Universidade do Estado do Rio de Janeiro
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Carla Bruna Pietrobon

O desmame precoce e a exposição à nicotina na lactação programa para esteatose pancreática que compromete a função das células beta e a homeostase glicêmica em ratos de ambos os sexos

Rio de Janeiro
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Tese apresentada, como requisito parcial para obtenção do título de Doutor, ao Programa de Pós-Graduação em Biociências, da Universidade do Estado do Rio de Janeiro.

Orientador: Prof. Dr. Egberto Gaspar de Moura

Coorientadora: Profª. Dra. Patrícia Cristina Lisboa da Silva

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Rio de Janeiro
2020

DEDICATÓRIA

Dedico esta, bem como todas as minhas conquistas primeiramente a Deus, aos meus pais, a minha família, ao meu marido, meus amigos e orientadores pela amizade, incentivo, apoio e dedicação. Tudo isso foi essencial para cumprir mais uma etapa da minha vida.

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RESUMO

PIETROBON, Carla Bruna. **O desmame precoce e a exposição à nicotina na lactação programa para esteatose pancreática que compromete a função das células beta e a homeostase glicêmica em ratos de ambos os sexos.** 2020. 128f. Tese (Doutorado em Biociências) Instituto de Biologia Roberto Alcântara Gomes, Universidade do Estado do Rio de Janeiro, Rio de Janeiro, 2020.

Estudos demonstram que o desmame precoce (DP) e a exposição à nicotina durante a amamentação levam a alterações endócrino-metabólicas, como obesidade e diabetes *mellitus* tipo 2 (DM2) na prole adulta. Demonstramos que ratos machos adultos submetidos a estes dois modelos de programação apresentam fenótipos similares em relação à homeostase glicêmica, sugerindo resistência à insulina. Dessa forma, investigamos os mecanismos pelos quais a homeostase glicêmica dos machos é alterada em ambos os modelos e propomos estudar pela primeira vez as fêmeas. Modelo de programação pelo desmame precoce: Ratas *Wistar* lactantes foram separadas em 3 grupos: DP não farmacológico (NPEW): as mães foram envolvidas com uma bandagem adesiva nos 3 últimos dias de lactação; DP farmacológico (PEW): as mães receberam bromocriptina (1mg/Kg PC/dia) nos 3 últimos dias da lactação; e Controle (C): cujos filhotes foram desmamados em PN21. Um filhote de cada ninhada/sexo/grupo foi eutanasiado em PN45 e o restante dos animais em PN180. Em PN45 apenas os machos DP apresentaram aumento da secreção de insulina (vs C). Em PN180: os machos PEW apresentaram intolerância à glicose (OGTT), bem como redução na secreção de insulina, da glicoquinase e aumento de GLUT2, enquanto que os machos NPEW apresentaram apenas redução da secreção de insulina e da glicoquinase. As fêmeas desmamadas precocemente tiveram menor secreção de insulina (vs C). A prole apresentou menor adiponectina/gordura visceral, bem como, esteatose pancreática. No músculo esquelético os machos NPEW mostraram redução na expressão do receptor de insulina, pAKT e GLUT4, enquanto que os machos PEW exibiram redução apenas no GLUT4, em contrapartida, as fêmeas não apresentaram qualquer alteração. Modelo de programação pela exposição materna à nicotina durante a lactação: Em PN2 foram implantadas minibombas nas ratas *Wistar* lactantes, as quais foram separadas em 2 grupos: Nicotina (NIC, 6 mg/kg/dia, durante 14 dias) e Controle (CON, solução salina por 14 dias). Os animais foram eutanasiados em PN180. A prole de ambos os sexos apresentou intolerância à glicose (OGTT), aumento do IRI, redução da razão adiponectina/gordura visceral e maior acúmulo de gordura no pâncreas. Os machos NIC apresentaram hiperinsulinemia, enquanto que as fêmeas foram hiperglicêmicas sem alterações na insulina. Nas ilhotas pancreáticas, os machos NIC exibiram redução de PDX-1, e as fêmeas NIC aumento de PDX-1 e GLUT2 com redução do receptor adrenérgico α2. No músculo os machos NIC apresentaram redução na expressão do receptor de insulina, pAKT e GLUT4, enquanto queas fêmeas NIC exibiram apenas redução de GLUT4. Ambos os modelos de programação induzem alterações relacionadas com a homeostase glicêmica. Observamos que os machos do grupo DP ou NIC são afetados negativamente e de forma semelhante, enquanto que o DP parece não prejudicar de forma tão acentuada a homeostase glicêmica das fêmeas, o que é diferente do observado modelo de exposição à nicotina, onde a prole do sexo feminino é tão prejudicada quanto os machos. Apesar do dimorfismo sexual observado entre os diferentes insultos, ambos contribuem para a pandemia da obesidade e DM2, uma vez que predispõe os descendentes ao aumento das chances para o desenvolvimento destas doenças.

Palavras-chave: Amamentação. Desmame precoce. Nicotina. Programação metabólica.

Diabetes *mellitus* do tipo 2.

ABSTRACT

PIETROBON, Carla Bruna. **Early weaning and nicotine exposure during lactation programs for pancreatic steatosis, which compromise beta cell function and glucose homeostasis in rats of both sexes.** 2020. 128f. Tese (Doutorado em Biociências) Instituto de Biologia Roberto Alcântara Gomes, Universidade do Estado do Rio de Janeiro, Rio de Janeiro, 2020.

Studies showed that early weaning (EW) and icotine exposure during breastfeeding lead to endocrine-metabolic changes, such as obesity and type 2 diabetes mellitus (T2DM) in adult offspring. We demonstrated that adult male rats submitted to these two programming models present similar phenotypes in relation to glycemic homeostasis, suggesting insulin resistance. Thus, we investigate the mechanisms by which male glycemic homeostasis is altered in both models and propose to study females for the first time. **Early weaning programming model:** Lactating Wistar rats were separated into 3 groups: Non-pharmacological EW (NPEW): mothers were wrapped with an adhesive bandage at 3 last days of lactation; Pharmacological EW (PEW): mothers received bromocriptine (1mg / kg BW / day) in the last 3 days of lactation; and Control (C): whose pups were weaned in PN21. One pup of each litter/sex/group was euthanized in PN45 and the rest of the animals were euthanized in PN180. At PN45 only EW males showed increased insulin secretion (vs C). At PN180: PEW males showed glucose intolerance (OGTT), as well reduced in insulin secretion, glucokinase, and increased GLUT2, while NPEW males showed only reduced in insulin secretion and glucokinase. Early weaned females had lower insulin secretion (vs C). Adult offspring had a lower adiponectin/visceral fat as well pancreatic steatosis. In skeletal muscle, NPEW males showed reduction in insulin receptor, pAKT and GLUT4 expression, while PEW males showed reduction only GLUT4 expression. In contrast, females showed no alteration. **Programming model for maternal nicotine exposure during breastfeeding:** At PN2, minipumps were implanted in lactating Wistar rats, which were separated into 2 groups: Nicotine (NIC, 6 mg/kg/day for 14 days) and Control (CON, saline solution for 14 days). The animals were euthanized in PN180. Adult offspring of both sexes presented glucose intolerance (OGTT), increased IRI, reduced adiponectin/visceral fat ratio and greater accumulation of ectopic fat in the pancreas. Males of NIC group had hyperinsulinemia, while females were hyperglycemic with no changes in insulin. In pancreatic islets, NIC males exhibited PDX-1 reduction and NIC females increased PDX-1 and GLUT2 with reduced α2 adrenergic receptor. In muscle, NIC males showed reduction in insulin receptor, pAKT and GLUT4 expression, while NIC females showed only reduction of GLUT4. Both programming models induce changes related to glycemic homeostasis. We observed that males from the EW or NIC group are negatively and similarly affected, whereas the female EW does not appear to have such a higher impairment in glycemic homeostasis, which is different in the model of nicotine exposure, where female offspring are as harmed as males. Despite the sexual dimorphism observed between the different insults, both contribute to the obesity and T2DM pandemic, since it predisposes the offspring to increased chances for the development of these diseases.

Keywords: Breastfeeding. Early weaning. Nicotine. Metabolic programing. Type 2 diabetes mellitus.

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LISTA DE ABREVIATURAS E SIGLAS

AC	Adenilato ciclase
ADP	Adenosina difosfato
AdRβ3	Receptor adrenérgico beta 3
AKT/PKB	Proteína quinase B
AMPc	Adenosina monofosfato cíclico
AMPK	Proteína quinase ativada por AMP
AMPKp	Proteína quinase ativada por AMP fosforilada
ARC	Núcleo arqueado
ATP	Adenosina trifosfato
CART	Fator de transcrição cocaína-anfetamina dependente
C/EBP-β	CCAAT/ proteína de ligação ao intensificador beta
CRH	Hormônio liberador de corticotrofina
CTX-1	Telopeptídeo de colágeno tipo I C-terminal
D2R	Receptor de dopamina tipo 2
DAG	Diacilglicerol
DAT	Transportador de dopamina
DM	Diabetes *mellitus*
DMG	Diabetes *mellitus* gestacional
DM1	Diabetes mellitus tipo 1
DM2	Diabetes mellitus tipo 2
DM3	Diabetes mellitus tipo 2
DNA	Ácido desoxirribonucleico
DOHAD	Origens Desenvolvimentistas da Saúde e da Doença
FAS	Ácido graxo sintase
FDA	*Food Drug and Administration*
GCK	Glicoquinase
GFAP	Fibras da proteína ácida fibrilar glial
GLP1	Peptídeo semelhante ao glucagon 1
GLP1r	Receptor do peptídeo semelhante a glucagon 1
GLUT2	Transportador de glicose tipo 2
GLUT4	Transportador de glicose tipo 4

GPx	Glutationa peroxidase
GRα	Receptor de glicocorticóide alfa
GSK-3	Glicogênio sintase quinase-3
HOMA-IR	Modelo de avaliação da homeostase da resistência à insulina
IBA-1	Molécula adaptadora de ligação a cálcio ionizada 1
IDF	*International Diabetes Federation*
IL-6	Interleucina 6
IP3	Inositol-1,4,5 trifosfato
IR	Receptor de insulina
IRI	Índice de resistência à insulina
IRS	Substrato do receptor de insulina
IRS-1	Substrato do receptor de insulina 1
JAK2	Janus kinase 2
LH	Hipotálamo lateral
MCP-1	Proteína-1 quimioatrativa de monócitos
MDA	Malondialdeído
MODY	Maturity-Onset Diabetes of the Young
MS	Ministério da Saúde do Brasil
mTOR	Complexo 2 do alvo da rapamicina
Munc18	*Mammalian uncoordinated-18*
NAc	Núcleo acumens
NAFPD	Doença pancreática gordurosa não alcoólica
NPY	Neuropeptídio Y
ObR	Receptor de leptina
OMS	Organização Mundial da Saúde
PDX-1	Fator de transcrição homeobox pancreático e duodenal 1
PDK-1	Proteína quinase dependente de fosfoinositídeos-1
PE	Núcleo Periventricular
PGC1α	Co-ativador-1 do receptor ativado por proliferador do peroxissoma alfa
PI3K	Fosfatidilinositol 3-quinase
PIP3	Fosfatidilinositol 3,4,5 trifosfato
PKA	Proteína quinase A

PKC	Proteína quinase C
PLC	Fosfolipase C
PN	Pós natal
POMC	Pró-opiomelanocortina
PPARγ	Receptores ativados por proliferador de peroxissoma gama
PVN	Núcleo paraventricular
RI	Resistência à insulina
RNA	Ácido ribonucléico
SBD	Sociedade Brasileira de Diabetes
SNA	Sistema nervoso autônomo
SNAP-25	Proteína sinaptossomal associada de 25 kDa
SNP	Sistema nervoso parassimpático
SNARE	Soluble N-ethylmaleimide-sensitive factor activing protein receptor
SNS	Sistema nervoso simpático
SOCS3	Supressor da sinalização de citocinas 3
SOD	Superóxido dismutase
T3	Triiodotironina
T4	Tiroxina
TAB	Tecido adiposo branco
TAM	Tecido adiposo marrom
TH	Tirosina hidroxilase
TNF-α	Fator de necrose tumoral α
TRH	Hormônio liberador de tireotrofina
TSH	Tireotrofina
UCP-1	Proteína desacopladora 1
VAMP	Proteína associada a membrana vesicular
VDR	Receptor de vitamina D
VIGITEL	Sistema de Vigilância de Fatores de Risco e Proteção para Doenças Crônicas por Inquérito Telefônico
α-MSH	Hormônio estimulante de alfa-melanócitos
11βHSD1	11β-hidroxiesteróide desidrogenase tipo 1
25(OH)D	1,25 Diihidroxivitamina D

LISTA DE SÍMBOLOS

%	Porcentagem
±/	Mais ou menos
β	Beta
mL	Mililitro
rpm	Rotações por minuto
α	Alfa
g	Grama
mg	Miligrama
l	Litro
dL	Decilitro
δ	Delta
µg	Micrograma
kg	Kilograma
°c	Graus celsius
H	Hora
Min	Minuto
<	Maior
>	Menor
n	Número
vs	Versus

SUMÁRIO

INTRODUÇÃO

Obesidade

A prevalência mundial de obesidade quase triplicou entre 1975 a 2016 e passou a ser considerada uma pandemia nas últimas décadas, que afeta tanto países desenvolvidos quanto países em desenvolvimento (WHO 2018a). A obesidade passou a ser amplamente reconhecida como uma das ameaças mais desafiadoras para o setor público de saúde, que acarreta em intervenções contínuas com cargas significativas aos gastos públicos para o tratamento desta doença crônica, visto que ela já afeta grande parte da população mundial (FINUCATE et al., 2011; KREDEL; SIEGMUND, 2014; SHARMA et al., 2017).

Estudos epidemiológicos realizados pela Organização Mundial de Saúde (OMS, em inglês World Health Organization - WHO) confirmaram que em 2016 mais de 1,9 bilhões de adultos apresentavam excesso de peso, destes, aproximadamente 650 milhões eram obesos, sendo 11% da população do sexo masculino e 15% do sexo feminino (WHO 2018a). No Brasil, uma pesquisa realizada pela VIGITEL (Sistema de Vigilância de Fatores de Risco e Proteção para Doenças Crônicas por Inquérito Telefônico) confirmou que a frequência de adultos obesos é de aproximadamente 19,8%, sendo ligeiramente maior entre as mulheres (20,7%) do que entre os homens (18,7%), enquanto que o sobrepeso no País gira em torno de 56% (MS, 2019).

A obesidade é decorrente de uma condição de desequilíbrio energético, onde a ingestão de energia, principalmente armazenada em forma de triglicerídeos excede o seu gasto (SINGLA; BARDOLOI; PARKASH, 2010; GONZÁLEZ-MUNIESA et al., 2017). As mudanças no estilo de vida atual, que envolvem o aumento da ingestão de alimentos altamente calóricos, associados ao estilo de vida sedentário, com redução da prática de atividade física, são consideradas as principais causas da epidemia da obesidade (SCHUSTER 2010; SHARMA et al., 2017). Porém a susceptibilidade ao desenvolvimento desta doença é extremamente complicada, pois envolvem outras condições além da questão ambiental que podem predispor o indivíduo ao aumento excessivo de peso corporal, como fatores neuroendócrinos, psicossociais e genéticos (WELLMAN; FRIEDBERG, 2002; SCHUSTER 2010).

A obesidade tem se tornado um fator de risco preocupante, pois, além de ocasionar problemas psicossociais, como baixa autoestima, depressão e exclusão do indivíduo da sociedade (SCHERER; SANTOS, 2011; CAREY et al., 2014) ela também está relacionada com

o desenvolvimento de diversas doenças, incluindo hipertensão, osteoartrite, alguns tipos de câncer, doenças cardiovasculares, doença hepática gordurosa não alcoólica, dislipidemia, resistência à insulina (RI) e diabetes mellitus do tipo 2 (DM2) (HOTAMISLIGIL; ERBAY, 2008; GUTIERREZ; PUGLISI; HASTY; 2009; SCHUSTER 2010; JUNG; CHOL, 2014; SHARMA et al., 2017).

A obesidade está diretamente relacionada ao mecanismo de RI e ao desenvolvimento do DM2, uma vez que neste quadro metabólico, o acúmulo excessivo de triglicerídeos no tecido adiposo resulta na expansão do adipócito, seja por hipertrofia ou hiperplasia, que pode acarretar em impactos que são prejudicais a saúde, como a secreção acentuada de ácidos graxos, sinalização anormal do tecido adiposo (SCHUSTER 2010) e aumento da produção de excessiva de citocinas pró-inflamatórias (ROCHA; FOLCO, 2011). Na obesidade, quando ocorre expansão da massa adiposa, ocorrem processos inflamatórios, envolvidos com a secreção principalmente do fator de necrose tumoral alfa (TNF-α).

O TNF-α juntamente com a interleucina 6 (IL-6) e resistina, que são citocinas pró-inflamatórias, participam da indução e manutenção do estado inflamatório subagudo, relacionado com a obesidade. Este processo inflamatório acarreta doenças secundárias a longo prazo interferindo na progressão de outras doenças relacionadas com a síndrome metabólica, incluindo a RI (SHOELSON et al., 2006). A RI é caracterizada pela diminuição da sensibilidade à insulina, principalmente no músculo esquelético e tecido adiposo que em longo prazo aumentam os riscos de desenvolvimento de DM2 (GUTIERREZ; PUGLISI; HASTY; 2009; AL-GOBLAN; AL-ALFI; KHAN, 2014).

Além disso, levando em consideração a característica dos adipócitos em se adaptar frente à demanda de armazenamento de energia excessiva, no quadro de obesidade, os triglicerídeos passam a ser acumulados no parênquima, com a formação de gotículas de gordura dentro de células teciduais que não sejam adipócitos, como no fígado, coração, músculo e pâncreas, levando ao seu acúmulo ectópico (LONGO et al., 2019). No pâncreas, o acúmulo ectópico de gordura é chamado especificamente de doença pancreática gordurosa não alcoólica (NAFPD) ou esteatose pancreática (PINNICK et al., 2008; CATANZARO et al., 2016).

Esteatose pancreática

O acúmulo de gordura no pâncreas é uma característica clínica importante associada ao aumento do risco para o desenvolvimento da síndrome metabólica, incluindo DM2 e câncer de

pâncreas (ZHOU et al., 2016). Dados epidemiológicos sobre prevalência de esteatose pancreática ainda são escassos. A faixa de prevalência observada em um estudo populacional asiático demonstrou que a esteatose pancreática está entre 16 a 35% dos indivíduos (ZHOU et al., 2016). Dentro da população pediátrica hospitalizada a prevalência estimada é de 10% (PHAM et al., 2016). Ainda não existem estudos em outras populações.

O primeiro relato de gordura pancreática foi realizado em 1933 por Ogilvie, onde após a análise, encontrou maior gordura pancreática (~17%) em cadáveres obesos em comparação com os pâncreas de cadáveres magros (~ 9%) (OGILVIE, 1933). Quase 40 anos mais tarde, Olsen e cols (1978) realizaram um estudo com 394 autópsias, sendo os cadáveres separados em três grupos: abaixo, normal e acima do peso. Estes pesquisadores encontraram uma forte correlação entre teor de gordura pancreática, idade e sua relação com a obesidade.

Diferentemente da esteatose hepática, em que não há infiltração de adipócitos no tecido, mas sim alterações nos processos envolvidos com a homeostase lipídica, como aumento da lipogênese, exportação de lipídeos ou oxidação, a esteatose pancreática é caracterizada pela infiltração e aumento do número de adipócitos (CATANZARO et al., 2016) que leva ao acúmulo intracelular de lipídeos no tecido pancreático exócrino (SAKAI; TAYLOR; CHOUHAN, 2018). A associação entre esteatose pancreática, RI e disfunção das células-β pancreáticas tem sido investigada em estudos epidemiológicos e experimentais a fim de compreender os mecanismos fisiopatológicos envolvidos (YIN et al., 2004; TUSHUIZEN et al., 2007; WANG et al., 2014). Contudo, evidências reais que suportem a ideia de que a infiltração de gordura pancreática seja responsável pela disfunção das células-β pancreáticas ainda é discutida, uma vez que o acúmulo de lipídeos intra-ilhotas não é observado (VAN RAALTE; VAN DER ZIJL; DIAMANT, 2010).

As células adiposas infiltradas no pâncreas possuem a capacidade de secretar diversas citocinas pró-inflamatórias, resultando em inflamação localizada, associada ao aumento do estresse oxidativo (JUNG; CHOI, 2014). Além disso, os macrófagos recrutados produzem interleucina 1β e mieloperoxidase, as quais exacerbam o processo inflamatório. Esses eventos levam à lipotoxicidade e, consequentemente, danos às células acinares e células-β pancreáticas (SAKAI; TAYLOR; CHOUHAN, 2018). Esta é a teoria mais aceita para

explicar a relação do acúmulo de gordura ectópica no pâncreas com o aumento das chances para o desenvolvimento de pancreatite (SMITS; GEENEN, 2008), câncer de pâncreas (TAKAHASHI et al., 2018), disfunção das células-β pancreáticas e DM2 (MARTÍNEZ et al., 2006; SAKAI; TAYLOR; CHOUHAN, 2018).

A esteatose pancreática tem sido relacionada com a perda da massa e função das células-β pancreáticas (KHARROUBI et al., 2004), além da redução da secreção de insulina (HENI et al., 2010). Um estudo utilizando mini porcos Bama demonstrou que a administração de uma dieta rica em gordura e açúcar promove a longo prazo esteatose pancreática, aumento do estresse oxidativo e apoptose das células-β pancreáticas (ZHAO et al., 2015). Camundongos C57BL/6 adultos alimentados com ração de alto teor de gordura (60%) durante um período de 16 semanas apresentaram hiperglicemia, com hipertrofia das ilhotas e esteatose pancreática (FERNANDES-SANTOS et al., 2009).

Em humanos foi relatado associação positiva entre o aumento da infiltração de gordura no pâncreas e RI, sugerindo que a esteatose pancreática agrava esta condição (PATEL et al., 2013). Em uma coorte de 8.097 indivíduos, Wang e cols (2014) demonstraram que havia o risco aumentado para o desenvolvimento de diabetes em pacientes que apresentavam acúmulo de gordura no pâncreas em relação a pacientes cujos pâncreas não apresentavam acúmulo de gordura. Além disso, foi relatado que indivíduos recém diagnosticados com DM2 apresentam quantidades significativamente maior de gordura pancreática em comparação com pacientes saudáveis (CHAI et al., 2016). Após análise do conteúdo de gordura pancreática em homens com e sem DM2, Tushuizen e cols (2007) demonstraram que o conteúdo médio de gordura pancreática em pacientes diabéticos foi 20% maior em relação aos pacientes não diabéticos. Além disso, a gordura pancreática observada nestes pacientes estava relacionada com a disfunção das células-β pancreáticas.

A alta incidência de DM2 e obesidade, com suas co-morbidades, passaram a ser atribuídas não apenas com as mudanças no estilo de vida e abundância de alimentos calóricos, mas também com insultos no início da vida, que programam o metabolismo do indivíduo para o desenvolvimento de doenças endócrino-metabólicas a longo prazo. Esse fenômeno é denominado programação metabólica (PATTI, 2013; REMACLE et al., 2011; KERELIUK; BRAWERMAN; DOLINSK., 2017).

Programação metabólica

A nutrição e o metabolismo materno em diferentes fases da vida podem afetar o fenótipo dos filhos, causando um imprint para o desenvolvimento de doenças na vida adulta (MCMILLEN; ROBINSON, 2005; ALFARADHI et al., 2014). Barker, no início da década de

1980 foi o primeiro pesquisador a propor a “hipótese do fenótipo poupador” para tentar explicar a relação entre o ambiente fetal e as doenças evidenciadas na vida adulta. De acordo com essa teoria, a subnutrição materna durante o desenvolvimento embrionário pode programar o feto a economizar nutrientes para garantir o desenvolvimento de órgãos críticos, como cérebro e coração restringindo os nutrientes para órgãos menos críticos, tais como pâncreas e rim. Assim, o ambiente fetal, por meio da exposição materna a situações nutricionais e/ou ambientais inadequadas, pode desencadear o processo chamado de programação metabólica (BARKER, 1995; HALES; BARKER, 2001).

Atualmente esse conceito é conhecido como as “Origens Desenvolvimentistas da Saúde e da Doença” (em inglês: Developmental Origins of Health and Disease - DOHaD), a qual postula que insultos ambientais durante um período crítico do desenvolvimento podem acarretar em adaptações que promovem alterações ao longo da vida (EBERLE; AMENTE, 2012; VILLARES, 2016; MANDY; NYIRENDA, 2018). O indivíduo pode ser programado por diferentes insultos, de acordo com o ambiente que ele se encontra, isso envolve o estado nutricional materno, seja pela desnutrição, sobrepeso ou obesidade, além de doenças maternas, como DM gestacional ou até mesmo a exposição a fatores exógenos, que incluem o estresse, tabagismo, álcool, disruptores endócrinos, entre outros (MARCINIAK et al., 2017; ELLSWORTH et al., 2018).

Pesquisas apontam que a base molecular da programação está relacionada com mudanças epigenéticas, caracterizadas por alterações mitóticas hereditárias na expressão gênica sem alterar a sequência do DNA (PATTI, 2013; VAISERMAN, 2015; NAVARRO et al., 2017), modificando padrões de transcrição e interferindo na acetilação ou desacetilação das histonas e metilação do DNA (BIANCO-MIOTTO et al., 2017; BAROUKI et al., 2018). Recentemente, sabe-se que alterações na expressão de microRNAs, pequenas moléculas de RNA não codificantes, podem modular a estabilidade e/ou a eficiência da transdução do sinal do RNA mensageiro, podendo interferir também na plasticidade ontogenética (BOCK; EL- OSTA, 2017; NAVARRO et al., 2017).

As janelas críticas da vida que podem levar a programação incluem desde a pré-concepção até a adolescência (CERF, 2015). Dentre estes períodos, a lactação de forma semelhante à vida intrauterina, é considerada uma janela suscetível, visto que este período é caracterizado pela maturação das funções celulares, incluindo o desenvolvimento neurológico e cognitivo da criança (KWON; KIM, 2017; MARCINIAK et al., 2017).

Existe uma gama de estudos epidemiológicos e experimentais que buscam entender os efeitos da programação ocasionada por diferentes insultos, como obesidade materna, restrição

alimentar, fatores ambientais entre outros durante a gestação e/ou associada à lactação sobre o metabolismo dos descendentes. Estes trabalhos demonstram que insultos impostos nestas fases críticas da vida podem contribuir para o aumento da prevalência de sobrepeso e obesidade na infância até a vida adulta (ASHINO et al., 2012; REYNOLDS; SEGOVIA; VICKERS, 2017), intensificar as comorbidades associadas a esta síndrome, incluindo estresse psicossocial (PAINTER, et al., 2006), esteatose hepática (GUPTA et al., 2011), aumento do risco de desenvolvimento de alguns tipos de câncer, hipertensão (JUONALA et al., 2011) e doenças renais crônicas (GLASTRAS et al., 2018). Além disso, a nutrição materna durante a gestação afeta diretamente a homeostase glicêmica, levando a intolerância à glicose, hiperglicemia, disfunção das células-β com redução da sua massa e alterações na secreção de insulina estimulada por glicose na vida da prole adulta (WINICK; NOBLE, 1966; RAYCHAUNDHURI et al., 2008; DE LOS RÍOS et al, 2018).

A programação decorrente apenas de uma exposição durante o período em que a mãe está amamentando, pode resultar em alterações metabólicas, estruturais e fisiológicas do bebê, tendo como resposta aumento na suscetibilidade para o desenvolvimento de doenças (MARCINIAK et al., 2017; ELLSWORTH et al., 2018). Além disso, os órgãos envolvidos na manutenção da homeostase glicêmica, como cérebro, ilhotas pancreáticas e hepatócitos continuam diferenciando-se durante o período de lactação, portanto são vulneráveis a insultos de programação com efeitos duradouros sobre a homeostase glicêmica (ELLSWORTH et al., 2018).

Dessa forma, considerando o vasto número de estudos que se concentram principalmente na gestação como período de imprinting e a escassez de estudos focando a lactação como um importante período de desenvolvimento para humanos, o nosso grupo de pesquisa vem investigando ao longo de quase 20 anos o fenômeno de programação para obesidade em roedores, causada por insultos impostos exclusivamente durante o período de aleitamento materno e suas consequências em longo prazo. Dentre os modelos animais estudados, destacamos os que serão abordados neste trabalho: o desmame precoce e a

exposição materna à nicotina durante a lactação (MOURA et al., 2009; OLIVEIRA et al., 2009; LIMA et al., 2011).

Aleitamento materno

Amamentar vai além de apenas nutrir uma criança, é um processo que envolve uma relação profunda de mãe e filho, que influenciam na saúde física e psicológica materna, que refletem no estado nutricional, imunológico, fisiológico e no desenvolvimento cognitivo e emocional da criança (MS, 2015; WHO 2018b). Levando em consideração a importância do aleitamento materno, baseado em estudos desenvolvidos pelo grupo da Universidade Federal de Pelotas (HORTA; VICTORA, 2013; VICTORA et al., 2016) e com objetivo de promover melhoras na saúde da população a OMS, juntamente com o Ministério da Saúde do Brasil, recomendam que as crianças sejam amamentadas exclusivamente com o leite materno até os seis meses de vida. Após este período, é aconselhável a inclusão de outros alimentos suplementares na dieta do bebê, porém sem a interrupção do aleitamento materno até pelo menos 2 anos de idade (MS, 2015; WHO 2018b).

O aleitamento exclusivo nos primeiros meses de vida é considerado um objetivo de saúde pública global, pois é considerada a intervenção com maior potencial para reduzir a morbidade e mortalidade infantil. Uma estimativa demonstrou que o aleitamento ideal pode prevenir mais de 820 mil mortes infantis, com menos de 5 anos de idade em todo o mundo (UNICEF, 2007; VICTORA et al., 2016).

O leite materno é considerado a maneira mais apropriada para fornecer alimentos ideais e assim, atender às necessidades nutricionais do bebê garantindo o seu crescimento e desenvolvimento adequado, pois além de conter carboidratos, proteínas, lipídeos e minerais apropriados para o bebê, também possui outras substâncias nutritivas e de defesa que não são encontrados em outros tipos de leite (MS, 2015). Bebês que são amamentados exclusivamente no início da vida apresentam 98% de proteção entre o nascimento e seis meses pós-parto (WHO, 2018b), reduzem os riscos de doenças gastrointestinais e alérgicas (KRAMER; KAKUMA, 2012), apresentam menor risco cardiometabólico (WONG et al., 2018) diminuem as chances de desenvolvimento de diabetes (HORTA; VICTORA, 2013) e obesidade na idade adulta (YAN et al., 2014).

Apesar dos inúmeros benefícios causados pelo aleitamento materno, dados mundiais comprovam que a amamentação exclusiva ainda é muito baixa, apenas 40% dos lactantes com até seis meses de idade são amamentados exclusivamente (WHO, 2018b). Assim, quando ocorre a privação do aleitamento materno, a criança deixa de receber os nutrientes essenciais para seu desenvolvimento, acarretando impactos negativos a longo prazo, como sobrepeso e

obesidade, além de alterações no perfil lipídico, pressão arterial, e DM2 (ONIS et al., 2013; MS, 2015; WHO 2013).

No Brasil, a “epidemia do desmame” iniciou na década de 1970, devido o intenso processo de urbanização, o aumento do número de mulheres que ingressavam no mercado de trabalho e as incansáveis propagandas e publicidades para comercialização do leite industrializado em todo o mundo (VENANCIO; SALDIVA; MONTEIRO, 2013). A fim de tentar minimizar este fato, o Brasil desde a década de 80 desenvolveu inúmeras estratégias dentro do Programa Nacional de Incentivo ao Aleitamento Materno, para estimular a amamentação exclusiva, como a produção e vigilância das normas nacionais para comercialização de alimentos para lactantes (MS, 1998), fundação da Rede Brasileira de Bancos de Leite Humano (GIUGLIANI, 2002), implementação e apoio aos Hospitais Amigos da Criança (REA, 2003), criação de políticas públicas, incluindo a elaboração da Estratégia Amamenta e Alimenta Brasil (PASSANHA et al., 2013).

Além disso, foram criadas leis voltadas para a mulher trabalhadora que amamenta. Em 2008, a licença de maternidade que era apenas de 4 meses passou a ser prorrogada por mais 2 meses, tornando assim, o tempo de maternidade condizente com a nova lei e as diretrizes da OMS (Lei n°11.770). Segundo o artigo 396 da Consolidação das Leis de Trabalho, toda mulher tem o direito de amamentar seu filho durante a jornada de trabalho até que este complete 6 meses de vida, incluindo duas pausas remuneradas de meia hora cada um para este processo (UNICEF, 2007).

Recentemente, foi lançada a campanha anual de incentivo à amamentação, ação que marca o início da Semana Mundial de Amamentação (SMAM), no Brasil, que ocorreu na primeira semana de agosto de 2019, em mais de 170 países (MS, 2019). O destaque deste ano foi dado à importância do amparo de toda a rede de apoio, inclusive família, amigos, profissionais de saúde e empresários às mães que estão amamentando (MS, 2019).

De acordo com as estatísticas nacionais, todas estas estratégias proporcionaram grandes avanços nos índices de aleitamento maternos exclusivos observados desde a década de 80 (BOCCOLINI et al., 2017). Entretanto, pesquisas têm provocado sinais de alerta no país em relação aos números de aleitamentos exclusivos, uma vez que se observou a estagnação destes números e não a progressão como esperado na última pesquisa Nacional da Saúde (PNS) 2013 em comparação com a PNS-2006 (IBGE, 2013). Levando em consideração estes dados, é necessário reforçar e intensificar as ações já implementadas e desenvolver novas políticas de proteção, incentivo e apoio a amamentação materna, na tentativa de progredir a duração do aleitamento materno e evitar a interrupção precoce (BOCCOLINI et al., 2017).

Dessa forma, com o objetivo principal de entender os mecanismos fisiopatológicos decorrentes da interrupção da amamentação antes do recomendado, nosso laboratório desenvolveu dois modelos animais de programação pelo desmame precoce: farmacológico e não farmacológico, sendo que a diferença entre eles é a forma de interromper o aleitamento materno. Vale ressaltar que em ambos os modelos, os filhotes permanecem em contato com a mãe até o desmame, diminuindo a influência do estresse de separação (BONOMO et al., 2005; LIMA et al., 2011). Dessa forma, ao comprovar o mecanismo, pretendemos inequivocamente alertar as autoridades sanitárias da importância do aleitamento exclusivo.

Modelo de programação pelo desmame precoce farmacológico

O primeiro modelo de desmame precoce que estudamos foi o farmacológico, o qual é realizado através do tratamento materno com bromocriptina, um fármaco pertencente à classe dos agonistas dos receptores da dopamina, capaz de atravessar a barreira hematoencefálica e agir por meio de receptores D2 das células lactotróficas da adeno-hipófise inibindo a expressão do gene e secreção de prolactina, boqueando a produção de leite materno (LANDGRAF et al., 1977; FRIIS; PAULSON; HERTZ, 1979).

A bromocriptina foi utilizada inicialmente para o tratamento de doenças como Parkinson (COOLS, 2006) e depois para a acromegalia, galactorréia, hipogonadismo (KARASHIMA et al., 1986; RASMUSSEN, 1990; BOYD, 1995 XUE; LI; WANG, 2010;

KRYSIAK; SZKRÓBKA; OKOPIEŃ, 2018). Observou-se que este fármaco pode ser eficaz para hipertermia central (NATTERU et al., 2017), melhora do funcionamento sexual feminino reduzindo levemente sintomas depressivos em mulheres (KRYSIAK; SZKRÓBKA; OKOPIEŃ, 2018), e participando do controle bioquímico do aldosteronismo primário (WU et al., 2017). Este fármaco também passou a ser utilizado no tratamento do DM2. Sob a apresentação de mesilato de bromocriptina (Cycloset®) este fármaco foi aprovado pela agência americana Food and Drug Administration (FDA) e prescrito como tratamento auxiliar para o DM2, que deve ser associado ao controle alimentar e prática de atividade física (QUIANZON; CHEIKH, 2012). Estudos prévios mostraram os efeitos positivos da bromocriptina no controle da hiperglicemia, contribuindo para a melhora na tolerância à glicose e agindo como sensibilizador da insulina, melhorando o quadro clínico do paciente diabético (PIJL et al., 2000; CHAMARTHI; CINCOTTA, 2017).

Em nosso modelo de programação, ratas lactantes recebem intraperitonealmente 1mg de bromocriptina administrada em 2 doses por dia durante os 3 últimos dias que antecedem o desmame. Sabe-se que roedores machos programados dessa forma apresentam alterações aos 21 dias de vida, como perda de peso e redução no acúmulo de gordura, acompanhado de hiperleptinemia (BONOMO et al., 2005; ALBUQUERQUE MAIA et al., 2013).

Na vida adulta, a prole desmamada precocemente, de ambos os sexos, apresenta aumento do acúmulo de tecido adiposo branco (TAB) (BONOMO et al., 2007; PIETROBON & BERTASSO et al., 2019), com expansão da área dos adipócitos (PEIXOTO-SILVA et al., 2014; PIETROBON & BERTASSO et al., 2019). Ademais, os machos apresentam hiperleptinemia, resistência ao efeito anorexígeno da leptina, sem alterações na ingestão alimentar, além de hipotireoidismo central (BONOMO et al., 2007; BONOMO et al., 2008). Moura et al., (2009) observaram, hipoprolactinemia, dislipidemia e hiperfunção da adrenal em machos adultos, marcada por hipercorticosteronemia e aumento do conteúdo de catecolaminas adrenais. Fêmeas adultas também apresentam maior corticosterona sérica, porém exibem redução de catecolaminas adrenais (MIRANDA et al., 2019; PEIXOTO et al., 2019). A prole de ambos os sexos apresentam comprometimento da termogênese (PEIXOTO et al., 2019).

A tabela 1 resume as principais características na vida adulta do modelo de programação pelo tratamento materno com bromocriptina no final da lactação.

Tabela 1 - Principais resultados do modelo de programação pelo desmame precocefarmacológico (prole adulta) – (continua)

Artigo	Parâmetros avaliados
Bonomo et al., 2007 (♂ PN180)	✓ Aumento peso corporal; ✓ Aumento TAB retroperitoneal; ✓ Aumento gordura total; ✓ Normofagia; ✓ Aumento de leptina plasmática; ✓ Resistência ao efeito anorexigênico da leptina;
Bonomo et al., 2008 (♂ PN180)	✓ Redução de TSH, T3 e T4; ✓ Menor atividade hipofisária da desiodase tipo 2;
Moura et al., (2009) (♂ PN180)	✓ Aumento proteína; ✓ Aumento colesterol e triglicerídeo; ✓ Redução de prolactina e adiponectina; ✓ Hiperglicemia; ✓ Resistência à insulina; ✓ Aumento da corticosterona sérica e do conteúdo de catecolaminas na adrenal;
Passos et al., 2011) (♂ PN180)	✓ Redução peso relativo dos rins; ✓ Glomeruloesclerose; ✓ Fibrose peritubular; ✓ Redução clearance de creatinina; ✓ Aumento de proteinúria; ✓ Aumento de potássio e creatinina sérico;
Albuquerque Maia et al., 2014) (♂ PN180)	✓ Aumento 25(OH)D; ✓ Aumento do marcador sérico de reabsorção óssea (CTX1); ✓ Maior densidade óssea (coluna vertebral, fêmur e vértebra lombar); ✓ Maior número trabecular, rigidez e carga de ruptura; ✓ Menor separação trabecular, máxima deformação de ruptura;
Fraga et al., (2014) (♂ PN180)	✓ Distúrbios comportamentais: ✓ Redução memória/aprendizado; ✓ Aumento ansiedade;

Tabela 1 - Principais resultados do modelo de programação pelo desmame precoce farmacológico (prole adulta) – (conclusão)

Peixoto-Silva et al., (2014) (♂ PN180)		✓ Hipertrofia dos adipócitos; ✓ Aumento insulina e HOMA-IR; ✓ Aumento triglicerídeo hepático; ✓ Redução MDA plasmático e hepático; ✓ Redução SOD e aumento de GPx plasmático; ✓ Aumento SOD e da atividade da catalase no fígado; ✓ Preservação da arquitetura dos hepatócitos;
Younes-Rapozo et al., (2015) (♂ PN180)		✓ Aumento NPY no PVN; ✓ Aumento de astrócitos no ARC, PVN e LH; ✓ Aumento da densidade de GFAP no ARC, PVN e LH; ✓ Inflamação hipotalâmica;
Pietrobon & Bertasso et al., (2019) (♂ e ♀ PN180)	♂	✓ Maior massa adiposa (PN90-PN180); ✓ Hipertrofia adipócitos retroperitoneal;
	♀	✓ Maior ganho de peso corporal; ✓ Maior acúmulo de gordura visceral; ✓ Maior massa adiposa (PN90-PN180); ✓ Normofagia; ✓ Hipertrofia adipócitos retroperitoneal; ✓ Redução de estradiol sérico; ✓ Normoleptinemia;
Miranda et al., (2019) (♂ e ♀ PN180)	♂	✓ Redução da expressão de GRα no tecido adiposo subcutâneo; ✓ Aumento da expressão de C/EBPβ no tecido adiposo subcutâneo e redução na sua expressão no tecido adiposo visceral;
	♀	✓ Aumento de corticosterona sérica; ✓ Aumento da expressão de 11βHSD1 no tecido adiposo subcutâneo e redução da expressão de FAS e PPARγ no tecido adiposo visceral;

Peixoto et al., (2019) (♂ e ♀ PN180)	♂	✓ Menor atividade do SNS no TAM; ✓ Redução da expressão de PGC1α e da razão AMPKp/AMPK no TAM; ✓ Redução da expressão de TH na adrenal;
	♀	✓ Menor atividade do SNS no TAM; ✓ Redução da expressão de biomarcadores da termogênese no TAM; ✓ Redução do conteúdo de catecolaminas na adrenal;

Legenda: **AMPK:** Proteína quinase ativada por AMP; **AMPKp:** Proteína quinase ativada por AMP fosforilada; **ARC:** Núcleo arqueado; **C/EBPβ:** Proteína beta intensificadora de ligação a CCAAT; **CTX1:** Telopeptídeo de colágeno tipo I C-terminal; **FAS:** Ácido graxo sintase; **GFAP:** Fibras da proteína ácida fibrilar glial; **GPx:** Glutationa peroxidase; **GR*α*:** Receptor de glicocorticóide alfa; **HOMA-IR:** Modelo de avaliação da homeostase da resistência à insulina **LH**: Hipotálamo lateral; **MDA:** Malondialdeído; **NPY:** Neuropeptídeo Y; **PGC1*α*:** Co-ativador-1 do receptor ativado por proliferador do peroxissoma alfa; **PN:** Pós natal; **PPARγ:** Receptores ativados por proliferador de peroxissoma gama; **PVN:** Núcleo paraventricular; **SOD:** Superóxido dismutase; **SNS:** Sistema nervoso simpático; **TAB:** Tecido adiposo branco; **TAM:** Tecido adiposo marrom; **TH:** Tirosina hidroxilase; **TSH:** Tireotrofina; **T3:** Triiodotironina; **T4:** Tiroxina; **11βHSD1:** 11β-hidroxiesteróide desidrogenase tipo 1; **25(OH)D:** 1,25 Diihidroxivitamina D.

Fonte: A autora, 2020.

Em relação à homeostase glicêmica destes animais do sexo masculino, até o presente momento, sabe-se que os mesmos apresentam hiperglicemia, normoinsulinemia, e hipoadiponectinemia, sugerindo RI (MOURA et al., 2009; PEIXOTO-SILVA et al., 2014). Peixoto-Silva e cols (2014) observaram que aos 90 dias de vida, os animais programados apresentavam intolerância à glicose, porém tal alteração não se manteve aos 180 dias.

Modelo de programação pelo desmame precoce não farmacológico

Diferente do modelo anterior, este modelo experimental consiste em criar uma barreira física no corpo da mãe para interromper a amamentação. As ratas lactantes são levemente anestesiadas e cuidadosamente enfaixadas com bandagens adesivas, de maneira que todas as tetas fiquem cobertas, impedindo o acesso dos filhotes ao leite materno nos 3 últimos dias da lactação e obrigando-os a consumirem ração padrão a partir do 18° dia de vida pós-natal (LIMA et al., 2011). Descrito inicialmente por Lima e cols (2011), os primeiros resultados indicaram que tal manipulação resulta em modificações precoces nos filhotes machos, como redução de peso corporal, percentual de gordura total, hipoleptinemia, hipoglicemia e hipoinsulinemia ao desmame.

A prole adulta apresenta aumento do acúmulo de gordura corporal, hiperfagia e hiperleptinemia (LIMA et al.; 2011; PIETROBON & BERTASSO et al., 2019), sendo que os machos apresentam ainda resistência central à leptina e hipoprolactinemia (LIMA et al., 2011). Os machos exibem disfunção da adrenal, sem mudanças de glicocorticóides e hormônios tireoidianos (LIMA et al., 2013). Especificamente no TAB, foi observado hipertrofia dos adipócitos viscerais e subcutâneos dos machos, além de aumento de leptina neste tecido e citocinas inflamatórias (LIMA et al., 2013; LIMA et al., 2014; NOBRE et al., 2016; PEIXOTO-SILVA et al., 2017), enquanto que as fêmeas apresentam hipertrofia dos adipócitos retroperitoneais (PIETROBON & BERTASSO et al., 2019). Quando o fígado destes animais foi estudado, observou-se que os machos programados apresentam aumento do estresse oxidativo e microesteatose hepática (FRANCO et al., 2013).

A tabela 2 descreve as principais características já descritas em nosso laboratório da prole adulta submetida ao desmame precoce não farmacológico.

Tabela 2 - Principais resultados do modelo de programação pelo desmame precoce não farmacológico (prole adulta) – (continua)

Artigo	Parâmetros avaliados
Lima et al., (2011) (♂ PN180)	✓ Sobrepeso; ✓ Hiperfagia; ✓ Aumento gordura total e visceral; ✓ Aumento de leptina plasmática; ✓ Redução JAK2 e STAT3p, aumento SOCS3 no hipotálamo; ✓ Hiperglicemia e IRI; ✓ Menor adiponectina e prolactina;
Younes-Rapozo et al., (2012) (♂ PN180)	✓ Aumento NPY no hipotálamo; ✓ Redução CART no hipotálamo;
Lima et al., (2013) (♂ PN180)	✓ Hipertrofia de adipócitos subcutâneo e visceral; ✓ Aumento leptina no TAB visceral; ✓ Aumento do conteúdo de catecolaminas na adrenal; ✓ Aumento receptor β3 no TAB visceral; ✓ Sem alterações nos glicocorticoides e nos hormônios tireoidianos;
Franco et al., (2013) (♂ PN180)	✓ Aumento pressão sistólica e diastólica; ✓ Aumento triglicerídeo e LDL-c plasmático; ✓ Aumento triglicerídeo hepático ✓ Aumento MDA plasmático e hepático; ✓ Redução SOD plasmático e GPx hepático; ✓ Microesteatose hepática;

Albuquerque Maia et al., 2014) (♂ PN180)	✓ Aumento 25(OH)D; ✓ Aumento de CTX1; ✓ Aumento da massa óssea; ✓ Maior número trabecular, rigidez e carga de ruptura; ✓ Menor separação trabecular, máxima deformação de ruptura;
Nobre et al., (2016) (♂ PN180)	✓ Aumento área de adipócitos; ✓ Redução calbindina intestinal; ✓ Redução do VDR no TAB; ✓ Aumento C/EBP-β e PPARγ no TAB ✓ Aumento leptina, redução ObR e SOCS3 no TAB; ✓ Aumento IL-6, MCP-1 e TNFα e redução de IL-10 no TAB;

Tabela 2 - Principais resultados do modelo de programação pelo desmame precoce não farmacológico (prole adulta) – (conclusão)

Artigo	Parâmetros avaliados
Lima et al., (2011) (♂ PN180)	✓ Sobrepeso; ✓ Hiperfagia; ✓ Aumento gordura total e visceral; ✓ Aumento de leptina plasmática; ✓ Redução JAK2 e STAT3p, aumento SOCS3 no hipotálamo; ✓ Hiperglicemia e IRI; ✓ Menor adiponectina e prolactina;
Younes-Rapozo et al., (2012) (♂ PN180)	✓ Aumento NPY no hipotálamo; ✓ Redução CART no hipotálamo;
Lima et al., (2013) (♂ PN180)	✓ Hipertrofia de adipócitos subcutâneo e visceral; ✓ Aumento leptina no TAB visceral; ✓ Aumento do conteúdo de catecolaminas na adrenal; ✓ Aumento receptor β3 no TAB visceral; ✓ Sem alterações nos glicocorticoides e nos hormônios tireoidianos;
Franco et al., (2013) (♂ PN180)	✓ Aumento pressão sistólica e diastólica; ✓ Aumento triglicerídeo e LDL-c plasmático; ✓ Aumento triglicerídeo hepático ✓ Aumento MDA plasmático e hepático; ✓ Redução SOD plasmático e GPx hepático; ✓ Microesteatose hepática;
Albuquerque Maia et al., 2014) (♂ PN180)	✓ Aumento 25(OH)D; ✓ Aumento de CTX1; ✓ Aumento da massa óssea; ✓ Maior número trabecular, rigidez e carga de ruptura; ✓ Menor separação trabecular, máxima deformação de ruptura;
Nobre et al., (2016) (♂ PN180)	✓ Aumento área de adipócitos; ✓ Redução calbindina intestinal; ✓ Redução do VDR no TAB; ✓ Aumento C/EBP-β e PPARγ no TAB ✓ Aumento leptina, redução ObR e SOCS3 no TAB; ✓ Aumento IL-6, MCP-1 e TNFα e redução de IL-10 no TAB; ✓

Legenda: **CART:** Fator de transcrição cocaína-anfetamina dependente; **C/EBP-β:** CCAAT/ proteína de ligação ao intensificador beta; **CTX1:** Telopeptídeo de colágeno tipo I C-terminal; **GLP-1:** Peptídeo semelhante a glucagon 1; **GLP-1r:** Receptor do peptídeo semelhante a glucagon 1; **GPx:** Glutationa peroxidase; **GRα:** Receptor de glicocorticóide alfa; **IL-6:** Interleucina 6; **IL-10:** Interleucina 10; **IRI:** Índice de resistência à insulina; **JAK2:** Janus kinase 2; **LDL-c:** Lipoproteína de baixa densidade; **MCP-1:** Proteína-1 quimioatrativa de monócitos; **MDA:** Malondialdeído;

NPY: Neuropeptídeo Y; **OBRb:** Receptor de leptina; **PN:** Pós natal; **PPARγ:** Receptores ativados por proliferador de peroxissoma; **SNS:** Sistema nervoso simpático; **SOD:** Superóxido dismutase; **SOCS3:** Supressor da sinalização de citocinas 3; **STAT3p:** Transdutor de sinal e ativador da transcrição 3 fosforilado; **TAB:** Tecido adiposo branco; **TAM:** Tecido adiposo marrom; **TH:** Tirosina hidroxilase; **TNF-α:** Fator de necrose tumoral α; **VDR:** Receptor de vitamina D; **11βHSD1:** 11β-hidroxiesteróide desidrogenase tipo 1; **25(OH)D:** 1,25 Diihidroxivitamina D.
Fonte: A autora, 2020.

Quanto à homeostase glicêmica, os machos programados pelo desmame precoce não farmacológico apresentam hiperglicemia, sem alterações na insulina, e hipoadiponectinemia, sugerindo o desenvolvimento de RI (LIMA et al., 2011). Até o momento, os resultados indicam que estes animais apresentam o mesmo fenótipo de roedores submetidos ao desmame precoce farmacológico (LIMA et al., 2011; NOBRE et al., 2012; LIMA et al., 2014).

Além dos insultos nutricionais no início da vida que podem levar a programação metabólica, outras exposições maternas, como as poluições ambientais, agrotóxicos, estresse, álcool e tabagismo, durante a janela da lactação, também podem influenciar os fenótipos metabólicos da prole na vida adulta (LI; GONZALEZ; ZHANG, 2012; ELLSWORTH et al.,2018). Assim, nosso grupo desenvolveu um terceiro modelo de programação a fim de mimetizar os efeitos tardios do tabagismo materno no início da vida, onde há cerca de 14 anos investiga-se os efeitos a longo prazo da exposição precoce e involuntária da prole exclusivamente à nicotina, principal componente aditivo do cigarro, via leite materno.

Tabagismo

Segundo a OMS, o tabagismo deixou de ser considerado um vício e passou a ser classificada como uma doença crônica causada pela dependência química dos fumantes à nicotina, principal componente ativo presente nos produtos do cigarro (WHO, 2019a). O uso do tabaco esteve relacionado com aproximadamente 100 milhões de mortes em todo o mundo no século XX. Atualmente, o ato de fumar se tornou uma epidemia, onde aproximadamente 5,4 milhões de pessoas morrem todos os anos de doenças relacionadas ao tabaco. A menos que uma ação urgente seja tomada, estima-se que em 2030 haverá mais de 8 milhões de mortes relacionadas ao tabaco, sendo que 80% destas mortes estarão em países em desenvolvimento (WHO, 2019a).

A prevalência do tabagismo é maior em homens que em mulheres, visto que estas começaram a fumar mais tarde. No entanto, nas ultimas décadas foi observado um declínio na

curva do tabagismo para os homens ao passo que, houve aumento para as mulheres, e a ideia de que o ato de fumar é uma epidemia masculina não existe mais (REGUEIRA; SUÁREZ-LUGO; JAKIMCZUK, 2010; WHO, 2019a). Nos EUA estima-se que aproximadamente 30% e 35% de mulheres e homens, respectivamente, fumam em idade reprodutiva (The practice committee of the american society for reproductive medicine, 2012). Segundo dados recentes da VIGITEL, no Brasil, a frequência de adultos fumantes em 2018 foi de 9,3%, sendo a prevalência de 12,1% em homens e 6,9% em mulheres (MS, 2018).

O tabaco é o único produto de consumo legal que mata mais da metade daqueles que usam intencionalmente. Dentre as doenças crônicas não transmissíveis relacionadas ao cigarro às doenças cardiovasculares são as principais causas de mortes anuais, seguida de doença pulmonar obstrutiva crônica (enfisema pulmonar, bronquite crônica), câncer de pulmão, pneumonia e acidente vascular cerebral (DOWNEY, 1990; INCA, 2018a). É importante ressaltar ainda, que o tabagismo prejudica também todos aqueles expostos a fumaça do cigarro, pois, sua inalação involuntária pode acarretar desde reações alérgicas a curto prazo e infarto agudo do miocárdio, câncer e doenças respiratórias a longo prazo (INCA, 2018b).

Entre os fumantes passivos, estima-se que aproximadamente 700 milhões de crianças entram em contato com a fumaça do cigarro todos os anos. A exposição à fumaça ambiental do tabaco gera efeitos negativos no desenvolvimento das crianças, especialmente no crescimento fetal, causando baixo peso ao nascer, parto prematuro, além dos efeitos relacionados com o trato respiratório, podendo levar até a morte (INCA, 2018b).

O uso do tabaco é comum em todo o mundo devido aos baixos custos para o consumidor, falta de consciência sobre os seus efeitos e políticas públicas ineficientes. Em 2014, aproximadamente 5,8 trilhões de cigarros foram fumados em todo o mundo (ERIKSEN et al., 2015). A fumaça do cigarro é uma mistura de aproximadamente 7.000 substâncias tóxicas, incluindo propriedades mutagênicas e carcinogênicas (INCA, 2018a; WHO 2019a). Dentre estes compostos encontra-se a nicotina e o alcatrão, este último é uma mistura complexa de sustâncias, como o arsênio, níquel, benzopireno, cádmio, resíduos de agrotóxicos, substâncias radioativas, como o polônio 210, acetona, naftalina e até fósforo P4/P6, substâncias usadas em veneno para matar rato. Dentre todas as substâncias encontradas para compor o alcatrão, mais de 40 são comprovadamente cancerígenas (LI, 2016; INCA, 2018a).

A nicotina é considerada uma droga psicoativa e o principal agente causador da dependência da fumaça do cigarro (LE FOLL; GOLDBERG, 2009; WHO 2019b). Age no sistema nervoso central e por isso está inserida no Código Internacional de Doença (CID-11) no grupo de transtornos mentais e de comportamento (WHO, 2019b). A nicotina ao entrar em

contato com a circulação arterial se liga em receptores colinérgicos nicotínicos, que são canais iônicos dependentes de ligante que normalmente são ligados à acetilcolina (CHANGEUX, 2010). A ligação da nicotina ao seu receptor permite a entrada de cálcio intracelular, resultando em um dos seus efeitos, a liberação de neurotransmissores (DAJAS-BAILADOR; WONNACOTT, 2004; NEAL; BENOWITZ, 2010). Dessa forma, a nicotina é capaz de estimular a secreção de catecolaminas, provocando vasoconstrição, aumentando a frequência cardíaca e causando hipertensão arterial (SLOTKIN; SEIDLER, 1975), além de induzir a psicoestimulação e recompensa, reduzir o estresse e ansiedade (HUGHES, 2007).

Outro fator preocupante em relação ao tabagismo é o seu uso por mulheres em idade reprodutiva, inclusive durante a gestação. Sabe-se que aproximadamente 10% das mulheres relatam fumar neste período, sendo o cigarro a droga mais utilizada por gestantes (RYDELL et al., 2014; NAPIERALA et al., 2016). O ato de fumar durante a gravidez é considerado um grave problema de saúde pública, visto que, acarreta em prejuízos tanto para mãe quanto para o feto, como dificuldades de desenvolvimento, parto prematuro, mortalidade infantil e neonatal, além do risco duas vezes maior de desenvolver dependência à nicotina e obesidade na vida adulta (BUKA; SHENASSA; NIAURA, 2003; OKEN; LEVITAN; GILLMAN, 2008;NAPIERALA et al., 2016; FONSECA et al., 2018).

Embora tenha sido demonstrado que muitas mulheres param de fumar durante o período gestacional (GIGLI; BINNS; ALFONSO, 2006, POLANSKA et al., 2007) alguns estudos revelam que a maioria das mulheres que interromperam este hábito, o retomam na lactação (HANNOVER et al., 2008; ROCKHILL et al., 2016). Assim, o tabagismo durante a amamentação é prejudicial não só para as mães, mas também para o bebê que é exposto involuntariamente aos componentes do cigarro via leite materno. Frente a isto, nosso grupo desenvolveu um modelo de programação de exposição pela nicotina a fim de investigar seus impactos durante a lactação.

Modelo de programação pela exposição materna à nicotina durante a lactação

Neste modelo experimental, as ratas lactantes são expostas à nicotina, onde são implantadas minibombas osmóticas do 2° ao 16° dia da lactação. Estas liberam na corrente sanguínea 6mg/kg de nicotina por dia durante 14 dias consecutivos, o equivalente a um

fumante pesado. Observamos que as lactantes expostas à nicotina apresentaram grande quantidade de cotinina, o principal metabólito da nicotina, no soro e no leite, comprovando a eficácia do modelo.

Aos 15 dias de vida, período que corresponde o final da exposição materna a nicotina, foi detectado a presença de cotinina no soro da prole do sexo masculino (OLIVEIRA et al., 2010a), maior massa de gordura visceral, hiperleptinemia, hipotireoidismo primário (OLIVEIRA et al., 2010a; SANTOS-SILVA et al., 2010; OLIVEIRA et al., 2011) com aumento de corticosterona plasmática e do conteúdo total de catecolaminas adrenais, apesar que nas mães, a concentração de corticosterona sérica foi normal, ao final da exposição (OLIVEIRA et al., 2010a). Ao desmame não foi detectado a presença de cotinina e todas as alterações observadas em PN15 foram normalizadas (OLIVEIRA et al., 2009; OLIVEIRA et al., 2010a; OLIVEIRA et al., 2011).

Os machos adultos programados pela exposição à nicotina na lactação apresentam obesidade com maior acúmulo de gordura visceral (OLIVEIRA et al., 2010b; SANTOS-SILVA et al., 2010; YOUNES-RAPOSO et al., 2015), normofagia (OLIVEIRA et al., 2009), apesar de apresentarem hiperleptinemia sérica (PINHEIRO et al., 2011) e resistência central à leptina (PINHEIRO et al., 2011). Além destas alterações, roedores adultos exibem hipotireoidismo secundário (OLIVEIRA et al., 2009; YOUNES-RAPOSO et al., 2013; LISBOA et al., 2015), hipercorticosteronemia, bem como, aumento do conteúdo de catecolaminas adrenais (PINHEIRO et al., 2011). O fígado dos machos é comprometido; este tecido apresenta aumento do estresse oxidativo e esteatose hepática (CONCEIÇÃO et al., 2015). As fêmeas adultas desenvolvem dislipidemia, sem alterações na morfologia hepática (BERTASSO et al., 2019).

Os resultados já descritos neste modelo de programação estão listados na tabela abaixo (Tabela 3).

Tabela 3 - Principais resultados do modelo de programação pela exposição materna à nicotina (prole adulta) – (continua)

Oliveira et al., (2009) (♂ PN180)	✓ Sobrepeso; ✓ Maior gordura corporal; ✓ Normofagia; ✓ Hiperleptinemia; ✓ Menor T4 livre, TSH e atividade D1 hepática;
Oliveira et al., (2010) (♂ PN180)	✓ Normoglicemia; ✓ Aumento de insulina plasmática; ✓ Hipertrofia dos adipócitos visceral e subcutâneo; ✓ Menor ObR, JAK2 e pSTAT3 e maior SOCS3;
Pinheiro et al., (2011) (♂ e ♀ PN180)	♂ ✓ Maior conteúdo de catecolaminas e TH na adrenal; ✓ Menor liberação de catecolamina (*in vitro*); ✓ Aumento de corticosterona; ✓ Maior AdRβ3 no TAB; ✓ Maior leptina no TAB visceral; ♀ ✓ Maior proteína corporal; ✓ Menor ADRβ3 no TAB; ✓ Menor leptina TAB subcutâneo; ✓ Maior leptina no músculo;
Younes-Rapozo et al., (2013) (♂ PN180)	✓ Aumento NPY, POMC e α-MSH no hipótalamo; ✓ Aumento CRH e redução do no PVN; ✓ Redução CART no PVN; ✓ Redução de TRH no hipotálamo;

Tabela 3 - Principais resultados do modelo de programação pela exposição materna à nicotina (prole adulta) – (conclusão)

Pinheiro et al., (2015) (♀ PN180)	✓ Preferência por dieta rica em açúcar; ✓ Maior ansiedade e menor atividade locomotora; ✓ Menor D2R e DAT no NAc; ✓ Menor D2R no ARC;
Conceição et al., (2015) (♂ PN180)	✓ Aumento triglicerídeo hepático; ✓ Aumento de MDA, carbonilação de ptn hepática; ✓ Redução de atividade GPx e aumento de SOD hepática; ✓ Microesteatose hepática;
Younes-Rapozo et al., (2015) (♂ PN180)	✓ Aumento astrócitos no ARC, PVN e LH; ✓ Aumento GFAP no ARC e PVN; ✓ Aumento IBA1 no PVN; ✓ Redução IL10 no TAB e plasma;

Legenda: **α-MSH:** Hormônio estimulante de alfa-melanócitos; **AdRβ3:** Receptor adrenérgico beta 3; **ARC:** Núcleo arqueado; **CART:** Fator de transcrição cocaína-anfetamina dependente; **CRH:** Hormônio liberador de corticotrofina; **DAT:** Transportador de dopamina; **D1:** Desiodase tipo 1; **D2:** Desiodase tipo 2; **D2R:** Receptor de dopamina tipo 2; **GFAP:** Proteína ácida fibrilar glial; **GPx:** Glutationa peroxidase; **IBA-1:** Molécula adaptadora de ligação a cálcio ionizada 1; **IL-10:** Interleucina 10; **JAK2:** Janus kinase 2; **LH:** Hipotálamo lateral; **MDA:** Malondialdeído; **NAc:** Núcleo acumens; **NPY:** Neuropeptídeo Y; **ObR:** Receptor de leptina; **PE:** Núcleo Periventricular; **PN:** Pós natal; **POMC:** Pró-opiomelanocortina; **PVN:** Núcleo paraventricular; **SOCS3:** Supressor da sinalização de citocinas 3; **SOD:** Superóxido dismutase; **STAT3p:** Transdutor de sinal e ativador da transcrição 3 fosforilado;**T4:** Tiroxina; **TAB:** Tecido adiposo branco; **TH:** Tirosina hidroxilase; **TRβ1:** Receptor de hormônio tireoidiano β1; **TRH:** Hormônio liberador de tireotrofina; **TSH:** Tireotrofina; **UCP-1:** Proteína desacopladora 1.

Fonte: A autora, 2020.

A adiponectina plasmática destes animais não é alterada, entretanto a concentração deste hormônio em razão do TAB visceral é reduzida (OLIVEIRA et al., 2010b), o que foi demonstrado guardar relação com a sensibilidade à insulina (PARK et al., 2005). Quanto à homeostase glicêmica foi descrito que estes animais são normoglicêmicos e hiperinsulinêmicos (OLIVEIRA et al., 2010b). Vale destacar que os machos adultos expostos à nicotina no início da vida apresentam um fenótipo muito similar aos animais machos submetidos ao desmame precoce, apesar de serem diferentes modelos de programação durante a lactação, no que se diz respeito à homeostase glicêmica ambos modelos causam RI, portanto outros estudos são necessários para compreender e elucidar mecanismos envolvidos em tais alterações. Assim este trabalho buscou investigar os mecanismos pelo qual esta homeostase é alterada. Além disso, todos os resultados encontrados referem-se apenas a prole de machos, e, portanto, não sabemos

ainda como é a resposta das fêmeas em relação à homeostase glicêmicasubmetidas a estes modelos de programação.

Secreção da insulina pelas células-β pancreáticas

A insulina é secretada pelo pâncreas endócrino, uma glândula mista que apresenta papéis fundamentais na regulação da digestão de macronutrientes e na homeostase. Localizada atrás do estômago, dentro da cavidade abdominal, este órgão é formado por duas regiões: a região exócrina, composta pelos ácinos, responsáveis pelas secreções digestivas que contém enzimas como, amilase, lipase pancreática e tripsinogênio que são secretadas no duodeno e a região endócrina, constituída pelas ilhotas pancreáticas ou ilhotas de Langherans, envolvidas na secreção de alguns hormônios que participam da regulação do metabolismo, principalmente regulando a homeostase glicêmica (CABRERA et al., 2006; RÖDER et al., 2016).

O pâncreas humano possui entre um a dois milhões de ilhotas pancreáticas, organizadas em torno de capilares sanguíneos, para os quais secretam seus hormônios. As ilhotas são formadas por quatro grupos celulares: 1) células alfa (□), que representam aproximadamente 25% do volume total e secretam glucagon; 2) células beta (β), que constituem cerca de 60% do total e são responsáveis por secretar insulina, amilina e peptídeo C; 3) células delta (δ), que correspondem a 10% do total e secretam somatostatina e 4) células PP, que equivalem a 5% do tecido e secretam polipeptídeo pancreático (BRISSOVA et al.,2005; CABRERA et al., 2006).

A homeostase glicêmica é balanceada por mecanismos intracelulares, ajustada de

acordo com as flutuações de determinados nutrientes e pela sinalização hormonal. Estes fatores controlam a utilização de glicose por tecidos específicos, como fígado, músculo esquelético, TAB e cérebro, os quais contribuem diretamente para manutenção da concentração de glicose na corrente sanguínea dentro dos valores adequados (glicemia de jejum <99 mg/dL – Unidade convencional ou < 6,1 mmol/L – Unidade internacional) (ASSOCIAÇÃO AMERICANA DE DIABETES, 2010; SHARABI et al., 2015).

Quando a concentração de glicose sanguínea atinge valores muito baixos, em situações como no sono ou entre as refeições, as células-α aumentam a secreção de glucagon, promovendo a glicogenólise hepática (RAMNANAN et al., 2011). Além disso, este hormônio estimula a gliconeogênese hepática e renal e inibe a síntese de glicogênio, a fim de aumentar a concentração de glicose no sangue durante um período de jejum prolongado (SHARABI et al., 2015). Em contrapartida, altas concentrações de glicose na corrente sanguínea, tais como

ocorrem após uma refeição, estimulam a secreção de insulina pelas células-β pancreáticas (FREYCHET et al., 1988; RÖDER et al., 2016).

A regulação da secreção de insulina é constantemente determinada por diferentes nutrientes, especialmente a glicose. Além disso, a secreção é modulada direta ou indiretamente, por alguns hormônios, neurotransmissores e agentes farmacológicos (CHANDRA; LIDDLE, 2009; MOLINA et al., 2014). Este controle multifatorial permite que as células-β pancreáticas secretem insulina em tempos e quantidades adequados, possibilitando a regulação da concentração de nutrientes no sangue em diferentes situações fisiológicas, como jejum, refeição, exercício físico, gestação e lactação, atendendo a demanda metabólica em cada condição específica (BOSCHERO, 1996; FU; GILBERT; LIU, 2013).

A glicose é o secretagogo fisiológico mais importante na secreção de insulina

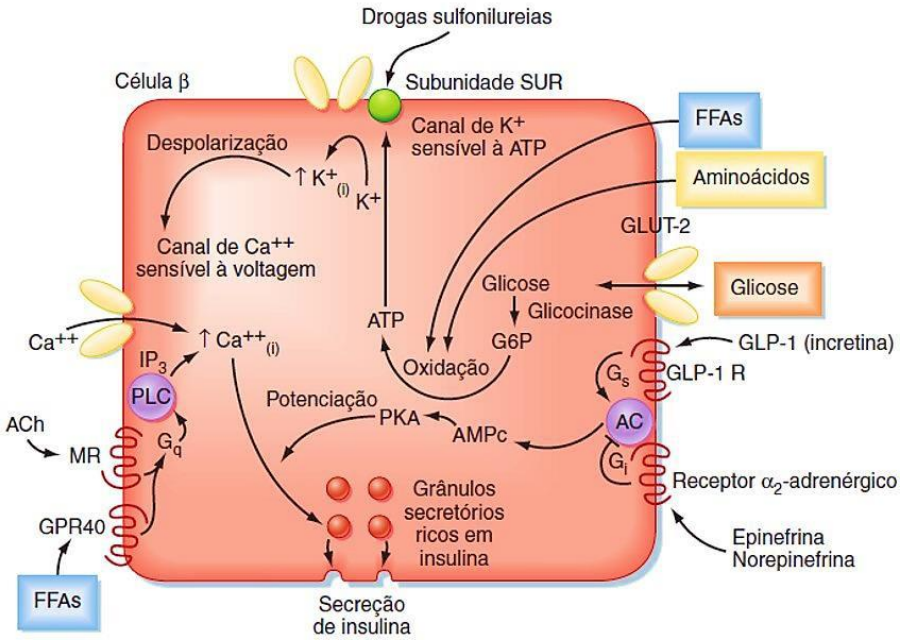
(RUTTER et al., 2015). O mecanismo de secreção de insulina tem início quando a glicose é transportada para o interior da célula-β, através de uma proteína integral de membrana, chamada transportador de glicose do tipo 2 (GLUT 2). Esta proteína possui um elevado Km (entre 15 e 20 mmol/L, portanto não saturável em concentrações fisiológicas de glicose) permitindo que o transporte de glicose aumente rapidamente quando a glicemia se eleva (SUCKALE; SOLIMENA, 2008).

No interior da célula-β a glicose é fosforilada à glicose-6-fosfato pela ação da enzima glicoquinase (GCK), logo a glicose-6-fosfato é submetida à glicólise, gerando o piruvato, ainda no citoplasma. Este é então metabolizado em acetil-CoA pela ação das enzimas piruvato desidrogenase e piruvato carboxilase na mitocôndria, permitindo a formação de ATP (MATSCHINSKY, 1995, KOMATSU et al., 2013). O aumento da relação entre ADP/ATP intracelular resulta no fechamento de canais de K+ ATP-dependentes. A redução do efluxo de K+ leva a despolarização da membrana, que resulta na abertura de canais de Ca2+ sensíveis à voltagem e o influxo deste íon para o interior da célula. Em decorrência do aumento Ca2+ intracelular e a despolarização suplementar da membrana plasmática ocorre o processo de fusão dos grânulos de insulina com a membrana plasmática e, subsequentemente a exocitose do seu conteúdo (AHRÉN; SAUERBERG; THOMSEN, 1999; RUTTER, 2001; KOMATSU et al., 2013; GODINE; GHASEMI, ZAHEDIASL, 2015).

A metabolização da glicose nas células-β também pode ativar enzimas que vão gerar outros mensageiros intracelulares que amplificam a secreção de insulina. Uma destas enzimas é a adenilato ciclase (AC), que ao clivar o ATP, gera adenosina monofosfato cíclico (AMPc), que então ativa a proteína quinase A (PKA). A PKA pode intensificar a secreção de insulina pela fosforilação do canal de Ca2+, sensível à voltagem, aumentando a concentração deste íon

no interior da célula (CHRISTIE; ASHCROFT, 1984; GILON et al., 2002). Ademais, a estimulação das células-β pela glicose resulta na ativação da enzima fosfolipase C (PLC), que promove a hidrólise de fosfolipídios de membrana, resultando na formação de inositol 1-4-5-trifosfato (IP3) e diacilglicerol (DAG). O IP3, por sua vez, ativa os canais de cálcio presentes na membrana do retículo endoplasmático, resultando na saída desse íon da organela e, consequentemente, aumentando a concentração de Ca2+ no citossol. O DAG produz efeito semelhante em relação à concentração de Ca2+ no meio intracelular, devido à ativação dos canais de cálcio sensíveis à voltagem presentes na membrana plasmática, resultando na passagem desse íon para o interior da célula. Além disso, o DAG ativa a proteína quinase C (PKC), responsável pela ativação de proteínas dos grânulos secretórios, que em conjunto com íons Ca2+, promovem a ativação do sistema de microtúbulos e microfilamentos, culminando na exocitose de insulina (PRENTKI; MATSCHINSKY, 1987; RÖDER et al., 2016; STRAUB; SHARP, 2002) (Figura 1).

Figura 1 - Regulação da secreção de insulina pelos substratos energéticos glicose (secretagogo primário); aminoácidos, ácidos graxos livres (FFAs) e pelos neurotransmissores e hormônios acetilcolina (ACh), norepinefrina, epinefrina e peptídeo 1 tipo glucagon (GLP-1)



Fonte: Berny e Levy, 6ªed, pág 684, 2009.

As ilhotas pancreáticas são ricamente inervadas por ramos do sistema nervoso autônomo (SNA), ou seja, tanto estímulos parassimpáticos quanto simpáticos podem interferir na função

das células-β (AHRÉN, 2000; PRATES et al., 2017) (Figura 1). A ação parassimpática nas ilhotas pancreáticas ocorre através da liberação de acetilcolina (Ach), a qual pode se ligar a quatro subtipos de receptores muscarínicos expressos no pâncreas (m1AchR-m4AchR) (MIGUEL et al., 2002; MIRANDA et al., 2014). Entretanto sua ligação com o receptor m3AchR, acoplado a proteína Gq catalisam a clivagem de fosfolipídios que membrana, levando a ativação de PKC, cujo resultado é a secreção de insulina estimulada pela glicose fortemente intensificada (KOMATSU et al., 2013; TIWARI et al., 2013; THORENS, 2014). Enquanto, a ligação da Ach aos seus receptores do tipo M2 ou M4 vai desencadear um efeito inibitório da secreção de insulina atrávez da ativação do seu receptor acoplado a proteína Gi e a inibição da atividade da AC (MIGUEL et al., 2002; GRASSIOLLI; GRAVENA; MATHIAS, 2007).

A inervação simpática é responsável por transmitir sinais inibitórios da secreção de insulina induzida pela glicose (PORTE, 1969). O tônus simpático age nas células-β pela liberação e ação da noradrenalina (ZHAO et al., 2010), que atua por meio da ativação do receptores alfa-2 adrenérgicos (α2AR), encontrado em três isoformas, α-2A, α-2B e α-2C e receptores beta-adrenérgicos (βAR), nas isoformas β-1, β-2 e β-3, todos estes receptores são acoplados à proteína G (AHRÉN et al., 2006; FAGERHOLM; HAAPARANTA; SCHEININ, 2011). Em relação à sinalização adrenérgica em células-β, a ligação da noradrenalina com o α-2A age via proteína Gi, inibindo PKA e como consequência promove a inibição da secreçãode insulina (FAGERHOLM; HAAPARANTA; SCHEININ, 2011). Por outro lado, sua ligação com βAR aumenta a secreção de insulina estimulada pela glicose. Entretanto, o α-2A é predominantemente expresso nas ilhotas pancreáticas (AHRÉN et al., 1981; AHRÉN et al., 2006; FAGERHOLM; HAAPARANTA; SCHEININ, 2011; KOMATSU et al., 2013).

Ainda, nas células-β o fator de transcrição homeobox pancreático e duodenal 1 (PDX-1) desempenha um papel essencial no adequado desenvolvimento inicial do pâncreas, bem como, na manutenção deste tecido ao longo da vida (AHLGREN et al., 1998). O aumento da expressão desta proteína durante a formação das células endócrinas é fundamental para garantir a diferenciação adequada das células-β (GAO et al., 2008). No início da vida de roedores, o PDX-1 é expresso em todo o epitélio pancreático, enquanto que na vida adulta se restringe apenas às células-β (AHLGREN et al., 1998; GAO et al., 2014). Na idade adulta, o PDX-1 é considerado um dos principais reguladores do gene da insulina, além de participar da regulação da expressão da GCK e GLUT2, influenciando na manutenção da homeostase glicêmica (AHLGREN et al., 1998; ABUZGAIA; HARDY; ARANY, 2015).

O processo de fusão dos grânulos de insulina com a membrana plasmática células-β depende do remodelamento do citoesqueleto e subsequente ativação de proteínas específicas da

família SNARE (soluble N-ethylmaleimide-sensitive factor activing protein receptor) (OTTO; HANSON; JAHN, 1997; UNGERMANN; LANGOSCH, 2005; HAN;

PLUHACKOVA; BÖCKMANN, 2017). Estas proteínas classificadas em duas subfamílias: as t-SNARE, compostas por proteínas na membrana plasmática da célula alvo, sendo as principais a sintaxina e a SNAP-25 (proteína sinaptossomal associada de 25 kDa) e as v- SNARE, situadas nas membranas das vesículas de secreção, como a proteína associada a membrana vesicular (VAMP), também conhecidas como sinaptobrevina (SUTTON et al., 1998; LIANG et al., 2017). Tais proteínas encontram-se em membranas opostas até agruparem-se e formarem um complexo heterotrimérico estável composto por 4 hélices paralelas denominado complexo nuclear ou complexo SNARE, ligando assim, as vesículas à membrana plasmática de modo análogo ao de um zíper, através da reorganização dos fosfolipídios e consequente fusão (SUTTON et al., 1998; LOU; SHIN, 2016).

Diferentes isoformas de sintaxina já foram descritas, bem como duas variantes da sintaxina 1, conhecidas como 1A e 1B, porém apenas a isoforma 1A é expressa em ilhotas de roedores (NAGAMATSU et al., 1996; TORREJÓN-ESCRIBANO et al., 2011). Estas proteínas possuem uma porção transmembrana C-terminal, um domínio SNARE central e uma região N-terminal longa, que pode assumir duas conformações, aberta ou fechada (FERNANDEZ et al., 1998). Na presença de SNAP-25 ou VAMP esta região permanece aberta e favorece a formação do complexo SNARE (LIANG et al., 2017) A SNAP-25, diferente das outras t-SNARE não apresenta uma porção transmembrana e sua fixação na membrana ocorre por dois domínios SNARE, separados por uma região central rica em resíduos de cisteínas (OYLER et al., 1989). A VAMP2 é constituída por 120 resíduos de aminoácidos, que compõem uma região C-terminal transmembrana, um domínio SNARE central e uma região N-terminal (DANIEL et al., 1999).

O influxo de Ca2+ é fundamental para a secreção de insulina e também alguns estudos vêm demonstrando a participação do Ca2+ estimulando a formação do complexo SNARE (TREXLER; TARASKA, 2017). Entretanto, as proteínas que formam este complexo não apresentam receptores para Ca2+ e, portando é necessário a presença de um sensor de Ca2+ para este processo. Dentre os diferentes sensores de Ca2+, a sinaptotagmina, uma proteína integral de membrana é encontrada nas ilhotas pancreáticas (GAO et al., 2000; DOLAI et al., 2016). Tem sido proposto que a ligação do Ca2+ com a sinaptotagmina levaria a sua interação com a sintaxina 1A induzindo e/ou acelerando a formação do complexo SNARE levando, finalmente a fusão das membranas e consequente extrusão de insulina (LI et al., 1995; MA et al., 2013).

Foi descrito que a proteína Munc18-a ou Munc18-1 (mammalian uncoordinated-18-1) regula a formação do complexo SNARE nas células-β através da sua interação com a sintaxina 1A (SHEN et al., 2007). A munc18-a se liga a região N-terminal da sintaxina 1A quando esta proteína assume a conformação fechada e dessa forma, evita a sua ligação com outras proteínas com complexo SNARE (PEVSNER et al., 1994).

Mecanismo de ação da insulina

A insulina é o principal hormônio anabólico, essencial para manutenção da homeostase glicêmica, que em altas concentrações no período de absorção alimentar, estimula processos anabólicos de armazenamento de reservas energéticas. Além disso, a insulina tem importante função de inibir grande parte dos processos catabólicos do organismo (CHANG; CHIANG; SALTIEL, 2004; SALTIEL, 2016). Isso ocorre através de sua capacidade em estimular a captação periférica de glicose em tecidos alvos, tais como tecido adiposo e muscular. Ainda, este hormônio induz a lipogênese nos adipócitos e no fígado e, reduz a lipólise, além de aumentar a síntese e inibir a degradação proteica. Além disso, a fim de reduzir a produção de glicose, a insulina reduz a gliconeogênese e glicogenólise hepática (CARVALHEIRA; ZECCHIN; SAAD, 2002; WILCOX, 2005; SALTIEL, 2016; RÖDER et al., 2016).

A ação da insulina inicia-se pela sua ligação ao receptor de insulina (IR), que faz parte da família das tirosinas quinases (Figura 2). Considerado uma glicoproteína heterotetramérica, o IR é formado por duas subunidades α, inteiramente extracelular que apresenta o domínio de ligação da insulina, e duas subunidades β que possui a atividade tirosina quinase, responsável pela transmissão do sinal intracelular desencadeado pela ligação da insulina do IR. Estas subunidades são unidas por ligações dissulfeto. Na ausência da insulina, a subunidade α do IR exerce influência inibitória sobre a porção intracelular, bloqueando a atividade tirosina quinase da subunidade β (KAHN, 1985). Quando a insulina se liga a subunidade α, ocorre rápida mudança conformacional do receptor, levando a ativação da atividade quinase da subunidade β, que resulta em autofosforilação do IR, aumentando de forma constante a atividade tirosina quinase, que fosforila proteínas citoplasmáticas ancoradas ao mesmo, denominadas substrato do receptor de insulina (IRS) (WHITE et al., 1988).

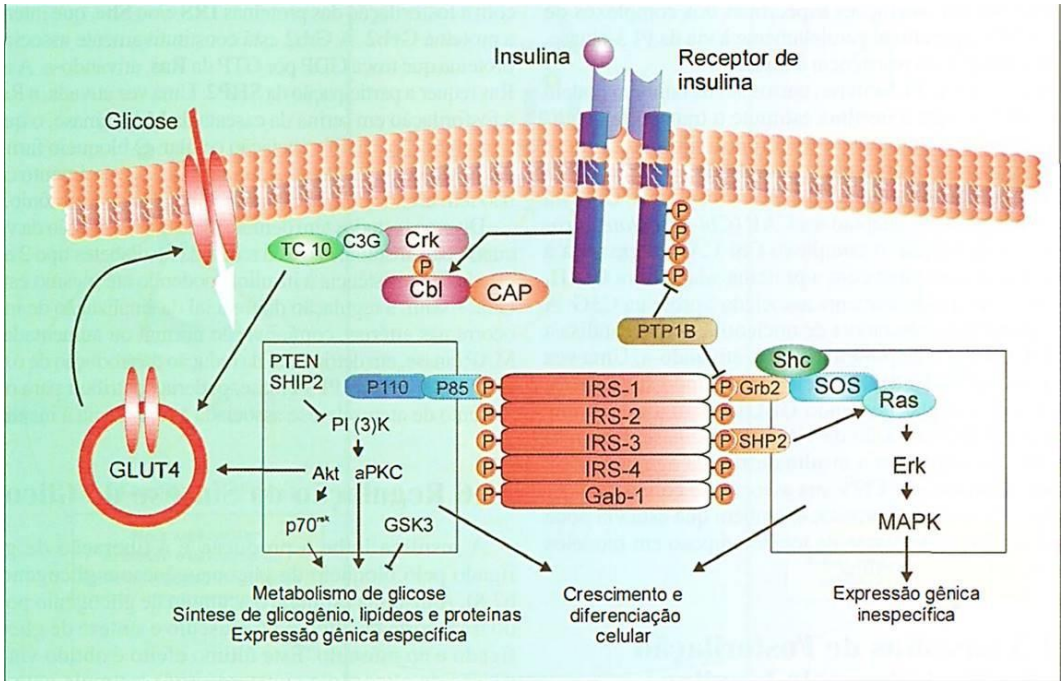
Os IRS fosforilados associam-se à subunidade regulatória p85 da fosfatidilinositol 3-quinase (PI3K) (SALTIEL; PESSIN, 2002). Essa enzima possui ainda uma subunidade

catalítica p110, que catalisa a fosforilação de fosfoinositois, gerando o segundo mensageiro fosfatidilinositol 3,4,5 trifosfato (PI(3,4,5)P3 ou PIP3), responsável pela ativação e recrutamento da proteína quinase dependente de fosfoinositídeos-1 (PDK-1) próximo à membrana plasmática (WHITE; KAHN, 1994; CHANG; CHIANG; SALTIEL, 2004;

BAYASCAS, 2010). A PI3K é encarregada de realizar a ativação de várias quinases, entre elas a proteína quinase B (PKB/AKT) (CHOU et al., 1998; CHANG; CHIANG; SALTIEL, 2004).

A AKT é uma proteína serina/treonina quinase (ZECCHIN; CARVALHEIRA; SAAD, 2004; KLEMENT et al., 2012) que regula o crescimento, proliferação, metabolismo, migração, ciclo celular, funções e capacidade de sobrevivência celular (DATTA; BRUNET; GREENBERG, 1999; WOODGETT, 2001), bem como as ações metabólicas da insulina, como transporte de glicose e síntese de glicogênio (WILCOX, 2005; MARTYN; KANEKI; YASUHARA, 2008). Para se tornar completamente ativa, a AKT depende de um mecanismo regulatório de dupla ação, a translocação da proteína para a membrana plasmática e subsequente fosforilações nos segmentos Thr308 e Ser473 (ANDJELKOVIĆ et al., 1997; BELLACOSA et al., 1998). A AKT exibe um domínio de homologia plecstrina, o qual interage com PIP_3, produto da fosforilação da PI3K (ALTOMARE et al., 1998; SHAW; COHEN; ALESSI, 1998; VIVANCO; SAWYERS, 2002). Essa interação permite a translocação da AKT até a membrana plasmática, onde é fosforilada no segmento Thr308 pela PDK1. Para sua máxima eficiência, a AKT requer uma segunda fosforilação no resíduo serina 473, realizada pelo complexo 2 do alvo da rapamicina em mamíferos (mTOR2) (VANHAESEBROECK; ALESSI, 2000; TANIGUCHI; EMANUELLI; KAHN, 2006; SALTIEL, 2016).

Figura 2 - Via de sinalização da insulina em tecidos insulinodependentes



Fonte: Aires, 3ªed, pág 1039, 2008.

Um dos alvos da AKT ativa é a glicogênio sintase quinase-3 (GSK-3), uma serina/treonina que, quando fosforilada, acaba sendo desativada e, por consequência aumenta a síntese de glicogênio no fígado (COHEN; ALESSI; CROSS, 1997; BOUSKILA et al., 2008). Além disso, a AKT tem um importante papel sobre o transportador de glicose tipo 4 (GLUT 4), pois estimula sua translocação até a membrana celular, participando do transporte de glicose insulinodependente no músculo e tecido adiposo (ALESSI et al., 1997; SALTIEL; KAHN, 2001; ZECCHIN; CARVALHEIRA; SAAD, 2004) (Figura 2).

Em resposta à insulina, as vesículas de GLUT4 são mobilizadas do interior da célula para a membrana plasmática, onde se acoplam e se fundem por meio de interações mediadas pela formação do complexo SNARE, proteínas localizadas na membrana plasmática, sintaxina 4 e SNAP23, e proteínas localizadas na vesícula, a VAMP2 (BRYANT; GOVERS; JAMES, 2002; KIOUMOURTZOGLOU; GOULD; BRYANT, 2014). A VAMP2 é fosforilada na presença de insulina, que por sua vez interage com a sintaxina 4 e SNAP23 levando a formação de um complexo ternário, que permite a fusão das vesículas de GLUT4 com a membrana plasmática (WATSON; PESSIN, 2001). Em condições basais a munc18c permanece ligada com sintaxina 4, impedindo sua interação com a VAMP2 (D'ANDREA-MERRINS et al., 2007). A munc18c foi identificada como alvo direto do IR, tornando-se rapidamente fosforilado em resposta à insulina, de maneira independente da ativação da via PI3K/AKT (JEWELL et al., 2011). Quando fosforilado, a munc18c sofre uma mudança

conformacional expondo seu domínio de ligação com o complexo ternário e, dissocia-se da sintaxina 4, permitindo sua interação com a VAMP2, levando a fusão das vesículas com a membrana plasmática (WATSON; PESSIN, 2001; RAMALINGAM; YODER; THURMOND, 2014).

Portanto, alterações ou defeitos nos mecanismos de secreção e ação da insulina podem acarretar na interrupção do transporte e utilização da glicose em tecidos alvos, proporcionando o acúmulo da mesma da corrente sanguínea, caracterizando hiperglicemia no indivíduo, levando ao posterior desenvolvimento de DM2 (SBD, 2018).

Diabetes *mellitus* tipo 2

O diabetes *mellitus* (DM) é uma doença metabólica crônica e complexa, de múltiplas etiologias e fatores de risco heterogêneos, dependentes da condição social, comportamental, ambiental e genética. No quadro metabólico do DM, a hiperglicemia crônica é considerada o fator primário responsável pelas complicações desta doença (CHEN et al., 2015; SBD, 2018). O DM tem alcançado proporções epidêmicas em todo o mundo, gerando um sério e importante problema de saúde pública, a qual atinge não só países em desenvolvimento mais também países já desenvolvidos (SHAW; SICREE; ZIMMET, 2010; FERREIRA; PITITTO, 2015).

Dados recentes da International Diabetes Federation (IDF) demonstraram que aproximadamente 425 milhões de pessoas em todo o mundo apresentam a doença. Em 2045 mais de 438 milhões de pessoas entre 20-64 anos e 191 milhões de pessoas entre 65-79 anos irão desenvolver DM2 em todo o mundo (IDF, 2017). Na América Central e do Sul, esta organização prevê aumento de 62% até 2045, representando 42 milhões de indivíduos diabéticos nesta região (IDF, 2017). No Brasil, o último levantamento realizado pela Sociedade Brasileira de Diabetes (SBD), realizado em 2017, constatou que a população brasileira diabética é estimada em 13 milhões, deixando o país na 4ª posição no ranking mundial em número de casos, ficando atrás apenas da China, Índia e Estados Unidos (SBD, 2019). Além disso, o percentual de casos de diabetes passou de 5,5% para 8,9% entre os anos de 2006-2017, e o maior número de casos foi registrado no Estado do Rio de Janeiro (SBD, 2019).

Dentre os tipos de DM, destacam-se três grupos principais: 1) DM tipo 1 (DM1), uma doença autoimune, caracterizado pela destruição total das células-β, que normalmente manifesta-se na infância, sendo responsável por 5-10% de todos os casos de DM; 2) DM tipo

2 (DM2), responsável por pelo menos 90% dos casos de DM, resultado da ineficácia da ação da insulina e diminuição progressiva da sua secreção. O principal fenômeno fisiopatológico é a RI associada à hiperglicemia; 3) DM gestacional (DMG), definida por qualquer grau de intolerância à glicose, diagnosticada com hiperglicemia observada pela primeira vez na gestação (IDF, 2017; AMERICAN DIABETES ASSOCIATION, 2018).

Existem outros subtipos específicos de diabetes, como a diabetes tipo MODY (Maturity-Onset Diabetes of the Young), caracterizada por manifestação precoce, em geral abaixo dos 25 anos de idade, representando 1 a 2% de todos os casos de DM e, está associado a defeitos genéticos marcados por herança autossômica dominante. Este tipo de DM envolve falhas primárias na secreção de insulina, com graus variáveis de disfunções nas células-β. Além disto, defeitos genéticos na ação da insulina, causados por leprechaunismo, síndrome de Rabson-Mendenhall e síndrome de Berardinelli-Seip, também podem cursar com o desenvolvimento de DM (SBD, 2018; JANG, 2020). Algumas classes de DM são consideradas secundárias, como doenças do pâncreas exócrino, pancreatite, trauma, pancreatectomia e carcinoma pancreático podem causar diabetes. Endocrinopatias, como acromegalia, síndrome de Cushing, glucagonoma, feocromocitoma, relacionados com os hormônios contrarreguladores da ação da insulina que devido à ação glicogênica inerente aos hormônios endógenos secretados excessivamente nessas condições podem ser causas de diabetes (SBD, 2018). Recentemente tem se descrito a DM tipo 3, a qual vem sendo intimamente relacionada com a doença de Alzheimer. Os cérebros de pacientes com esta doença apresentam expressão reduzida de insulina e de seus receptores neurais, o que leva a falha em toda sua cascata de sinalização, caracterizando RI central. Esta falha, por sua vez, afeta o metabolismo central e as funções cognitivas que são anormalidades bastante conhecidas na doença de Alzheimer (LESZEK et al., 2017).

O DM2 é o tipo de diabetes que apresenta maior incidência entre todos os indivíduos diabéticos, e está associado à epidemia da obesidade (HOSSAIN; KAWAR; EL NAHAS, 2007; BOLES; KANDIMALLA; HEMACHANDRA, 2017). As chances para o desenvolvimento de DM2 aumentam 50% em indivíduos obesos com IMC entre 33 e 35 kg/m² (SILVEIRA, 2003). O desenvolvimento de DM2 e sua ligação com a obesidade pode ser decorrente ainda de outros fatores de risco, como o sedentarismo e fatores nutricionais, classificados como fatores de riscos modificáveis. Contudo, existem também os fatores de risco não modificáveis, como idade, problemas durante a gestação, etnia e histórico familiar de DMG (ALBERTI; ZIMMET; SHAW, 2007; IDF, 2017). Ao longo do desenvolvimento desta doença, o DM2 está relacionado com outras complicações, como doenças periodontais

e cardiovasculares, além de retinopatia e nefropatia diabética (FLETCHER; GULANICK; LAMENDOLA, 2002; VALK; BRUIJN; BAJEMA, 2011; FAERCH; BERGMAN; PERREAULT, 2012; IDF, 2017).

A maioria dos pacientes, se não todos, antes de desenvolver DM2 passam por um processo intermediário para a progressão da doença. Esta fase é denominada pré-diabetes, no qual estes indivíduos apresentam inicialmente intolerância à glicose, ou seja, concentrações de glicemia basal mais altas que o normal (99 e 126 mg/dL), porém não altas o suficiente para serem classificados como DM ou um teste de tolerância à glicose anormal (TABÁK et al., 2012; SBD, 2019). Quando este quadro é identificado, se realizadas alterações apropriadas no estilo de vida associada ou não, a intervenções farmacológicas esta progressão para o DM2 pode ser atrasada ou, até mesmo impedida (TUOMILEHTO et al., 2001).

As alterações na secreção da insulina que ocorrem em resposta ao DM2 podem ser decorrentes de alterações na arquitetura normal das ilhotas pancreáticas, tais como, aumento da massa, redução da proliferação celular, e até mesmo apoptose das células-β (PICK et al., 1998; TOMITA, 2016). O pâncreas de seres humanos, bem como o de roedores, possui a capacidade de aumentar a massa de células-β e consequentemente a secreção de insulina a fim de compensar a RI, uma vez que a massa de células-β é dinâmica e capaz de se adaptar a condições fisiológicas e patológicas para manter a normoglicemia (MEZZA et al., 2014). processo é considerado essencial para manutenção da homeostase glicêmica e prevenção daprogressão do DM2 (BLANDINO-ROSANO et al., 2012).

Inicialmente na obesidade, ocorre o aumento da massa de células-β em resposta a RI. Esta fase é denominada de compensação, caracterizada por promover adaptações estruturais das células-β, tais como proliferação celular, hiperplasia e hipertrofia, a fim de manter a euglicemia nestes indivíduos através do aumento da secreção de insulina (CERF, 2015). No entanto, o aumento substancial na demanda por insulina pode causar redução das células-β através de toxicidade causada pela glicose e/ou por lipídeos, pelo estado de inflamação crônica e aumento no estresse oxidativo (TOMITA, 2016). Contrariamente a compensação, esta fase é caracterizada pela descompensação das células-β, onde ocorre a reversão das adaptações estruturais destas células, com redução na massa de células-β, redução da proliferação celular, hipoplasia, hipotrofia, levando à hiperglicemia. A fase de descompensação pode progredir para uma fase de aceleração, caracterizada como o estado metabólico crítico que antecede a disfunção das células-β, com perda severa na massa destas células ao longo do tempo, devido à redução cada vez mais acentuada, associada ao

aumento de fibrose que pode levar a morte celular das mesmas (CHO et al., 2011; CERF, 2015; TOMITA, 2016).

Diante destes dados, levando em consideração os impactos negativos causados pela obesidade e DM2 para a saúde do indivíduo, e, que ambas as doenças estão relacionados com insultos no início da vida (PATTI, 2013; REMACLE et al., 2011; KERELIUK; BRAWERMAN; DOLINSK., 2017), estudos são necessários para compreender e elucidar mecanismos envolvidos na programação de tais doenças a fim de prevenir esses altos índices.

Como descrito anteriormente, ambos os modelos experimentais de programação pelo desmame precoce (farmacológico e não farmacológico) e da exposição à nicotina durante a lactação apresentam fenótipos similares, como sobrepeso e RI, podendo cursar com o desenvolvimento do DM2. A nossa hipótese é que os animais submetidos a estes modelos de programação apresentem comprometimento do pâncreas, com disfunção nas ilhotas pancreáticas, resultando em alteração na secreção de insulina além da redução das proteínas envolvidas com a sinalização periférica da insulina, principalmente no músculo.

Assim, este trabalho buscou investigar os mecanismos pelos quais a homeostase glicêmica é alterada nestes modelos de programação. Vale destacar que todos os resultados encontrados relacionados com a homeostase glicêmica, em ambos os modelos de programação, referem-se apenas aos machos, e, portanto, não sabemos se existe dimorfismo sexual nestes modelos de programação.

1 OBJETIVOS

1.1 Objetivo geral

Investigar os efeitos do desmame precoce e da exposição à nicotina durante a lactação sobre os mecanismos responsáveis pelas alterações na homeostase glicêmica em descendentes machos e fêmeas. Esse trabalho está organizado sob a forma de 3 manuscritos, resultantes dos experimentos realizados durante os 3 anos do doutorado.

1.2 Objetivos específicos

Avaliar os efeitos do desmame precoce farmacológico e não farmacológico sobre os descendentes machos e fêmeas aos 45 e 180 dias de vida sobre:

a) Parâmetros corporais, tolerância à glicose, secreção de insulina em ilhotas isoladas com estímulo de glicose, expressão de proteínas envolvidas neste mecanismo secretório nas células-β pancreáticas, bem como a sinalização deste hormônio no músculo esquelético, além da avaliação da presença ou ausência de esteatose pancreática.

Avaliar os efeitos da exposição materna à nicotina sobre os descendentes machos e fêmeas aos 120 e 180 dias de vida sobre:

b) Parâmetros corporais e plasmáticos, tolerância à glicose, expressão de proteínas envolvidas na secreção de insulina, bem como a sinalização deste hormônio no músculo esquelético, além da avaliação da presença ou ausência de esteatose pancreática.

2 MATERIAIS E MÉTODOS, RESULTADOS E DISCUSSÃO

2.1 Modelo experimental I: Desmame precoce farmacológico e desmame precoce não-farmacológico

A metodologia, resultados e as discussões referentes a este modelo de programaçãoestão apresentados nos 2 artigos abaixo:

2.1.1 Artigo 1 – Early weaning induces short-and long-term effects on pancreatic islets inWistar rats of both sexes

Carla Bruna Pietrobon[1]; Rosiane Aparecida Miranda[1]; Iala Milene Bertasso[1]; Paulo Cezarde Freitas Mathias[2]; Maria Lúcia Bonfleur[3]; Sandra Lucinei Balbo[3]; Marise Auxiliadora de Barros Reis[4]; Márcia Queiroz Latorraca[4]; Vanessa Cristina Arantes[4]; Elaine de Oliveira[1]; Patrícia Cristina Lisboa[1]; Egberto Gaspar de Moura[1]*.

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Early weaning induces short- and long-term effects on pancreatic islets in Wistar rats of both sexes

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Key points

- The World Health Organization recommends exclusive breastfeeding until 6 months of age as an important strategy to reduce child morbidity and mortality.
- Studies have associated early weaning with the development of obesity and type 2 diabetes in adulthood.
- In our model, we demonstrated that early weaning leads to increased insulin secretion in adolescent males and reduced insulin secretion in adult offspring.
- Early weaned males exhibit insulin resistance in skeletal muscle.
- Early weaning did not change insulin signalling in the muscle of female offspring.
- Taking into account that insulin resistance is one of the primary factors for the development of type 2 diabetes mellitus, this work demonstrates the importance of breastfeeding in the fight against this disease.

Abstract Early weaning (EW) leads to short- and long-term obesity and diabetes. This phenotype is also observed in experimental models, in which early-weaned males exhibit abnormal insulinaemia in adulthood. However, studies regarding the effect of EW on pancreatic islets are rare. We investigated the mechanisms by which glycaemic homeostasis is altered in EW models through evaluations of insulin secretion and its signalling pathway in offspring. Lactating Wistar rats and their pups were divided into the following groups: non-pharmacological EW (NPEW): mothers were wrapped with an adhesive bandage on the last 3 days of lactation; pharmacological EW (PEW): mothers received bromocriptine to inhibit prolactin (1 mg/kg body mass/day) on the last 3 days of lactation; and control (C): pups underwent standard weaning at PN21. Offspring

Carla Bruna Pietrobon has a Bachelor's in Biology. She obtained her MSc in 2017 at the State University of Western Paraná, Brazil. She is currently a PhD student in the Bioscience Program of the State University of Rio de Janeiro, Brazil. She investigates different models of metabolic programming in rats and the impact on the offspring metabolism in adult life, with emphasis on insulin secretion by pancreatic β-cells and the peripheral action of insulin. Part of the current study was awarded at the 3rd Meeting of Ibero-American DOHaD (Developmental Origins of Health and Disease), 2018, in the Basic Science Research Category.

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of both sexes were euthanized at PN45 and PN180. At PN45, EW males showed higher insulin secretion (*vs.* C). At PN170, PEW males exhibited hyperglycaemia in an oral glucose tolerance test (*vs.* C and NPEW). At PN180, EW male offspring were heavier; however, both sexes showed higher visceral fat. Insulin secretion was lower in EW offspring of both sexes. Males from both EW groups had lower glucokinase in islets, but unexpectedly, PEW males showed higher GLUT2, than did C. EW males exhibited lower insulin signalling in muscle. EW females exhibited no changes in these parameters compared with C. We demonstrated distinct alterations in the insulin secretion of EW rats at different ages. Despite the sex dimorphism in insulin secretion in adolescence, both sexes showed impaired insulin secretion in adulthood due to EW.

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Introduction

Obesity is a complex, multifactorial disease with major impacts on public health and is related to the development of several comorbidities, such as type 2 diabetes mellitus (T2DM) (Han & Boyko, 2018). T2DM is characterized by chronic hyperglycaemia and is associated with insulin resistance (IR), which results from defects in the secretion and/or action of insulin (International Diabetes Federation, 2011). Insulin acts through the tyrosine kinase receptor, which phosphorylates insulin receptor substrates (IRS1), leading to the activation of phosphoinositide 3-kinase (PI3K) and protein kinase B (PKB)/AKT, which are responsible for glucose uptake in peripheral tissues, such as skeletal muscle and adipose tissue (Kahn, 1985).

Recently, it has been observed that pandemic obesity and T2DM are related not only to changes in lifestyle but also to nutritional patterns in early life (Meng *et al.* 2018). According to the World Health Organization (2018), malnutrition is one of the most common causes of child morbidity and mortality in all countries; high rates of malnutrition may be due to the interruption of breastfeeding, which deprives the child of nutritional and hormonal components essential for development (Victora *et al.* 2016; World Health Organization, 2018). This change in early life causes a metabolic programming, defined as an insult or disturbance that occurs in a critical period of life that produces long-term consequences for development (Barker, 1995). Lactation is considered a critical window susceptible to such insults; therefore, maternal nutritional or environmental alterations at this stage may negatively affect the individual, leading to an altered endocrine-metabolic profile and favouring the onset of diseases in adult life (Ong & Gues, 2018). Experimental studies have shown that the interruption of breastfeeding programs offspring for metabolic syndrome in adulthood (Bonomo *et al.* 2007; Moura *et al.* 2009; Lima *et al.* 2011). In two different models of early weaning (EW), our group demonstrated reduced body fat and hypoglycaemia in neonatal rats (Bonomo *et al.* 2005; Lima *et al.* 2011). In the first model, described as pharmacological EW (PEW), suppression of lactation occurs through the inhibition of prolactin by maternal treatment with bromocriptine (BRO), a type 2 dopaminergic receptor agonist, in the last 3 days of lactation (Ben-Jonathan & Hnasko, 2001; Bonomo *et al.* 2005). The second model consists of wrapping of the maternal body with an adhesive bandage to interrupt breastfeeding, characterized as non-pharmacological EW (NPEW) (Lima *et al.* 2011).

Studies that investigate the glycaemic homeostasis of animals subjected to EW are scarce. Both EW models were performed only in males and indicate that these males have insufficient plasma insulin (Moura *et al.* 2009; Lima *et al.* 2011). It is known that glycaemic homeostasis is directly controlled by the ability of pancreatic β-cells to properly secrete insulin and by the effects of this hormone on peripheral tissues. The mechanism of insulin secretion starts when glucose is transported into β-cells via the type 2 glucose transporter (GLUT2). Glucose is phosphorylated to glucose-6-phosphate by the action of the enzyme glucokinase (GCK) and is then metabolized, resulting in ATP production. The increase in the ATP/ADP ratio results in the closure of ATP-dependent K^+ channels, leading to depolarization of the membrane; this depolarization opens voltage-sensitive Ca^{2+} channels, resulting in an influx of this ion, increasing its intracellular concentration and culminating in the exocytosis of secretory insulin granules (Prentki & Matschinsky, 1987). In addition, pancreatic duodenal homeobox-1 (PDX1) is involved in the initial development of the pancreas, in the differentiation of β-cells and in maintaining this tissue throughout life (Jonsson *et al.* 1994). PDX1 regulates the expression of the insulin, GLUT2 and GCK genes, influencing glucose homeostasis (Abuzgaia, 2015).

To promote the exocytosis of insulin granules, cytoskeletal remodelling through the activation of specific proteins is necessary (Otto, 1997). These proteins are known as SNARE (soluble *N*-ethylmaleimide-sensitive

factor activating protein receptor) proteins and include syntaxins, SNAP25 (synaptosome-associated protein of 25 kDa) and VAMP2 (vesicle-associated membrane protein), which form a SNARE complex responsible for vesicle exocytosis (Sutton *et al.* 1998). Syntaxin may further interact with Munc18, inhibiting the formation of the SNARE complex (Otto, 1997; Sutton *et al.* 1998). The SNARE complex is also involved with type 4 glucose transporter (GLUT4) vesicle translocation and fusion in muscle and adipocytes (St-Denis & Cushman, 1998).

Thus, our hypothesis is that animals subjected to EW develop pancreatic impairment and pancreatic β-cell dysfunction, resulting in impaired insulin secretion and reduced expression of the proteins involved in insulin signalling, especially GLUT4, in the skeletal muscle, contributing to IR. Therefore, we propose to investigate the short- and long-term alterations caused by the interruption of breastfeeding in males, which have an abnormal insulinaemia. In addition, for the first time, we propose to study females in the two EW models to determine if there is a sex dimorphism in the investigated parameters.

Methods

Ethical approval

All experimental procedures were approved by the Animal Care and Use Committee of the Biology Institute of the State University of Rio de Janeiro (CEUA/014/2017), and were in accordance with principles adopted by Brazilian Law no. 11.794/2008. The experimental sizes were determined to minimize both the number of animals used and the animals' suffering, following the ethical doctrine of the three 'Rs' – reduction, refinement and replacement. Our study complies with the animal ethics checklist as outlined by Grundy (2015).

Animals

Three-month-old Wistar female rats were acclimated in a controlled environment at room temperature (23 ± 1°C) and with artificial light/dark cycles (lights on 07.00 h and lights off 19.00 h). For the mating period, virgin females were placed in cages with sexually active adult male rats (3 females/1 male) for 2 weeks. Pregnancy was confirmed by the detection of spermatozoa in vaginal smears. During the gestational and lactational periods, all females received free access to water and standard chow (Nuvilab, Sogorb, São Paulo, SP, Brazil).

At birth, i.e. the first postnatal day (PN0), sex determination was evaluated by anogenital distance, and the litters were adjusted to six pups per dam, three males and three females, to avoid the effects of litter size on programming. Next, the lactating rats were divided into three groups (Fig. 1): (1) non-pharmacological EW (NPEW, $n = 9$), in which the lactating rats were lightly anaesthetized with xylazine and ketamine (2:1) and wrapped with an adhesive bandage covering all teats for the last 3 days of lactation (Lima *et al.* 2011); (2) pharmacological EW (PEW, $n = 9$), in which the lactating rats intraperitoneally received two doses of 0.5 mg bromocriptine (Parlodel; Novartis, São Paulo, SP, Brazil) diluted in methanol–saline (1:1) each day (08.00 h and 20.00 h) for the last 3 days of lactation (Bonomo *et al.* 2005); and (3) standard weaning (C, control, $n = 10$), in which the dams breastfed their pups until PN21. During the EW period, the pups had free access to standard chow. It is important to emphasize that 1 day for a rodent is equivalent to ~9 days for a human; thus, the standard breastfeeding

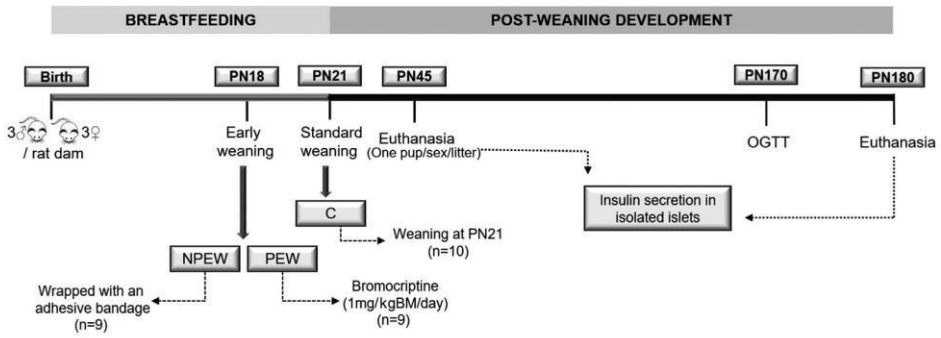

Figure 1. Two experimental models of early weaning
After birth the litter was reduced to three males and three females. Groups: C, control, standard weaning (PN21); NPEW, non-pharmacological early weaning; PEW, pharmacological early weaning. One male and one female from each litter per group was euthanized at PN45. The other animals were euthanized at PN180. OGTT, oral glucose tolerance test; PN, postnatal day.

for rats, 21 days, corresponds to 6 months of exclusive breastfeeding for human children (Quinn, 2005). In this sense, 3 days of experimental EW for a rat corresponded to 1 month for a human baby.

Until weaning, pups' body masses were monitored every day. From weaning to PN180, the animals received water and standard chow. At PN45, one rat of each sex per litter was euthanized. The remaining pups were followed until PN180, and pups' body masses were recorded weekly.

Nuclear magnetic resonance (NMR) for small living animals was used to evaluate the total fat mass at PN45 and PN180. Animals were scanned using Whole Body Composition Analyzer NMR equipment (Bruker Minispec LF90 TD-NMR, Rheinstetten, Germany). A quality control check of internal voltages, temperature, magnets and NMR parameters was performed using a standard provided by the manufacturer. Non-anaesthetized animals were placed in a clear plastic cylinder and were immobilized by the insertion of a tight-fitting plunger into the cylinder. Then, the cylinder was inserted into the chamber of the NMR for scanning for 2 min. The data are expressed as grams fat mass and grams lean mass.

An oral glucose tolerance test (OGTT) was performed at PN170. After 12 h of fasting, a blood sample was collected to determine basal glycaemia (time 0) using a glucometer (Onetouch Ultra®, Johnson & Johnson, São Paulo, Brazil). Glucose solution (50%) was injected into sterile saline via an oral probe at 2 g/kg body mass (BM), and glycaemia was measured after 15, 30, 60 and 120 min.

Euthanasia occurred after 12 h of fasting; the animals were anaesthetized with a non-lethal dose of 2:1 solution of xylazine (Xilazin®, 100 mg/kg BM) and ketamine (Cetamin®, 50 mg/kg BM), and blood was collected by cardiac puncture in heparinized tubes. Blood samples were centrifuged (1500 *g* for 20 min at 4°C) to obtain plasma, which was stored at −20°C. The visceral fat mass (VFM) was quickly determined by weighing the retroperitoneal, gonadal and mesenteric white adipose tissues. The insulin concentration (PN45 and PN180) was determined by ^{125}I-labelled insulin radioimmunoassay (RIA) using a gamma counter (PerkinElmer, Shelton, CT, USA) with human insulin as the standard and an antibody against rat insulin as the tracer. The insulin intra- and inter-assay variation coefficients were 9% and 12%, respectively. The detection limits ranged from 0.002 ng/mL to 200 ng/mL.

Adiponectin was measured by an ELISA kit (Millipore Corp., Billerica, MA, USA) according to the manufacturer's instructions. The concentration of adiponectin is expressed in ng/mL. Plasma adiponectin was normalized by total VFM, and this ratio was used to evaluate IR (Park *et al.* 2005).

Islet isolation and insulin secretion

Islets were isolated from fasted offspring at PN45 and PN180 by collagenase (0.8 mg/mL; Sigma-Aldrich, St Louis, MO, USA, 5% bovine serum albumin (BSA) and 0.6% HEPES according to a previous report (Pietrobon *et al.* 2019). After digestion of the exocrine pancreas, the islets were collected with a micropipette under a microscope to exclude any contaminating tissues. For the static incubation, groups of four islets were preincubated with 5.6 mM glucose for 30 min in Krebs–bicarbonate (KRB) solution (120 mM NaCl, 5 mM KCl, 25 mM $NaHCO_3$, 2.56 mM $CaCl_2$, 1.13 mM $MgCl_2$) supplemented with 5.6 mM glucose and 0.3% BSA and equilibrated with 95% O_2–5% CO_2, pH 7.4, at 37°C. Next, islets were incubated for 1 h with 5.6, 8.3, 11.1, 16.7, 22.0 or 27.0 mM glucose, and the supernatants were collected and stored at −20°C for insulin measurement by RIA. After incubation, approximately 300 islets were stored in a −80°C freezer for further analysis via western blotting.

Western blotting

For protein expression analysis, the samples were homogenized in tissue-specific buffers as follows: muscle samples in RIPA buffer (20 mM Tris–HCl, 10 mM NaF, 1% NP40, 150 mM NaCl, 5 mM EDTA, 0.1% SDS) and isolated islet samples in T-PER™ Tissue Protein Extraction Reagent (78510, Sigma-Aldrich). A cocktail of protease inhibitors (complete EDTA-free; Roche Applied Science, Mannheim, Germany) was added to all homogenates. Muscle homogenates were centrifuged at 15,294 *g* for 15 min at 4°C (Eppendorf 5417R Refrigerated Centrifuge, Hampton, CT, USA), and islet samples were centrifuged at 15,294 *g* for 5 min at 4°C.

The total protein content in the homogenates was determined using a specific kit (BCA, Protein Assay Reagent, Thermo Fisher Scientific, Waltham, MA, USA), and the cell lysates were denatured in sample buffer (50 mM Tris–HCl, pH 6.8, 1% SDS, 5% 2-mercaptoethanol, 10% glycerol, 0.001% bromophenol blue). The samples were separated by gel electrophoresis (SDS-PAGE, 8.5–12%) and transferred to nitrocellulose membranes (Hybond ECL; GE Healthcare, Piscataway, NJ, USA). Rainbow markers (GE Healthcare, Uppsala, Sweden) were run in parallel to estimate the molecular masses. The membranes were blocked with 5% BSA in Tween-tris-buffered saline(TBS) buffer (containing 20 mM Tris–HCl, pH 7.5, 500 mM NaCl and 0.1% Tween-20). Then, the membranes were incubated overnight at 4°C with the following primary antibodies: PDX1 (Abcam, Cambridge, MA, USA, cat. no. ab47267, RRID:AB_777179; 1:3,000), GLUT2 (Thermo Fisher Scientific, cat. no. 720238, RRID: AB_2633212; 1:200), GCK (Santa Cruz Biotechnology, Dallas, TX, USA, cat.

no. sc-17819, RRID:AB_627722; 1:500), estrogen receptor α (ERα; Millipore, cat. no. 04–1564, RRID: AB_10618636; 1:500), syntaxin 1A (Santa Cruz Biotechnology, cat. no. sc-12736, RRID:AB_2271330; 1:200), SNAP25 (Santa Cruz Biotechnology, cat. no. sc-65232, RRID:AB_632413; 1:200), synaptotagmin VII (Santa Cruz Biotechnology, cat. no. sc-15420, RRID:AB_2199665; 1:200), Munc18a (Sigma-Aldrich, cat. no. M4438, RRID:AB_260531; 1:500), syntaxin 4 (Santa Cruz Biotechnology, cat. no. sc-101301, RRID:AB_2255579; 1:200), SNAP23 (Santa Cruz Biotechnology, cat. no. sc-166244, RRID:AB_2286322; 1:100), Munc18c (Santa Cruz Biotechnology, cat. no. sc-14566, RRID:AB_2271160; 1:200), IR-β (Santa Cruz Biotechnology, cat. no. sc-711, RRID:AB_631835; 1:500), IRS1 (Santa Cruz Biotechnology, cat. no. sc-559, RRID:AB_631842; 1:1,000), pIRS1 (Santa Cruz Biotechnology, cat. no. sc-17201, RRID:AB_653280; 1:1,000), $AKT_{1/2/3}$ (Santa Cruz Biotechnology, cat. no. sc-8312, RRID:AB_671714; 1:500), $pAKT_{1/2/3}$ (Santa Cruz Biotechnology, cat. no. sc-271966, RRID:AB_10715102; 1:500), and GLUT4 (Sigma-Aldrich, cat. no. G4048, RRID:AB_1840900; 1:500).

After this incubation, the membranes were washed 3 times with Tween-TBS, followed by 1 h of incubation with the appropriate secondary antibody, anti-rabbit (Sigma-Aldrich, cat. no. SAB4600068, RRID:AB_2336059), anti-mouse (Santa Cruz Biotechnology cat. no. sc-2377, RRID:AB_634819) or anti-goat (Santa Cruz Biotechnology, cat. no. sc-2352, RRID:AB_634812) horseradish peroxidase-conjugated secondary antibody using 1% bovine serum albumin in Tween-TBS (1:10,000) at room temperature.

After another series of washes, the targeted proteins were detected by enhanced chemiluminescence (Clarity^{TM} and Clarity Max^{TM} Western ECL Blotting Substrates, cat. no. 170–5061, Bio-Rad Laboratories, Hercules, CA, USA). The images were scanned, and bands were quantified by densitometry using ImageJ 1.34s software (Wayne Rasband, National Institutes of Health, Bethesda, MD, USA). To normalize the data, we used glyceraldehyde-3-phosphate dehydrogenase (GAPDH; Cell Signaling Technology, Danvers, MA, USA, cat. no. 5174, RRID:AB_10622025; 1:10,000) and β-actin (Santa Cruz Biotechnology, cat. no. sc-47778 HRP, RRID:AB_2714189; 1:10,000).

Statistical analysis

The results are expressed as the means ± SEM. Data were analysed using the statistical program GraphPad Prism 6.0 (GraphPad Software, Inc., La Jolla, CA, USA). The results were analysed using one-way ANOVA followed by the Newman–Keuls *post hoc* test, separately considering the effects of programming in male and female rat offspring. Differences were considered significant when $P < 0.05$.

Results

Body parameters of the NPEW and PEW offspring

At PN21, the NPEW and PEW males showed lower BM compared to the C group (–14% NPEW and –15% PEW, respectively, $P < 0.05$, Table 1). EW females also had lower levels of this parameter (*vs.* C; –16% NPEW, $P < 0.01$; –13% PEW, $P < 0.05$, Table 1). At PN45 (Table 1), males and females did not exhibit alterations in either body mass or the accumulation of VFM.

At PN180, NPEW and PEW males showed higher BM compared to the C group (+13% and +15%, $P < 0.01$, Table 1). EW promoted an increase in total VFM in adult males compared to the C group (+55% NPEW, $P < 0.01$; +70% PEW, $P < 0.001$, Table 1). In females, BM was not changed in adult life (Table 1). However, the NPEW and PEW females had higher VFM (*vs.* C; +55% and +56%, respectively, $P < 0.01$, Table 1).

Insulin secretion and protein expression in islets isolated from adolescent offspring

At PN45, the islets of NPEW and PEW males showed higher secretion of insulin after stimulation with glucose at concentrations of 5.6 mM (+163% and +157%, respectively, $P < 0.01$ and $P < 0.001$, Fig. 2*A*) and 11.1 mM (+110% and +101%, respectively, $P < 0.05$, Fig. 2*A*) compared to the C group. With 16.7 mM glucose, the islets of PEW males secreted 201% and 91% more insulin than those of the C and NPEW groups, respectively ($P < 0.0001$ and $P < 0.01$, respectively, Fig. 2*A*). When islets were stimulated with supraphysiological glucose concentrations, the islets of the PEW animals exhibited an increase in insulin secretion compared to the C group (+189% in 22.0 mM, $P < 0.05$ and +127% in 27.0 mM, $P < 0.01$, Fig. 2*A*). Islets of adolescent EW females showed no difference in *in vitro* insulin secretion among the groups (Fig. 2*B*).

Islets of the adolescent offspring of both sexes did not show any modifications in the expression of proteins involved in insulin secretion (Fig. 3).

Insulin signalling pathway in the skeletal muscle of adolescent offspring

No modifications were observed in the expression of the main proteins involved in the insulin signalling pathway, such as IR-β, total $AKT_{1/2/3}$, $pAKT_{1/2/3}$ and GLUT4, in the skeletal muscle of adolescent EW rats of both sexes (Fig. 4).

Table 1. Effects of non-pharmacological and pharmacological early weaning on the biometric parameters of the pups at weaning, adolescence (PN45) and adulthood (PN180)

	Male			Female		
	C ($n = 10$)	NPEW ($n = 9$)	PEW ($n = 9$)	C ($n = 10$)	NPEW ($n = 9$)	PEW ($n = 9$)
PN21						
Body mass (g)	49.89 ± 1.4	43.1 ± 2.2$^{\#}$	42.4 ± 1.9*	47.2 ± 1.0	39.4 ± 1.9$^{\#\#}$	41.0 ± 2.1*
PN45						
Body mass (g)	194.1 ± 4.3	181.6 ± 6.8	199.1 ± 5.8	163.0 ± 1.9	147.7 ± 4.4	151.4 ± 5.5
VFM (100 g/BM)	1.29 ± 0.10	1.35 ± 0.12	1.31 ± 0.12	1.51 ± 0.10	1.33 ± 0.12	1.35 ± 0.10
PN180						
Body mass (g)	415.4 ± 7.3	469.1 ± 11.6$^{\#\#}$	480.1 ± 13.5**	258.7 ± 4.8	262.9 ± 6.0	261.8 ± 8.8
VFM (100 g/BM)	3.25 ± 0.23	5.07 ± 0.30$^{\#\#}$	5.54 ± 0.50***	2.86 ± 0.27	4.44 ± 0.32$^{\#\#}$	4.47 ± 0.33**

Groups: C, control, standard weaning; NPEW, non-pharmacological early weaning; PEW, pharmacological early weaning. Values represent means ± SEM of different litter/sex/group ($n = 9$–10). One-way ANOVA followed by Newman–Keuls *post hoc* test. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, NPEW *vs.* C; $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ PEW *vs.* C. BM, body mass; VFM, visceral fat mass.

Insulin secretion and protein expression in the isolated islets in adult offspring

At PN170, the animals were subjected to an OGTT. The males exhibited an increase in blood glucose 30 min after the administration of glucose, followed by a gradual decrease (Fig. 5*A*). The area under the curve (AUC) of the OGTT showed an increase of approximately 10% in the glucose concentration of PEW males compared to the C and NPEW groups ($P < 0.05$ and $P < 0.01$, respectively, Fig. 5*B*). Females did not exhibit any difference in this parameter (Fig. 5*C* and *D*).

At PN180, in isolated islets, the basal insulin secretion levels were similar among the males of all the groups, and this similarity remained in the presence of 8.3, 11.1 and 16.7 mM glucose (Fig. 6*A*). In the presence of 22.0 and 27.0 mM glucose, the isolated islets of NPEW males released less insulin (–50% and –36%, respectively, $P < 0.05$, Fig. 6*A*) than the islets of the C group. The PEW males showed reduced insulin secretion when stimulated with 27.0 mM glucose compared to those isolated from C males (–44%, $P < 0.01$, Fig. 6*A*).

The insulin secretion levels when stimulated with 5.6, 8.3, 11.1 and 16.7 mM glucose were similar among females. However, when stimulated with 22.0 and 27.0 mM glucose, islets of the NPEW females secreted 51% and 37% less insulin than those of C females ($P < 0.01$ and $P < 0.05$, respectively, Fig. 6*B*). The PEW group exhibited a reduction in insulin secretion of ∼34% when stimulated only with 22.0 mM glucose (*vs.* C; $P < 0.05$, Fig. 6*B*).

Regarding the expression levels of some proteins related to insulin secretion in β-cells, we observed an increase of ∼38% in GLUT2 expression in islets of PEW males compared to the C group ($P < 0.05$, Fig. 7*A*). The CGK

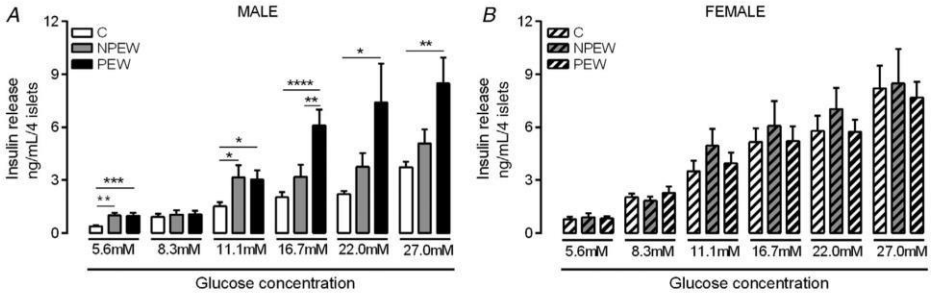

Figure 2. Insulin secretion in isolated islets in adolescent offspring
Insulin secretory dose–response curve in males (*A*) and females (*B*). Groups of 4 islets were incubated for 1 h with 5.6, 8.3, 11.1, 16.7, 22.0 and 27.0 mM glucose. Data are reported as means ± SEM obtained from 24 groups of islets in 3 independent experiments with 6 rats per sex per group. Groups: C, control, standard weaning; NPEW, non-pharmacological early weaning; PEW, pharmacological early weaning. One-way ANOVA followed by Newman–Keuls *post hoc* test. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$.

contents were lower in both EW groups (*vs.* C; –27% of NPEW and –28% of PEW, respectively, $P < 0.05$, Fig. 7*A*). PDX1 expression was not altered among the groups (Fig. 7*A*). We did not observe changes in the expression levels of proteins involved with the exocytosis of insulin granules in the β-cells of males (Fig. 7*B*).

Furthermore, adult females did not show significant differences in the expression levels of the proteins studied in the pancreatic islets (Fig. 7*C* and *D*).

Insulin signalling pathway in the skeletal muscle of adult offspring

Skeletal muscle from the NPEW males showed reductions in the expression of IR-β, pAKT and GLUT4 (*vs.* C; –27%, –17% and –14%, respectively, $P < 0.05$, Fig. 8*A*), which are proteins involved in insulin signalling in skeletal muscle. Skeletal muscle from the PEW males showed a reduction of only GLUT4 compared to the C group (–11%, $P < 0.05$, Fig. 8*A*). The other proteins were not affected in the skeletal muscle from the EW males (Fig. 8*A*). However, we did not observe differences in the protein expression levels of syntaxin 4, SNAP23 and Munc18c, which are involved in the secretory process of GLUT4 in skeletal muscle (Fig. 8*B*).

Skeletal muscle from the EW adult females did not show any modifications in the expression levels of these proteins (Fig. 8*C* and *D*).

Adult EW rats from both sexes showed no differences in plasma adiponectin (Fig. 9*A* and *C*), despite a lower adiponectin/VFM (*vs.* C; –44% NPEW and –47% PEW male, $P < 0.01$, Fig. 9*B*, and –25% NPEW and –31% PEW female, $P < 0.05$, respectively, Fig. 9*D*).

Oestrogen receptor α in the islets and muscle of female offspring

The protein expression of ERα was determined in the pancreatic islets (both ages) and skeletal muscle (only at PN180) in female offspring. We did not observe any alterations in ERα content in these tissues of EW females (Fig. 10).

Discussion

The main findings of this work are directly related to the pancreatic β-cell functions of animals programmed by EW. First, we observed increases in insulin secretion stimulated by different glucose concentrations in the islets isolated from males in both EW models at 45 days of life.

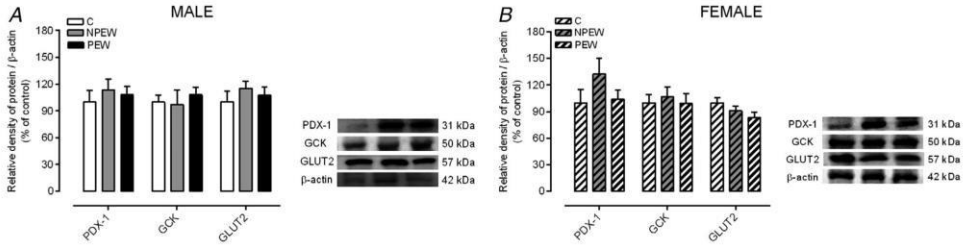

Figure 3. Expression of proteins involved in insulin secretion in pancreatic β-cells in adolescent offspring
PDX-1, GCK, GLUT2 protein expressions of males (*A*) and females (*B*). β-Actin content used as internal control for protein normalization. Groups: C, control, standard weaning; NPEW, non-pharmacological early weaning; PEW, pharmacological early weaning. Values represent means ± SEM of different litter per sex per group ($n = 5$).

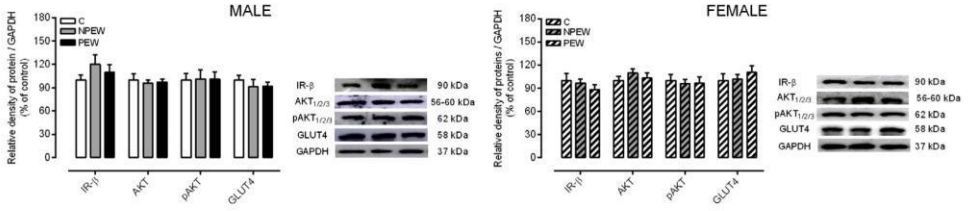

Figure 4. Insulin signalling pathway in skeletal muscle in adolescent offspring
IR-β, AKT, pAKT and GLUT4 protein expression of males (*A*) and (*B*) females. GAPDH content we used as internal control for protein normalization. Groups: C, control, standard weaning; NPEW, non-pharmacological early weaning; PEW, pharmacological early weaning. Values represent means ± SEM of different litter per sex per group ($n = 5$–6).

However, the islets isolated from EW females at the same age showed no changes in this parameter. In adulthood, the islets isolated from NPEW and PEW males showed deficiencies of insulin secretion when exposed to high glucose concentrations and changes in the expression of proteins involved with this secretory process in pancreatic β-cells; in addition, these groups exhibited IR in skeletal muscle. Interestingly, the adult NPEW and PEW females only showed reductions in insulin secretion in isolated islets, without showing the other modifications observed in males at the same age.

The rodent pancreas, similar to that of humans, has the ability to increase its mass of β-cells and, consequently, the level of insulin secretion under both physiological and pathological conditions, ensuring normoglycaemia (Mezza *et al.* 2014). This compensation mechanism promotes structural adaptations in β-cells, such as cell proliferation, hyperplasia and hypertrophy, to improve their function (Cerf, 2015). In our study, in adolescence, we showed a functional compensation in response to EW only in males, which showed increased insulin secretion, since the islets in mammals continue to differentiate during the postnatal period, becoming vulnerable to insults (Ellsworth *et al.* 2018). The islets of adolescent females did not present any alterations in insulin secretion or ERα protein expression. Recently, Pietrobon *et al.* (2019) demonstrated that EW females showed no changes in plasma oestradiol in adolescence. That there are differences in pancreatic insulin secretion between males and females has already been described in the literature. In addition, it is known that sexual maturation in adolescence increases insulin needs due to the rapid growth and development that occur during this particular phase of life (Amiel *et al.* 1986). Some studies have addressed the role of sex steroids on pancreatic β-cells (Nadal *et al.* 2009; Mauvais-Jarvis, 2016). Oestrogens act on the pancreatic islets by binding to their α-type receptors, resulting in increased biosynthesis and glucose-stimulated

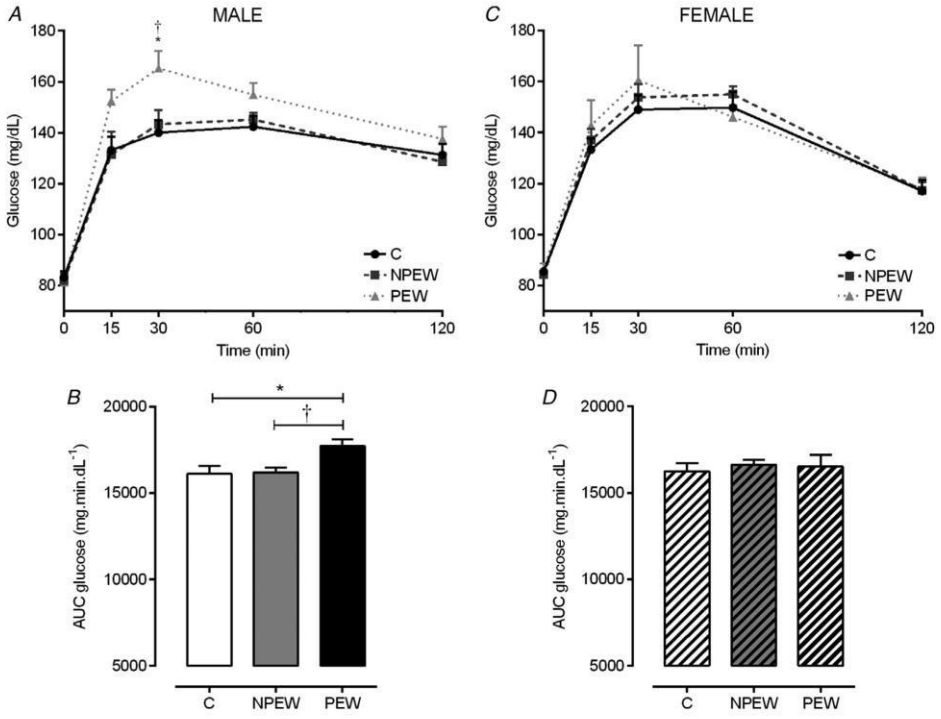

Figure 5. Oral glucose tolerance test (OGTT) in adult offspring
A and *C*, OGTT in males (*A*) and females (*C*). *B* and *D*, area under curve (AUC) of OGTT in males (*B*) and females (*D*). Groups: C, control, standard weaning; NPEW, non-pharmacological early weaning; PEW, pharmacological early weaning. Values represent means ± SEM of different litter per sex per group (n = 9–10). One-way ANOVA followed by Newman–Keuls *post hoc* test. *PEW *vs.* C, †NPEW *vs.* PEW, $P < 0.05$.

insulin secretion (Nadal *et al.* 2009). In situations that require more insulin, such as in adolescence, oestrogens may help β-cells to adapt to this metabolic need without causing losses in glycaemic homeostasis (Livingstone & Collison, 2002). Because oestradiol levels are higher in females during puberty (Cameron, 2004), we believe that this adaptation mechanism explains the fact that EW females do not exhibit changes in insulin secretion during adolescence. Therefore, the observed differences between males and females at this age may be related to the

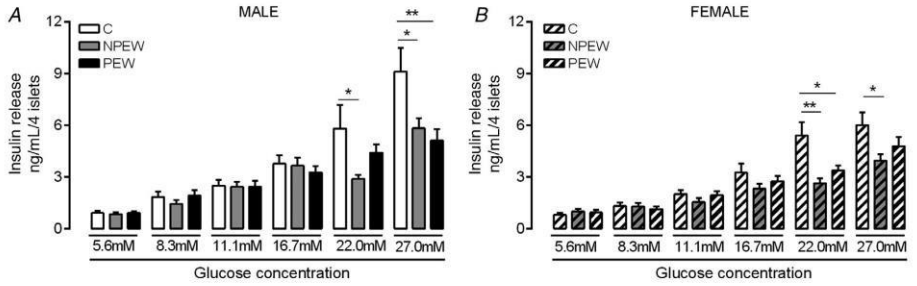

Figure 6. Insulin secretion in isolated islets in adult offspring
Insulin secretory dose–response curve in males (*A*) and females (*B*). Groups of 4 islets were incubated for 1 h with 5.6, 8.3, 11.1, 16.7, 22.0 and 27.0 mM glucose. Data are reported as means ± SEM obtained from 24 groups of islets in 3 independent experiments with 6 rats per sex per group. Groups: C, control, standard weaning; NPEW, non-pharmacological early weaning; PEW, pharmacological early weaning. One-way ANOVA followed by Newman–Keuls *post hoc* test. $^{*}P < 0.05$, $^{**}P < 0.01$.

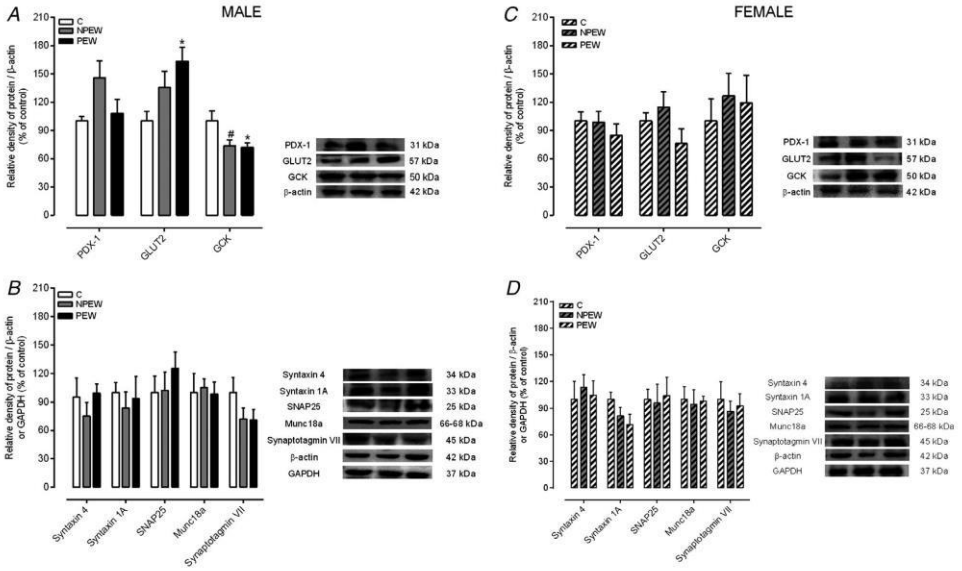

Figure 7. Protein expression in pancreatic islets in adult offspring
A and *C*, proteins involved with insulin secretion (PDX1, GLUT2 and GCK) of males (*A*) and females (*C*). *B* and *D*, protein involved in the exocytosis of insulin granules (syntaxin 4, syntaxin 1A, SNAP25, Munc18a, synaptotagmin VII) in β-cells of males (*B*) and females (*D*) with their respective representative bands. GAPDH and β-actin content we used as internal control for protein normalization. Five to six rats were used from different litter per sex per group. Groups: C, control, standard weaning; NPEW, non-pharmacological early weaning; PEW, pharmacological early weaning. One-way ANOVA followed by Newman–Keuls *post hoc* test. #NPEW *vs.* C, *PEW *vs.* C, $P < 0.05$.

hormonal changes characteristic of this period (Benassi *et al.* 1991).

In adulthood, we observed that PEW males exhibited glucose intolerance. Models of programming by protein restriction during lactation result in reduced sensitivity to secretory stimuli in pancreatic β-cells (Ferreira *et al.* 2003; Oliveira *et al.* 2011). In fact, adult rats programmed by mothers that were subjected to protein restriction during lactation have lower insulin secretion (Latorraca *et al.* 1998; Venci *et al.* 2018). The results of the present study using programming models of EW corroborate this same idea. In the literature, it has already been suggested that the reduction of insulin secretion in the programming model of protein restriction may be attributed to an imbalance of autonomic nervous system activity (Oliveira *et al.* 2011; Venci *et al.* 2018). Thus, a similar mechanism may be occurring in our programming model. Perhaps both EW groups undergo changes in the autonomic nervous system, resulting in reduced parasympathetic activity or enhanced sympathetic activity. Further studies need to be performed to confirm our hypothesis.

Skeletal muscle is responsible for absorbing 70–80% of postprandial glucose, which performs a central role in maintaining glycaemic homeostasis (Jornayvaz *et al.* 2010). GLUT4 is considered a limiting step in this process; therefore, defects in this transporter may result in IR and glucose intolerance (Carnagarin *et al.* 2015). In our model, we did not observe differences in the skeletal muscle of adolescent animals in the two sexes, which may suggest that this tissue has not yet been affected by greater insulin release at this age. In adulthood, EW programming in females caused a deficiency only in β-cell function without affecting the peripheral insulin effects.

Recently, our group demonstrated that PEW females have reduced serum oestrogen levels in adulthood (Pietrobon & Bertasso *et al.* 2019), which could contribute to the reduction in insulin secretion in this group, despite the lack of alterations in ERα expression in islets. Epidemiological and experimental studies suggest that oestradiol protects insulin production in diabetic females (Louet *et al.* 2004). Oestrogen directly stimulates insulin secretion by pancreatic β-cells and improves peripheral insulin sensitivity (Mauvais-Jarvis *et al.* 2016). We did not perform *in vivo* experiments to investigate the direct effect of oestrogen in insulin secretion in the EW model, which is a limitation of our study. Even so, this alteration may not be sufficient to trigger glucose intolerance or IR in these animals. However, the inability of the islets to respond to high glucose concentrations (which correspond to a highly palatable diet) suggests that EW females exposed to a high-sugar or high-fat diet could develop IR and even progress to T2DM.

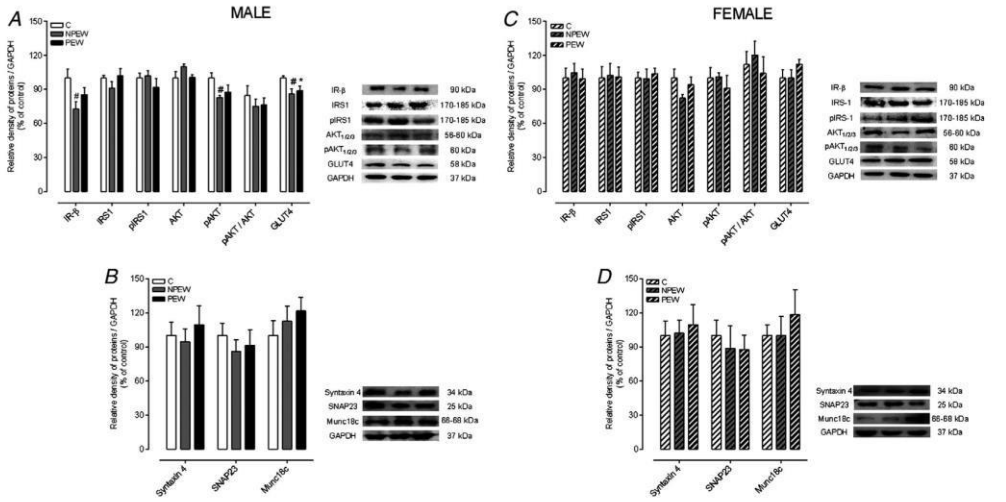

Figure 8. Protein expression in skeletal muscle of adult offspring
A and *C*, insulin signalling pathway (IR-β, IRS1, pIRS1, AKT, pAKT, pAKT/AKT and GLUT4) in males (*A*) and females (*C*). *B* and *D*, protein expressions involved exocytosis of GLUT4 vesicles (syntaxin 4, SNAP23 and Munc18c) in males (*B*) and females (*D*) with their respective representative bands. GAPDH content we used as internal control for protein normalization. We used 5–6 rats from different litter per sex per group. Groups: C, control, standard weaning; NPEW, non-pharmacological early weaning; PEW, pharmacological early weaning. One-way ANOVA followed by Newman–Keuls *post hoc* test. #NPEW *vs.* C, *PEW *vs.* C, $P < 0.05$.

At PN180, EW males presented a reduction in GLUT4 expression in muscle, and NPEW males still showed reduced IR-β and pAKT expression, indicating an onset of IR in these animals. Inadequate regulation of proteins involved with insulin signalling, including IR-β, IRS, PI3K and AKT, has been associated with IR or glucose intolerance (Deshmukh, 2016). There are sex-specific differences in the activation of these pathways in the liver and muscle (Chamson-Reig *et al.* 2009). Men are more resistant to insulin than women (Ludvik *et al.*

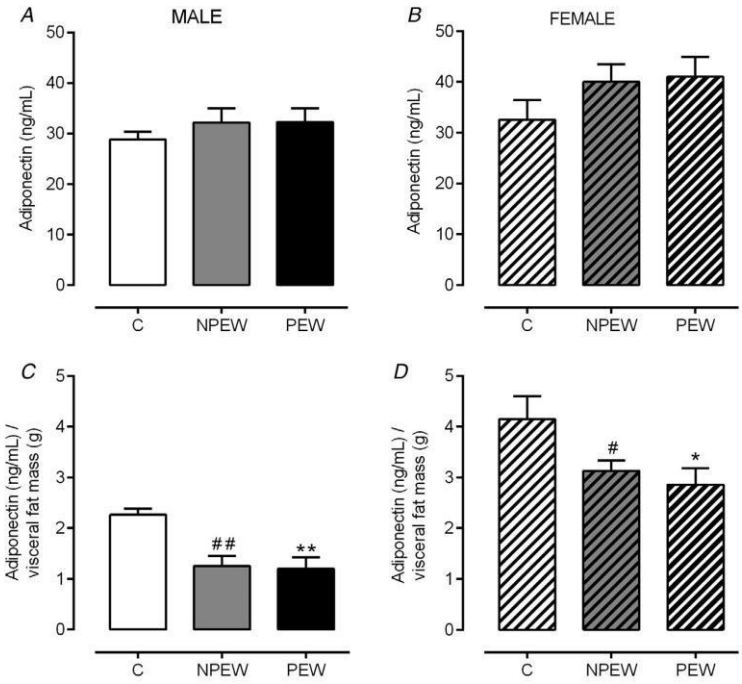

Figure 9. Plasma adiponectin in adult offspring
A and *B*, adiponectin levels of males (*A*) and females (*B*). *C* and *D*, adiponectin levels/VFM of males (*C*) and females (*D*). Groups: C, control, standard weaning; NPEW, non-pharmacological early weaning; PEW, pharmacological early weaning. Values represent mean ± SEM of different litter per sex per group (n = 7–8). One-way ANOVA followed by Newman–Keuls *post hoc* test. #P < 0.05, ##P < 0.01, NPEW *vs.* C; *P < 0.05, **P < 0.01, PEW *vs.* C.

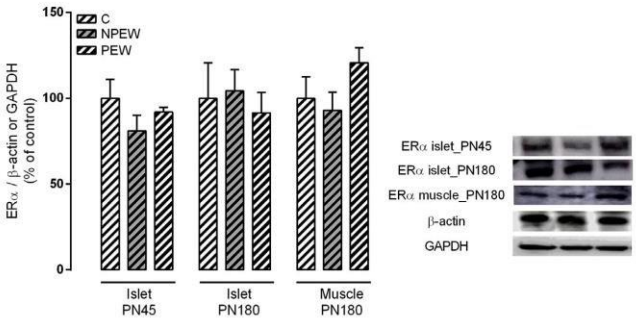

Figure 10. Protein expression of oestrogen receptor α (ERα) of female offspring
ERα content in pancreatic islet (PN45 and PN180) and skeletal muscle (PN180) with their respective representative bands. β-Actin or GAPDH content was used as internal control for protein normalization. Groups: C, control, standard weaning; NPEW, non-pharmacological early weaning; PEW, pharmacological early weaning. Values represent means ± SEM of different litter per group (n = 5–7).

1995; Badri *et al.* 2018). Female rats whose mothers received a protein-poor diet during gestation and lactation became deficient in insulin secretion, while the males became more resistant to this hormone under similar conditions (Chamson-Reig *et al.* 2009). In the literature, maternal malnutrition *in utero* decreases the expression of the GLUT4 gene in the offspring muscle, favouring the methylation of the GLUT4 promoter in the muscle, which may lead to IR and glucose intolerance in adulthood (Wang *et al.* 2016).

The current findings of insulin signalling in adult males and females in EW groups demonstrate a sexual dimorphism. At least in part, this difference could be explained by the protective effect of oestrogen in females, although ERα expression was unaltered in the skeletal muscle in the EW groups. In premenopausal women, elevated oestradiol levels are observed, which is related to the lower prevalence of glucose intolerance compared to men of the same age (Zhu *et al.* 2014). On the other hand, the beneficial effects of oestrogen are reduced after menopause in humans or in ovariectomized rats, which is accompanied by increases in visceral fat pad production, glucose intolerance and pancreatic β-cell failure, abruptly reducing insulin secretion and leading to an increased risk to T2DM (Brussaard *et al.* 1997; Le May *et al.* 2006; Mauvais-Jarvis, 2016).

Prolactin also showed an important role in the maintenance of glycaemic homeostasis during the gestational and lactational periods, leading to β-cell proliferation and increased insulin secretion (Huang *et al.* 2009). This hormone increases the expression of GCK in β-cells by regulating insulin secretion and increasing sensitivity to this hormone (Weinhaus *et al.* 2007; Park *et al.* 2012). The males of both EW groups exhibited hypoprolactinaemia at 180 days of age (Moura *et al.* 2009; Lima *et al.* 2011). Therefore, the lower serum prolactin levels of these animals could contribute to the reduction in the expression of GCK in the pancreatic islets and, consequently, the reduced insulin secretion and the detection of IR in the skeletal muscle.

Our results highlight the importance of exclusive breastfeeding in the first 6 months of life in humans, since one of the future consequences of EW is IR development, which contributes to the T2DM pandemic in adulthood. Increasingly, it is necessary to reinforce and enhance the actions already implemented and to develop new policies for the protection, encouragement and support of breastfeeding in an attempt to increase the duration of breastfeeding and to avoid its early interruption.

Conclusion

For the first time, we have shown that males subjected to EW have distinct alterations in insulin secretion at different ages, which were not observed in females, probably due to the protective effect of oestrogen on islets and skeletal muscle. These results demonstrate a sexual dimorphism in this model. We suggest that the increased insulin secretion of isolated islets stimulated with different glucose concentrations in early life could compensate for the EW and that such changes could lead to hyposecretion of insulin in adult male animals. In addition, we demonstrated that EW affects males more than females, since the males exhibited IR in muscle, a metabolic condition that could progress to the development of T2DM.

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Additional information

Competing interests

The authors declare that they have no competing interest.

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Author contributions

C.B.P., P.C.L. and E.G.M. designed the study. C.B.P. and P.C.L. wrote the manuscript. C.B.P., R.A.M., I.M.B. and E.O. were responsible for animal programming, morphology and biochemical procedures, which were performed at State University of Rio de Janeiro. P.C.F.M. was responsible for the insulin RIA at State University of Maringa. M.L.B. and S.L.B. were responsible for the purchase of type V collagenase and for the training to isolate pancreatic islets. M.A.B.R., M.Q.L. and V.C.A. were responsible for some western blot analyses, which were performed at Federal University of Mato Grosso. All authors contributed to the final version of the manuscript. All authors have read and approved the final version of this manuscript and agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. All persons designated as authors qualify for authorship, and all those who qualify for authorship are listed.

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Keywords

breastfeeding, metabolic programming, obesity, type 2 diabetes mellitus, weaning

Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Statistical Summary Document

2.1.2 Artigo 2 – Early weaning leads to pancreatic steatosis in adult Wistar rats of both sexes

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EARLY WEANING LEADS TO PANCREATIC STEATOSIS IN ADULT WISTAR RATS OF BOTH SEXES.

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Model or Method:	Small animals < Animal
Topic:	Diabetes < Outcome/System, Obesity < Outcome/System
Abstract:	Exclusive breastfeeding up to 6 months of age protects against endocrine-metabolic diseases during development. In fact, epidemiological and experimental data have shown that early interruption of breastfeeding is a risk factor for obesity and type 2 diabetes mellitus (T2DM). In rats, we previously reported that early weaning (EW) models showed increases in abdominal fat and reduced insulin secretion in adults. Concerning ectopic fat, nonpharmacological early weaning (NPEW) induces liver microsteatosis in male offspring, while pharmacological early weaning (PEW) does not cause this dysfunction. Therefore, the current study was designed to investigate whether these animals also developed lipid accumulation in the pancreas, leading to the development of steatosis. At birth, litters were culled to obtain 3 male and 3 female pups. Lactating Wistar rats and their pups were separated into the following groups: control group - dams whose pups obtained milk throughout breastfeeding; NPEW group - dams were subjected to bandage-interrupted suckling for the last 3 days of breastfeeding; PEW group - dams were treated with bromocriptine (0.5 mg/twice a day by ip) 3 days before standard weaning. Offspring were killed on PN180. The pancreas was collected for histological evaluation. Adult EW offspring of both sexes had significantly increased adipocyte infiltration in the pancreas, leading to lipid deposition, although the islets showed a normal architecture. We found that both EW models induced the development of ectopic fat accumulation in the pancreas, which may contribute to pancreatic dysfunction later in life in both male and female progeny.

EARLY WEANING LEADS TO PANCREATIC STEATOSIS IN ADULT WISTAR RATS OF BOTH SEXES

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Running head: Precocious weaning and pancreatic steatosis

Abstract

Exclusive breastfeeding up to 6 months of age protects against endocrine-metabolic diseases during development. In fact, epidemiological and experimental data have shown that early interruption of breastfeeding is a risk factor for obesity and type 2 diabetes mellitus (T2DM). In rats, we previously reported that early weaning (EW) models showed increases in abdominal fat and reduced insulin secretion in adults. Concerning ectopic fat, nonpharmacological early weaning (NPEW) induces liver microsteatosis in male offspring, while pharmacological early weaning (PEW) does not cause this dysfunction. Therefore, the current study was designed to investigate whether these animals also developed lipid accumulation in the pancreas, leading to the development of steatosis. At birth, litters were culled to obtain 3 male and 3 female pups. Lactating Wistar rats and their pups were separated into the following groups: control group - dams whose pups obtained milk throughout breastfeeding; NPEW group - dams were subjected to bandage-interrupted suckling for the last 3 days of breastfeeding; PEW group - dams were treated with bromocriptine (0.5 mg/twice a day by ip) 3 days before standard weaning. Offspring were killed on PN180. The pancreas was collected for histological evaluation. Adult EW offspring of both sexes had significantly increased adipocyte infiltration in the pancreas, leading to lipid deposition, although the islets showed a normal architecture. We found that both EW models induced the development of ectopic fat accumulation in the pancreas, which may contribute to pancreatic dysfunction later in life in both male and female progeny.

Introduction

The World Health Organization (WHO) recommends exclusive breastfeeding for the first six months of life, as this practice is associated with numerous benefits to child health, in addition to reducing high rates of infant morbidity and mortality. The introduction of water or any food in the first months of life is considered early weaning (EW).[1] Clinical and epidemiological studies correlate the interruption of breastfeeding with the development of endocrine-metabolic diseases in adulthood, such as obesity and type 2 diabetes mellitus (T2DM).[1,2]

As a result of obesity, in addition to adipocyte hypertrophy and hyperplasia due to the demand for excessive energy storage, triglycerides begin to accumulate in the parenchyma, and the formation of fat droplets within non-adipocyte tissues, such as the liver, heart, muscle and pancreas, is observed, leading to the development of steatosis. In the pancreas, ectopic fat accumulation is specifically referred to as nonalcoholic fatty pancreatic disease (NAFPD).[3]

Experimental models in rodents confirm the negative effects of the early interruption of breastfeeding observed in humans. Our group found evidence that offspring of both sexes in two different models of EW without maternal deprivation and with nonpharmacological early weaning (NPEW) or pharmacological early weaning (PEW) presented with hyperplasia and hypertrophy of visceral adipocytes in adulthood, and only the males had insulin resistance.[4-6] Recently, we demonstrated that NPEW and PEW animals also have impaired pancreatic function, with reduced in vitro insulin secretion.[7] We know that only NPEW offspring showed increases in oxidative stress and steatosis in the liver, unlike PEW offspring, which seem to preserve their hepatic function.[4,5] We emphasize that these findings were investigated only in EW males.

Considering that EW offspring of both sexes present increased visceral fat and impaired insulin secretion, in the current study, we decided to evaluate the late effects of EW on the possible ectopic deposition of lipids in the pancreas. Our hypothesis is that early weaned animals are prone to developing pancreatic steatosis and that this disorder is involved in tissue dysfunction.

Experimental Methods

Animal care and all experimental procedures were approved by the Institutional Ethical Committee for the use of laboratory animals of the Institute of Biology Institute, State University of Rio de Janeiro (CEUA/036/2018).

Rat early weaning models

At birth, denoted as postnatal day 1 (PND1), the litters were culled to obtain six pups / dam (three males and three females). The lactating Wistar rats were divided into three groups: 1) nonpharmacological EW (NPEW, n=6), in which the lactating rats were wrapped with an adhesive bandage covering all teats for the last 3 days of the lactation period; 2) pharmacological EW (PEW, n=6), in which dams received two doses of 0.5 mg bromocriptine i.p. (Parlodel; Novartis, São Paulo, SP, Brazil) diluted in methanol-saline (1:1) for the last 3 days of lactation; 3) standard weaning (C, control, n=5), in which the dams breastfed their pups until PND21. One rat day is equivalent to ~ 9 human days; therefore, 3 days of EW for rats corresponded to ~ 1 month of human life. Pups were separated from their dams only on PND21 and fed standard chow until PN180, when the analyses were performed.

Pancreas morphology

On the day of sacrifice, the pancreases of 5-6 animals per litter / group were fixed in 4% paraformaldehyde for 24 h, processed and embedded in Paraplast® Plus (Sigma-Aldrich Chemicals, St Louis, MO, USA). Consecutive 5-µm serial sections were obtained, and the slides were stained with haematoxylin and eosin (H&E). Ten images / animal / group were randomly captured (TIFF format, 36-bit colour, 1,360×1,024 pixels) using an Olympus DP71 camera attached to an Olympus BX40 light microscope (Olympus, Japan), and the quantification of pancreatic steatosis was performed by stereology. Pancreatic fat (interlobular, intralobular and perilobular) was quantified by the volume density (Vv) using a point test system superimposed on the tissue image (36 points) and calculated as Vv = PP / PT, where PP represents the number of points that reached the fat cells and PT is the total number of test points.[8]

Statistical analysis

Data represent the mean ± SEM. GraphPad Prism version 6.0 for Windows (GraphPad Software, Inc., San Diego, CA, USA) was used for the statistical analysis and generation of graphics. The results were analysed by one-way ANOVA followed by the Newman-Keuls post-test, which separately considered the effects of programming on males and females. A value of $P < 0.05$ was considered statistically significant.

Results

As depicted in Fig. 1, EW induced fat accumulation in pancreatic tissue (measured as the percentage of the total adipocyte area occupied in the tissue) in the interlobular, intralobular and perilobular regions in adult males (+ 977% NPEW and + 1,910% PEW, * $P < 0.05$ and ** $P < 0.01$, respectively) and females (+ 1,402% NPEW and + 1050% PEW, ** $P < 0.01$) compared with the respective controls. Independently

of the degree of adipocyte infiltration, the islets of EW animals exhibited a normal architecture (Fig. 1b and d show results for males and females, respectively).

Discussion

Pancreatic lipid accumulation is an important clinical indicator related to the increased risk of metabolic disorders such as T2DM. Unlike the liver, in which the accumulation of triglycerides occurs in intracellular droplets, in the pancreas, the infiltration of lipids that are stored in adipocytes in the parenchyma of this tissue occurs.[2,3]

Liver steatosis is known to be due to factors associated with obesity, while pancreatic steatosis is directly related to the body mass index and visceral fat accumulation.[2] Accordingly, our data showed that EW male and female offspring had increased visceral fat, adipocyte hypertrophy[4-6] and pancreatic steatosis. Histological analysis showed that ectopic lipid accumulation in the pancreatic parenchyma occurred independently of sex or the EW model used. Although PEW males did not develop hepatic steatosis[5], they exhibited the large infiltration of adipocytes into the pancreas, which is characteristic of pancreatic steatosis. It is possible that PEW males are able to maintain liver function to the detriment of other peripheral tissues, such as the pancreas. Interestingly, Pinick et al. (2008) demonstrated that animals exposed to a high-fat diet have increased lipid deposition in the pancreas but not in the liver.[3]

The impact of the ectopic deposition of fat in the pancreas has been widely investigated in order to understand the underlying mechanisms involved. Adipocytes impair the pancreatic function of both acinar cells and pancreatic islets by a paracrine effect by increasing the secretion of different cytokines by infiltrating adipocytes, which

triggers an inflammatory response associated with increased oxidative stress in this tissue. These events lead to lipotoxicity and, consequently, damage to acinar and β cells.[8-9] Thus, pancreatic steatosis contributes to the loss of β-pancreatic cell mass and function[11], in addition to being related to the reduction of insulin secretion.[12] In our EW models, ectopic lipid accumulation may have contributed to islet dysfunction, which was recently described by our group, as EW male and female offspring showed decreased glucose-stimulated insulin secretion.[7]

Conclusion

Clinical and experimental studies suggest that pancreatic steatosis is an early indicator of metabolic syndrome and is closely associated with T2DM in obese individuals. Here, we demonstrate that our EW models, which mimic the interruption of exclusive breastfeeding at 5 months of life, showed ectopic fat accumulation in the pancreas. This steatosis can be considered the cause of pancreatic islet dysfunction in the early weaned offspring of both sexes. To the best of our knowledge, this is the first report in the literature showing that pancreatic steatosis is induced by the early weaning.

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Conflict of Interest

None.

Ethical Standards

The authors assert that all procedures contributing to this work comply with the ethical standards of the relevant national guides on the care and use of laboratory animals (Brazilian Law 11.794/ 2008 that regulates the procedures for the scientific use of animals) and has been approved by the institutional committee (Institutional Ethical Committee for the use of laboratory animals of the Institute of Biology Institute, State University of Rio de Janeiro - CEUA/036/2018).

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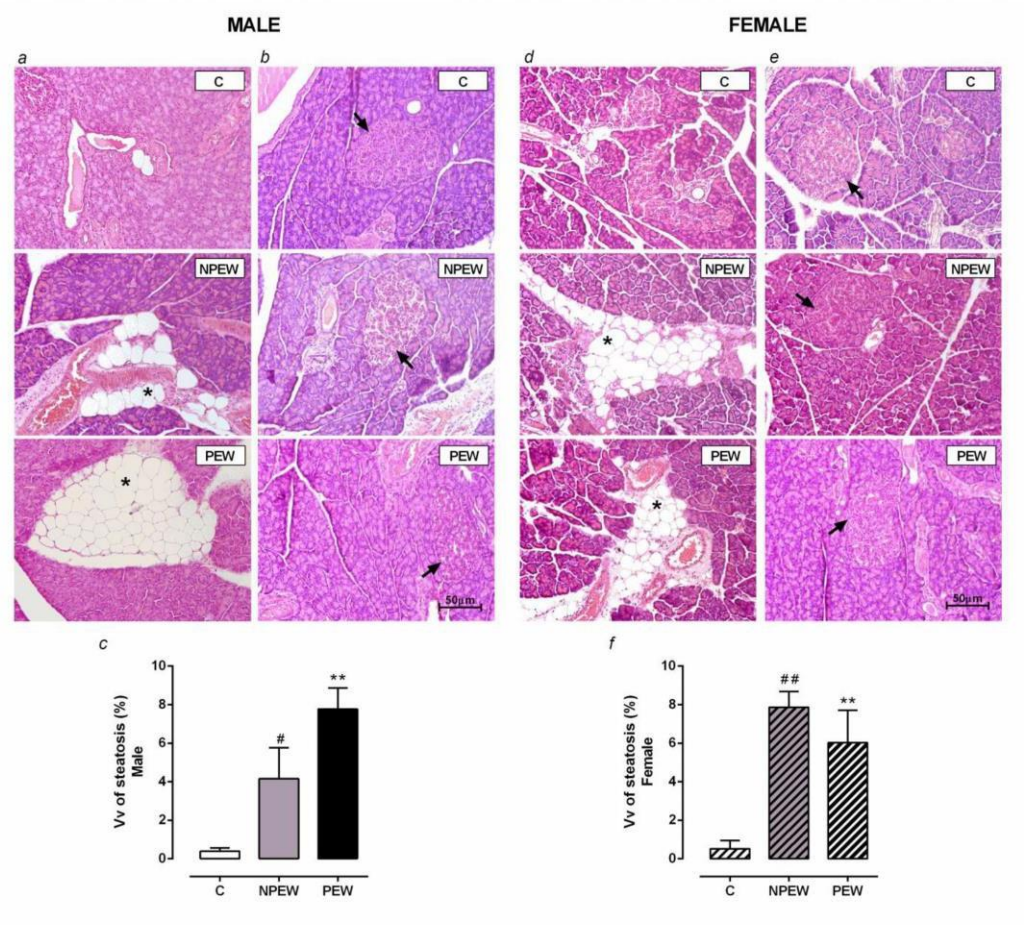
Figure captions

Fig. 1 Representative photomicrographs of pancreatic tissue (a and d) showing lipid accumulation via H&E staining (scale bar: 50 μm - magnification 20X) in adult offspring. Groups: C: control, standard weaning (PN21); NPEW: nonpharmacological EW; PEW: pharmacological EW. The male and female EW groups both showed high adipocyte infiltration (asterisk) in pancreatic tissue (a and d). The percentages of males (c) and females (f) with steatosis. Representative images of histological islets in adult offspring (b and d). The arrows indicate the normal architecture of islets in male and female EW rats through qualitative analysis (scale bar: 50 μm - magnification 20X). We used n=5-6 animals per sex from different litters in each group. One-way ANOVA followed by the Newman-Keuls post-test. * $P < 0.05$; ** $P < 0.01$. # NPEW vs C; * PEW vs C.



F1

290x263 (300 x 300 DPI)

2.2 **Modelo experimental II: Exposição materna à nicotina**

A metodologia, resultados e a discussão referente a este modelo de programação estão apresentados no artigo abaixo:

2.2.1 Artigo 3 - Pancreatic steatosis in rats by nicotine exposure during breastfeeding

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ORIGINAL ARTICLE

Pancreatic steatosis in adult rats induced by nicotine exposure during breastfeeding

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Abstract

Purpose Maternal nicotine exposure negatively impacts offspring's health and metabolism, leading to obesity and insulin resistance. Here we investigated the pancreatic islet function, glycemic homeostasis, and insulin signaling in adult rat offspring that were nicotine-exposed during breastfeeding.

Methods For this, lactating Wistar rat dams were divided into two groups: Nicotine (implanted with osmotic minipumps containing 6 mg/Kg, NIC) and Control (saline, CON). Solutions were released from postnatal (PN) day 2–16. At PN110 and PN170, 10 offspring per litter/sex/group were submitted to the oral glucose tolerance test (OGTT). PN180 offspring were killed and glycemia, insulinemia, adiponectinemia, pancreas morphology as well as pancreatic islet protein expression (related to insulin secretion) and skeletal muscle (related to insulin action) were evaluated. Males and females were compared to their respective controls.

Results Adult NIC offspring of both sexes showed glucose intolerance in the OGTT. Despite normoglycemia, NIC males showed hyperinsulinemia while females, although normoinsulinemic, had hyperglycemia. Both sexes showed increased IRI, reduced adiponectin/visceral fat mass ratio and higher ectopic deposition of lipids in the pancreatic tissue adipocytes. In pancreatic islets, NIC males showed lower PDX-1 expression while females had higher PDX-1 and GLUT2 expressions plus lower α2 adrenergic receptor. In the muscle, NIC offspring of both sexes showed reduction of GLUT4 expression; NIC males also had lower insulin receptor and pAKT expressions.

Conclusions Thus, glycemic homeostasis and peripheral insulin signaling in adult offspring of both sexes are affected by nicotine exposure through the milk, increasing the risk for type 2 diabetes development.

Keywords Glycemia · Lactation · Nicotine · Pancreatic beta cell · Programming

Introduction

Type 2 diabetes mellitus is considered a pandemic, mainly caused by insulin resistance (IR), which is closely associated with obesity [1]. However, in recent years, experimental and epidemiological evidence have shown that the growing incidence of type 2 diabetes and the obesity pandemic may also be due to insults during critical early life periods [2]. In the 1980s, Barker raised the "thrifty phenotype hypothesis" to explain the relationship between the fetal environment and the diseases found in adulthood [3]. This concept is now known as "Developmental Origins of Health and Disease" (DOHaD), which postulates that specific environmental insults during a critical period of early life can induce developmental plasticity by programming the individual for disease development [4].

Supplementary information The online version of this article (https://doi.org/10.1007/s12020-020-02579-9) contains supplementary material, which is available to authorized users.

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The period of breastfeeding is considered a window of susceptibility to metabolic programming, because organs involved in the maintenance of glycemic homeostasis, such as the brain, liver and pancreatic islets continue to differentiate during this period. These organs have been shown to be vulnerable during this period since programming insults can have negative, long-lasting and sometimes late-emerging effects on glycemic homeostasis [5].

Smoking exposure during critical periods of development is related to metabolic programming. It is well known that cigarette smoke contains several compounds that can be harmful to the developing individual. Studies show that smoking during pregnancy is dangerous for both mother and fetus [6]. Although it has been shown that many pregnant women quit smoking, some studies show that most women who quit smoking during gestation resume it during breastfeeding, erroneously believing that smoking during this period is no longer harmful to the baby. Unfortunately, babies are not only exposed to second-hand smoke but also to those components of tobacco smoke that will be present in the mother's milk, nicotine included [7].

Nicotine is a psychoactive substance and is the main agent leading to tobacco dependence [8]. Nicotine is considered an endocrine disruptor, interfering with the action of hormones [9]. To mimic the late-emerging effects of maternal smoking during early life, our research group has developed a programming model to investigate the effects of early, involuntary exposure to nicotine via breast milk on the offspring. In this experimental model, lactating rats are exposed to nicotine through implantation of osmotic minipumps that release a controlled amount of nicotine (equivalent to a heavy smoker) into the bloodstream [10]. In this model, lactating rats show a large amount of cotinine, the main metabolite of nicotine, in serum and milk, confirming the efficacy of the model [11]. Male animals programmed by exposure to nicotine during lactation are overweight and have increased visceral fat accumulation, hyperleptinemia, central leptin resistance, and hepatic steatosis at adulthood [12, 13]. However, the female offspring show no alteration of body mass (BM), adiposity, and leptinemia [14, 15]. Regarding glycemic homeostasis, male offspring are normoglycemic and hyperinsulinemic, suggesting IR. They also have lower plasma adiponectin/adipose tissue ratio [13]. It is worth mentioning that the aforementioned results were not studied in females.

The present study aimed to investigate the mechanisms involved in the IR that has been previously observed in the male offspring of this programming model [12], and to investigate, for the first time, the glycemic homeostasis of the female offspring. Our hypothesis is that animals early-exposed to nicotine will develop pancreatic dysfunction and alterations in proteins involved with peripheral insulin signaling in the muscle, which is responsible for 70–80% uptake of postprandial glucose.

Materials and methods

The Institutional Ethical Committee for the Use of Laboratory Animals of the Biology Institute of the State University of Rio de Janeiro approved all experimental procedures (CEUA/038/2018). Experimental procedures were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Experimental model of maternal exposure to nicotine

Three-month-old virgin female Wistar rats were caged with male rats at a 3:1 ratio for 2 weeks. After the detection of pregnancy, dams were allocated in individual cages with free access to water and standard chow for rodents. At birth, litters were adjusted to six pups, three males and three females, to maximize lactation performance. According to the manufacturer's recommendation, in order to ensure proper operation of the osmotic minipumps (continuous and homogeneous release of solutions), they were filled with the solution of interest and immersed in saline solution for 24 h prior to implantation. Thus, implantation of osmotic minipumps was performed at postnatal (PN) day 2. Lactating dams were randomly assigned to one of two groups: **Nicotine group (NIC):** 10 dams were lightly anaesthetized with thiopental (30 mg/Kg; Thiopentax, Itapira, SP, Brazil), a 3 × 6 cm area on the back was shaved and an incision was performed to allow for the subcutaneous insertion of the osmotic minipumps (Alzet, 2ML2, California, USA). Minipumps were prepared with nicotine free-base diluted in a saline solution (NaCl 0.9%) to deliver a dose rate of 6 mg/Kg of nicotine per day during 2 weeks [10]. This protocol produces plasma nicotine concentrations similar to those found in typical smokers [16]. **Control group (CON):** 10 dams were implanted, using the procedures indicated above, with osmotic minipumps containing only NaCl 0.9%.

After weaning (PN21), NIC and CON pups received free access to water and standard rodent chow. The offspring's BM was monitored every 4 days until PN180, when animals were euthanized: After a 12 h-fasting period, animals were anesthetized with thiopental (Thiopentax, 30 mg/Kg) and blood was collected by cardiac puncture in tubes with heparin. Blood samples were centrifuged (1500 *g* for 20 min at 4 °C) to obtain plasma, which was kept at −20 °C. Afterwards, the retroperitoneal, gonadal and mesenteric white adipose tissues were collected, weighted and the sum of these tissues was used as the visceral fat mass (VFM) variable. The pancreas and soleus skeletal muscle were quickly removed to be used in the specific techniques described below.

Glycemic homeostasis

The oral glucose tolerance test (OGTT) was performed at PN110 and PN170. A blood sample was collected, after a 12 h-fasting period, for the determination of basal glycemia (time 0) using a glucometer (ONETOUCH ULTRA®, Johnson & Johnson, São Paulo, Brazil). Glucose solution (50%) was injected in sterile saline via an oral probe at 2 g/Kg BM and glycemia was measured after 15, 30, 60 and 120 min. Fasting glycaemia was analyzed at PN180 in blood samples obtained from the tail vein using reagent strips that were read in a glucometer. Plasma insulin and adiponectin concentrations were measured by Elisa Kit (Millipore Corporation, MA, USA) according to the manufacturer's instructions. Plasma adiponectin was normalized by total VFM [17]. Insulin sensitivity was assessed using the insulin resistance index (IRI), which was calculated as follow: fasting glucose (mg/mL) × fasting insulin (mUI/mL).

Pancreas morphology

Due to the lack of standardized nomenclature in the literature, the term pancreatic steatosis was used in the current study to describe all types of accumulation of fat in the pancreas, as suggested by Smits & Van Geenen [18].

The pancreases of seven animals per litter/sex/group were removed, weighed and quickly fixed in 4% paraformaldehyde for 24 h. Then, the entire tissue was incorporated into Paraplast® Plus (Sigma-Aldrich Chemicals, St Louis, MO, USA) and prepared for light microscopy. Consecutive 5 µm serial sections were obtained and the slides were stained with hematoxylin-eosin. Ten images/animal/group were captured randomly (magnification ×20, Leica Microsystems CTR6000, Wetzlar, Germany) and quantification of pancreatic steatosis was performed by stereology. Pancreatic fat (interlobular, intralobular and perilobular) was quantified by volume density (Vv) using a point test system superimposed on the tissue image (36 points), calculated as: Vv = PP/PT, where PP represents the number of points that reach the fat cells and PT is the total number of test points.

Western blotting

The pancreatic islets were isolated using the collagenase method [19]. The islets were collected using a stereomicroscope and approximately 300 islets were stored in a −80 °C freezer until the Western blot analysis was carried out.

For protein expression analysis, samples of isolated islets of six animals per litter/sex/group were resuspended and lysed by sonication (two times, 10 s pulses, Sonic Dismembrator Model 100, Thermo Fisher Scientific) in T-PER™ Tissue Protein Extraction Reagent (78510, Sigma St. Louis, MO, USA) buffer. The cocktail of protease inhibitors (complete EDTA-free, Roche Applied Science, Mannheim, Germany) was added and samples were centrifuged at 15,294 *g* for 5 min at 4 °C (Eppendorf 5417R Refrigerated Centrifuge). The skeletal muscle of 6–7 animals per litter/sex/group were homogenized in RIPA buffer, (20 mM TRIS HCl, 10 mM NaF, 1% NP40, 150 mM NaCl, 5 mM EDTA, 0.1% SDS) containing a protease inhibitor cocktail and centrifuged twice at 15,294 *g* for 15 min at 4 °C. The total protein content was determined using a BCA Protein Assay Kit (Thermo Scientific, IL, USA) and cell lysates were treated with Laemmli sample buffer (50 mM Tris-HCl, pH 6.8, 1% SDS, 5% 2-mercaptoethanol, 10% glycerol, 0.001% bromophenol blue).

Total protein extracts were separated by gel electrophoresis (SDS-PAGE, 8.5–12%) at 150 V/60 A. The proteins were then transferred from the gel to a nitrocellulose membrane (Hybond ECL; Amersham Pharmacia Biotech, NJ, USA) by the Trans-Blot® Turbo™ Transfer System (Bio-Rad®, Hercules, CA, USA) at 2.5 A/15 V/45 min. Membranes were blocked with 5% BSA in Tween-TBS buffer (containing 20 mM Tris-HCl, pH 7.5; 500 mM NaCl and 0.1% Tween-20) for 1 h with continuous shaking. Then, membranes were incubated with different primary antibodies (described in Table 1) overnight at 4 °C. Then, membranes were washed 3 times with Tween-TBS, followed by 1 h incubation with the appropriate secondary antibody at room temperature. After this period, membranes were incubated with streptavidin-horseradish peroxidase conjugated HRP (RPN1231V, GE Healthcare Life Sciences, USA) when necessary. After another series of washes, targeted proteins were detected by enhanced chemiluminescence (Clarity™ and Clarity Max™ Western ECL Blotting Substrates, cat 170-5061, Bio-Rad, California, USA). Images were scanned and bands were quantified by densitometry, using Image J 1.34 s software (Wayne Rasband, National Institute of Health, MA, USA). The glyceraldehyde-3-phosphate dehydrogenase protein content (GAPDH, 5174, Cell Signaling, Massachusetts, USA; diluted 1:1000 in TTBS) and B-actin (Santa Cruz Biotechnology®, CA, USA; diluted 1:1000 in TTBS) were used for the normalization of the data.

Statistical analysis

The results are expressed as mean ± SEM. Data were analyzed using the statistical program GraphPad Prism 6.0 (GraphPad Software, Inc., La Jolla, CA, USA). Unpaired Student's *t* test were used separately when analyzing the programming effects in males and females. Differences were considered significant when $P < 0.05$.

Table 1 Antibodies used in western blotting

	Primary antibody	Secondary antibody
PDX-1 (31 kDa)	Anti-pancreatic and duodenal homeobox 1 antibody—Rabbit polyclonal (1:5000) (Cat # ab47267, RRID: AB_777179; Abcam, São Paulo, BR)	Anti-rabbit (1:10000) (Cat # SAB4600068, RRID: AB_2336059; Sigma-Aldrich, Darmstadt, Germany)
GLUT2 (57 kDa)	Anti-glucose Transporter 2 antibody—Rabbit polyclonal (1:200) (Cat # 720238, RRID: AB_2633212; Thermo Fisher, Massachusetts, EUA)	Anti-rabbit (1:10000) (Cat # SAB4600068, RRID: AB_2336059; Sigma-Aldrich, Darmstadt, Germany)
GCK (50 kDa)	Anti-glucokinase antibody—Mouse monoclonal (1:500) (Cat # sc-17819, RRID: AB_627722; Santa Cruz Biotechnology®, CA, USA)	Anti-mouse (1:10000) (Cat# sc-2377, RRID: AB_634819; Santa Cruz Biotechnology®, CA, USA)
AdRα2 (45 kDa)	Anti-α2 Adrenergic Receptor antibody—Rabbit polyclonal (1:500) (Cat# A271, RRID: AB_2225050; Sigma-Aldrich, Darmstadt, Germany)	Anti-rabbit (1:10000) (Cat # SAB4600068, RRID: AB_2336059; Sigma-Aldrich, Darmstadt, Germany)
AdRβ2 (46 kDa)	Anti-β2 Adrenergic Receptor antibody—Rabbit polyclonal (1:500) (Cat# SAB4500576, RRID: AB_10743973; Sigma-Aldrich, Darmstadt, Germany)	Anti-rabbit (1:10000) (Cat # SAB4600068, RRID: AB_2336059; Sigma-Aldrich, Darmstadt, Germany)
mAChRM3 (75 kDa)	Anti-Muscarinic Acetylcholine Receptor M3 antibody—Rabbit polyclonal (1:500) (Cat# M9568, RRID: AB_262100; Sigma-Aldrich, Darmstadt, Germany)	Anti-rabbit (1:10000) (Cat # SAB4600068, RRID: AB_2336059; Sigma-Aldrich, Darmstadt, Germany)
mAChRM4 (75 kDa)	Anti-Muscarinic Acetylcholine Receptor M4 antibody—Rabbit polyclonal (1:500) (Cat# sc-9109, RRID: AB_2080211; Santa Cruz Biotechnology®, CA, USA)	Anti-rabbit (1:10000) (Cat # SAB4600068, RRID: AB_2336059; Sigma-Aldrich, Darmstadt, Germany)
Syntaxin 1 A (33 kDa)	Anti-syntaxin 1 A antibody—Mouse monoclonal (1:200) (Cat # sc-12736, RRID: AB_2271330; Santa Cruz Biotechnology®, CA, USA)	Anti-mouse (1:5000) (Cat# sc-2377, RRID: AB_634819; Santa Cruz Biotechnology®, CA, USA)
SNAP25 (25 kDa)	Anti-SNAP25 antibody—Mouse monoclonal (1:200) (Cat # sc-65232, RRID: AB_632413; Santa Cruz Biotechnology®, CA, USA)	Anti-mouse (1:5000) (Cat# sc-2377, RRID: AB_634819; Santa Cruz Biotechnology®, CA, USA)
Synaptotagmin VII (45 kDa)	Anti-synaptotagmin VII antibody—Goat polyclonal (Cat # sc-15420, RRID: AB_2199665; Santa Cruz Biotechnology®, CA, USA)	Anti-goat (1:10000) (Cat # sc-2352, RRID: AB_634812; Santa Cruz Biotechnology®, CA, USA)
Munc18a (66-68 kDa)	Anti-munc18a antibody—Rabbit polyclonal (1:200) (Cat # M4438, RRIDAB_260531; Sigma-Aldrich, Darmstadt, Germany)	Anti-rabbit (1:10000) (Cat # SAB4600068, RRID: AB_2336059; Sigma-Aldrich, Darmstadt, Germany)
IR-beta (90 kDa)	Anti-insulin receptor beta antibody—Rabbit polyclonal (1:500) (Cat # sc-711, RRID: AB_631835; Santa Cruz Biotechnology®, CA, USA)	Anti-rabbit (1:10000) (Cat # SAB4600068, RRID: AB_2336059; Sigma-Aldrich, Darmstadt, Germany)
$AKT_{1/2/3}$ (56-60 kDa)	Anti-serine/threonine protein kinase B antibody—Rabbit polyclonal (1:500) (Cat # sc-8312, RRID: AB_671714; Santa Cruz Biotechnology®, CA, USA)	Anti-rabbit (1:10000) (Cat # SAB4600068, RRID: AB_2336059; Sigma-Aldrich, Darmstadt, Germany)
$pAKT_{1/2/3}$ (56-60 kDa)	Anti-phosphorylated serine/threonine protein kinase B antibody—Rabbit polyclonal (1:500) (Cat # sc-271966, RRID:AB_10715102; Santa Cruz Biotechnology®, CA, USA)	Anti-rabbit (1:10000) (Cat # SAB4600068, RRID: AB_2336059; Sigma-Aldrich, Darmstadt, Germany)
GLUT 4 (58 kDa)	Anti-Glucose Transporter 4 antibody–Rabbit polyclonal (1:500) (Cat # G4048, RRID:AB_1840900; Sigma-Aldrich, Darmstadt, Germany)	Anti-rabbit (1:10000) (Cat # SAB4600068, RRID: AB_2336059; Sigma-Aldrich, Darmstadt, Germany)
Syntaxin 4 (34 kDa)	Anti-syntaxin 4 antibody–Mouse monoclonal (1:200) (Cat # sc-101301, RRID: AB_2255579; Santa Cruz Biotechnology®, CA, USA)	Anti-mouse (1:5000) (Cat# sc-2377, RRID: AB_634819; Santa Cruz Biotechnology®, CA, USA)
SNAP23 (25 kDa)	Anti-SNAP23 antibody—Rabbit polyclonal (1:200) (Cat # sc-166244, RRID: AB_2286322; Santa Cruz Biotechnology®, CA, USA)	Anti-rabbit (1:10000) (Cat # SAB4600068, RRID: AB_2336059; Sigma-Aldrich, Darmstadt, Germany)
Munc18c (66-68 kDa)	Anti-munc18a antibody—Goat polyclonal (1:200) (Cat # sc-14566, RRID: AB_2271160; Santa Cruz Biotechnology®, CA, USA)	Anti-goat (1:10000) (Cat # sc-2352, RRID: AB_634812; Santa Cruz Biotechnology®, CA, USA)
GAPDH (37 kDa)	Anti-glyceraldehyde 3-phosphate dehydrogenase antibody—Rabbit polyclonal (1:1000) (Cat# 5174, RRID: AB_10622025; Cell Signaling, Massachusetts, EUA)	Anti-rabbit (1:10000) (Cat # SAB4600068, RRID: AB_2336059; Sigma-Aldrich, Darmstadt, Germany)
Bl-Actin (42 kDa)	Anti-beta actin antibody—Mouse polyclonal (1:1000) (sc-47778 HRP, RRID:AB_2714189, Santa Cruz Biotechnology®, CA, USA)	Anti-mouse (1:10000) (Cat# sc-2377, RRID: AB_634819; Santa Cruz Biotechnology®, CA, USA)

PDX-1 Pancreatic and duodenal homeobox 1, *GLUT2* Glucose transporter 2, *GCK* Glucokinase, *AdRα2* α2 adrenergic receptor, *AdRβ2* β2 adrenergic receptor, *mAChRM3* Muscarinic acetylcholine receptor subtype 3, *mAChRM4* Muscarinic acetylcholine receptor subtype 4, *IR-beta* Insulin receptor beta, $AKT_{1/2/3}$ Serine/threonine protein kinase B, $pAKT_{1/2/3}$ Phosphorylated serine/threonine protein kinase B, *GLUT4* glucose transporter 4, *GAPDH* Glyceraldehyde 3-phosphate dehydrogenase

Table 2 Effects of nicotine exposure during lactation on biometric parameters of the adult offspring (PN180)

	MALE		FEMALE	
	CON	NIC	CON	NIC
Body mass (g)	521 ± 10	597 ± 14***	290 ± 8	303 ± 7
VFM (100 g/BM)	5.5 ± 0.3	7.3 ± 0.5**	5.4 ± 0.4	6.0 ± 0.5
Pancreas (g)	1.8 ± 0.1	1.6 ± 0.1	1.2 ± 0.1	1.2 ± 0.1
Pancreas (g/100 g BM)	0.32 ± 0.01	0.32 ± 0.04	0.38 ± 0.02	0.40 ± 0.02

Values represent mean ± SEM of different litters per sex per group ($n = 10$). Student's t tests were used separately for male and female rat offspring

Groups: *CON* Control, *NIC* Nicotine. VMF (visceral fat mass), BM (body mass)

$^{**}P < 0.01$; $^{***}P < 0.001$

Results

Somatic parameters of the offspring

At PN180, NIC males showed increased BM and VFM [15% ($P < 0.01$) and 33% ($P < 0.001$), respectively] when compared to CON group (Table 2), while NIC females did not have changes in these parameters (Table 2). The absolute and relative mean pancreases masses were similar between groups (Table 2).

Oral glucose tolerance test (OGTT) and glucose homeostasis

At PN110, after 30 min of glucose administration, we observed an increase in glucose in all groups, which was followed by a gradual reduction, without significant differences between them (Fig. 1a, c). The area under curve (AUC) of the OGTT was unchanged in both NIC males and NIC females (Fig. 1b, d).

At PN170, NIC males showed increased blood glucose at 60 and 120 min after glucose administration when compared to CON males ($P < 0.05$ and $P < 0.01$, respectively, Fig. 1e). The AUC of OGTT is compatible with the aforementioned data, showing an 18% increase in NIC males when compared to the CON group ($P < 0.05$, Fig. 1f). NIC females had higher glucose levels at 30, 60 and 120 min after glucose administration ($P < 0.05$, $P < 0.01$ and $P < 0.05$, respectively, Fig. 1g). Regarding the AUC, NIC females showed increased glycemia during OGTT compared to the CON ones (11%, $P < 0.01$, Fig. 1h).

At PN180, NIC males whose mothers were exposed to nicotine during lactation showed no difference in fasting glycemia (Fig. 2a), but had higher insulinemia (51% vs CON, $P < 0.05$, Fig. 2b) and IRI (66% vs CON, $P < 0.01$, Fig. 2c). In addition, these animals showed no differences in plasma adiponectin (Fig. 2d), although they showed a lower adiponectin/VFM when compared to CON males (−39%, $P < 0.01$, Fig. 2e).

Regarding NIC females at PN180, we observed hyperglycemia (12%, $P < 0.05$, Fig. 2f), normoinsulinemia (Fig. 2g), higher IRI (80%, $P < 0.01$, Fig. 2h), hypoadiponectinemia (−24%, $P < 0.05$, Fig. 2i) and reduced adiponectin/VFM when compared to CON females (−53%, $P < 0.01$, Fig. 2j).

Pancreas parameters in adult offspring

Concerning the histological analysis of the pancreas (Fig. 3), NIC males showed increased fat accumulation in the pancreatic tissue compared to CON males (183%, $P < 0.01$, Fig. 3b). NIC females also showed increased pancreatic steatosis when compared to CON females (438%, $P < 0.001$, Fig. 3d).

The expression of proteins related to the insulin secretion are depicted in Fig. 3. NIC males showed a reduction only of PDX1 expression when compared to CON males (−20%, $P < 0.05$, Fig. 3e). The other proteins were not altered (Fig. 3e). NIC females had an increase of GLUT2 and PDX1 expressions (33%, $P < 0.05$ in both cases, Fig. 3f) and a reduction of α2 adrenergic receptor in islets (−25%, $P < 0.05$, Fig. 3f) in relation to CON females. The expressions of the other proteins were unaltered (Fig. 3f).

Insulin signaling pathway in the skeletal muscle of adult offspring

The main proteins involved with insulin signaling in skeletal muscle are shown in Fig. 4. NIC males showed reduced expression of IR-beta (−32%, $P < 0.05$, Fig. 4a), GLUT4 (−24%, $P < 0.05$, Fig. 4a) and less degree of phosphorylation of AKT (pAKT) (−19%, $P < 0.05$, Fig. 4a) and in relation to the CON males, although there was no change in the expression of total AKT (Fig. 4a). The evaluation of total pAKT/AKT ratio from NIC animals was not changed (CON male: 98.9 ± 11.7; NIC male: 81.4 ± 11.6; CON female: 87.5 ± 8.7; NIC female: 79.7 ± 8.1). In addition, NIC males did not show changes in the expression of SNARE proteins (Syntaxin 4, SNAP23 and Munc18c), a complex involved in GLUT4 transport (Fig. 4a). The skeletal muscle of NIC females only showed a reduction of GLUT4 expression when compared to CON females (−37%, $P < 0.05$, Fig. 4b). The other proteins were not affected in this tissue (Fig. 4b).

Discussion

In the present study we demonstrated that changes in the glycemic homeostasis previously found [12] in male rats

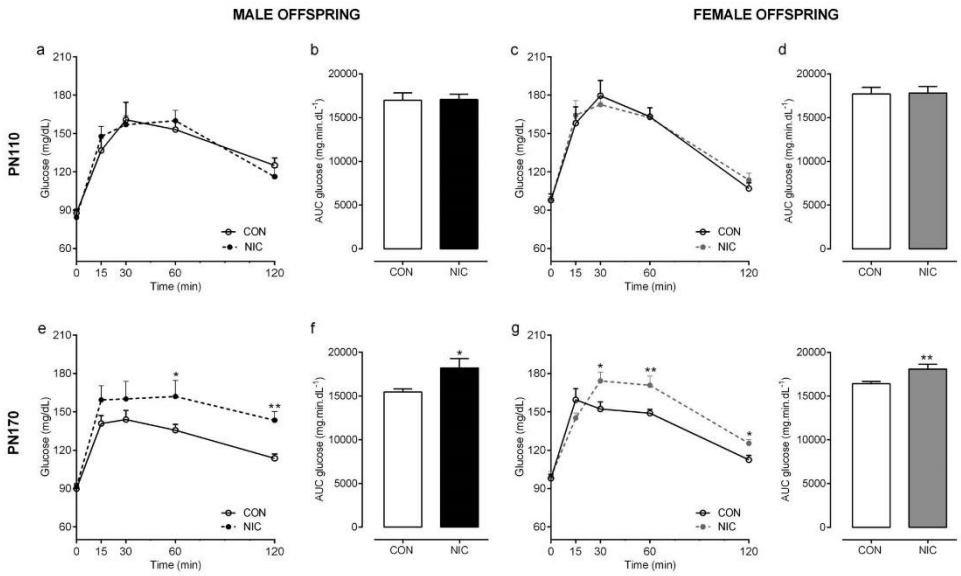

Fig. 1 Oral glucose tolerance test (OGTT) at PN110 and PN170. OGTT (**a** and **e**) and area under curve (AUC) of OGTT (**b** and **f**) of male rat offspring. OGTT (**c** and **g**) and AUC of OGTT (**d** and **h**) of female rat offspring. Groups: CON: Control, NIC: Nicotine. Values represent mean ± SEM of different litters per sex per group ($n = 10$). Student's t tests were used for the comparisons. $^{*}P < 0.05$, $^{**}P < 0.01$

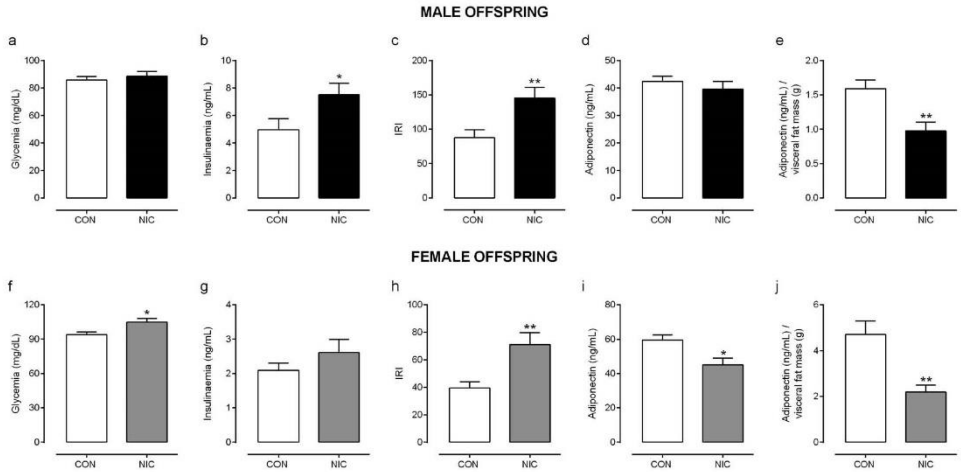

Fig. 2 Glycemia (**a**), Insulinemia (**b**), IRI (**c**), Serum adiponectin (**d**) and Serum adiponectin/visceral fat mass (**e**) of male rat offspring at PN180. Glycemia (**f**), Insulinemia (**g**), IRI (**h**), Serum adiponectin (**i**) and Serum adiponectin /visceral fat mass (**j**) of female rat offspring at PN180. Groups: CON: Control, NIC: Nicotine. Values represent mean ± SEM of different litters per sex per group ($n = 10$). Student's t tests were used for the comparisons. $^{*}P < 0.05$, $^{**}P < 0.01$

programmed by early exposure to nicotine during lactation occur only after PN110, since OGTT, which is considered an indicative test for IR, was unchanged at that age. At PN170, animals of both sexes had glucose intolerance and, at PN180, they showed pancreatic steatosis and IR, characterized by reduced GLUT4 in the skeletal muscle,

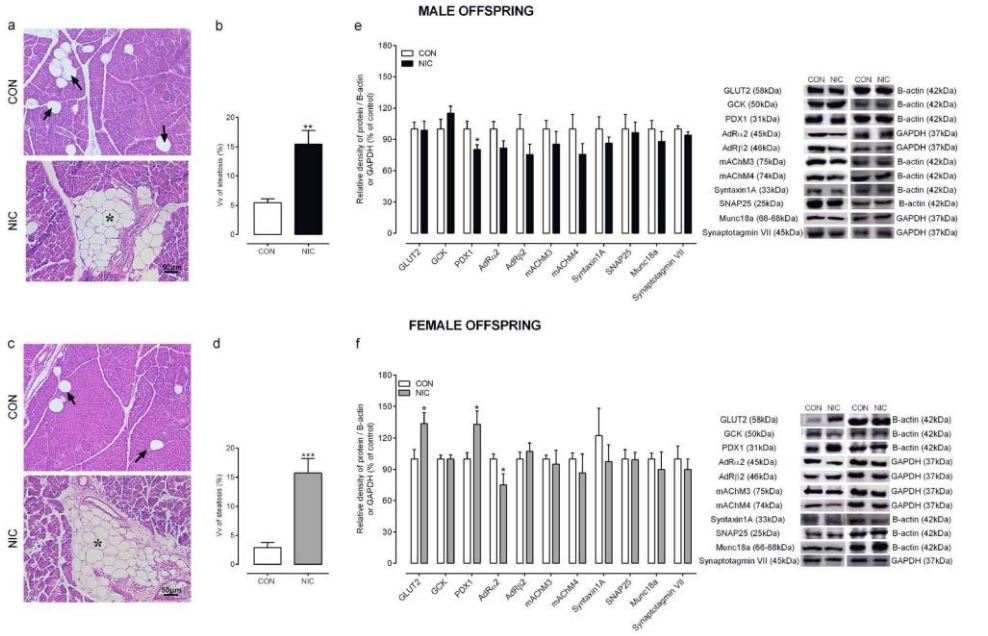

Fig. 3 Pancreas parameters adult offspring. Representative photomicrographs of pancreatic tissue (**a** and **c**) of adult rat offspring show lipid accumulation with H&E staining (scale bar: 50 µm - magnification ×10). Groups: CON: Control, NIC: Nicotine. NIC groups have a large adipocyte infiltration (asterisk). The arrows indicate lipid droplets that are found in the exocrine pancreas of CON animals. Percentage of steatosis in male (**b**) and female (**d**) offspring. We used different litters per sex per group ($n = 7$). Protein expression involved in insulin secretion (PDX1, GLUT2, GCK, AdRα2, AdRβ2, mAChM3, mAChM4) and in the exocytosis of insulin granules (Sintaxin 1A, SNAP25, Munc18a, Synaptotagmin VII) by beta cells in male (**e**) and female (**f**) offspring with their respective representative bands. GAPDH or B-actin contents were used as internal controls for protein normalization. Values represent mean ± SEM of different litters per sex per group ($n = 6$). Student's t tests were used for the comparisons. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$

increased IRI and reduced adiponectin/VFM ratio. Specifically, males also had hyperinsulinemia, reduced expression of PDX1 in the pancreatic islets and decreased expression of the insulin receptor and less degree of phosphorylation of AKT in muscle, while females had hyperglycemia, hypoadiponectinemia, increased expression of GLUT2 and PDX1 and reduced α2-adrenergic receptor in the islets.

IR associated with progressive beta cell dysfunction is characteristic of the development of type 2 diabetes [18]. Unlike hepatic steatosis, pancreatic steatosis is histologically characterized by infiltration, increased number of adipocytes in this tissue [20, 21] and increased intracellular lipids [22]. Here, males and females exposed to nicotine during breastfeeding showed increased lipids accumulation in fat cells in the pancreas. Although controversial, the effect of ectopic fat associated with IR and pancreatic beta cell dysfunction has been investigated in clinical and experimental studies [21, 23]. Pancreatic steatosis may potentiate the metabolic syndrome, resulting in hyperglycemia, reduction of insulin secretion [24], loss of mass and function of pancreatic beta cells [25]. The pancreatic infiltrated fat cells also could be associated with the increased oxidative stress and proinflammatory cytokines production resulting in localized inflammation and fibrosis, impairing the architecture [26, 27] and causing inflammation in the pancreatic islets [28], apoptosis of pancreatic beta cells [26, 27] and pancreatic dysfunction [21–23].

In humans, a positive association between increased lipid infiltration in the pancreas and IR has been reported, suggesting that pancreatic fat enhances this condition [22–32]. In a cohort of 8097 individuals, Wang et al. [30] demonstrated that there was increased risk of developing diabetes in patients with pancreatic fat accumulation. Also, it has been reported that individuals newly diagnosed with type 2 diabetes have significantly higher amounts of pancreatic fat when compared to healthy patients [31]. After analysis of pancreatic fat content in men with and without type 2 diabetes, Tushuizen et al. [32] demonstrated that the average pancreatic fat content in diabetic patients was 20% higher than in nondiabetic patients. In addition, the pancreatic fat

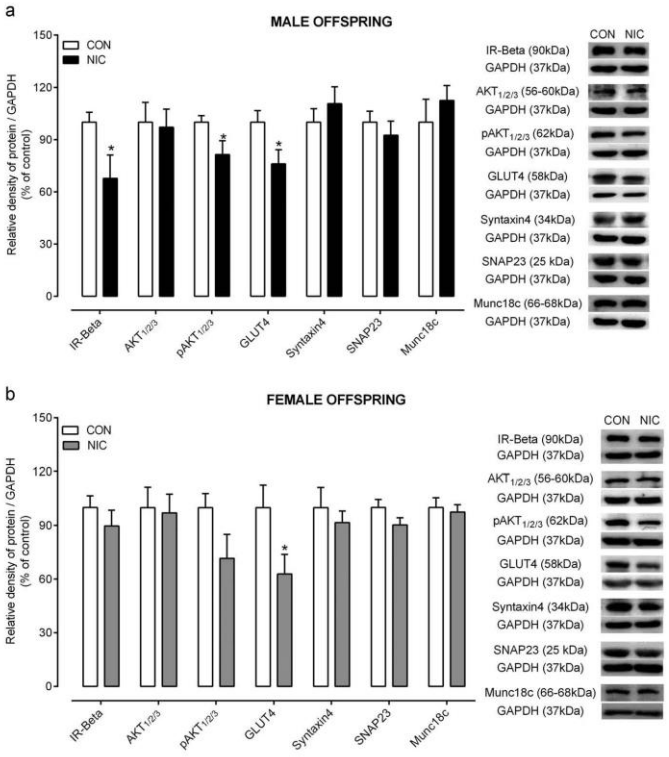

Fig. 4 Insulin signaling pathway in the skeletal muscle of adult offspring. IR-beta, AKT, pAKT, GLUT4, Syntaxin 4, SNAP23 and Munc18c protein expression at PN180 in male (**a**) and female (**b**) rat offspring with their respective representative bands. Groups: CON: Control, NIC: Nicotine. GAPDH content we used as internal control for protein normalization. Values represent mean ± SEM of different litters per sex per group ($n = 6$–7). Student's t tests were used for the comparisons. $^*P < 0.05$

observed in these patients was associated with the pancreatic beta cell dysfunction [31, 32]. It appears that may occur a combined destructive effect between increased free fatty acids (FAs) and pancreatic beta cell function, associated with increased lipotoxicity and increased local chemokine production, resulting in long-term beta cell injury and/or death [18, 21, 23]. However, the role of pancreatic steatosis on beta cell function is not fully understood and remains contradictory in the literature. Not all human studies have found a relationship between pancreatic adipocyte infiltration and pancreatic beta cell function in individuals with impaired glucose metabolism [33–35]. These contradictory results can be attributed to differences in applied methodology, number of individuals evaluated, age, and ethnicity of the population studied.

In addition to local inflammation possibly caused by adipocyte infiltration, pancreatic beta cells can be affected by a lipotoxic mechanism, since it seems that the islet cells have no adipocyte infiltration and only the exocrine pancreas show the ectopic fat. It is well described in the literature that the adipocyte-stored triglycerides are composed of different FAs, which have positive and negative effects on insulin secretion and on the survival of beta cells [36]. For example, monounsaturated FAs, are generally associated with protective effects, participating in the regulation of normoglycemia, increased insulin sensitivity, and prevention of apoptosis [37]. However, saturated FAs are widely associated with lipotoxicity, associated with reduced beta cell proliferation, insulin gene expression and induction of cell death [38, 39]. Chronic exposure to FAs results in disturbances in the regulation of lipid metabolism, which contribute to decreased function and beta cells, through lipotoxicity and, consequently, inducing T2DM [37, 40]. Here, we hypothesize that accumulated pancreatic lipids may act as a source of FAs or other lipid-derived metabolites, which can gradually affect the beta cell function and insulin secretion through lipotoxic pathways, which seems to be more pronounced in NIC females. In fact, the percentage of adipocytes infiltrating the female pancreas (+438%) is more pronounced than the percentage of male steatosis (+183%). Therefore, NIC female is more affected; its beta cell does not produce enough insulin resulting in hyperglycemia, while NIC male is able to release more insulin, perhaps due to less fat in the pancreas, maintaining

normoglycemia. This hypothesis was also based on available literature. Some authors suggest a relationship between pancreatic steatosis and the severity of pancreatitis [18, 41, 42]. Although we propose that the endocrine dysfunction observed in animals exposed to NIC could in part be related to mild pancreatitis, lipotoxicity or increased local chemokine production and the infiltration of immune cells through a paracrine effect mediated by adipocytes that are infiltrated in the pancreas, further studies are needed to clarify this idea. This is a limitation of our study, since we only evaluated the percentage of adipocytes that were infiltrated in the pancreatic tissue.

It is worth mentioning that there are few and controversial studies in the literature on exocrine dysfunction caused by pancreatic steatosis. Three mechanisms have been described that can cause exocrine pancreatic disorder in patients with pancreatic steatosis: acinar cell lipotoxicity, negative paracrine effect mediated by infiltrated adipocytes, or direct destruction of acinar cells [21, 43]. Therefore, further research in animal models is needed to assess the influence of pancreatic steatosis on the exocrine pancreas, especially using immunostaining techniques.

Epidemiological data demonstrate the negative impacts of smoking during pregnancy and the increased risk of developing type 2 diabetes at adulthood [44]. A clinical study showed that newborns of mothers who reported smoking in the middle and late gestation had hyperglycemia, reduced fetal IGF-I concentration with changes in fetal pancreatic beta cell function [45]. In animals, maternal cigarette smoke exposure during pregnancy and/or lactation caused glucose intolerance in the offspring and reduced insulin sensitivity [46], besides leading to reduction in size and number of pancreatic islets [47]. In addition to overweight and increased visceral fat, male offspring whose mothers were exposed to nicotine concentrations equivalent to women who smoke moderately during pregnancy and lactation had glucose intolerance and IR, effects that may be mediated by reduced pancreatic beta cell mass in early life [47, 48] and possibly associated with greater susceptibility for the development of metabolic syndrome in the adult offspring.

In our programming model of nicotine exposure exclusively during lactation, males had normoglycemia, but had glucose intolerance and increased IRI. Normoglycemia is possibly being maintained by the hyperinsulinemia. In humans, most diabetic patients initially have glucose intolerance, which is considered the intermediate phase in type 2 diabetes progression [49]. To compensate for IR, pancreatic beta cells adapt to different situations to improve function and maintain glycemic homeostasis. In this phase, structural adaptations occur in the beta cells that lead to hyperfunction, i.e., increase insulin secretion in response to hyperglycemia, ensuring glycemic homeostasis, even if only for a temporary period [50]. A limitation in the present study was that the area and mass of the pancreatic islets were not evaluated, parameters that were assessed by other authors in models of pregnancy + lactation exposure [47, 48].

Unlike males, NIC females had hyperglycemia, which may be due to beta cell failure to produce sufficient amounts of insulin to maintain normoglycemia. Increased metabolic insulin demand may lead to reduced pancreatic beta cell function [50]. Chronic hyperglycemia can lead to pancreatic beta cell depletion, probably by glucotoxicity of the beta cell [51], a phase that characterizes a critical metabolic state that precedes beta cell dysfunction leading to cell death [50]. This difference observed on the glycemic profile between adult men and women in these two human studies [50, 51] may be related to the amount of adipocytes infiltrated in pancreatic tissue, in which women are most affected, because they showed higher percentage of adipocytes infiltrated. However, we know that pancreatic steatosis is only one of the factors that contribute to the development of T2DM. To support this idea, a limitation of our work was not to have assessed insulin secretion in vitro, therefore, further studies are needed to support our hypothesis.

Proper functioning of pancreatic islets depends on a number of regulators, among them PDX-1, which is considered one of the most important transcription factors involved in the regulation, development and maintenance of pancreatic beta cells, and that controls the expression of the GLUT2, GCK, and insulin [52]. A change in metabolic state reduces PDX1 transcription, mediating a cascade of modifications that culminate in the silencing of this gene [53]. Here, we demonstrated that early life nicotine exposure reduces PDX1 expression in male pancreatic islets, although it did not alter GLUT2 and GCK expressions. Downregulation of PDX1 expression in beta cells may underlie the pathogenesis of beta cell failure and type 2 diabetes [54]. NIC females showed increased expressions of PDX1 and GLUT2, mainly due to the hyperglycemia. However, due a primary impairment in insulin production, the compensatory increase in GLUT2 was unable to increase insulin secretion.

Pancreatic islets are innervated by branches of the autonomic nervous system and sympathetic innervation is responsible for inhibiting glucose-induced insulin release [55]. In adult females, nicotine exposure via breast milk also seems to change the autonomic nervous system that controls insulin secretion, since these animals show a reduction in $\alpha 2$ adrenergic receptor expression. This reduction could be a compensatory effect to the lower insulin production.

Adiponectin is a fat tissue hormone that increases insulin sensitivity, as well as has antioxidant and anti-inflammatory actions [56]. The concentration of adiponectin in the umbilical cord of newborns of smoking women and in

children exposed to cigarette smoke was significantly lower when compared to children of healthy mothers [57]. The present study lends support to the idea that a correlation exists between glucose intolerance, IR and hypoadiponectinemia, since NIC offspring of both sexes had a reduction in adiponectin/VFM and NIC females had a reduction in plasma adiponectin.

Especially in the skeletal muscle, IR is considered the primary defect in the progression of this disease. Glucose uptake in the muscle is substantially higher than in the adipose tissue or liver [58]; the former tissue is the predominant site of postprandial insulin-mediated glucose uptake through GLUT4 stimulation and translocation to the plasma membrane. Thus, after a meal, ~80% of the glucose uptake occurs in the muscle [58, 59]. Insulin signaling in muscle depends on it binding to its receptor, which activates a cascade of protein phosphorylation, including insulin receptor substrate (IRS), phosphatidylinositol 3-kinase, and a serine/threonine protein kinase (AKT). AKT stimulates GLUT4 translocation, therefore, participating in insulin-dependent glucose transport in the muscle and adipose tissue [60]. Thus, the deregulation of critical proteins in the insulin signaling pathway is related to the pathogenesis of IR and type 2 diabetes [61]. Specifically, muscle GLUT4 defects contribute to glucose intolerance and IR [58, 59]. In our model, NIC rats of both sexes showed a reduction in GLUT4. In addition, NIC males also had decreased muscle insulin receptor and pAKT. Alteration in the expression of these proteins may be contributing to IR in animals programmed by nicotine exposure during lactation.

Most studies investigating the negative effects of nicotine only consider pregnancy or pregnancy plus breastfeeding, i.e., there are few studies that evaluate the effects of exposure to tobacco smoke exclusively during the early PN period. As already mentioned, many women resume smoking during lactation, and here we highlight the deleterious effects of early nicotine exposure, causing pancreatic steatosis, beta-cell dysfunction and IR in the offspring of both sexes. Maternal smoking during breastfeeding is still a serious public health problem, as this attitude contributes to the type 2 diabetes pandemic.

Conclusion

We evidence that smoking during breastfeeding predisposes the progeny of both sexes to the development of type 2 diabetes at adulthood, since we demonstrated that early, involuntary nicotine-only exposure via breast milk led to the development of pancreatic steatosis, glucose intolerance and IR. In our programming model, it seems that the female offspring are more prone to develop severe diabetes earlier in life. However, further studies are needed to understand how pancreatic steatosis can impair beta cell function by lipotoxicity, cytotoxicity or both and contributes to type 2 diabetes or whether its presence is only a marker of pancreatic beta cell dysfunction. In addition, we need to understand how the females are more prone to pancreatic beta cell failure.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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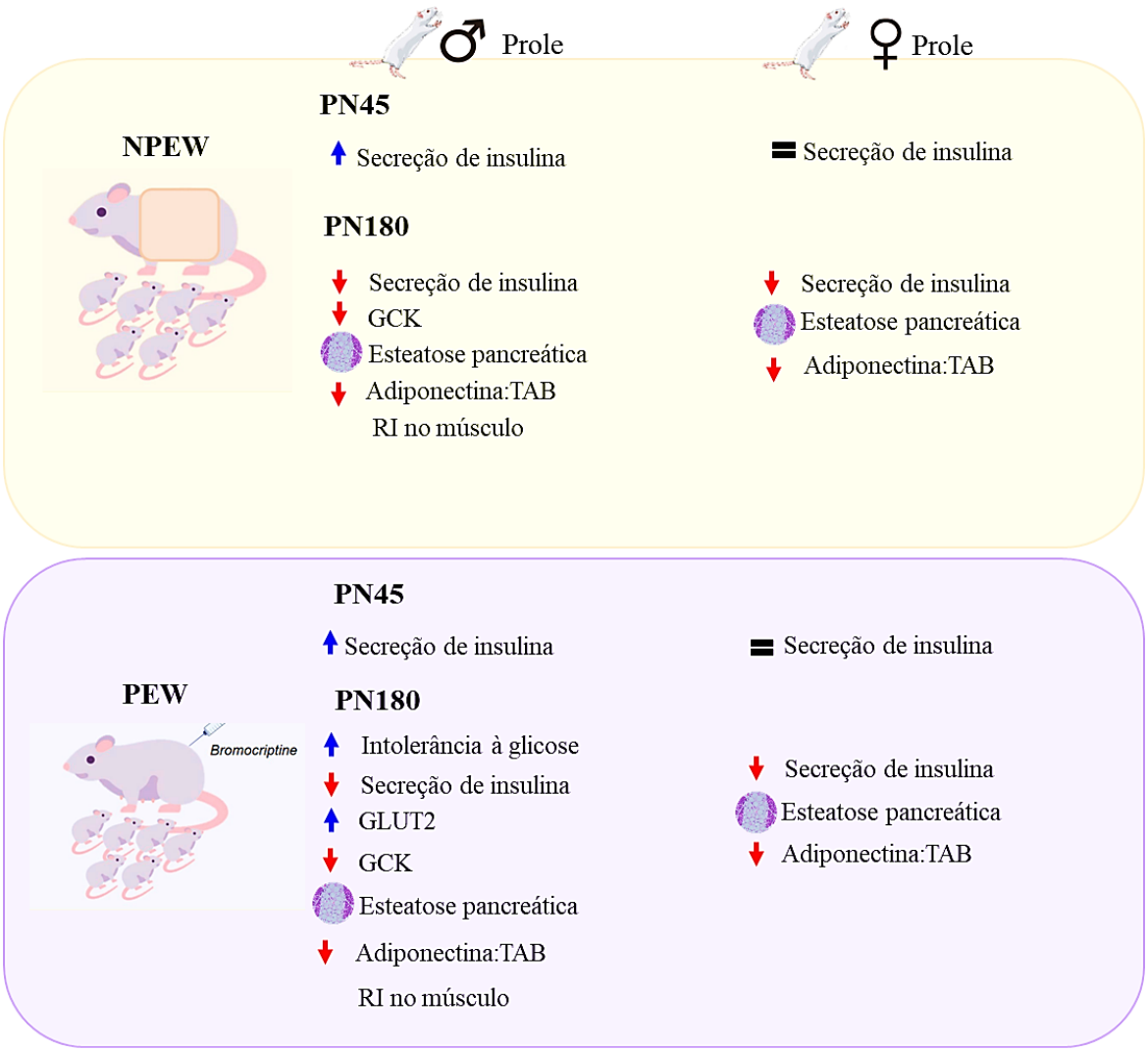
3 RESUMO DOS RESULTADOS

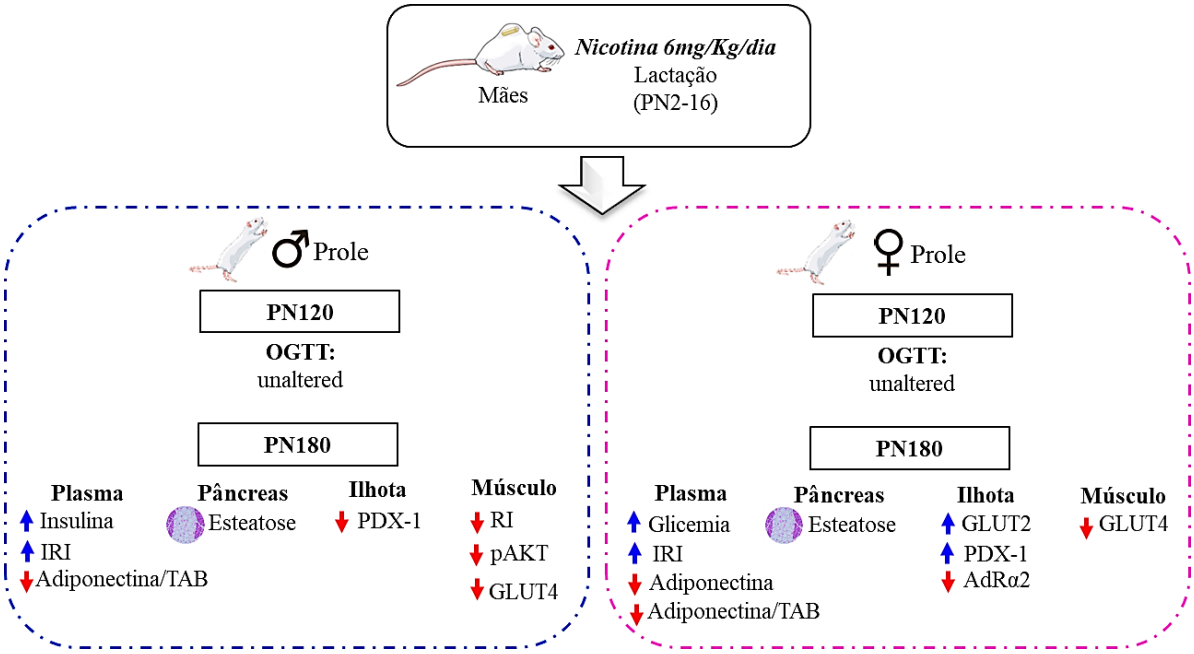
Figura 3 - Principais alterações relacionadas à homeostase glicêmica no modelo de programação pelo desmame precoce (artigo 1 e 2)



Legenda: **GCK:** Glicoquinase; **GLUT2:** Transportador de glicose tipo 2; **NPEW:** Desmame precoce não farmacológico (non-pharmacological early weaning); **PEW:** Desmame precoce farmacológico (pharmacological early weaning); **PN:** Pós natal; **TAB:** Tecido adiposo branco; **RI:** Resistência à insulina;

Fonte: A autora, 2020.

Figura 4 - Principais alterações relacionadas à homeostase glicêmica no modelo de programação pela exposição à nicotina durante a lactação (artigo 3)



Legenda: **AdRα2:** Receptor adrenérgico alfa 2; **GLUT2:** Transportador de glicose tipo 2; **GLUT4:** Transportador de glicose tipo 4; **IRI:** Índice de resistência à insulina; **OGTT:** Teste oral de tolerânciaà glicose; **pAKT:** Proteína quinase B fosforilada; **PDX-1:** Fator de transcrição homeobox pancreáticoe duodenal 1; **PN:** Pós natal; **RI:** Resistência à insulina; **TAB:** Tecido adiposo branco;

Fonte: A autora, 2020

CONSIDERAÇÕES FINAIS

Com a pandemia do diabetes é essencial identificar as janelas de suscetibilidade que contribuem para a programação metabólica. Dentre estas janelas, nossos dados destacam que a lactação é uma importante janela crítica de programação, a qual exposições nutricionais (desmame precoce) e ambientais (exposição à nicotina), cursam com alterações endócrino-metabólicas semelhantes, que ao longo da vida levam ao desenvolvimento de DM2.

No primeiro modelo de programação abordamos a importância do aleitamento materno exclusivo, o qual é considerado a estratégia mais eficaz e natural de vínculo, afeto, proteção e nutrição da mãe para o bebê. A alimentação da criança única e exclusiva com o leite materno tem repercussões ao longo de toda a vida do indivíduo. A partir dos nossos resultados, observamos que a interrupção precoce do aleitamento materno, a qual em nosso modelo de programação equivale a um mês de vida do bebê humano, ou seja, a amamentação exclusiva se dá apenas com cinco meses e não seis meses como o recomendado pela OMS, impacta diretamente o funcionamento das células-β pancreáticas.

Pela primeira vez estudamos a homeostase glicêmica destes animais durante a adolescência e observamos que apenas os machos programados pelo desmame precoce apresentaram aumento da secreção de insulina. Na vida adulta, os machos apresentam deficiência na secreção de insulina, bem como, alterações na expressão de proteínas envolvidas com o mecanismo secretório nas células-β, esteatose pancreática e RI periférica. Curiosamente, as fêmeas adultas programadas pelo desmame precoce apresentaram apenas redução na secreção de insulina e esteatose pancreática, sem apresentar as demais alterações observadas em machos na mesma idade. Vale ressaltar que o desmame precoce afeta adversamente, de maneira dependente do sexo, o fenótipo da prole, uma vez que os machos apresentam alterações distintas na secreção de insulina em diferentes idades, o que não foi observado nas fêmeas, provavelmente devido ao efeito protetor do estrogênio nas ilhotas e no músculo esquelético. Além disso, o desmame precoce afeta mais os machos que as fêmeas uma vez que os mesmos exibiram RI no músculo, uma condição metabólica que pode progredir para o desenvolvimento de DM2.

Dentro do contexo da amamentação, os pesquisadores e profissionais da saúde ainda discutem os impactos do tabagismo materno em relação ao bem-estar da criança que pode ser exposta tanto pela fumaça do cigarro quanto aos seus compostos, inclusive a nicotina, via leite materno. Devido à pequena quantidade de estudos que avaliam os efeitos diretos da nicotina,

oriunda do tabagismo materno, sobre a saúde do bebê, com o nosso terceiro modelo de programação, conseguimos demonstrar os impactos causados sobre a homeostase glicêmica da prole em consequência desta atitude. Diferentemente do modelo anterior, em que os machos são afetados de maneira mais intensa pelo desmame precoce, a exposição à nicotina durante a lactação afeta ambos os sexos, aumentando as chances da prole em desenvolver DM2 na vida adulta, uma vez que tanto machos quanto fêmeas adultas apresentaram esteatose pancreática, intolerância à glicose e RI.

Especificamente os machos do grupo NIC também apresentaram hiperinsulinemia, redução da expressão do PDX-1 nas ilhotas pancreáticas e redução da expressão do IR e pAKT no músculo esquelético, enquanto que as fêmeas apresentaram hiperglicemia, normoinsulinemia, hipoadiponectinemia, aumento da expressão de GLUT2 e PDX-1 e redução da expressão do receptor adrenérgico α2 nas ilhotas. Sabe-se que o PDX-1 desempenha adequadamente seu papel dependendo de outros fatores de transcrição que controlam a sua expressão nas células-β, inclusive a própria insulina participa desta regulação. Ademais, nos machos, outros fatores de transcrição do gene da insulina podem estar compensando a baixa expressão do PDX-1 observada, isso explicaria o fato destes animais ainda apresentarem aumento da insulina plasmática. Apesar dos resultados da expressão deste fator de transcrição serem distintos entre machos e fêmeas, alterações na sua expressão são relacionadas diretamente com disfunções nas células-β, associada ao diabetes, embora pouco se sabe sobre como o PDX1 regula a sobrevivência, diferenciação e a função das células-β. Acreditamos que o papel do PDX-1 deve ser considerado em estudos futuros na tentativa de desenvolver novos agentes que tenham como alvo a prevenção e tratamento do diabetes. Uma limitação deste trabalho foi não ter avaliado a secreção de insulina in vitro e não ter estudado no período de adolescência, como fizemos no modelo de desmame precoce. Assim, com os resultados encontrados neste modelo, acreditamos que as fêmeas são mais propensa a desenvolver falência pancreática mais cedo na vida.

Vale ressaltar que a maioria dos estudos que investigam os impactos da nicotina leva em consideração apenas a gravidez e/ou gravidez/lactação, ou seja, poucos estudos avaliam os efeitos da exposição ao tabagismo exclusivamente durante o período pós-natal. Como já mencionado, muitas mulheres retomam o hábito de fumar durante a lactação, e com o conjunto dos nossos dados, destacamos os efeitos deletérios da exposição precoce à nicotina exclusivamente na lactação. Assim, mais estudos são necessários para compreender e minimizar os impactos causados pela exposição precoce ao tabagismo, principalmente por estar relacionada com a pandemia do DM2.

Diante de todos os achados encontrados neste trabalho, destacamos que pela primeira vez foi observado a presença de esteatose no pâncreas tanto de fêmeas quanto de machos adultos que foram submetidos ao desmame precoce ou expostos à nicotina durante a lactação. Atribuímos que este acúmulo ectópico de gordura no pâncreas pode ser uma das possíveis causas das disfunções que foram observadas nas ilhotas pancreáticas. Este fenótipo de esteatose pancreática é considerado um indicativo da síndrome metabólica (ZHOU et al., 2016) e assim, com base nestes resultados, esperamos contribuir e despertar novos interesses para o desenvolvimento de outras pesquisas, inclusive clínicas e epidemiológicas para o diagnostico precoce de esteatose pancreática, visto que ela está associada ao desenvolvimento de DM2 e posteriormente ao câncer de pâncreas.

Nosso trabalho demonstra que cada vez mais é necessário reforçar as ações já implementadas e desenvolver novas políticas de proteção, incentivo e apoio ao aleitamento materno e a interrupção do tabagismo não apenas no periodo gestacional, mas também durante a amamentação no intuito de minimizarmos a exposição a fatores de impressão, a fim de prevenir o surgimento de doenças crônicas na vida adulta como obesidade e diabetes.

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ANEXO A - Aprovação do Comitê de Ética Modelo experimental I

COMISSAO DE ETICA PARA O CUIDADO E USO DE ANIMAIS EXPERIMENTAIS (CEUA)

CERTIFICADO

Certificamos que a proposta intitulada **"O desmame precoce afeta a homeostase glicêmica e função das células beta pancreáticas a curto e longos prazos"**, registrada com o nº 036/2018, sob a responsabilidade de **Egberto Gaspar de Moura** - que envolve a produção, manutenção ou utilização de animais pertencentes ao filo Chordata, subfilo Vertebrata (exceto humanos), para fins de pesquisa científica (ou ensino) - 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 PARA O CUIDADO E USO DE ANIMAIS EXPERIMENTAIS (CEUA) do Instituto de Biologia Roberto Alcantara Gomes da UERJ, em reunião de 31/07/2018.

Finalidade	( ) Ensino (X) Pesquisa Científica
Vigencia da autorização	31/07/2022
Espécie/linhagem/raça	Rato Wistar
Nº de animais	220
Peso/Idade	150-600 g / 45 – 180 dias
Sexo	Macho e femea
Origem	Biotério setorial

Rio de Janeiro, 31 de Julho de 2018.

Prof. Dr. Alex C. Manhães
Coordenador
CEUA/IBRAG/UERJ

Profa. Dra. Patricia C. Lisboa
Vice-Coordenadora
CEUA/IBRAG/UERJ

ANEXO B - Aprovação do Comitê de Ética Modelo experimental II

COMISSAO DE ETICA PARA O CUIDADO E USO DE ANIMAIS EXPERIMENTAIS (CEUA)

CERTIFICADO

Certificamos que a proposta intitulada **"Avaliação da homeostase glicêmica e função das células beta pancreáticas em ratos adultos programados pela exposição materna à nicotina na lactação"**, registrada com o nº 038/2018, sob a responsabilidade de **Egberto Gaspar de Moura** - que envolve a produção, manutenção ou utilização de animais pertencentes ao filo Chordata, subfilo Vertebrata (exceto humanos), para fins de pesquisa científica (ou ensino) - 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 COMISSAO DE ETICA PARA O CUIDADO E USO DE ANIMAIS EXPERIMENTAIS (CEUA) do Instituto de Biologia Roberto Alcantara Gomes da UERJ, em reunião de 31/07/2018.

Finalidade	( ) Ensino (X) Pesquisa Científica
Vigencia da autorização	31/07/2022
Espécie/linhagem/raça	Rato Wistar
Nº de animais	150
Peso/Idade	200-600 g / 60 – 180 dias
Sexo	Macho e femea
Origem	Biotério setorial

Rio de Janeiro, 31 de Julho de 2018.

Prof. Dr. Alex C. Manhães
Coordenador
CEUA/IBRAG/UERJ

Profa. Dra. Patricia C. Lisboa
Vice-Coordenadora
CEUA/IBRAG/UERJ

ANEXO C - Comprovação de submissão do 2º artigo científico

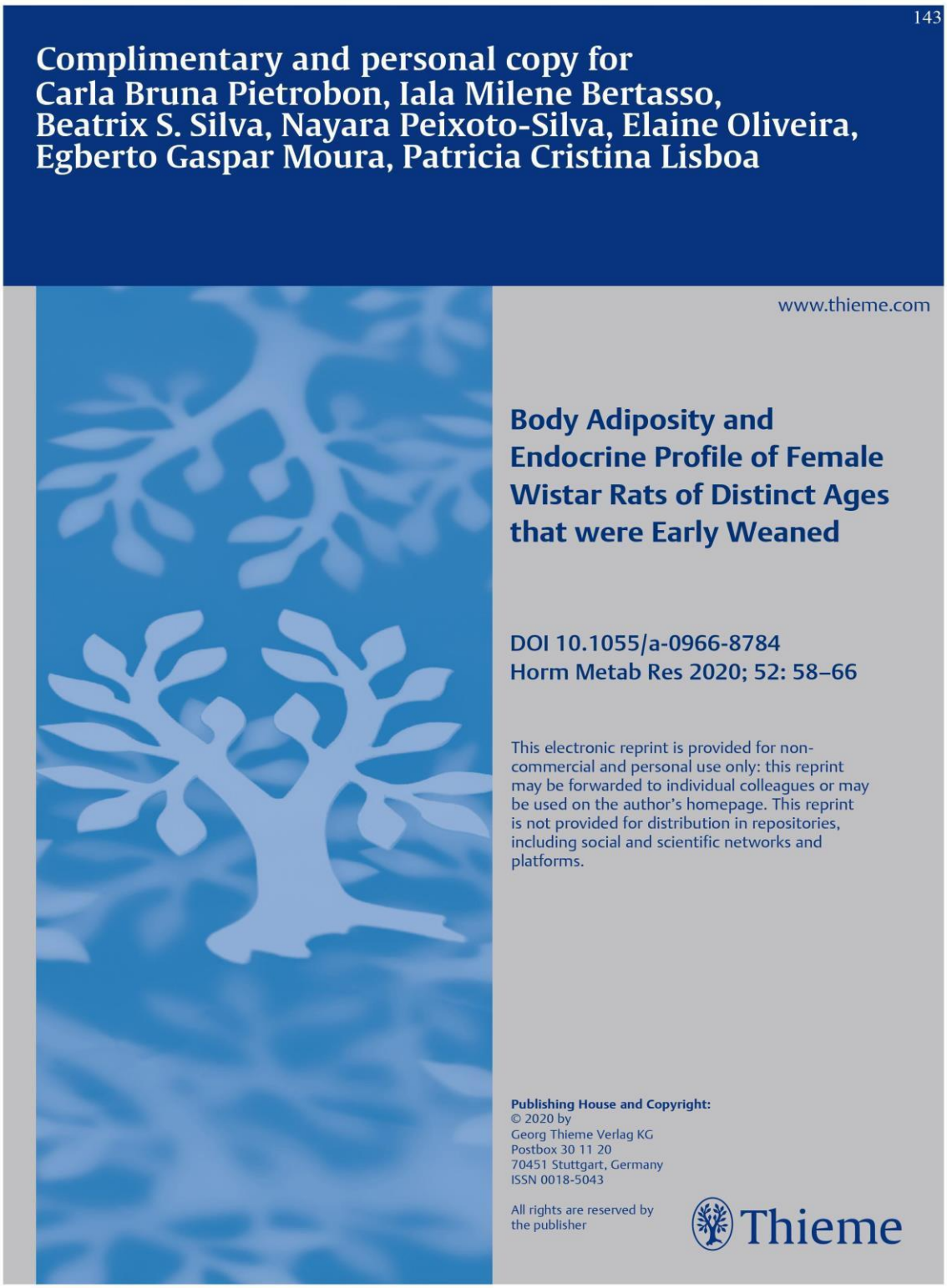
Submission Confirmation

Thank you for your submission

Submitted to	Journal of Developmental Origins of Health and Disease
Manuscript ID	DOHaD-01-20-BR-1271
Title	EARLY WEANING LEADS TO PANCREATIC STEATOSIS IN ADULT WISTAR RATS OF BOTH SEXES.
Authors	Pietrobon, Carla Lisboa, Patricia Bertasso, Iala Moura, Egberto
Date Submitted	17-Jan-2020

Author D

ANEXO D - Comprovantes de publicação de artigos científicos como primeira autora e coautora durante o período do doutorado



Body Adiposity and Endocrine Profile of Female Wistar Rats of Distinct Ages that were Early Weaned

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Body Adiposity and Endocrine Profile of Female Wistar Rats of Distinct Ages that were Early Weaned

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ABSTRACT

Early weaning (EW) is a risk factor for metabolic syndrome. Male rats that were precociously weaned present neonatal malnutrition and, in adulthood, developed overweight, accumulation of body fat, dyslipidemia, changes in glycemic homeostasis, hyperleptinemia, and increase of vitamin D. As metabolic profile of early-weaned females is not known, we investigated the endocrine-metabolic parameters in adolescence and adult female rats of 2 different EW models. Wistar lactating rats and pups from both sexes were separated into 3 groups: non-pharmacological EW (NPEW), dams were involved with a bandage interrupting suckling in the last 3 days of lactation; pharmacological EW (PEW), dams were bromocriptine-treated (0.5 mg/twice a day via intraperitoneal injection) for 3 days before weaning; and control, dams whose pups ate milk throughout lactation. At 21 days-old, NPEW and PEW females had lower body weight. At 180 days-old, NPEW and PEW females showed higher feed efficiency, weight gain, body fat percentage, and greater accumulation of gonadal and retroperitoneal fat depots associated with adipocyte hypertrophy. NPEW females also showed hyperphagia. Only NPEW females presented hyperleptinemia. Plasma thyroid hormones and vitamin D were unchanged among EW females. Regarding sex hormones, at 45 days-old, no change was found in EW females, while at 180 days-old, PEW females had hypoestrogenemia. EW increases the risk for obesity in female rats in adulthood, as already demonstrated for males, although through distinct mechanisms involving some hormones.

Introduction

Exclusive breastfeeding in the first months of life is considered as the most potent intervention against child morbidity and mortality, and a global public health goal [1–3]. Despite several benefits related to breastfeeding during the first six months of life, exclusive breastfeeding during this period occurs in only 41 % of cases worldwide and in 38.6 % of Brazilian's population [3]. The "weaning epidemic" is associated with reduced intellectual performance as well as changes in lipid profile, cardiovascular development, blood pressure, type 2 diabetes mellitus, overweight, and obesity throughout life regardless of gender [4–10].

Considering the nutritional status and its important correlation with development, Hales and Barker [11] proposed the theory of the "thrifty phenotype" suggesting that the development of the fetus is sensitive to the nutritional environment. Therefore, according to the hypothesis of the developmental origins of health and disease (DOHaD), the metabolism of an individual has the capacity to adapt to different environmental factors, leading to a great genetic variability and survival capacity to adverse conditions [12, 13]. Such metabolic programming by insults during the critical windows of development, promotes permanent changes throughout the life [14, 15]. Among the critical developmental periods, lactation presents great programming potential, since it is characterized by the neurological and cognitive development of the child, suggesting that adverse conditions at this stage of life

* CBP and IMB contributed equally to this study.

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Effects of maternal bisphenol A on behavior, sex steroid and thyroid hormones levels in the adult rat offspring

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ABSTRACT

Aims: Bisphenol A (BPA), an endocrine disruptor used in industrial applications, has been detected in both placenta and milk. We studied the effects of BPA exposure during pregnancy and lactation on body composition, palatable food intake, biochemical, hormonal and behavioral profiles of young and adult Wistar rat offspring.
Main methods: Female rats were divided into: control, BPA10 (10 μg/kg/day) and BPA50 (50 μg/kg/day). BPA was administered by gavage to dams from gestation until the end of lactation. Euthanasia occurred at weaning [postnatal day (PN) 21] or adulthood (PN180).
Key findings: At weaning, BPA10 female pups had higher plasma cholesterol and triacylglycerol. BPA10 male pups showed lower plasma T3. BPA10 pups of both sexes had higher plasma progesterone, testosterone and estradiol. At adulthood, females of both BPA groups had lower food intake and higher insulinemia, whereas males had lower visceral fat, lower progesterone and testosterone concentrations. BPA10 females and males had lower T4 levels, while only males showed lower estradiol. BPA50 females showed lower fat mass, higher lean mass and lower corticosteronemia, while males had lower food intake. In the feeding study, BPA10 males ate more fat at 30 min, while BPA10 females and males ingested less fat after 12 h. BPA10 females showed hyperactivity while both groups showed less exploration.
Significance: Maternal exposure to BPA during gestation and lactation, even at low doses, induces life-long changes in the regulation of metabolic homeostasis of the progeny, affects sex steroids and thyroid hormones levels, compromises behavior, but does not lead to obesity or dyslipidemia.

1. Introduction

Adverse events during the intrauterine and postnatal periods can modify gene expression and lead to permanent changes in metabolism and hormonal regulation [1,2]. Maternal exposure, during pregnancy and breastfeeding, to insults such as inadequate nutrition, social stress and environmental pollutants can induce obesity and its comorbidities later in life [3–5]. Studies in humans have identified that environmental pollutants are potential risk factors in the development of several diseases [6,7]. Substances defined as endocrine disrupting chemicals (EDCs) or "exogenous agents" have been associated with numerous health problems, such as reproductive dysfunction, obesity, diabetes, among others disturbances [8].

Bisphenol A (BPA) is one of the most used EDCs worldwide since it is a starting material for the synthesis of plastics (such as toys, tools, flame retardants, antioxidants and pesticides). It has bioaccumulation characteristics [8–10] and an annual production that exceeds 3.5 million tons [11]. The U.S. Environmental Protection Agency (EPA) and the U.S. Food and Drug Administration (FDA) established a BPA reference dose of 50 μg/kg BW/day [12,13], while the European Food Safety Authority (EFSA) estimated as tolerable a daily intake (TDI) of 4 μg BPA/kg BW/day [14].

BPA is considered a xenoestrogen, capable of binding to endogenous estrogen receptors [15,16]. Besides, it can bind to receptors of testosterone [17,18], thyroid hormones [19,20] and glucocorticoids [21]. BPA has been detected in amniotic fluid, neonatal blood, placenta,

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Short and long-term effects of bisphenol S (BPS) exposure during pregnancy and lactation on plasma lipids, hormones, and behavior in rats☆

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ABSTRACT

Bisphenol S (BPS) has replaced bisphenol A (BPA), a known non-persistent endocrine disrupting chemical, in several products. Considering that little is known regarding BPS effects, especially during critical windows of ontogenetic development, and that BPA, which is quite similar to BPS, is know to be transferred to the offspring via the placenta and milk, in the present study we investigated the behavioral, biochemical and endocrine profiles of Wistar rats born from dams that were BPS-exposed [groups: BPS10 (10 μg/kg/day), BPS50 (50 μg/kg/day)] during pregnancy and lactation. Due to the non-monotonic dose-response effect of bisphenol, the data of both BPS groups were directly compared with those of the controls, not to each other. Males and females were analyzed separately. At weaning, male BPS50 offspring had hypotriglyceridemia and hyperthyroxinemia, whereas BPS50 females showed higher 25(OH)D levels. At adulthood, BPS offspring of both sexes had lower food intake. BPS males showed lower visceral adiposity. BPS50 females had smaller fat droplets in brown adipocytes. BPS males showed higher anxiety and higher locomotor activity, while BPS10 females showed lower exploration. During a food challenge test at adulthood, BPS males consumed more high-fat diet at 30 min. BPS10 females initially (at 30 min) consumed more high-fat diet but, after 12 h, less of this diet was consumed. BPS50 males had hypertriglyceridemia and lower plasma T3, while BPS females showed lower plasma T4. BPS10 females had lower progesterone, whereas BPS50 females had higher plasma 25(OH)D. Maternal BPS exposure has adverse effects on the triacylglycerol, hormones levels and behavior of the progeny. Furthermore, the increased preference for the fat-enriched diet suggests an increased risk for obesity and its health consequences in the long term.

1. Introduction

One of the most studied non-persistent Endocrine Disrupting Chemicals (EDCs) is bisphenol A (BPA), initially used as a synthetic estrogen, but currently used in the manufacture of polyvinyl-chloride films (PVC), food packaging (Dziewirska et al., 2018; Novo et al., 2018; Son et al., 2018), metallic cans and plastic packaging of food and beverage, medical devices, dental materials, computers, thermal papers, among others (Zbucka-Kretowska et al., 2018). BPA acts as an endogenous estrogen by interacting with estrogen receptors (Alonso-Magdalena et al., 2008). BPA may be associated with a higher risk of developing diabetes, obesity, cardiovascular disease and sexual dysfunction (Bernier and Vandenberg, 2017), even at levels equal to or lower than 50 μg BPA/kg body weight/day for humans, a level used as reference for daily human BPA consumption by the EPA (Environmental Protection Agency) (Altamirano et al., 2015; García-Córcoles et al., 2018) and FDA (Food Drug Administration). However, the European Food Safety Authority (EFSA) has estimated an acceptable daily intake of only 4 μg

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ORIGINAL CONTRIBUTION

Early weaning alters the thermogenic capacity of brown adipose tissue in adult male and female rats

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Abstract

Purpose Early weaning (EW) is a risk factor for obesity development. Brown adipose tissue (BAT) hypofunction is related to obesity onset. Here, we evaluated whether sympathetic nervous system (SNS) activity in BAT and the thermogenic function of BAT are decreased in adulthood in obese rats from two EW models.

Methods At the time of birth, lactating Wistar rats and their pups (three males and three females) were separated into three groups: the control group, in which pups consumed milk throughout lactation; the non-pharmacological EW (NPEW) group, in which suckling was interrupted with a bandage during the last 3 days of lactation; and the pharmacological EW (PEW) group, in which dams were treated with bromocriptine (0.5 mg/twice a day) 3 days before weaning. The offspring were sacrificed on PN180.

Results Adult male rats from both EW models exhibited lower BAT SNS activity. Female rats from the PEW group showed a decrease in BAT SNS activity. The protein levels of UCP1 were lower in the NPEW males, while PGC1α levels were lower in both PEW and NPEW males. Both groups of EW females showed reductions in the levels of β3-AR, TRβ1, and PGC1α. The UCP1 protein level was reduced only in the NPEW females. The EW groups of both sexes had lower AMPK protein levels in BAT. In the hypothalamus, only the PEW females showed an increase in AMPK protein levels. In both groups of EW males, adrenal catecholamine was increased and tyrosine hydroxylase was decreased, while in EW females, adrenal catecholamine was decreased.

Conclusions Early weaning alters the thermogenic capacity of BAT, which partially contributes to obesity in adulthood, and there are sex-related differences in these alterations.

Keywords Breastfeeding · Early weaning · Bromocriptine · Developmental plasticity · Metabolic syndrome · Autonomic function

Introduction

Environmental and nutritional changes during periods of great plasticity, such as intrauterine life and/or lactation, can cause metabolic disorders in offspring. This phenomenon is known as metabolic programming [1–4]. Early weaning can lead to metabolic programming. Epidemiological data have shown that exclusive breastfeeding up to 6 months of age is protective against the development of obesity in adulthood [5–8]. In rats, we have shown the long-term adverse effects of breast milk deprivation, which include programming for metabolic syndrome [9–12]. In a pharmacological early weaning (PEW) model, bromocriptine, a type 2 dopamine agonist that inhibits prolactin production/secretion, is administered to mothers during the last 3 days of lactation, and this programs adult male offspring for obesity, hyperleptinemia, insulin resistance, dyslipidaemia, central hypothyroidism, and high concentrations of adrenal hormones [9, 10]. Lima et al. [11, 12] developed a non-pharmacological early weaning (NPEW) model in which a bandage is applied to mothers during the last 3 days of lactation. They found that adult males are programmed for obesity, higher body fat, insulin resistance, hyperleptinemia, and hypertriglyceridemia [11, 12]. The aforementioned metabolic and hormonal alterations

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ORIGINAL ARTICLE

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Early weaning leads to specific glucocorticoid signalling in fat depots of adult rats

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Abstract

Purpose Early weaning (EW) is a stressful condition that programmes a child to be overweight in adult life. Fat mass depends on glucocorticoids (GC) to regulate adipogenesis and lipogenesis. We hypothesised that the increased adiposity in models of EW was due to a disturbed HPA axis and/or disrupted GC function.

Methods We used two experimental models, pharmacological early weaning (PEW, dams were bromocriptine-treated) and non-pharmacological early weaning (NPEW, dams' teats were wrapped with a bandage), which were initiated during the last 3 days of lactation. Offspring from both genders was analysed on postnatal day 180.

Results Offspring in both models were overweight with increased visceral fat mass, but plasma corticosterone was increased in both genders in the PEW group but not the NPEW group. NPEW males had increased GRα expression in visceral adipose tissue (VAT), and GRα expression decreased in PEW males in subcutaneous adipose tissue (SAT). Females in both EW groups had increased 11βHSD1 expression in SAT. PEW males had increased C/EBPβ expression in SAT. PEW females had lower PPARγ and FAS expression in VAT than the NPEW females. We detected a sex dimorphism in VAT and SAT in the EW groups regarding 11βHSD1, GRα and C/EBPβ expression.

Conclusions The accumulated adiposity induced by EW exhibited distinct mechanisms depending on gender, specific fat deposition and GC metabolism and action. The higher proportion of VAT/SAT in both sets of EW males may be related to the action of GC in these tissues, and the higher conversion of GC in SAT in females may explain the differences in the fat distribution.

Keywords Early weaning · Glucocorticoids · Adipogenesis and lipogenesis

Introduction

The interruption of exclusive breastfeeding is related to a susceptibility to chronic diseases in adulthood, such as obesity and associated comorbidities. This phenomenon is called "metabolic programming", and it describes the influence of early nutritional, hormonal and environmental insults during critical phases of development; the phases include gestation and lactation period, and disruptions at these points could predispose individuals to long-term non-communicable diseases [1, 2].

Breastfeeding inhibition deprives children of the natural bioactive factors present in milk, including hormones, growth factors and essential nutrients [3], and it induces several dysfunctions [4]. The World Health Organisation [5] suggests the initiation of complementary feeding at 6 months of age. The interruption of lactation before this period is considered "early weaning" (EW), which is a stressful status that is prejudicial to babies primarily because it is associated with gastrointestinal and airways diseases, under-nutrition, obesity and diabetes [6].

Our group has two well-established models of programming based on EW, in which milk yield is reduced during the last 3 days of lactation via the pharmacological inhibition of prolactin using administration of a type 2 dopaminergic receptor agonist, bromocriptine [7], or the non-pharmacological interruption of lactation using a bandage that covers all of the mothers' teats [7, 8], which causes malnutrition in pups and leads to several distinct

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Programming of hepatic lipid metabolism in a rat model of postnatal nicotine exposure – Sex-related differences☆

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ABSTRACT

Maternal nicotine exposure during lactation induces liver damage in adult male rats. However, the mechanism in males is unknown and females have not been tested. Here, we determined the liver lipid composition and lipogenic enzymes in male and female offspring at two ages in a model of postnatal nicotine exposure. Osmotic minipumps were implanted in lactating Wistar rat dams at postnatal day (PND) 2 to release 6 mg/kg/day of nicotine (NIC group) or saline (CON group) for 14 days. Offspring received a standard diet from weaning until euthanasia at PND120 (1 pup/litter/sex) or PND180 (2 pups/litter/sex). At PND120, NIC males showed lower plasma triglycerides (TG), steatosis degree 1, higher hepatic cholesterol (CHOL) ester, free fatty acids, monoacylglycerol content as well as acetyl-coa carboxylase-1 (ACC-1) and fatty acid synthase (FAS) protein expression in the liver compared to CON males. At this age, NIC females had preserved hepatocytes architecture, higher plasma CHOL, higher CHOL ester and lower total CHOL content in the liver compared to CON females. At PND180, NIC males showed steatosis degrees 1 and 2, higher TG, lower free fatty acids and total CHOL content in the liver and an increase in ACC-1 hepatic protein expression. NIC females had higher plasma TG and CHOL levels, no change in hepatic morphology, lower CHOL ester and free fatty acids in the liver, which also showed higher total ACC-1 and FAS protein expression. Maternal nicotine exposure induces long-term liver dysfunction, with an alteration in hepatic cytoarchitecture that was aggravated with age in males. Concerning females, despite unchanged hepatic cytoarchitecture, lipid metabolism was compromised, which deserves further attention.

1. Introduction

Nonalcoholic Fatty Liver Disease (NAFLD) is an inclusive term for a group of non-alcohol-related progressive liver disorders (Angulo, 2002) that has become a serious public health problem worldwide, affecting more than half billion people worldwide (Younossi et al., 2018) and currently being the main cause of liver transplantation and carcinoma (Pais et al., 2016; Michelotti et al., 2013). Steatosis is the first manifestation of NAFLD, which is characterized by excessive accumulation of triglycerides (TG). In normal situations, this accumulation is not hepatotoxic and represents a defensive mechanism to balance free fatty acids excess (Koliwad et al., 2010; Yamaguchi et al., 2007). However, increased TG concentration happens simultaneously with toxic metabolites generation, lipotoxicity, liver injury, activation of inflammatory cascades and fibrogenesis (Neuschwander-Tetri, 2010). In this case, steatosis

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