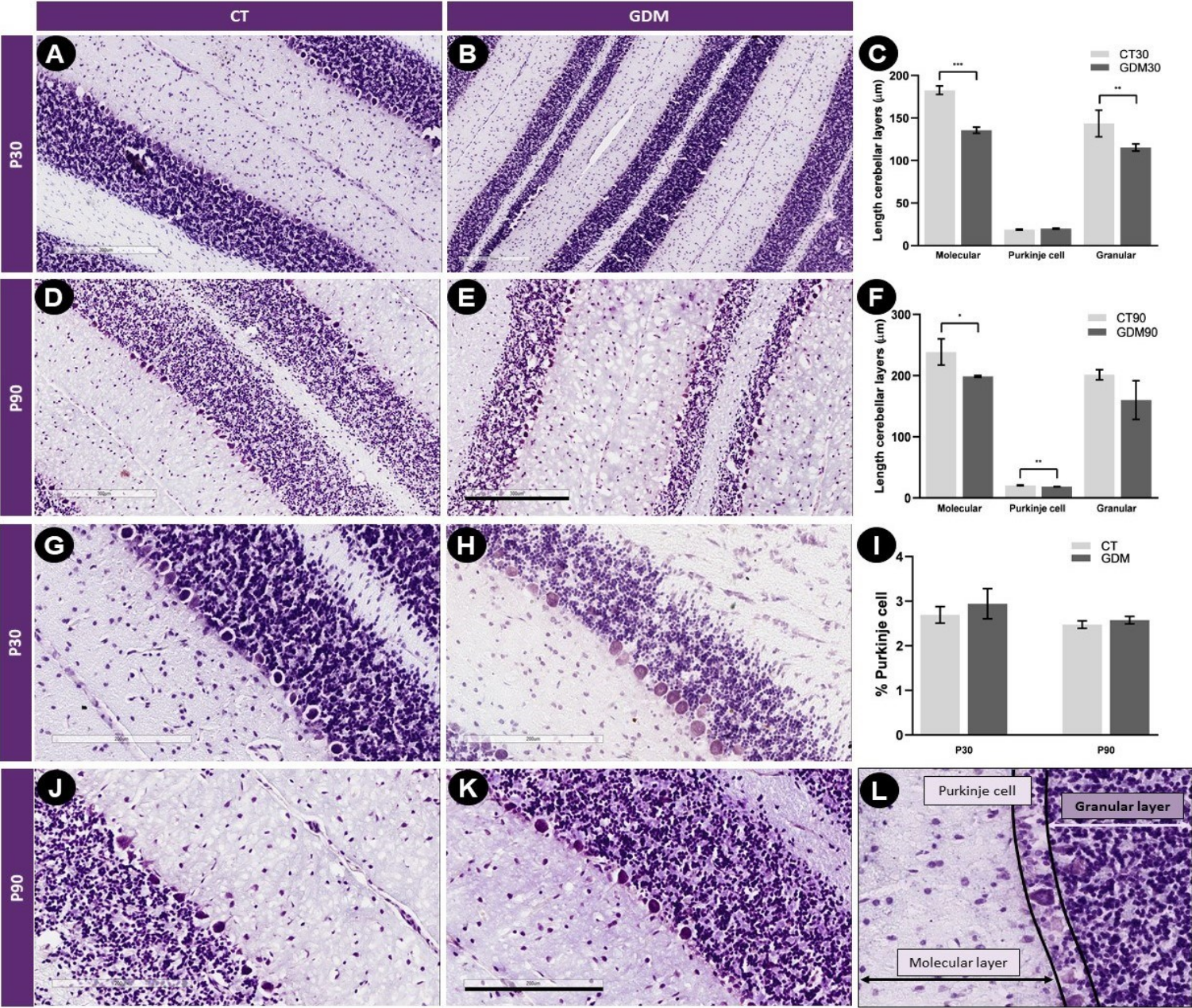


Figure 3 – Morphological evaluation of the cerebellum of puppies at 30 and 90 days of age. **A** and **B**: Photomicrographs of the CT (**A**) and GDM (**B**) groups, of the cerebellum of 30-day-old Wistar rat pups (P30; scale = 300µm). **C**: Average graph that compares the length of the cerebellar layers (Molecular, Purkinje cells and Granular) between the CT and GDM groups, the pups in the GDM group had thinner layers. **D** and **E**: Photomicrographs of the CT (**D**) and GDM (**E**) groups, of the cerebellum of 90-day-old Wistar rat pups (P90). **F**: Average graph that compares the length of the cerebellar layers (Molecular, Purkinje cells and Granular) between the CT and GDM groups, the pups in the GDM group also had thinner layers. **G** and **H**: Photomicrographs of the CT (**G**) and GDM (**H**) groups, of the cerebellum of 30-day-old Wistar rat pups (P30). **I**: Average graph comparing the number of Purkinje cells in a determined length (µm) between the CT and GDM groups, with no differences between the groups. **J** and **K**: Photomicrographs of the CT (**J**) and GDM (**K**) groups, of the cerebellum of 90-day-old Wistar rat pups (P90). **L**: Photomicrograph showing in detail the regions that were measured. Scale = 300 µm (A, B, D, E) and 200 µm (D, H, J, K). Unpaired t-test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.0001$.

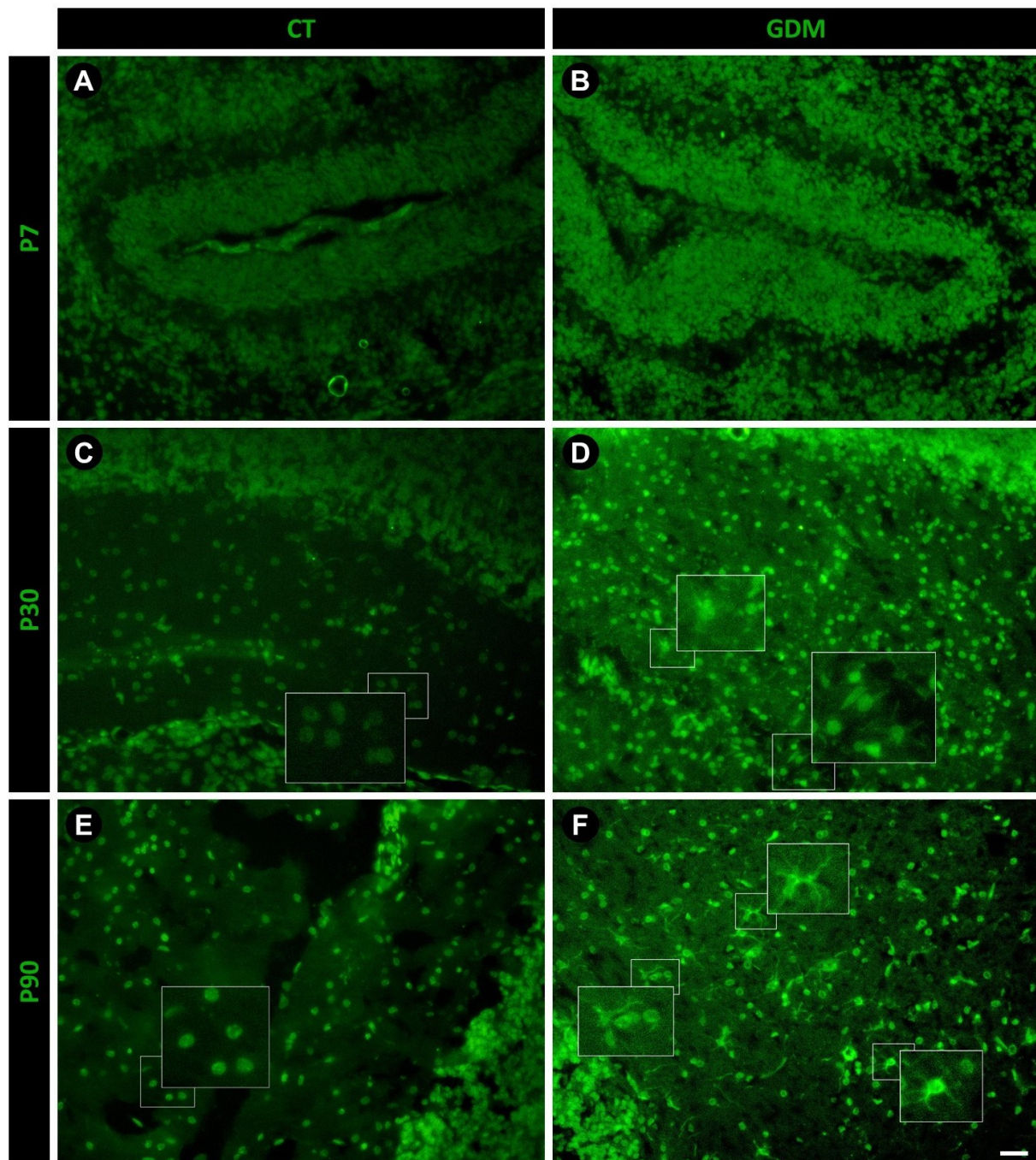


Figure 4 – Morphological evaluation of the cerebellum of pups at seven, 30 and 90 days of age, by immunofluorescence with mouse anti-GFAP antibody, at 20x magnification. **A** and **B**: Photomicrographs of the cerebellum of seven-day-old pups (P7), from the CT (**A**) and GDM (**B**) groups. **C** and **D**: Photomicrographs of the cerebellum of 30-day-old pups (P30), from the CT (**C**) and GDM (**D**) groups. **E** and **F**: Photomicrographs of the cerebellum of pups at 90 days of age (P90), from the CT (**E**) and GDM (**F**) groups. By qualitative assessment, it is noted that the GDM group at both ages' present markings of astrocytes in hypertrophy, characteristic of astrogliosis. Scale = 10 μ m. Squares magnified 2x.

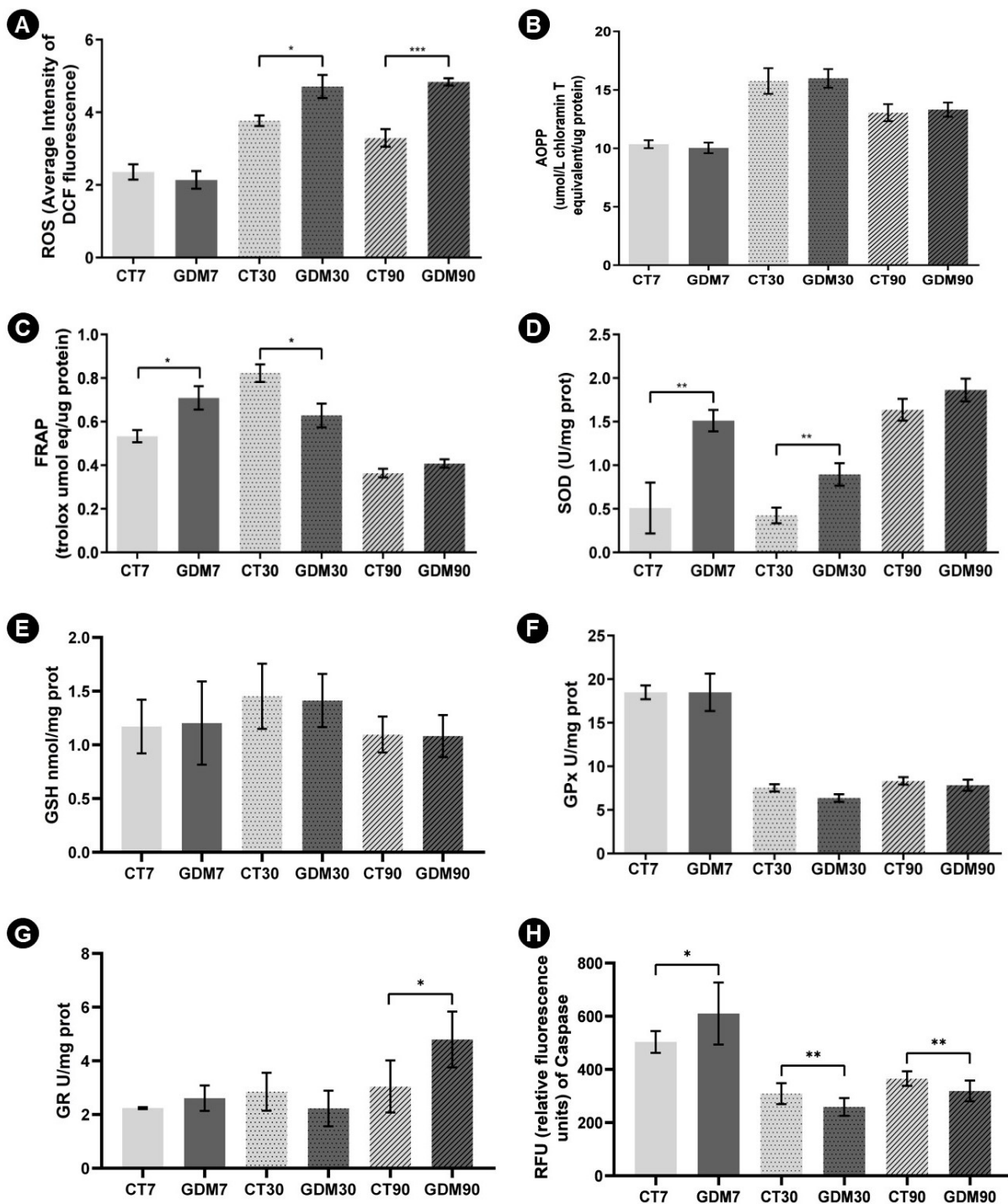


Figure 5 – Evaluation of the redox balance and caspase assay of the cerebellum of puppies at seven, 30 and 90 days of age, in the control (CT) and gestational diabetic (GDM) groups, using graphs of means and standard error. **A:** Graph of oxidant activity measurements, by quantifying reactive oxygen species (ROS); **B:** Graph of protein damage measurements through the determination of advanced protein oxidation products (AOPP); **C:** Graph of measurements of total antioxidant activity using the iron reduction method (FRAP); **D:** Graph of measurements of primary defense against oxidative stress, by superoxide dismutase

(SOD); **E, F and G:** Graphs of measurements of defense against oxidative stress, by the equivalents of reduced (GSH), oxidized (GPx) and reductase (GR) glutathione, respectively. The GDM group showed higher rates of oxidative stress, at the ages of P30 and P90, but without signs of protein damage. In relation to the antioxidant system, at P7 the FRAP levels were increased, while at P30 the antioxidant activity was decreased, in the evaluation of antioxidant enzymes, SOD presented was increased at the ages of P7 and P30 and glutathione was increased only at P90. **H:** Graph of detection of fluorescence emitted by active caspase-3 in protein extract from cerebellum. It is noted that caspase-3 activation was elevated in the GDM group at P7, while at P30 and P90 it was less active. Unpaired t-test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.0001$.

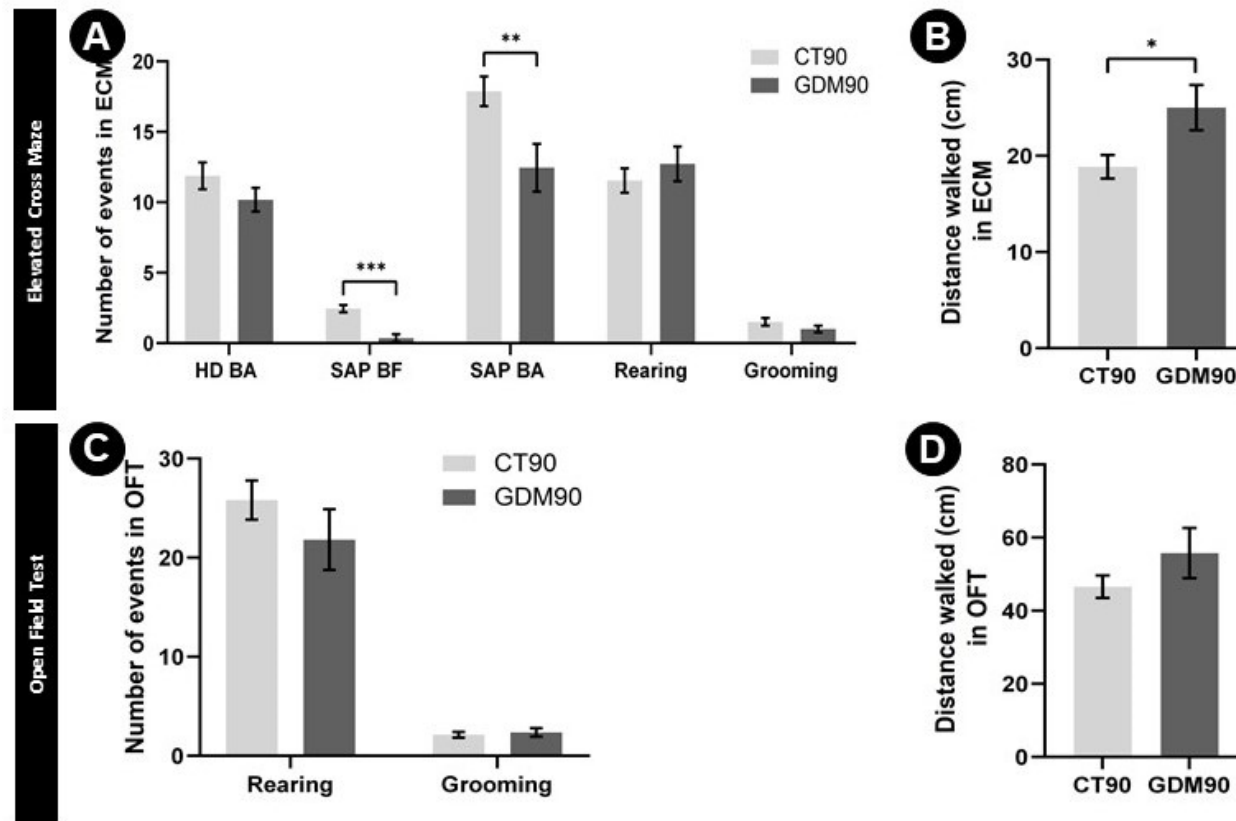


Figure 6 – Graphs of behavioral tests performed with puppies at 90 days of age (P90), in the control (CT) and gestational diabetic (GDM) groups. **A** and **B**: Parameters evaluated in the elevated plus maze (ECM). Number of exploratory events: dive in open arms (HD BA), stretched out in closed arms (SAP BF), stretched out in open arms (SAP BA), rearing, grooming (**A**) and distance covered (**B**). **C** and **D**: Parameters evaluated in the open field test (OFT), number of exploratory events: rearing, grooming (**C**) and distance covered (**D**). It is noted that in ECM the GDM group explored the apparatus less and covered a greater distance, when compared to the CT group. Unpaired t-test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.0001$.

REFERÊNCIAS

- 1) AMARAL, A. C. S. et al. Complicações neonatais do diabetes mellitus gestacional–DMG. **Rev Med Minas Gerais**, v. 22, n. 5, p. S40-S42, 2012.
- 2) AMERICAN DIABETES ASSOCIATION. 2. Classification and diagnosis of diabetes: standards of medical care in diabetes—2021. **Diabetes care**, v. 44, n. Supplement_1, p. S15-S33, 2021. <https://doi.org/10.2337/dc21-S002>
- 3) ANCIUTI, A. N. Avaliação da plasticidade neural, níveis glicêmicos e peso da prole de ratas diabéticas. 51p. Dissertação de Mestrado, PROGRAMA DE PÓS-GRADUAÇÃO EM BIOQUÍMICA, UFRGS, 2016.
- 4) ARLEO, Angelo et al. Consensus Paper: Cerebellum and Ageing. *The Cerebellum*, p. 1-31, 2023. <https://doi.org/10.1007/s12311-023-01577-7>
- 5) CHANDNA, A. R. et al. Chronic maternal hyperglycemia induced during mid-pregnancy in rats increases RAGE expression, augments hippocampal excitability, and alters behavior of the offspring. **Neuroscience**, v. 303, p. 241-260, 2015. <https://doi.org/10.1016/j.neuroscience.2015.06.063>
- 6) DAMIANI, Daniel et al. Aspectos neurofuncionais do cerebelo: o fim de um dogma. **Arquivos Brasileiros de Neurocirurgia: Brazilian Neurosurgery**, p. 039-044, 2016. <http://dx.doi.org/10.1055/s-0035-1570498>
- 7) DE MORAIS RIBEIRO, C. N. Alterações no ambiente uterino por teratógenos e as consequentes complicações fetais. **Revista Plêiade**, v. 2, n. 4, p. 135-143, 2008.
- 8) DE SOUZA MELLO, A.; SANTOS, A. Q.; FUNCHAL, C. Correlação entre hiperglicemia e células do SNC, com enfoque na atividade glial. **Revista Neurociências**, v. 20, n. 2, p. 294-301, 2012.
- 9) FALEIROS, G. Q. A. et al. Diabetes Mellitus Gestacional: o controle glicêmico como elemento de controle de peso fetal. **Revista Eletrônica Acervo Saúde**, v. 13, n. 5, p. e7413-e7413, 2021. <https://doi.org/10.25248/reas.e7413.2021>
- 10) FELLIN, T.; CARMIGNOTO, G. Neurone-to-astrocyte signalling in the brain represents a distinct multifunctional unit. **The Journal of physiology**, v. 559, n. 1, p. 3-15, 2004.
- 11) FERREIRA, L. T.; et al. Diabetes melito: hiperglicemia crônica e suas complicações. **Arquivos Brasileiros de Ciências da Saúde**, v. 36, n. 3, 2011. <https://doi.org/10.7322/abcs.v36i3.59>
- 12) FERREIRA, C. L. R.; NICOLAU, R. A. Diabete experimental em ratos-revisão sistemática. **Encontro Latino Americano de Iniciação Científica, Encontro Latino Americano de Pós-Graduação: Anais de trabalhos completos XV Encontro Latino**

Americano de Iniciação Científica, XI Encontro Latino Americano de Pós-Graduação, p. 1-5, 2011.

13) FRANCIS, G. J.; et al. Motor End Plate Innervation Loss in Diabetes and the Role of Insulin: **Journal of Neuropathology & Experimental Neurology**, v. 70, n. 5, 2011. <https://doi.org/10.1097/NEN.0b013e318215669a>

14) GARCIA-SERRANO, Alba M.; DUARTE, João MN. Brain metabolism alterations in type 2 diabetes: what did we learn from diet-induced diabetes models?. **Frontiers in neuroscience**, v. 14, p. 229, 2020. <https://doi.org/10.3389/fnins.2020.00229>

15) GISPEN, W. H.; BIESSELS, G. Cognition and synaptic plasticity in diabetes mellitus. **Trends in Neurosciences**, v. 23, n. 11, p. 542–549, 2000.

16) GOLBERT, A.; CAMPOS, M. A. A. Diabetes melito tipo 1 e gestação: [revisão]. **Arquivos Brasileiros de Endocrinologia & Metabologia**, v. 52, n. 2, p. 307-314, 2008. <https://doi.org/10.1590/S0004-27302008000200018>

17) GOLDOWITZ, D.; HAMRE, K. The cells and molecules that make a cerebellum. **Trends in neurosciences**, v. 21, n. 9, p. 375-382, 1998. [https://doi.org/10.1016/S0166-2236\(98\)01313-7](https://doi.org/10.1016/S0166-2236(98)01313-7)

18) HAMI, J. et al. Some of the experimental and clinical aspects of the effects of the maternal diabetes on developing hippocampus. **World journal of diabetes**, v. 6, n. 3, p. 412, 2015. <https://doi.org/10.4239%2Fwj.d.v6.i3.412>

19) KIRSTEN, V. R.; SESTERHEIM, P.; SAITOVITCH, D. Modelos experimentais para o estudo do diabetes tipo 1. **Medicina (Ribeirão Preto. Online)**, v. 43, n. 1, p. 3-10, 2010.

20) KOSTOVIĆ, I.; SEDMAK, G.; JUDAŠ, M. Neural histology and neurogenesis of the human fetal and infant brain. **Neuroimage**, v. 188, p. 743-773, 2019. <https://doi.org/10.1016/j.neuroimage.2018.12.043>

21) LERCO, M. M. et al. Caracterização de um modelo experimental de Diabetes Mellitus, induzido pela aloxana em ratos: estudo clínico e laboratorial. **Acta Cirúrgica Brasileira**, v. 18, n. 2, p. 132-142, 2003.

22) MAGANHA, C. A. et al. Tratamento do diabetes melito gestacional. **Revista da associação médica brasileira**, v. 49, n. 3, p. 330-4, 2003. <https://doi.org/10.1590/S0104-42302003000300040>

23) MANTO, M. et al. Consensus paper: roles of the cerebellum in motor control—the diversity of ideas on cerebellar involvement in movement. **The Cerebellum**, v. 11, p. 457-487, 2012. <https://doi.org/10.1007/s12311-011-0331-9>

- 24) MASCARENHAS, F. N. A. P. et al. Prenatal effects of alprazolam treatment on the immature cerebellum of rats. **International Journal of Developmental Neuroscience**, v. 82, n. 8, p. 726-734, 2022. <https://doi.org/10.1002/jdn.10222>
- 25) MILECH, A. et al. Tratamento e acompanhamento do diabetes mellitus: **Diretrizes da Sociedade Brasileira de Diabetes**, 2006.
- 26) MILLIGAN, E. D.; WATKINS, L. R. Pathological and protective roles of glia in chronic pain. **Nature reviews neuroscience**, v. 10, n. 1, p. 23-36, 2009.
- 27) MONTANHA, F. P., AZEVEDO, M. G. P. Administração medicamentosa: Vantagens e desvantagens das diferentes vias. **Revista Científica Eletrônica de Medicina Veterinária**, n. 20, 2013.
- 28) NOMURA, R. M. Y. et al. Vitalidade Fetal em Gestações Complicadas com Diabetes Melito Pré-Gestacional: Um Estudo Longitudinal. **Revista Brasileira de Ginecologia e Obstetrícia**, v. 24, n. 2, p. 113-120, 2002.
- 29) PANTE, F. R. et al. Malformações congênitas do sistema nervoso central: prevalência e impacto perinatal. **Revista da AMRIGS**, v. 55, n. 4, p. 339-344, 2011.
- 30) PITITTO, B. A.; ALMADA FILHO, C. M.; CENDOROGLO, M. S. Déficit Cognitivo: mais uma Complicação do Diabetes Melito?. **Arquivos Brasileiros de Endocrinologia & Metabologia**, v. 52, n. 7, p. 1076-1083, 2008. <https://doi.org/10.1590/S0004-27302008000700003>
- 31) REMOR, A. P. et al. Differential effects of insulin on peripheral diabetes-related changes in mitochondrial bioenergetics: involvement of advanced glycosylated end products. **Biochimica et Biophysica Acta (BBA)-Molecular Basis of Disease**, v. 1812, n. 11, p. 1460-1471, 2011. <https://doi.org/10.1016/j.bbadis.2011.06.017>
- 32) RUDGE, M. V. C. et al. Hiperglicemia materna diária diagnosticada pelo perfil glicêmico: um problema de saúde pública materno e perinatal. **Revista Brasileira de Ginecologia e Obstetrícia**, v. 27, n.11, p. 691-697, 2005.
- 33) SANTOS, G. M. R. F. dos. **Diabetes gestacional induzido por estreptozotocina: contribuição do estresse oxidativo e da enzima NADPH oxidase no desenvolvimento de lesões cardiovasculares na prole**. 173P. Tese doutorado, Programa de Pós-Graduação em Bioquímica e Fisiologia, UFP, 2018.
- 34) SILVA, J. C. et al. Tratamento do diabetes mellitus gestacional com glibenclamida – fatores de sucesso e resultados perinatais. **Revista Brasileira de Ginecologia e Obstetrícia**, v. 29, n.11, p. 555-560, 2007.

- 35) SILVA, J. C. et al. Fatores relacionados a presença de recém-nascidos grandes para a idade gestacional em gestantes com diabetes mellitus gestacional. **Revista Brasileira de Ginecologia e Obstetrícia**, v. 31, n.1, p. 5-9, 2009.
- 36) SUN, H. et al. IDF Diabetes Atlas: Global, regional and country-level diabetes prevalence estimates for 2021 and projections for 2045. **Diabetes research and clinical practice**, v. 183, p. 109119, 2022. <https://doi.org/10.1016/j.diabres.2021.109119>
- 37) VILELA, W. R. Disfunção metabólica central e periférica associada ao diabetes Mellitus: impacto sobre a cognição e função mitocondrial. 2021.
- 38) XIANG, A. H. et al. Maternal type 1 diabetes and risk of autism in offspring. **Jama**, v. 320, n. 1, p. 89-91, 2018. <https://doi.org/10.1001/jama.2018.7614>
- 39) YONG, Y. et al. PACT/RAX regulates the migration of cerebellar granule neurons in the developing cerebellum. **Scientific reports**, v. 5, n. 1, p. 7961, 2015. <https://doi.org/10.1038/srep07961>

ANEXO 1 - Certificado de aprovação do CEUA/UFU



Universidade Federal de Uberlândia

– Comissão de Ética na Utilização de Animais –

**CERTIFICADO**

Certificamos que o projeto intitulado “Alterações sensitivas, motoras e cognitivas causadas pelo Diabetes gestacional: base celular e molecular”, protocolo nº 056/18, sob a responsabilidade de **Renata Graciele Zanon** – que envolve a produção, manutenção e/ou utilização de animais pertencentes ao filo Chordata, subfilo Vertebrata, para fins de pesquisa científica – encontra-se de acordo com os preceitos da Lei nº 11.794, de 8 de outubro de 2008, do Decreto nº 6.899, de 15 de julho de 2009, e com as normas editadas pelo Conselho Nacional de Controle da Experimentação Animal (CONCEA), e foi APROVADA pela COMISSÃO DE ÉTICA NA UTILIZAÇÃO DE ANIMAIS (CEUA) da UNIVERSIDADE FEDERAL DE UBERLÂNDIA, em reunião 28 de setembro de 2018.

(We certify that the project entitled intitulado “Alterações sensitivas, motoras e cognitivas causadas pelo Diabetes gestacional: base celular e molecular”, protocol 056/18, under the responsibility of – Renata Graciele Zanon involving the production, maintenance and/or use of animals belonging to the phylum Chordata, subphylum Vertebrata, for purposes of scientific research - is in accordance with the provisions of Law nº 11.794, of October 8th, 2008, of Decree nº 6.899 of July 15th, 2009, and the rules issued by the National Council for Control of Animal Experimentation (CONCEA) and it was approved for ETHICS COMMISSION ON ANIMAL USE (CEUA) from FEDERAL UNIVERSITY OF UBERLÂNDIA, in meeting of September 28th, 2018).

Vigência do Projeto	Início: 10/01/2019 Término: 31/12/2022
Espécie/Linhagem/Grupos Taxonômicos	Ratos wistar
Número de animais	132
Peso/Idade	5-10g/ 1 semana 100g/ 4 semanas 300g/ 8-10 semanas 300g/ 12 semanas
Sexo	Macho e Fêmea
Origem/Local	Centro de Bioterismo e Experimentação Animal (CBEA) da Universidade Federal de Uberlândia
Local onde serão mantidos os animais:	Biotério setorial ARFIS

Uberlândia, 19 de outubro de 2018

Prof. Dr. Lúcio Vilela Carneiro Girão
Coordenador da CEUA/UFU