Universidade Federal do Maranhão
Centro de Ciências Biológicas e da Saúde
Programa de Pós-Graduação em Ciências da Saúde

EXPOSIÇÃO PROLONGADA À DIETA RICA EM SACAROSE SUPRIME VIAS ADAPTATIVAS AO ESTRESSE DO RETÍCULO ENDOPLASMÁTICO EM HEPATÓCITOS E POTENCIALIZA A LIPOGÊNESE *DE NOVO* EM CAMUNDONGOS

KARLA FRIDA TORRES FLISTER

São Luís – MA
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Tese apresentada ao Programa de Pós-Graduação em Ciências da Saúde da Universidade Federal do Maranhão, como requisito para obtenção do título de Doutora em Ciências da Saúde.

Orientador: Prof. Dr. Antonio Marcus de Andrade Paes

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“A percepção do desconhecido é a mais fascinante das experiências. O homem que não tem os olhos abertos para o misterioso passará pela vida sem ver nada.”

Albert Einstein

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RESUMO

A doença hepática gordurosa não alcoólica (DHGNA) é definida como o acúmulo anormal de triglicerídeos no interior de hepatócitos (esteatose) na ausência de alcoolismo crônico. Em situações de estresse sustentado, esta esteatose pode evoluir para um padrão inflamatório e fibrótico, predispondo a doenças hepáticas mais severas. Nesse contexto, o presente estudo procurou investigar o papel das perturbações na lipogênese e do estresse do retículo endoplasmático (ERE) induzidos pelo consumo de sacarose e o desenvolvimento de DHGNA. Para isto, camundongos Swiss recém desmamados foram alimentados com uma dieta rica em sacarose (HSD) por 30, 60 e 90 dias e comparados ao grupo controle. O desenvolvimento de síndrome metabólica (MetS), o conteúdo de gordura hepática, análise histológica e as expressões no tecido hepático de genes relacionados a lipogênese e ERE foram avaliados nos grupos experimentais. A exposição a dieta HSD promoveu distúrbios metabólicos de forma progressiva com o tempo, com estabelecimento total da MetS após 60 dias, caracterizada por obesidade central, hiperglicemia, dislipidemia e resistência à insulina. Ademais, estes animais apresentaram elevação do conteúdo de gordura hepática e esteatose microvesicular. Após 30 dias de intervenção nutricional foi observado um equilíbrio entre a síntese de ácidos graxos (ChREBP e SCD1) e sua oxidação (PPARα) assim como expressão das vias adaptativas da UPR, caracterizadas pela expressão dos sensores da UPR (IRE1α, PERK e ATF6), chaperonas (GRP78 e PDI A1) e do fator de transcrição de defesa antioxidante (NRF2). Enquanto que a partir de 60 dias, observou-se de forma marcante aumento dos fatores de transcrição da lipogênese (ChREBP e SREBP-1c), elevação exponencial da síntese de ácidos graxos (SCD1) e ERE caracterizado por um padrão apoptótico (CHOP). Em conjunto, nossos resultados demonstraram que as alterações metabólicas induzidas pelo consumo da sacarose promoveram desregulação da síntese *de novo* de lipídeos (DNL) e ativação de vias adaptativas em resposta ao estresse do retículo, entretanto com a persistência da dieta observou-se falência destas vias seguida de ativação exacerbada da DNL, levando a crescente acúmulo de gordura nos hepatócitos e subsequente DHGNA.

Palavras-chave: Doença hepática gordurosa não alcoólica; Estresse do retículo endoplasmático; Lipogênese; Dieta rica em sacarose; Esteatose microvesicular; Síndrome metabólica.

ABSTRACT

Non-alcoholic fatty liver disease (NAFLD) is defined as the abnormal triglyceride accumulation into hepatocytes (steatosis) in the absence of chronic alcoholism. Upon unsolved stress, this steatosis evolve to inflammatory and fibrotic pattern, predisposing to worsen hepatic diseases. In the context, the present study sought to investigate the role of disrupted lipogenesis and endoplasmic reticulum (ER) stress sucrose intake-induced to NAFLD development. For this, post-weaned Swiss mice were fed a high-sucrose diet (HSD) for 30, 60 and 90 days and compared to control. Metabolic syndrome (MetS) development, liver fat content, histological analysis and hepatic gene expressions of lipogenesis and ER stress were assessed. Exposure to HSD promoted progressive metabolic disturbances in a time-dependent manner leading to full establishment of MetS upon 60 days, characterized by central obesity, hyperglicemia, dyslipidemia and insulin resistance. Moreover, these animals presented increased fat liver content and microvesicular steatosis. After 30 days of nutritional intervention were found a balance between fatty acids synthesis (ChREBP and SCD1) and oxidation (PPARα) as well as an UPR-adaptive pathways featured by gene expressions of UPR sensors (IRE1α, PERK and ATF6), chaperones (GRP78 and PDI A1) and antioxidant defense transcription factor (NRF2). Whereas since 60 days were marked by higher gene expression of lipogenesis transcription factors (ChREBP and SREBP-1c), exponential raise of fatty acids synthesis (SCD1), and ER stress characterized by an apoptotic (CHOP) pattern. In summary, our results demonstrated that metabolic changes induced by sucrose consumption promoted a disregulation of de novo lipogenesis (DNL) and activation of adaptive pathways in response to ER stress, however with diet maintainence, it was observed failure of these pathways followed by an exacerbated DNL activation, which lead to increased fat accumulation in hepatocytes and subsequent NAFLD.

KEYWORDS

Non-alcoholic fatty liver disease; Endoplasmic reticulum stress; Lipogenesis; High-sucrose diet; Microvesicular steatosis; Metabolic syndrome

LISTA DE SIGLAS E ABREVIATURAS

ACC	Acetil CoA carboxilase
Acetil coA	Acetil coenzima A
AG	Ácidos graxos
ALT	Alanina aminotransferase
AST	Aspartato aminotranferase
ATF4	Fator 4 de ativação transcricional
ATF6	Fator 6 de ativação transcricional
ATF6f	Fator 6 de ativação transcricional fosforilado
Bak	Assassina/antagonista do Bcl2
Bax	Proteína X associada ao Bcl2
Bcl2	Célula B de linfoma 2
BiP	Proteína ligante de imunoglobulina (ver GRP78)
C/EBP	Proteínas ligantes ao amplificador CCAAT
ChREBP	Proteína de ligação ao elemento responsivo a carboidrato
CHOP	Proteína homóloga da C/EBP
DGAT2	Diacilglicerol acetiltransferase
DHGNA	Doença hepática gordurosa não alcoólica
DNA	Ácido desoxirribonucleico
DNL	síntese *de novo de lipídeos*
EHNA	Esteato hepatite não alcóolica
eIF2α	Fator eucariótico iniciador de tradução 2, subunidade alfa
ERAD	Mecanismo de degradação associado ao retículo endoplasmático
ERE	Estresse do retículo endoplasmático
EROS	Espécies reativas de oxigênio
FAS	Ácido graxo sintase
GADD34	Indutor 34 de dano ao DNA
GRP78	Proteína regulada por glicose 78 (ver BiP)
IL-6	Interleucina 6
IMC	Índice de massa corpórea

Insig	Gene induzido por insulina
IRE1	Enzima 1 dependente de inositol
IκB	Inibidor do Kappa B
JNK	Quinase c-jun n-terminal
Keap1	Proteína kelch-associado como ECH-1
MTP	Proteína de transferência microssomal
NAS	Escore numérico de atividade
NF-κB	Fator nuclear kappa B
NRF2	Fator nuclear eritróide 2 relacionado ao fator 2
p53	Gene supressor tumoral p53
PDI	Proteína dissulfeto isomerase
PERK	Proteína quinase do retículo endoplasmático *PKR-like*
PKC	Proteína cinase C
PPARα	Receptor alfa ativado por proliferadores de peroxissomas
PPARγ	Receptor gama ativado por proliferadores de peroxissomas
RE	Retículo endoplasmático
RI	Resistência à insulina
RIDD	Mecanismo de decaimento regulado de RNAm Ire1 dependente
RNA	Ácido ribonucleico
RNAm	RNA mensageiro
SCAP	Proteína ativadora da clivagem de SRBP
SCD1	Estearoil-CoA desaturase
SM	Síndrome metabólica
SREBP-1c	Proteína 1c de ligação ao elemento responsivo de esterol
SREBP2	Proteína 2 de ligação ao elemento responsivo de esterol
TG	Triglicerídeos
TNF-α	Fator de necrose tumoral alfa
UPR	Resposta a proteínas mal enoveladas (*Unfolded protein response*)
VLDL	Lipoproteína de densidade muito baixa
XBP1	Proteína 1 de ligação à caixa X

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SUMÁRIO

1. INTRODUÇÃO

A doença hepática gordurosa não alcoólica (DHGNA) é uma condição clínica de fisiopatologia complexa e multifatorial, que engloba um espectro de lesões hepáticas caracterizadas inicialmente pelo acúmulo de gordura nos hepatócitos na ausência de alcoolismo crônico, infecções virais e outras doenças hepáticas (Tiniakos *et al.*, 2010). Este acúmulo de gordura ectópica hepática (esteatose) pode progredir para um estágio necro-inflamatório com ou sem presença de fibrose denominado de esteato hepatite não alcóolica (EHNA), o principal fator de risco para o desenvolvimento de outras doenças hepáticas mais graves, tais como cirrose, falência hepática e carcinoma hepatocelular (Dietrich e Hellerbrand, 2014; Povero e Feldstein, 2016).

A DHGNA têm crescido em proporções epidêmicas, acometendo cerca de 25% da população adulta mundial e é considerada a manifestação hepática da síndrome metabólica (SM) (Ahmed, 2015). A SM é definida como um conjunto de disfunções metabólicas interconectadas que aumentam os riscos para o desenvolvimento de doenças cardiovasculares e diabetes *mellitus* tipo 2 (Alberti *et al.*, 2005). Obesidade central, resistência à insulina (RI), dislipidemia aterogênica, DHGNA, hipertensão e distúrbios trombóticos são morbidades que compõem esta síndrome (Hoffman *et al.*, 2015). A DHGNA têm sido considerada um fator de risco precoce para o desenvolvimento de doenças cardiovasculares em indivíduos obesos (Vanwagner *et al.*, 2012; Anstee *et al.*, 2013).

Levantamentos epidemiológicos têm demonstrado que o aumento da prevalência de DHGNA e de outras alterações associadas a SM está diretamente associado ao crescente consumo de açucares de adição (sacarose, frutose ou xarope de milho), comumente utilizados como edulcorantes em refrigerantes e alimentos processados (Bray *et al.*, 2004; Moore *et al.*, 2014; Wijarnpreecha *et al.*, 2016).

O consumo destes açucares promove um balanço energético positivo com consequente aumento dos depósitos de gordura visceral, essenciais para o desenvolvimento da obesidade, RI e diabetes (Tappy e Le, 2012; Ma *et al.*, 2015; Stanhope, 2016). Adicionalmente, estes açucares atuam diretamente promovendo a ativação da síntese *de novo* de lipídeos (DNL), que por sua vez está relacionada à

desregulação do metabolismo de lipídeos e carboidratos e o desenvolvimento de esteatose hepática (Dinicolantonio *et al.*, 2017).

A ativação desregulada da DNL induzida pelo consumo excessivo de glicose e/ou frutose contribui para o desenvolvimento de DHGNA ao promover uma maior síntese de ácidos graxos, aumento do conteúdo intra-hepático de triglicerídeos (TG) e seus produtos intermédiarios lipotóxicos, além do aumento da produção e secreção de lipoproteína de densidade muito baixa (VLDL), que favorece o acúmulo ectópico de lipídeos no músculo e a instalação do quadro de RI periférica (Lombardo *et al.*, 1996; Hein *et al.*, 2010). Durante a desregulação da DNL ocorre a sobrecarga da β-oxidação mitocondrial, estresse oxidativo, dislipidemias e sinais inflamatórios, que favorecem a progressão de um esteatose simples para uma esteato-hepatite (Abid *et al.*, 2009; Choi *et al.*, 2017; Softic *et al.*, 2017).

Mesmo com o elevado número de estudos, os mecanismos moleculares envolvidos no desenvolvimento e progressão da DHGNA induzida por açucares ainda continuam obscuros. Neste contexto, o quadro de estresse do retículo endoplasmático (ERE) têm emergido como um possível alvo no estudo da patogênese de doenças hepáticas.

A desregulação do metabolismo lipídico no fígado, além das alterações metabólicas associadas a SM proporcionam perturbações na homeostase do retículo endoplasmático (RE), podendo levar a inibição de atividades de chaperonamento e de degradação proteassomal, causando desta forma acúmulo de proteínas mal enoveladas no lúmen do RE (Lake *et al.*, 2014). O desequilíbrio entre a produção destas proteínas mal enoveladas e a capacidade do retículo de erradicá-las define o estresse do retículo endoplasmático (ERE), uma condição celular favorável para o agravamento de diversas doenças (Rutkowski *et al.*, 2008).

Com o objetivo de preservar a integridade celular e restaurar a homeostase do RE induzido pelo estresse, uma série de eventos adaptativos conhecida como resposta a proteínas mal enoveladas (*Unfolded protein response* - UPR) são ativados. Dentre os principais eventos adaptativos, destacam-se a inibição da síntese global de proteínas, sintese de proteínas chaperonas, degradação proteassomal, síntese de enzimas antioxidantes e autofagia (Hetz, 2012). Entretanto, em condições de manutenção deste ERE por um tempo prolongado (por exemplo, doenças crônicas

como a DHGNA e SM), as mesmas proteínas sensoras da UPR responsáveis por iniciar uma sinalização pró-adaptativa ao estresse podem migrar para uma sinalização pró-apoptótica, levando a uma morte celular precoce (Rutkowski *et al.*, 2006).

Neste contexto, evidências atuais indicam que o consumo de dietas ricas em carboidratos associado ao aumento de ácidos graxos saturados provenientes da DNL podem desencadear ERE, contribuindo dessa forma para o desenvolvimento de estatose simples e à progressão da mesma para esteato-hepatite. No entanto os mecanismos pelos quais isto ocorre ainda não se encontram totalmente elucidados (Zhang *et al.*, 2012; Liu *et al.*, 2015; Balakumar *et al.*, 2016).

Desta forma, hipotetizamos em nosso estudo que as alterações metabólicas da SM induzidas pelo consumo de sacarose promovem a desregulação da DNL e ERE, que em conjunto contribuem para um maior acúmulo de gordura hepática e desenvolvimento de DHGNA e hepatopatias mais severas. A partir disso, nosso estudo tem como objetivo principal investigar a relação entre o consumo de dieta rica em sacarose em camundongos, ativação das vias de sinalização de lipogênese e estresse do retículo endoplasmático e o desenvolvimento de doença hepática gordurosa não alcoólica.

Esta caracterização é essencial para um maior entendimento das vias bioquímicas e moleculares responsáveis pelo acúmulo de gordura hepática, facilitando a elaboração de estratégias profiláticas e/ou terapêuticas mais eficazes no combate dessa doença.

2. REFERENCIAL TEÓRICO

2.1 Aspectos gerais da síndrome metabólica e doença hepática gordurosa não alcoólica

A síndrome metabólica (SM) é definida como um conjunto de disfunções metabólicas interconectadas que aumentam os riscos para o desenvolvimento de doenças cardiovasculares e diabetes *mellitus* tipo 2 (Alberti *et al.*, 2005). Obesidade central, resistência à insulina (RI), dislipidemia aterogênica, hipertensão e distúrbios trombóticos são morbidades que compõem esta síndrome (Hoffman *et al.*, 2015). Todas estas morbidades metabólicas são considerados fatores de risco para o desenvolvimento da doença hepática gordurosa não alcoólica (DHGNA) (Gastaldelli, 2010; Kim *et al.*, 2018; Lim e Bernstein, 2018), assim como a DHGNA é considerada um fator predisponente para o desenvolvimento dos componentes da SM (Lindenmeyer e Mccullough, 2018; Lonardo *et al.*, 2018).

A DHGNA é o espectro de lesões relacionadas ao acúmulo excessivo de lipídeos no citoplasma dos hepatócitos, em indivíduos sem histórico de ingestão de álcool relevante (Chalasani *et al.*, 2012; Anstee *et al.*, 2013). A DHGNA tem sido considerada uma doença multifatorial envolvendo fatores genéticos, ambientais e relacionados ao estilo de vida (dieta e sedentarismo), que apresenta um amplo espectro clínico-histológico que varia da esteatose simples, acúmulo de gordura sem evidência de lesão hepatocelular (balonização), até a esteatohepatite não alcoólica (EHNA) caracterizada pela presença de esteatose, infiltrado inflamatório e balonização com ou sem a presença de fibrose. A EHNA pode progredir para formas mais graves como cirrose, falência hepática e carcinoma hepatocelular (Tiniakos *et al.*, 2010; Satapathy e Sanyal, 2015; Marengo *et al.*, 2016).

A prevalência da DHGNA está aumentando em todo o mundo, paralelamente associada com o aumento da obesidade e diabetes tipo 2 (Younossi *et al.*, 2016; Jensen *et al.*, 2018). Estima-se que a prevalência da DHGNA na população geral varia entre 20-30%, enquanto em indivíduos com índice de massa corporal acima de 30 Kg/m^2 a prevalência varia entre 80-95% e, entre pacientes diabéticos do tipo 2 varia de 45 a 85% (Sayiner *et al.*, 2016; Dai *et al.*, 2017).

Na literatura científica são descritos dois modelos que tentam explicar as bases fisiopatológicas envolvidas com progressão da esteatose simples para EHNA. O primeiro modelo foi proposto em 1998 por Day & James, conhecido como modelo de dois impactos (*two hits*), no qual o desenvolvimento da EHNA envolve um insulto inicial que aumenta a predisposição para injúrias e desfechos celulares mais severos. Os insultos iniciais denominados de primeiro impacto (*first hit*) podem ser gerados pela obesidade, sedentarismo, consumo de dietas ricas em gorduras ou RI, que em conjunto provocam o acúmulo de ácidos graxos (AG) nos hepatócitos. Estes ácidos graxos aumentam a predisposição para à ocorrência do segundo impacto (*second hit*), caracterizado pelo estresse oxidativo, gerado pela elevação da beta oxidação e redução das defesas antioxidantes, promovendo uma ação lesiva e persistente no hepatócito, que favorece o surgimento do processo inflamatório e o desenvolvimento da EHNA (Day e James, 1998).

No entanto, essa teoria apresenta uma visão simplificada dos diversos fatores envolvidos no desenvolvimento da DHGNA. Atualmente, defende-se que a gênese da DHGNA envolve múltiplos fatores, atuando de forma sinérgica e simultânea em indivíduos geneticamente predispostos, resultando nos processos inflamatórios e fibróticos que contribuem para o desenvolvimento da EHNA (Buzzetti *et al.*, 2016). Esta teoria destaca a RI periférica como condição inicial para o acúmulo de AG no hepatócito (primeiro *hit*), uma vez que favorece a lipogênese e inibe a lipólise, aumentando excessivamente o aporte desse substrato para o fígado. Como resultado disto, o fígado esteatótico se torna vulnerável a múltiplos impactos (*multiple hits*), não apenas o estresse oxidativo, mais também ao estresse do retículo endoplasmático (ERE), as citocinas pró-inflamatórias produzidas pelo tecido adiposo e pela microbiota, que proporcionam o desenvolvimento das lesões hepatocelulares, inflamação e fibrose (Tilg e Moschen, 2010).

2.1.1 Alterações histológicas e diagnóstico da DHGNA

A maioria dos pacientes com DHGNA são assintomáticos, embora alguns indivíduos possam apresentar fadiga, hepatoesplenomegalia e dispepsia (Fierbinteanu-Braticevici *et al.*, 2017). O diagnóstico em geral ocorre com a detecção da elevação de enzimas hepáticas (aspartato aminotranferase-AST e alanina

aminotransferase-ALT) em exames de rotina. Outras formas de diagnóstico envolvem os métodos de imagem (ultrassonografia, espectroscopia por ressonância magnética) e a biopsia hepática (Shen e Lu, 2016; Hadizadeh *et al.*, 2017). A elevação das aminotransferases AST/ALT, assim como os exames de imagem não permitem a diferenciação entre a esteatose e a EHNA, sendo a biopsia hepática o padrão ouro para o diagnóstico, avaliação do tratamento e prognóstico da DHGNA (Xiao *et al.*, 2017).

A DHGNA é caracterizada histologicamente pela presença de esteatose em 5% dos hepatócitos e não requer definição de localização, enquanto a EHNA é caracterizada pela presença de esteatose e inflamação hepática com lesão hepatocitária (balonização), com ou sem fibrose (EASL-EASD-EASO Clinical Practice Guidelines for the management of non-alcoholic fatty liver disease, 2016a).

As alterações morfológicas da esteatose hepática manifestam-se com o acúmulo de gordura em pequenas (microvesicular) ou grandes gotículas (macrovesicular). A esteatose do tipo macrovesicular é a forma de maior predominância entre pacientes e é caracterizada pela presença de um grande e único vacúolo de gordura que desloca o núcleo do hepatócito para periferia, enquanto a esteatose microvesicular é formada por pequenos e numerosos vacúolos de gordura sem a descentralização do núcleo e reflete alterações mais graves do metabolismo celular relacionadas a prejuízos da β-oxidação mitocondrial (Figura 1). Na DHGNA a esteatose acomete primeiramente a zona 3 do ácino, e nos casos em que há evolução para cirrose esse achado histológico pode diminuir ou até mesmo desaparecer (Dietrich e Hellerbrand, 2014; Kleiner e Makhlouf, 2016).

A presença de degeneração hepática (balonização) é caracterizada por hepatócitos arredondados com citoplasma finamente granulares decorrentes de alterações da permeabilidade celular (Figura 2). A presença deste achado é fundamental para o diagnóstico de EHNA e está relacionada com o risco de progressão para fibrose. As células baloniformes podem conter em seu citoplasma agregados de proteínas do citoesqueleto formando inclusões homogêneas, eosinofílicas e perinucleares denominadas de Corpúsculos de Mallory, que são inclusões frequentemente circundadas por neutrófilos devido sua propriedade quimiotáxica. Outro achado são as megamitocôndrias, que aparecem como inclusões

citoplasmáticas em formas de agulhas ou arredondadas (Nalbantoglu e Brunt, 2014; Takahashi e Fukusato, 2014).

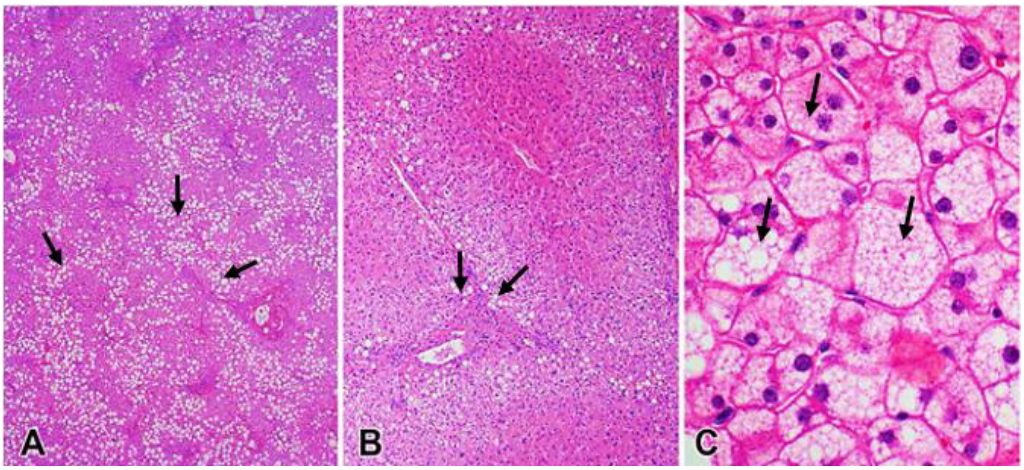

Figura 1. Esteatose microvesicular. A, padrão de esteatose microvesicular (40x); **B,** ampliação em 100x; e **C,** ampliação em 600x. **Fonte:** Kleiner e Makhlouf (2016).

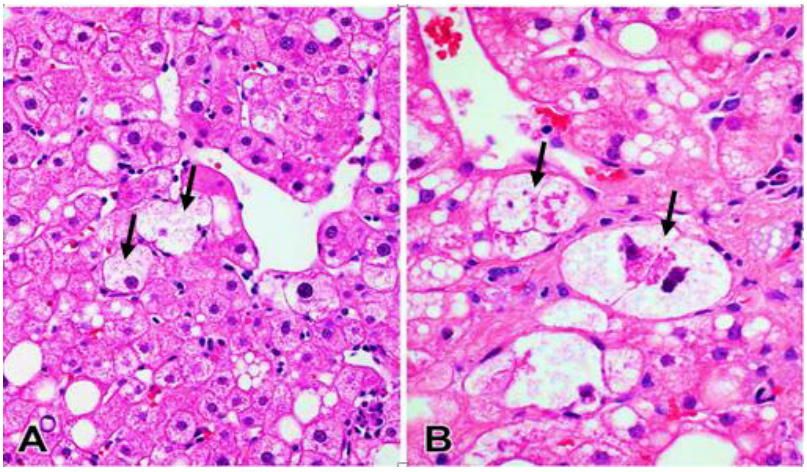

Figura 2. Hepatócitos com balonização. A, hepatócitos balonizados (400x); **B,** ampliação da lâmina A (600x). **Fonte:** Kleiner e Makhlouf (2016).

A inflamação na EHNA apresenta um padrão discreto e lobular com infiltrados inflamatórios caracterizados pela presença mista de células mono- e polimorfonucleares, enquanto o infiltrado portal pode ser discreto e composto por mononucleares (Figura 3) (Dietrich e Hellerbrand, 2014; Kleiner, 2017). Por fim, a fibrose é caracterizada pelo acúmulo de matriz extracelular composta principalmente por colágeno, é produzida pelas células estreladas hepáticas após a lesão celular e sendo inicialmente observada na região perivenular/perisinusoidal da zona 3 do ácino hepático formando um padrão de fibrose pericelular (forma de cerca) (Figura 4). A

medida que ocorre a progressão do dano hepático a fibrose se estende para as veias centrolobulares e espaços portais formando septos interligados culminando no desenvolvimento da cirrose. A esteatose microvesicular, Corpúsculos de Mallory e megamitocôndrias, inflamação portal e fibrose não são critérios obrigatórios para o diagnóstico de EHNA, no entanto a presença delas, especialmente de fibrose constitui um fator de risco para o desenvolvimento de cirrose (Enomoto *et al.*, 2015).

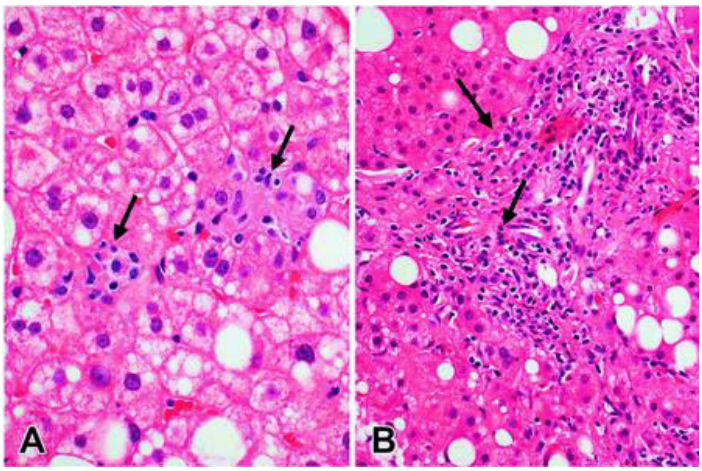

Figura 3. Hepatócitos com infiltrados inflamatórios. A, hepatócitos com inflamação lobular (600x); **B,** hepatócitos com inflamação lobular (400x). **Fonte:** Kleiner e Makhlouf (2016).

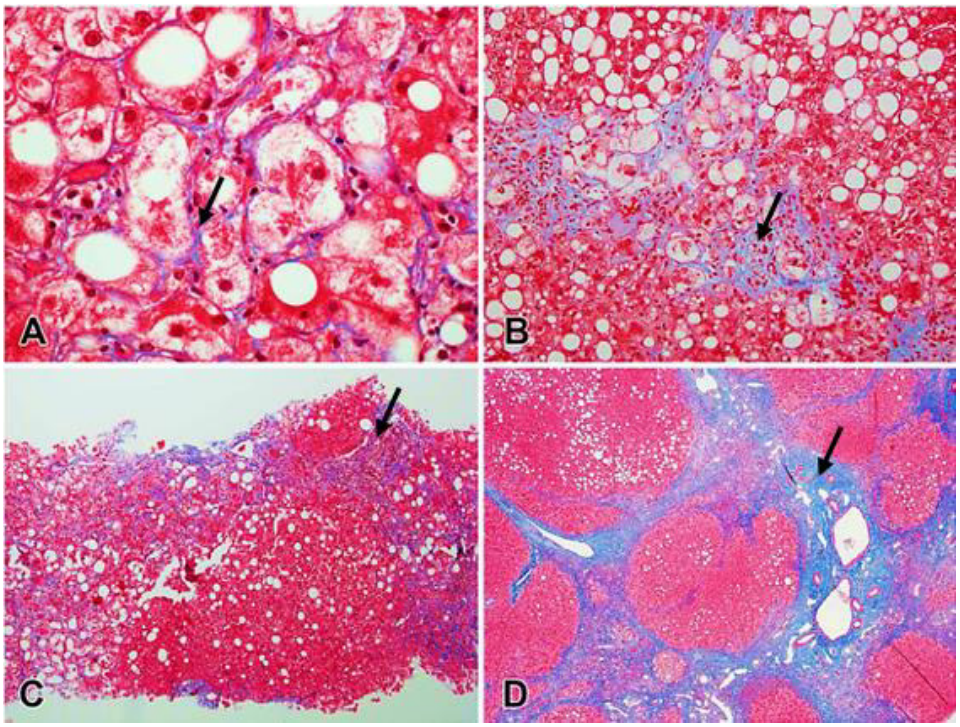

Figura 4.Hepatócitos com progressiva fibrose. A, fibrose perisinusoidal precoce (600x); **B,** fibrose perisinusoidal avançada (200x); **C,** extensiva rede de fibrose (100x); **D,** cirrose estabelecida (40x). **Fonte:** Kleiner e Makhlouf (2016).

Atualmente o sistema de gradação e classificação histológica da DHGNA descrito na literatura para estudos clínicos é o escore numérico de atividade (NAS), que é definido pela soma não ponderada dos escores para esteatose (0-3), inflamação lobular (0-3) e balonização (0-2). A fibrose não foi incluída como um componente de atividade inflamatória sendo considerada como resultado da atividade da doença. Os valores da soma podem variar de 0 a 8 em que NAS ≥ 5 é associado ao diagnóstico de EHNA, enquanto NAS ≤ 3 não-EHNA, ou seja, presença apenas de esteatose simples (Kleiner *et al.*, 2005; EASL-EASD-EASO Clinical Practice Guidelines for the Management of Non-Alcoholic Fatty Liver Disease, 2016b).

2.2 Dietas ricas em carboidratos e o desenvolvimento da DHGNA

O consumo excessivo de dietas ricas em carboidratos representa um importante fator nutricional que favorece um balanço energético positivo no organismo com consequente aumento dos depósitos de gordura corporal, que são essenciais para o desenvolvimento da obesidade, resistência à insulina e diabetes tipo 2, fatores diretamente envolvidos com o desenvolvimento da DHGNA (Stanhope, 2016; Burgeiro *et al.*, 2017; Schwarz *et al.*, 2017).

Com a maior ingesta destas dietas, o excesso de glicose na circulação estimula a maior secreção de insulina pelas células-β pancreáticas, que facilitam a entrada desse monossacarídeo nos tecidos insulino-depententes ou dependes do GLUT4 (ex: adiposo e muscular) (Samuel e Shulman, 2016). Entretanto, manutenção crônica de hiperglicemia resulta no processo de glicotoxicidade, onde ocorre uma sobrecarga mitocondrial para metabolizar esse excesso de glicose resultado em maior produção de espécies reativas de oxigênio e quadro de RI, fazendo com que a hiperinsulinemia que antes era transitória se torne crônica (Keane *et al.*, 2015).

Na RI ocorre aumento da atividade enzimática da lipase hormônio sensível sobre o tecido adiposo hipertrófico, aumentando assim a disponibilidade de ácidos graxos (AG) na circulação, seguida de sua maior captação pelo fígado, promovendo sobrecarga na β-oxidação mitocondrial. Em conjunto, esses fatores promovem o acúmulo excessivo de AG e metabólitos lipotóxicos (ceramidas e diacilgliceróis) envolvidos com a RI hepática e consequente aumento da gliconeogênese, que

associada a menor captação de glicose pelo músculo gera o quadro de hiperglicemia crônica e hiperinsulinemia compensatória (Chavez e Summers, 2012).

Vários trabalhos têm demonstrado a relação de dietas ricas em carboidratos com a indução dos componentes da SM e o desenvolvimento da DHGNA em modelos animais (Gutman *et al.*, 1987; Lombardo *et al.*, 1996; Lima *et al.*, 2016; Burgeiro *et al.*, 2017) e humanos (Ma *et al.*, 2015; Stanhope, 2016; Dinicolantonio *et al.*, 2017).

Além disso, o consumo excessivo de dietas ricas em carboidratos ativa diretamente a síntese *de novo* de lipídeos (DNL) de forma descontrolada, que está relacionada a desregulação do metabolismo de lipídeos e carboidratos e o desenvolvimento de esteatose hepática (Zago *et al.*, 2010; Dinicolantonio *et al.*, 2017; Iizuka, 2017). A DNL é responsável pela conversão do excesso de carboidratos da dieta em triacilgliceróis, que serão transportados para o tecido adiposo. Esta rota metabólica envolve a geração de acetil coenzima A (acetil-CoA) via glicólise seguida do processo de lipogênese, que converte a acetil-CoA em ácido graxos que serão esterificados a triacilglicerois ou exportados na forma de lipoproteína de densidade muito baixa (VLDL) (Wang, Y. *et al.*, 2015). As enzimas envolvidas em cada etapa desse processo são dinâmicamente reguladas por fatores transcricionais como a proteína de ligação ao elemento responsivo de esterol (SREBP) e a proteína de ligação ao elemento responsivo a carboidrato (ChREBP). (Weber *et al.*, 2004; Rui, 2014).

Os fatores de transcrição SREBP-1c e ChREBP promovem a transcrição de vários genes que codificam as enzimas envolvidas na síntese e esterificação de ácidos graxos como ácido graxo sintase (FAS), acetil CoA carboxilase (ACC), diacilglicerol acetiltransferase (DGAT2), estearoil-CoA desaturase (SCD1). A SREBP-1c é sintetizada como uma precursora inativa vinculada a proteína ativadora da clivagem de SRBP (SCAP) e a proteína do gene induzido pela insulina (Insig2a) formando o complexo inativo SREBP/SCAP/Insig2a ancorado à membrana do retículo endoplasmático (RE) (Moon, 2017). A ativação proteolítica no aparelho de Golgi do complexo para liberação de SREBP-1c envolve a hiperinsulinemia e o excesso de açucares de adição da alimentação (Weber *et al.*, 2004). Enquanto a ativação da ChREBP depende da sua translocação nuclear que ocorre após a desfosforilação mediada pela glicose por meio da enzima fosfatase 2A ou através do receptor X

hepático, o principal mediador da regulação lipídica hepática (Abdul-Wahed *et al.*, 2017).

Outro importante fator de transcrição envolvido com o desenvolvimento da DHGNA é o receptor ativado pelo proliferador de peroxissomo alfa (PPAR-α), que regula a beta-oxidação e o transporte dos ácidos graxos em nível mitocondrial, desempenhando um importante papel na transcrição de genes envolvidos com a absorção de ácidos graxos e o transporte reverso do colesterol, prevenindo o acúmulo hepático de gordura e a hipertrigliceridemia (Wang *et al.*, 2017). A redução da expressão do PPAR-α associada ao aumento da razão SREBP-1c/PPAR-α representa o desequilíbrio entre a síntese e a oxidação de ácidos graxos influenciando diretamente o desenvolvimento da esteatose hepática (Mansour, 2014). O consumo de dietas ricas em sacarose em modelos animais produziram diminuição dos níveis de PPAR-α associada ao quadro de esteatose hepática e hipertrigliciridemia (Hein *et al.*, 2010; Lucero *et al.*, 2015)

Assim a elevação da expressão desses fatores de transcrição lipogênicos associados a menor β-oxidação mitocondrial no fígado de alimentados com dietas ricas em carboidratos contribuem para o desenvolvimento da DHGNA, dislipidemia e instalação do quadro de RI (Lucero *et al.*, 2015; Burgeiro *et al.*, 2017).

2.3 Estresse do retículo endoplasmático (ERE) e ativação da resposta a proteínas mal enoveladas (UPR)

A grande maioria dos processos metabólicos exercidos pelo hepatócito ocorrem no RE, uma organela citoplasmática formada a partir de invaginações da membrana nuclear, que se ramificam em um sistema de túbulos e vesículas achatadas interligando o núcleo às demais organelas citoplasmáticas (Shibata *et al.*, 2006). O RE é estruturalmente classificado em RE rugoso e RE liso, de acordo com a presença ou ausência de ribossomos aderidos em suas paredes, respectivamente. Esta organela é considerada o maior reservatório de cálcio celular, e é responsável pela síntese, maturação e correto enovelamento das proteínas, além de exercer um papel importante na biossíntese de lipídios, montagem da bicamada lipídica, metabolismo de carboidratos e produção de enzimas de detoxificação (Dufey *et al.*, 2014; Smith e Wilkinson, 2017).

O RE rugoso do hepatócito é o principal sítio de síntese e enovelamento de proteínas plasmáticas, fatores da coagulação e apoliproteínas necessárias para formação das particulas de VLDL (Fagone e Jackowski, 2009; Lallukka *et al*., 2017). O processo de enovelamento proteico inicia-se logo após a translocação cotraducional das cadeias polipeptídicas lineares de proteínas nascentes produzidas pelos ribossomos, que no lúmen do RE são submetidas a ação de proteínas chaperonas e enzimas responsáveis pelo enovelamento final proteico por meio de diversos processos, com destaque para a clivagem do peptídeo sinal, n-glicosilação e deglicosilação, adição de pontes de dissulfeto e dobramento visando converter cadeias peptídicas lineares em estruturas terciárias ou quartenárias (Ron e Walter, 2007; Grek e Townsend, 2014).

Com o sucesso da maturação proteica após sucessivos ciclos de chaperonamento, a proteína nascente inicial agora já finalizada é exportada do RE e encaminhada para o aparelho de Golgi para processamento final e distribuição para o citosol (Barlowe e Miller, 2013). Entretanto, a maquinaria de controle de qualidade de enovelamento do RE é extremamente sensível, podendo ser prejudicada por diversas condições adversas, tais como estresse oxidativo, excesso de lipídeos e/ou carboidratos, desnutrição, síntese proteica excessiva e alterações na homeostase do cálcio, levando a perturbações no correto enovelamento proteico e gerando um maior número de proteínas mal enoveladas (*unfolded proteins*), que se acumulam e prejudicam a homeostase celular (Hetz, 2012). Neste cenário, estas proteínas mal enoveladas podem ser submetidas a novos processos de chaperonamento e/ou sofrer poliubiquitinação e serem encaminhadas para o processo de degradação proteassomal (ERAD) para destruição das mesmas (Mccaffrey e Braakman, 2016). O desequilíbrio entre a produção de proteínas mal enoveladas e redução da capacidade de correção/degradação das mesmas, gerando crescente acúmulo no lúmen é denominado de estresse do retículo endoplasmático (ERE) (Malhotra e Kaufman, 2007) (Figura 5).

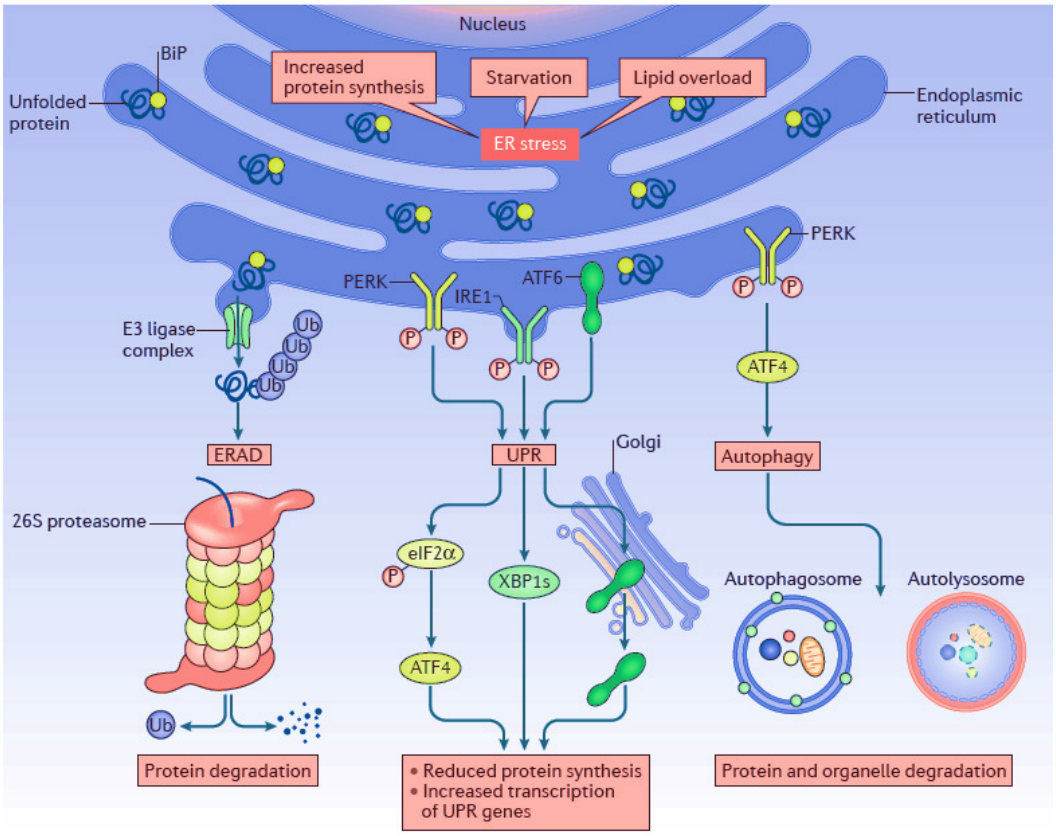

Figura 5. Respostas adaptativas iniciadas no retículo endoplasmático em situações de estresse. Situações de estresse como excesso de lipídeos e/ou carboidratos, desnutrição e síntese proteica desregulada são condições adversas que promovem um desequilíbrio no processo de enovelamento proteico, favorecendo o acúmulo luminal de proteínas mal enoveladas. Nesta situação, chaperonas são ativadas para corrigir o enovelamento incorreto. Em paralelo outras respostas adaptativas são ativadas, tais como: processo de degradação proteica via complexo ubiquitina-proteassoma (ERAD); processo de autofagia; e processo de resposta a proteínas mal enoveladas (UPR) via ativação de proteínas sensoras IRE1, PERK e ATF6. Em conjunto, estes processos visam garantir o correto enovelamento proteico, a destruição de proteínas mal enoveladas, degradação controlada da organela prejudicada e, por fim, redução da síntese proteica e garantia da homeostase da maquinaria de enovelamento do retículo endoplasmático. **Fonte:** Baiceanu, A. *et al.* (2016).

Na tentativa de controle do ERE, em paralelo ao chaperonamento e ERAD, o RE inicia a próxima linha de defesa denominada de resposta a proteínas mal enoveladas (*Unfolded protein response* - UPR), uma série de eventos adaptativos que visam preservar a integridade celular e restaurar a homeostase do RE em condições mais severas (Schroder e Kaufman, 2005; Fu *et al.*, 2012) (Figura 2).

As respostas adaptativas da complexa rede de sinalização da UPR se iniciam a partir da ativação de três vias: a quinase do retículo endoplasmático semelhante à proteína quinase do RNA (PERK), o fator 6 de ativação de transcrição (ATF6) e a enzima requerente do inositol 1 (IRE1). Estas três proteínas transmembrana apresentam um domínio sensível a presença de proteínas mal enoveladas e um

domínio citosólico que transmite os sinais transcricionais ou promove modificações translacionais no interior do RE (Rutkowski e Kaufman, 2004). Em condições de homeostasia, a chaperona proteína 78 regulada por glicose (GRP78) se encontra acoplada as proteínas sensoras, mantendo as mesmas inativadas. Em condições de ERE, na tentativa de promover um correto enovelamento proteico, ocorre um desacoplamento da GRP78, que se mobiliza para o lúmen do RE e promove a ativação das três vias da UPR (Pfaffenbach e Lee, 2011) (Figura 5).

Após a dissociação de GRP78, a PERK se dimeriza e autofosforila ativando sua função quinase, que fosforila o fator 2 de iniciação de tradução eucariótico (eIF2α) reduzindo assim a síntese global de proteínas no lúmen do RE. No entanto, paradoxalmente eIF2α codifica o fator de transcrição ATF4, que migra para o núcleo para promover respostas adaptativas, tais como a inibição da síntese global de proteínas, sintese de proteínas chaperonas, degradação proteassomal, síntese de enzimas antioxidantes e autofagia (Rutkowski e Kaufman, 2004) (Figura 6).

Adicionalmente, a PERK fosforila e ativa o fator 2 relacionado ao fator nuclear eritróide 2 (NRF2), que se encontra inativo no citoplasma ligado à proteína *Kelch-like ECH-associated protein 1* (Keap1) (Cullinan e Diehl, 2006). Após a dissociação da proteína repressora Keap1, o fator NRF2 migra para o núcleo da célula, promovendo síntese de enzimas antioxidantes (superóxido dismutase, catalase, glutationa peroxidase, peroxirredoxinas e tiorredoxina) e enzimas de detoxificação de fase II (glutationa-S-transferase, UDP-glucuronosil-transferase, etc.), que desempenham um papel importante na proteção da célula contra o dano oxidativo (Chambel *et al.*, 2015; Schmoll *et al.*, 2017).

A segunda via de sinalização da UPR é mediada pela IRE1 (isoformas α e β) uma proteína transmembrana com um domínio citosólico que apresenta atividade quinase e endoribonuclease. Após a ativação, IRE também sofre processo de dimerização e autofosforilação dos domínios quinases, ativando sua atividade endonucleásica luminal. A ativação do domínio endorribonuclease da IRE1α catalisa a remoção de um *intron* de 26 nucleotídeos do RNAm da proteína 1 de ligação (XBP1), resultando na expressão do fator de transcrição *XBP1 spliced*, que se transloca para o núcleo da célula de forma semelhante ao ATF4. Além disso, a função nucleásica de IRE1 é requerida para ativar o mecanismo de degradação de RNAm associado a

membrana, processo conhecido como mecanismo de decaimento regulado de RNA mensageiro IRE1-dependente (RIDD) (Gardner e Walter, 2011; Maurel *et al.*, 2014) (Figura 6).

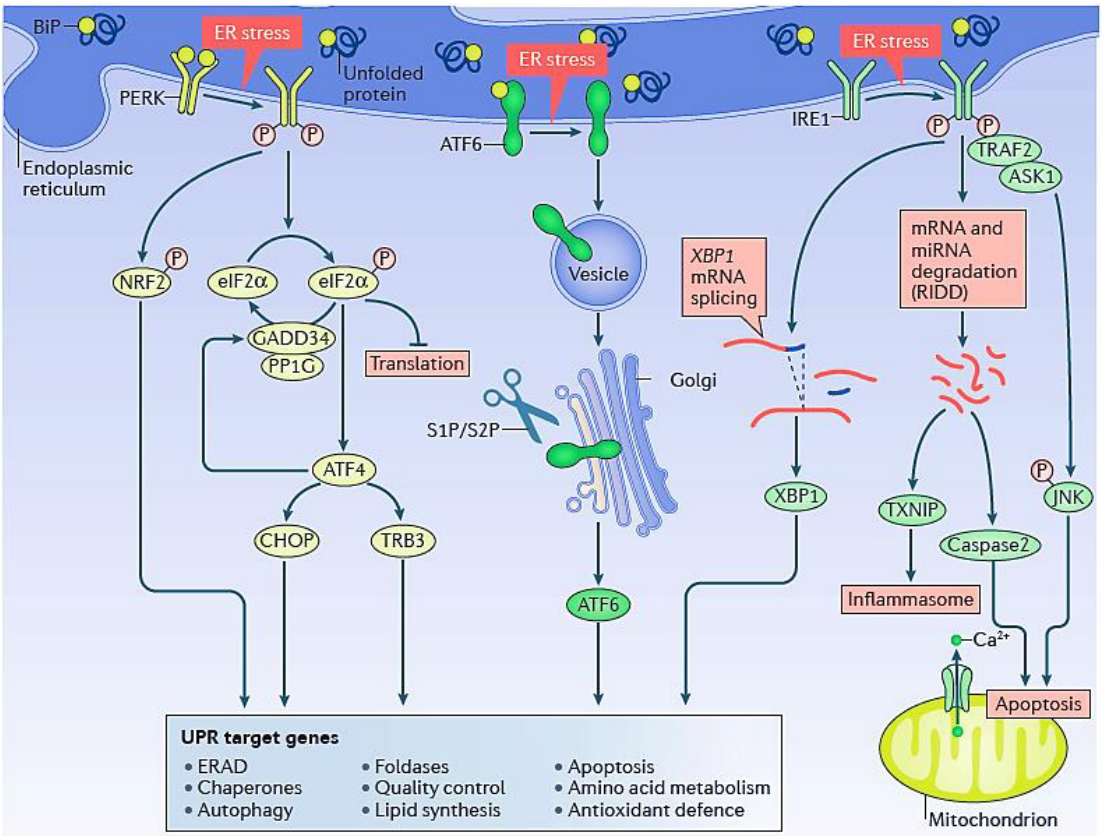

Figura 6. Respostas adaptativas e pró-apoptóticas desencadeadas no retículo endoplasmático em situações de estresse celular. Em situações de estresse, a chaperona GRP78/BiP se desacopla das proteínas sensoras transmembrana encontradas no lúmen do retículo endoplasmático. Ao fazer isto, estas proteínas sensoras se autofosforilam e dimerizam, iniciando uma cascata de sinalização adaptativa que visa controlar o acúmulo de proteínas mal enoveladas luminais. Estes processos são denominados de respostas a proteínas mal enoveladas (UPR). São três as proteínas sensoras: PERK, ATF6 e IRE1. Entretanto, em condições de estresse crônico, estas mesmas proteínas sensoras responsáveis inicialmente por uma sinalização adaptativa ao estresse pode migrar para um padrão apotótico. A PERK ativada inibe o processo de tradução proteica; ativa o fator Nrf2, relacionado a síntese de enzimas antioxidantes; e ativa o fator de transcrição ATF4, que no estresse agudo promove respostas adaptativas e no estresse crônico ativa a CHOP, responsável por apoptose. O ATF6 ativado migra para o aparelho de Golgi, onde é clivado pelas proteases S1P e S2P, liberando o fator de transcrição ATF6f que migra para o núcleo e realiza as mesmas funções exercidas pelo ATF4. Por fim, A IRE1 ativada promove o processo de ativação do fator de transcrição XBP1, responsável por funções adaptativas. Adicionalmente, ativa também o mecanismo de RIDD, que degrada RNAm's aberrantes. Em situações de estresse crônico, a IRE1 promove a ativação da JNK, que acelera processo de apoptose. Dentre as respostas adaptativas ativadas pelos fatores no núcleo, destacam-se: síntese de chaperonas, ativação da ERAD, autofagia, síntese de enzimas antioxidantes, transporte de aminoácidos, inibição da tradução global proteica e degradação de RNAm's aberrantes. **Fonte:** Baiceanu, A. *et al.* (2016).

A terceira via de sinalização da UPR é mediada pelo ATF6 (isoformas α e β) uma proteína transmembrana com uma porção carboxi-terminal com sinalização específica para clivagem no aparelho de Golgi voltada para o lúmen do RE e ligada a GRP78. Após ativação, o ATF6 se transloca para o aparelho de Golgi onde é clivado no fator de transcrição ativo (ATF6f) que migra para o núcleo (Shen e Prywes, 2005) (Figura 6). No fígado, ATF6 antagoniza os efeitos de SREBP2, o que possibilitaria às células lidarem com a condição de estresse e salvaguardar as reservas de energia (Zeng *et al.*, 2004).

Em conjunto, os fatores de transcrição nuclear ATF4, XBP1 *spliced* e ATF6f induzem diversas respostas adaptativas de controle ao ERE, com destaque para a síntese de proteínas chaperonas, ERAD, autofagia e regulação dos metabolismos lipídico e de aminoácidos. Ao mesmo tempo, outras respostas adaptativas independentes dos fatores de transcrição também são ativadas via UPR, como inibição da tradução proteica, síntese de enzimas antioxidantes e degradação de RNAm aberrantes (Manie *et al.*, 2014).

Em condições de manutenção do ERE por períodos prolongados, as respostas adaptativas da UPR podem não ser capazes de controlar a “expansão” de efeitos deletérios do lúmen do RE para a célula. Nestas condições de ERE crônico, as mesmas vias sensoras da UPR podem iniciar uma sinalização pró-apoptótica, com ativação de CHOP mediada por ATF4 e ATF6f, ativação de JNK mediada por IRE1α e de forma ainda não muito clara ativação de enzimas caspases e do gene p53, acelerando processos de morte celular por apoptose e/ou necrose (Rasheva e Domingos, 2009; Iurlaro e Munoz-Pinedo, 2016).

A CHOP está envolvida com o processo de apoptose através da supressão do fator anti-apoptótico célula B de linfoma 2 (Bcl-2); maior expressão de fatores pró-apoptóticos como o Bh3-*only,* proteínas X associada ao Bcl2 (BAX) e da assassina/antagonista de Blc2 (BAK), que alteram a permeabilidade da membrana do RE e permitem a translocação das caspases; aumenta expressão do indutor 34 de dano ao DNA (GADD34), que promove retorno de tradução proteica, produção de EROs e perda da homeostase do cálcio; e ativação de caspases. Adicionalmente, a CHOP também inibe a produção de GRP78 favorecendo o ERE (Rutkowski *et al.*, 2006; Guo *et al.*, 2017).

A UPR também pode ativar o fator nuclear de transcrição (NF-κB), através da supressão da transcrição do inibidor do Kappa B (IκB) por mecanismos que envolvem a PERK e IRE1α/JNK resultando na regulação de mediadores inflamatórios, como IL-6 e TNF-α, que contribuem para o desenvolvimento da esteato-hepatite não alcóolica (Urano *et al.*, 2000; Deng *et al.*, 2004)

2.4 Estresse do retículo endoplasmático e doença hepática gordurosa não alcóolica

O fígado durante a execução de suas funções fisiológicas ativa transitoriamente as vias da UPR, entretanto a ativação crônica dessas vias favorecem a desregulação do metabolismo lipídico culminando no desenvolvimento de esteatose e favorecendo a progressão da EHNA. Adicionalmente, o acúmulo de lipídeos no hepatócito é capaz de induzir o ERE, e por sua vez promover a DNL hepática, criando um ciclo vicioso (Baiceanu, Andrei *et al.*, 2016).

A manutenção do equilíbrio homeostático do RE no fígado durante a DNL, a síntese e armazenamento de TGs, síntese de fosfolipídeos de membrana e a montagem e secreção das partículas de VLDL são de suma importância para o correto metabolismo e transporte de lipídeos evitando assim o desenvolvimento da DHGNA e outros distúrbios metabólicos associados (Engin, 2017).

As três vias da UPR estão envolvidas com o metabolismo lipídico, portanto alterações nessas vias desempenham um importante papel no desenvolvimento da esteatose hepática (Bozaykut *et al.*, 2016). Animais com deleção hepática de IRE1α desenvolvem esteatose grave em resposta ao ERE induzido por agentes farmacológicos. Estes achados indicam que a IRE1α em condições de ERE agudo, atua na supressão gênica das proteínas ligantes ao amplificador CCAAT (C/EBPs), do receptor gama ativado por proliferadores de peroxissomas (PPAR-γ) e de enzimas envolvidas com a síntese de TG, modulando positivamente a lipogênese e favorecendo a secreção das partículas de VLDL (Zhang *et al.*, 2011). Em contraste, o ERE crônico têm demonstrado inibir o eixo IRE1α/XBP1/PDI, essencial para o funcionamento da proteína de transferência microssomal (MTP) e, desta forma, reduzindo a montagem de VLDL, agravando o desenvolvimento da DHGNA (Wang *et al.*, 2012).

A deleção específica de XBP1 no fígado de camundongos alimentados com dieta rica em carboidrato reduz a esteatose, pois nesse modelo ocorre a inibição da DNL sem ocorrer a alteração da montagem do VLDL. Estes dados evidenciam o papel da XBP1 na ativação direta das enzimas lipogênicas como FAS, SCD1, ACC1, ACC2 de forma independente de SREBP-1c (Lee *et al.*, 2008). Por outro lado, em um modelo de dieta rica em gordura, a ativação da DNL via XBP1 se mostrou diretamente ligada a interação com SREBP-1c (Kitteringham *et al.*, 2010; Ning *et al.*, 2011; Ramadori *et al.*, 2016). Adicionalmente, a ativação constante do mecanismo de IRE1α/RIDD durante o ERE foi descrito como um ativador de SREBP-1c por meio da degradação do RNAm responsável pela expressão de Insig-1 (Lee e Ye, 2004).

Apesar da vasta literatura sobre o efeito lipogênico da XBP1, um recente conduzido por Herrema *et al.* (2016) descreveu pela primeira vez a ação anti-lipogênica da XBP1 *spliced* em camundongos alimentados com dieta rica em gordura. Neste trabalho a superexpressão de XBP1 reduziu o conteúdo de TG hepático e seus intermediários lipotóxicos, especialmente o diacilglicerol, e reduziu a ativação da PKCε evitando o desenvolvimento de RI hepática. Porém, esse efeito não foi reproduzido em animais alimentados com dieta rica em frutose apesar da redução das enzimas lipogênicas e dos triglicerídeos plasmáticos.

A inibição da síntese global de proteínas promovida pelo braço PERK/eIF2α, prejudica a síntese de Insig, favorecendo a translocação de SRBP-1c para o núcleo e ativação das enzimas lipogênicas no fígado (Zhang *et al.*, 2012; Afrin *et al.*, 2016). O quadro de RI observado em modelos dietéticos contribui com a ativação contínua do braço da PERK/eIF2α promovendo a ativação de C/EBP, que consequentemente aumentam a gliconeogênese e a DNL, favorecendo ainda mais o quadro de RI periférica e de esteatose (Oyadomari *et al.*, 2008; Birkenfeld *et al.*, 2011). Adicionalmente, ativação de PERK/eIF2α/ATF4 em condições de estresse induzido por dieta rica em gordura aumenta a expressão do receptor de VLDL nos hepatócitos favorecendo a esteatose (Jo *et al.*, 2013).

O braço da PERK/eIF2α/ ATF4 apresenta um envolvimento na lipogênese em diferentes tecidos. A deleção de PERK inibe a expressão de enzimas lipogênicas como FAS e SCD1 em glândulas mamarias de ratos (Bobrovnikova-Marjon *et al.*, 2008). A desfosforilação sustentada de eIF2α no fígado de animais transgênicos

alimentados com dieta rica em gordura protege contra o desenvolvimento da obesidade e da esteatose hepática por meio de supressão de PPARγ, SREBP-1c, ACC e SCD1 (Oyadomari *et al.*, 2008). Resultados semelhantes são observados em animais com deleção de ATF4 alimentados com dieta rica em frutose (Xiao *et al.*, 2013). Um recente estudo demonstrou um papel protetor hepático da PERK/eIF2α/ATF4 ao reduzir os danos oxidativos pela eliminação do colesterol livre em condições basais e de ERE (Fusakio *et al.*, 2016).

Outra via ativada diretamente pela PERK envolve o sistema de defesa antioxidante mediado pelo fator NRF2, responsável por evitar o dano oxidativo, inibir lipogênese e melhorar o processo de β-oxidação mitocondrial em modelos dietéticos ricos em frutose ou em combinação com dietas ricas em gorduras (Kitteringham *et al.*, 2010; Ramadori *et al.*, 2016; Nigro *et al.*, 2017).

A ativação da CHOP associada ao estresse oxidativo são responsáveis por desencadear a morte celular através da ativação da apoptose na DHGNA e na EHNA (Adkins *et al.*, 2013; Lake *et al.*, 2014; Wang, H. *et al.*, 2015). O tratamento de animais alimentados com dieta rica em frutose com uma chaperona sintética promoveu a melhora do ERE, marcado por redução da expressão de CHOP e estresse oxidativo no fígado (Ren *et al.*, 2013). Por outro lado, a deleção de CHOP inibe o desenvolvimento de esteatose e fibrose em animais com dieta deficiente em metionina e colina (Tamaki *et al.*, 2008). Uma série de evidências têm demonstrado o papel da CHOP no desenvolvimento do hepatocarcinoma por meio da ativação de mecanismos inflamatórios e apoptóticos mediados pelo ERE (Dezwaan-Mccabe *et al.*, 2013; Willy *et al.*, 2015; Lei *et al.*, 2017).

A via do ATF6 desempenha um papel protetor no desenvolvimento da esteatose em modelo de ablação genética após estresse induzido por tunicamicina, levando a redução de PPARα, aumento de CHOP e redução da produção de VLDL (Yamamoto *et al.*, 2010). Quando estes animais foram alimentados com dieta rica em gordura, observou-se um quadro mais grave de esteatose (Usui *et al.*, 2012). ATF6 também atenua a lipogênese através da complexação com SREBP2, reduzindo a expressão de enzimas envolvidas com o metabolismo do colesterol (Zeng *et al.*, 2004).

3. OBJETIVOS

3.1. Objetivo Geral

- Investigar a relação temporal entre a ativação de vias de sinalização de lipogênese e estresse do retículo endoplasmático e o desenvolvimento de doença hepática gordurosa não alcoólica em camundongos com obesidade induzida por dieta rica em sacarose.

3.2. Objetivos Específicos

- Caracterizar o quadro de síndrome metabólica em camundongos com obesidade induzida por dieta rica em sacarose em diferentes períodos de exposição;
- Caracterizar histologicamente a evolução de doença hepática gordurosa não alcoólica;
- Investigar o efeito do consumo de dieta rica em sacarose na expressão gênica de fatores de transcrição e enzimas envolvidas na síntese *de novo* de lipídeos;
- Investigar o efeito do consumo de dieta rica em sacarose na expressão gênica de marcadores do estresse do retículo endoplasmático.

4. RESULTADOS

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ORIGINAL RESEARCH

Long-term exposure to high-sucrose diet downregulates hepatic endoplasmic reticulum-stress adaptive pathways and potentiates *de novo* lipogenesis in mice

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Running Title: HSD downregulates hepatic ER stress adaptive pathways.

ABSTRACT

Non-alcoholic fatty liver disease (NAFLD) is defined as the abnormal triglyceride accumulation into hepatocytes in the absence of chronic alcoholism. Upon unsolved stress, steatosis evolve to liver inflammation and fibrosis, predisposing to worsen hepatic diseases. The present study sought to investigate the role of disrupted *de novo* lipogenesis (DNL) and endoplasmic reticulum (ER) stress on NAFLD development. For this, post-weaned Swiss mice were fed a high-sucrose diet (HSD) for 30, 60 and 90 days and compared to control. Metabolic syndrome (MetS) development, liver fat content, histological analysis and hepatic gene expressions of lipogenesis and ER stress were assessed. Exposure to HSD promoted progressive metabolic disturbances in a time-dependent manner leading to full establishment of MetS upon 60 days, characterized by central obesity, hyperglicemia, dyslipidemia and insulin resistance. Moreover, these animals presented increased fat liver content and microvesicular steatosis. After 30 days of nutritional intervention it was found a balance between fatty acids synthesis (ChREBP and SCD1) and oxidation (PPARα), as well as upregulated UPR-adaptive pathways featured by increased gene expression of UPR sensors (IRE1α, PERK and ATF6), chaperones (GRP78 and PDIA1) and antioxidant defense (NRF2). However, from 60 days on HSD, there was marked increase in gene expression of lipogenesis transcription factors (ChREBP and SREBP-1c), exponential raise of fatty acids synthesis (SCD1), and ER stress moving toward a pro-apoptotic (CHOP) pattern. In summary, our study originally demonstrates a temporal relationship between DNL and ER stress pathways toward MetS and NAFLD development in rats fed a high-sucrose diet.

KEYWORDS

Non-alcoholic fatty liver disease; Endoplasmic reticulum stress; *De novo* lipogenesis; High-sucrose diet; Microvesicular steatosis; Metabolic syndrome.

1. INTRODUCTION

Non-alcoholic fatty liver disease (NAFLD) is an emerging obesity-related disorder, whose prevalence has doubled over the last 20 years and already affects 25% of global population and 90% of obese people [1]. NAFLD is characterized by abnormal intrahepatic triglyceride accumulation (steatosis) in the absence of chronic alcoholism, viral infections or others liver diseases. Upon hepatic inflammation, NAFLD progress to nonalcoholic steatohepatitis (NASH) with or without cellular ballooning and fibrosis, predisposing people to severe hepatic diseases, such as cirrhosis and hepatocarcinoma [2]. Besides being considered the hepatic manifestation of metabolic syndrome (MetS), NAFLD is also an important and independent risk factor for cardiovascular diseases [3, 4].

The pathophysiology of NAFLD is intimately associated with nutritional disorders resulting from either protein malnourishment or high fat/high sugar overfeeding [5]. Evidence suggests a positive correlation between added sugars, NAFLD and other MetS-related dysfunctions [6]. Added sugars, especially sucrose and fructose, contribute to increased circulating insulin and triglyceride levels, leading to increased hepatic *de novo* lipogenesis (DNL) and decreased insulin sensitivity in the liver. A recent meta-analysis showed a straight relationship between increased fructose intake and increased ectopic lipid accumulation, as

well as impaired hepatic function [7]. Moreover, sugars contribute more than fat to NAFLD development [8]. Indeed, added sugars intake is intrinsically related to steatosis [9], and NASH [10, 11], being considered a major candidate for causing NAFLD.

The molecular mechanisms involved in the onset and progression of NAFLD are still elusive, although recent evidence has proposed a role for the endoplasmic reticulum (ER) stress [12]. ER stress is triggered by unsolved transient overload of misfolded proteins inside ER lumen. As consequence, a series of adaptive signaling pathways, collectively known as unfolded protein response (UPR), is activated to reestablish ER homeostasis. However, under persistent ER stress, the UPR switches over from an adaptive to a pro-apoptotic pattern, accelerating cell death [13]. Considering the role of ER in lipid synthesis, disturbances in this organelle contribute to impaired lipid metabolism in liver [14]. Indeed, evidence shows that ER stress induced by hypercaloric diets [15] or pharmacologic stressors (tunicamycin) [16] lead to hepatic lipid accumulation, as well as more severe hepatic outcomes. On the other hand, it has also been proposed that steatosis is in fact the main trigger of hepatic ER stress [17]. Therefore, ER stress could be classified as both causative and consequence of lipid accumulation in liver, thus creating a positive feedback loop, which may promote NAFLD recrudescence [18].

In spite of the abovementioned mechanisms, processes concurring for the onset and progression of NAFLD caused by dietary and metabolic stressors are still elusive. Thus, in the present study we sought to investigate the role of disrupted ER homeostasis and DNL caused by high-sucrose intake on NAFLD

development. Data herein presented successfully demonstrate for the first time that high-sucrose diet induce metabolic alterations and early lipogenesis activation, which trigger adaptive UPR pathways. However, upon sustained sucrose overfeeding, ER stress progressed toward a pro-apoptotic pattern that in turn aggravated DNL upregulation and promoted deeper metabolic damages paralleling to NAFLD development.

2. MATERIALS AND METHODS

2.1. Animals and diets

Weaned male *Swiss* mice provided by the animal facility house of the Federal University of Maranhão (11.65 ± 0.57 g) were randomized into two groups: control mice (CTR; n = 21), fed a standard chow (Nuvital®, Nuvilab, Brazil) composed by 55.4% total carbohydrate (10% sucrose), 21% proteins, 5.2% total lipids, totaling approximately 350 kcal·100 g^{-1}; or high-sucrose diet mice (HSD, n = 21), fed a high-sucrose chow composed by 65% total carbohydrates (25% sucrose), 12.3% proteins, 4.3% total lipids, also approximately 350 kcal·100 g^{-1}, as previously described [19]. These groups were exposed to diets for 30, 60 and 90 days (euthanasia of 7 animals per period; HSD_{30}, HSD_{60} and HSD_{90}, respectively). Animals were maintained in a controlled room (21 ± 2 °C; 60% humidity and 12h light/dark cycle) with water and chow *ad libitum*. Throughout dietary interventional periods, body weight (g) and energy intake (Kcal/day/10g body weight) were assessed twice a week. Lee index (body weight $(g)^{1/3}$·naso-anal length $(cm)^{-1}$·100) was calculated every month for assessment of obesity development [20].

All procedures were performed in accordance with the rules of Brazilian Council for the Control of Animal Experimentation (CONCEA) and approved by the Ethical Committee on Animal Use and Welfare of the Federal University of Maranhão, under ruling number 23115.002832/2017-25.

2.2. Euthanasia and tissue collection

At defined periods, overnight fasted animals were anesthetized (40:10 mg/kg ketamine:xylazine solution) for blood collection by cardiac puncture and tissues upon laparotomy. Retroperitoneal, periepididymal and mesenteric white adipose tissue fat pads and liver were weighed for morphometric assessment and expressed as tissue mass (g) per 100 g body weight. Serum samples were used for the assessment of biochemical and hormonal profile, whereas liver samples were used for protocols of fat liver content measurement, histological analysis and gene expression.

2.3. Assessment of serum biochemical profile

For glucose levels, tail vein blood drops were collected and measured through glucometer (Accucheck Active®, Roche Diagnostic, Germany) after overnight fasting or post-prandial state. Serum samples were obtained by centrifugation (3500 rpm; 10min; 4 °C) and used for colorimetric measurement of triglycerides, total cholesterol (Labtest, Brazil) and free fatty acids (Sigma-Aldrich, USA) levels according to manufacturer's instructions. Serum insulin levels were assessed by immunoassay (Sigma-Aldrich, USA) according to manufacturer's instruction.

2.4. Assessment of insulin resistance

For oral glucose tolerance test (GTT), animals were submitted to 8h fasting prior to administration of oral gavage of glucose 4 g/kg. Tail vein blood drops were collected immediately before (time 0) and 15, 30, 60 and 120 min after glucose administration for glucose measurement. Data were expressed as area under glycemic curve (AUC). Similar procedure was carried out for intraperitoneal insulin tolerance test (ITT), excepting animals were fed, received 1 UI/kg insulin (Humulin 70/30®, Lilly, USA) and were evaluated for 0, 10, 15, 30 and 45 minutes. Glucose disappearance rate (kITT) was derived from ITT curve and calculated as percentage decline in glucose per minute [21]. Insulin sensitivity was inferred by calculation of homeostasis model assessment (HOMA) Index (HOMA-IR = fasting glucose (mM)·fasting insulinemia (μU/ml)·22.5^{-1}) [22] and TyG index (ln·(fasting glucose (mg/dL)·fasting triglyceride (mg/dL))·2^{-1}) [23].

2.5. Assessment of liver fat content

Liver fat accumulation was assessed from 500 mg liver samples, as previously described [24]. Briefly, samples were homogenized with chloroform/methanol solution (2:1) and resulting supernatant was paper-filtered and diluted in 0.9% NaCl (5:1). The organic phase was collected, airflow oven-dried at 40 °C for the measurement of the total fat content (mg) per tissue mass (g). The total fat was resuspended in Triton X/methanol (2:1) for colorimetric measurement of total cholesterol and triglycerides. Results were expressed as total cholesterol (mg) per tissue mass (g) and triglycerides (mg) per tissue mass (g).

2.6. Histological analysis

Liver samples were fixed for 1 day at room temperature in 10% buffered formalin, preserved in 70% ethanol and embedded in paraffin. Sections were prepared (5 μm thick) and stained with hematoxylin and eosin for visualization of hepatocyte morphology, and with Masson's trichrome for fibrosis assessment. The slides were analyzed according to score proposed by Kleiner et al [25] to classify steatosis and steatohepatitis.

2.7. Gene expression by real time PCR (qPCR)

Liver samples (n = 5) were used for RNA extraction using Trizol® (Invitrogen, Germany) as manufacturer's instructions. RNA samples (3 μg) were converted into cDNA using Super Script II Reverse Transcriptase® (Invitrogen, Germany). The qPCR amplification and Platinum® SYBR® Green qPCR SuperMix-UDG (Invitrogen, Germany) detection were performed using the 7500 Real time PCR Applied Biosystems®, USA. Reactions were incubated at 50 °C for 2 min and 95°C for 2 min followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. To melt curve stage, reactions were incubated at 95 °C for 15s, 60 °C for 60 min and 95 °C for 15 s. Primers were designed using Primer Express® software (Applied Biosystem, USA) and manufactured by Invitrogen, Brazil (Supplementary Table 1). All samples were normalized to the relative levels of GAPDH and results were expressed as the fold change (FC) values of $2^{-\Delta\Delta CT}$, as determined by real-time amplification.

2.8. Statistical analysis

Data were expressed as mean ± SEM and submitted to normality test (Shapiro-Wilk) followed by parametrical analysis through unpaired *t* test (one-tailed) or two-way ANOVA (Posttest Bonferroni) for a significance level of 5% ($p < 0.05$) using GraphPad Prism 7.0 software (GraphPad Software Inc., USA).

3. RESULTS

3.1. Exposure to HSD induces metabolic syndrome in a time-dependent way

Post-weaning exposure to HSD promoted progressive metabolic disturbances in a time-dependent manner leading to plenty establishment of MetS upon 60 days. HSD_{30} mice did not present altered body weight nor Lee index (Figure 1A and 1C), but had increased fat accumulation in both retroperitoneal and periepididymal fat pads (Figure 1D). Upon longer periods, HSD_{60} and HSD_{90} animals had significant increase of body weight, Lee index, as well as visceral and non-visceral fat pads, as compared to their controls (Figure 1A, 1C, 1D). Notoriously, all these changes occurred under lower energy intake by HSD mice throughout the interventional period (Figure 1B).

Serum biochemical profile assessment showed HSD mice had elevated fasting and fed glucose (Figure 2A), as well as cholesterol levels at all times (Figure 2B), whereas triglyceride and free fatty acids levels were increased only after 60 days of nutritional intervention (Figure 2B). HSD animals presented glucose intolerance at all times (Figure 2C-E), but had increased serum insulin levels (Figure 2F) and impaired insulin sensitivity only after a 60-day period (Figure

2D). In accordance, assessment of insulin resistance (HOMA-IR and TyG) indexes were increased only in HSD_{60} and HSD_{90} groups (Figure 2H and 2I). Noteworthy, most of morphometric and metabolic parameters were influenced by both HSD and nutritional intervention period, resulting in significant interaction between these variables (Supplementary Tables 2 and 3). This set of data importantly showed that early and sustained exposure to HSD time-dependently produced metabolic alterations leading to plenty MetS onset within a period as short as 60 days.

3.2. Exposure to HSD promotes hepatic lipid accumulation and microvesicular steatosis

Relative liver weight from HSD mice did not differ from their age-mated controls (Figure 3A). After 60 days of nutritional intervention, HSD animals presented higher hepatic total fat accumulation (Figure 3B), when triglyceride content increased by nearly 50% in comparison to CTR_{60} (Figure 3C). Upon 90 days of intervention, total cholesterol content also arose (Figure 3D) in addition to total fat and triglyceride content. These variables independently influenced the progression of hepatic lipid accumulation (Supplementary Table 4). Accordingly, histological analysis of HE-stained liver sections showed presence of microvesicular steatosis in HSD_{60} and HSD_{90} with no detectable cellular ballooning or inflammation (Figure 4). CTR mice did not present any hepatic degeneration throughout the study. This set of data strongly parallels ectopic lipid accumulation in the liver (Figure 3B) with the onset of insulin resistance (Figure 2D-I) and hypertriglyceridemia (Figure 2B), which were also detected only after 60 days under HSD consumption.

3.3. Exposure to HSD activates carbohydrate-derived *de novo* lipogenesis

To investigate the impact of sucrose intake on DNL, we first assessed the gene expression of lipogenesis transcription factors. ChREBP, which is activated upon carbohydrate intake, was overexpressed throughout the nutritional intervention period (Figure 5A), whereas SREBP-1c upregulation was observed only after 90 days of exposure to HSD (Figure 5A). Accordingly, SCD1 gene expression was also upregulated throughout the study's period (Figure 5B), as a possible result of ChREBP action on its promoter. On the other hand, HSD had no effect on FAS gene expression (Figure 5B). Concerning the contra regulatory role of fatty acid oxidation, we found increased gene expression of PPARα on HSD_{30} and HSD_{60}, which was blunted in HSD_{90}, as compared to their controls (Figure 5C). Interestingly, failure of PPARα response on HSD_{90} (Figure 5C) coincided with the later upregulation of SREBP-1c (Figure 5A) and a strong 6-fold increase of SCD1 gene expression (Figure 5B).

3.4. Exposure to HSD causes early hepatic ER stress

Finally, as an attempt to identify molecular pathways underlying the disruption of hepatic lipid homeostasis caused by high-sucrose intake, we assessed gene expression of ER stress markers involved in both pro-adaptive and pro-apoptotic UPR branches. Data in Figures 6A and 6B show that HSD_{30} mice had increased expression of UPR-sensors (IRE1α, PERK and ATF6) and chaperones (GRP78 and PDI 1A). NRF2, an important transcription factor related to antioxidant defense, was also upregulated (Figure 6B). In accordance, HSD_{30} mice had no change on CHOP expression (Figure 6C). On the other hand, a

completely different scenario was observed on HSD_{60} and HSD_{90} animals, featured by abolishment of UPR-driven pro-adaptive response. UPR-sensors, as well as PDI A1 expression, were downregulated in comparison to their age-mated controls, leading to attenuation of GRP78 and NRF2 expression (Figures 6A and 6B). Contrariwise, these animals presented a 1.7-fold increase of CHOP expression. In a whole, this set of data allow us to infer that sustained exposure to HSD induces an early activation of ER stress pro-adaptive pathways, which shortly transitioned to a pro-apoptotic pattern in parallel with the establishment of peripheral IR (Figures 2G – 2I) and dyslipidemia (Figures 2B).

4. DISCUSSION

The growing consumption of added sugars has been directly associated to the exponential rise of MetS and its comorbidities, particularly NAFLD [26]. Activation of lipogenic genes imposed by liver uptake of monosaccharides, mainly glucose and fructose, leads to the later, whereas precise mechanisms are still elusive [27]. In this context, the present study offers a body of evidence supporting a close interrelationship between DNL and ER stress towards NAFLD onset in HSD-fed mice. Firstly, post-weaning exposure to HSD triggered metabolic alterations leading to MetS after 60 days of nutritional intervention. Secondly, concurrent activation of carbohydrate-derived DNL and UPR pro-adaptive pathways in the liver preceded MetS onset. Thirdly, transition of ER stress pathways toward a pro-apoptotic pattern additionally upregulated DNL aggravating

MetS-associated disturbances on HSD mice upon 90 days of sucrose consumption.

Metabolism of sucrose-derived monosaccharides, glucose and fructose, is starkly different in spite of them to be 6-carbon isomers [28]. Fructose is primarily metabolized by the liver in an insulin-independent manner, while glucose is used as fuel source by virtually every cell in the body, as well as promotes strong insulin secretion, an effect not driven by fructose [27]. Both monossacharides are uptaken by hepatocytes and activate DNL transcription factors ChREBP and SREBP-1c, which together translate enzymes which convert that substrates into fatty acids and triglycerides [27, 29]. Sustained DNL activation promotes more ectopic lipid accumulation and VLDL secretion, that later lead to gluco- and lipotoxicity, insulin signaling impairment, oxidative stress and poor hepatic damages [30].

Post-weaning feeding of male Swiss mice with HSD progressively led to metabolic disturbances compatible with plenty establishment of MetS within a 60-day follow-up period. At 30 days, HSD mice showed glucose intolerance characterized by augmented blood glucose levels at both fasting and fed states allied to higher adipose tissue storage. At 60 days, HSD mice presented impaired insulin sensitivity assessed by insulin tolerance test, which led to additionally increased serum levels of lipids (total cholesterol, triglycerides and free fatty acids) as well as compensatory hyperinsulinemia. Metabolic alterations observed in HSD_{90} mice were similar to those found in HSD_{60}. In parallel to the establishment of insulin resistance, HSD mice began to present higher body mass and central obesity around 60 days of nutritional intervention. Notwithstanding, NAFLD onset also coincided with the triggering of peripheral insulin resistance. MetS

development herein described is corroborated by previous studies, which reported similar development of central obesity, hypertriglyceridemia, glucose intolerance, hyperinsulinemia and insulin resistance, besides applying higher supplementary sucrose concentrations (35 – 63%) for at least 12 weeks in rodents [31-34].

Assessment of liver morphometry, lipid content and histological analysis showed our $HSD_{60\text{-}90}$ mice presented hepatic ectopic fat deposition in parallel with triglycerides levels arise even without liver weight differences when compared to CTR. Evidence shows that sucrose exposure leads to higher liver mass [35-37], although some studies like ours did not observe any change [27, 38]. In agreement, histological slices revealed a predominance of multiple small lipid vesicles, defining a microvesicular steatosis without inflammation. Hepatic microvesicular steatosis is a clue of worse prognosis and has been associated with higher risk to ballooning, mitochondrial dysfunction, and more severe NAFLD phenotype [39]. It occurs because of impaired mitochondrial β-oxidation, which in turn leads to accumulation of fatty acids in the form of triglyceride and formation of small lipid droplets in hepatocyte's cytosol [40]. Noteworthy, other studies consistently support hepatic fat accumulation and cellular damages as a result of high-sucrose intake, driving hepatocytes to NAFLD-related outcomes such as isolated microvesicular steatosis [37], concomitant macro- and microvesicular steatosis [9, 41, 42] and NASH [43].

Our data consistently support that early-in-life introduction of HSD diet induces DNL. HSD-containing monosaccharides activate the transcription factor ChREBP, which in turn elevated SCD1 gene expression in all assessed ages. SCD1 is a key lipogenic enzyme which catalyzes the hepatic triglyceride synthesis

[44]. In contrast, only at 90 days of exposure was observed the raise of transcription factor SREBP-1c, which considerably contributed to the even greater increase of SCD1. Our findings are supported by several studies demonstrating that fructose can directly induce hepatic expression of ChREBP and SREBP-1c [27, 45, 46], whereas glucose primarily activate ChREBP [47, 48], although the late hyperinsulinemia also acts like a potent activator of SREBP-1c [49]. In the meantime, the expansion of the DNL was concomitant to gradual reduction of PPARα expression, which has a crucial role in beta-oxidation and exerts beneficial effects on obesity and management of hepatic steatosis [41]. Furthermore, PPARα suppresses the translocation of SREBP-1c to the Golgi apparatus, avoiding sequential cleavage and nuclear translocation, consequently resulting in inhibition of lipogenesis [50]. Thereby, our data suggest that HSD induces a switch-over from lipid oxidation lipogenesis in liver via suppression of PPARα and consequent over activation of SCD1, a pivotal mechanism involved in triglyceride storage and later hepatic steatosis.

As aforementioned, ER stress is a central driver of NAFLD onset and progression [18]. An impaired UPR signaling is associated to NAFLD development in distinct conditions, such as exposure to high-fat [51], high-fructose diet [52], and even in human obesity [53]. At 30 days of follow-up, our set of data allowed to sight the upregulation of UPR sensors, chaperones and antioxidant defense in response to impaired lipid metabolism induced by diet. UPR sensors and their network of associated chaperones efficiently coordinate cellular changes to maintain protein-folding in the ER and preserve the hepatic lipid homeostasis [54]. Indeed, overexpression of GRP78 has been shown to inhibit lipogenesis through decrease

of SREBP-1c activation, alleviating hepatic steatosis in *ob/ob* mice [55]. Other chaperones such as GRP94 and PDI also appear to promote the same effect [56]. Besides, PDI chaperonates VLDL particles assembly and secretion, contributing to diminish the fat accumulation in the liver [57]. In addition, growing evidence reports that NRF2 is not only involved in redox defense but also have a protective role against steatosis by repressing SREBP-1c expression, which enhances fatty acid oxidation and antagonizes inflammation in hepatocytes [58, 59]. Notwithstanding, several factors assessed in our study can explain the absence of CHOP over expression in the first 30-day period, such as increased expression of UPR sensors, chaperones and NRF2, although we cannot rule out the possibility of CHOP expression to be restrained by PPARα activation, protecting liver from injuries related to inflammation [60].

The ER stress pattern observed after 60 days of nutritional intervention was marked by failure of all adaptive UPR-signaling pathway herein assessed. It was allied to sustained CHOP overexpression, which resulted in exponential raise of DNL pathways, deepening the deleterious outcomes in the liver. On the other hand, it cannot be ignored that overexpressed DNL also contributes to maintenance of ER stress, generating a vicious cycle with higher negative impacts [12, 54]. In confluence with our data, exposure to fructose in different presentations lead to UPR signaling and later ER stress accompanied by lipogenic transcription factors expression and micro- or macrovesicular steatosis [52, 61, 62]. In agreement, ER stress treatment by use of the chemical chaperone phenylbutyric acid (PBA) has demonstrated a protective effect against hepatic lipid accumulation in fructose-evoked NAFLD [46, 63]. Our data are further supported by distinct

models of genetic ablation of either UPR sensors or ER quality control genes, which promote unsolved ER stress characterized by CHOP overexpression and consequent suppression of lipid homeostasis regulators, such as SREBP-1c and PPARα [16].

In conclusion, our study was successful in establish a temporal relationship between DNL and ER stress pathways toward MetS and NAFLD development. After 30 days of high-sucrose intake, even missing consistent MetS-related outcomes, adaptive UPR pathways were activated as an attempt to repair the ER homeostasis disruption caused by early DNL activation. However, upon long-term nutritional intervention, chronic ER stress led to a switch-over from an adaptive to a pro-apoptotic pattern, which further disrupted the balance between fatty acid synthesis and oxidation, allowing greater hepatic fat accumulation. To our knowledge, this is the first time that such transitional pattern of DNL and ER stress pathways is experimentally demonstrated for diet-induced NAFLD. Nevertheless, future studies should assess the outcomes from longer periods of high-sucrose feeding, as well as the consequences of similar nutritional interventions in older mice. Finally, our study importantly warns for the hazardous metabolic consequences of early introduction of added sugars in infant diet shortly after breastfeeding period, which may eventually be established already at young ages.

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DISCLOSURE STATEMENT

Authors declare the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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FIGURE LEGENDS

Figure 1. Morphometric parameters. A, body weight (BW, g); **B**, energy intake (Kcal/day/10g BW); **C,** Lee index ($g^{1/3}$ cm^{-1} × 100); **D**, relative weight (g/10g BW) of retroperitoneal, periepididymal and mesenteric fats assessed in mice fed a standard chow (CTR, n = 7) and high-sucrose diet (HSD, n = 7) for 30, 60 and 90-days from weaning. Points and bars represent mean ± SEM (unpaired t-test one-tailed). $^{ns}p > 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

Figure 2. Biochemical profile and insulin resistance. A, blood glucose levels (mg/dL) in fasting and fed states; **B,** serum total cholesterol, triglycerides and free fatty acids levels (mg/dL); **C-E,** blood glucose levels (mg/dL) during oral glucose tolerance test (GTT) and their respective AUC; **F,** serum insulin levels (µLU/mL)**;** **G,** blood glucose disappearance rate (KITT) after insulin tolerance test (ITT);; **H,** HOMA-IR index; **I,** TyG index assessed in mice fed a standard chow (CTR, n = 7) and high-sucrose diet (HSD, n = 7) for 30, 60 and 90-days, respectively, from weaning. Points and bars represent mean ± SEM (unpaired t-test one-tailed). $^{ns}p > 0.05$, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$.

Figure 3. Morphometric and lipid profile of liver. A, relative weight (g/100g BW) of liver; **B,** relative weight (mg/g of liver) of hepatic total fat; **C,** relative weight (mg/g of liver) of triglycerides in liver; **D,** relative weight (mg/g of liver) of total cholesterol in liver assessed in mice fed a standard chow (CTR, n = 7) and high-sucrose diet (HSD, n = 7) for 30, 60 and 90-days from weaning. Points and bars represent mean ± SEM (unpaired t-test one-tailed). $^{ns}p > 0.05$, $^{*}p < 0.05$, $^{**}p < 0.01$.

Figure 4. Histological analysis of liver. Sections of liver samples stained with H&E for visualization of hepatocytes morphologies and score for steatosis, ballooning and inflammation assessed in mice fed a standard chow (CTR, n = 7) and high-sucrose diet (HSD, n = 7) for 30, 60 and 90-days from weaning. Values represent mean ± SEM (unpaired t-test one-tailed). $*p < 0.05$.

Figure 5. Gene expression of lipogenic markers in liver. A-C, relative mRNA expressions of genes involved in lipogenesis (ChREBP and SREBP-1c); fatty acid synthesis (SCD1 and FAS); and fatty acid oxidation (PPARα) assessed in liver of mice fed a standard chow (CTR, n = 5) and high-sucrose diet (HSD, n = 5) for 30, 60 and 90-days, respectively, from weaning. All samples were normalized to the relative levels of GAPDH and results are expressed as the fold change values of $2^{-\Delta\Delta CT}$, as determined by real-time amplification. Bars represent mean ± SEM (unpaired t-test one-tailed). $^{ns}p > 0.05$, $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, $****p < 0.0001$.

Figure 6. Gene expression of endoplasmic reticulum stress markers in liver. A-C, relative mRNA expressions of UPR sensors (IRE1α, PERK and ATF6); chaperones (GRP78 and PDI 1A); antioxidant defense (NRF2); and apoptosis (CHOP) assessed in liver of mice fed a standard chow (CTR, n = 5) and high-sucrose diet (HSD, n = 5) for 30, 60 and 90-days, respectively, from weaning. All samples were normalized to the relative levels of GAPDH and results are expressed as the fold change values of $2^{-\Delta\Delta CT}$, as determined by real-time

amplification. Bars represent mean ± SEM (unpaired t-test one-tailed). $^{ns}p > 0.05$, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

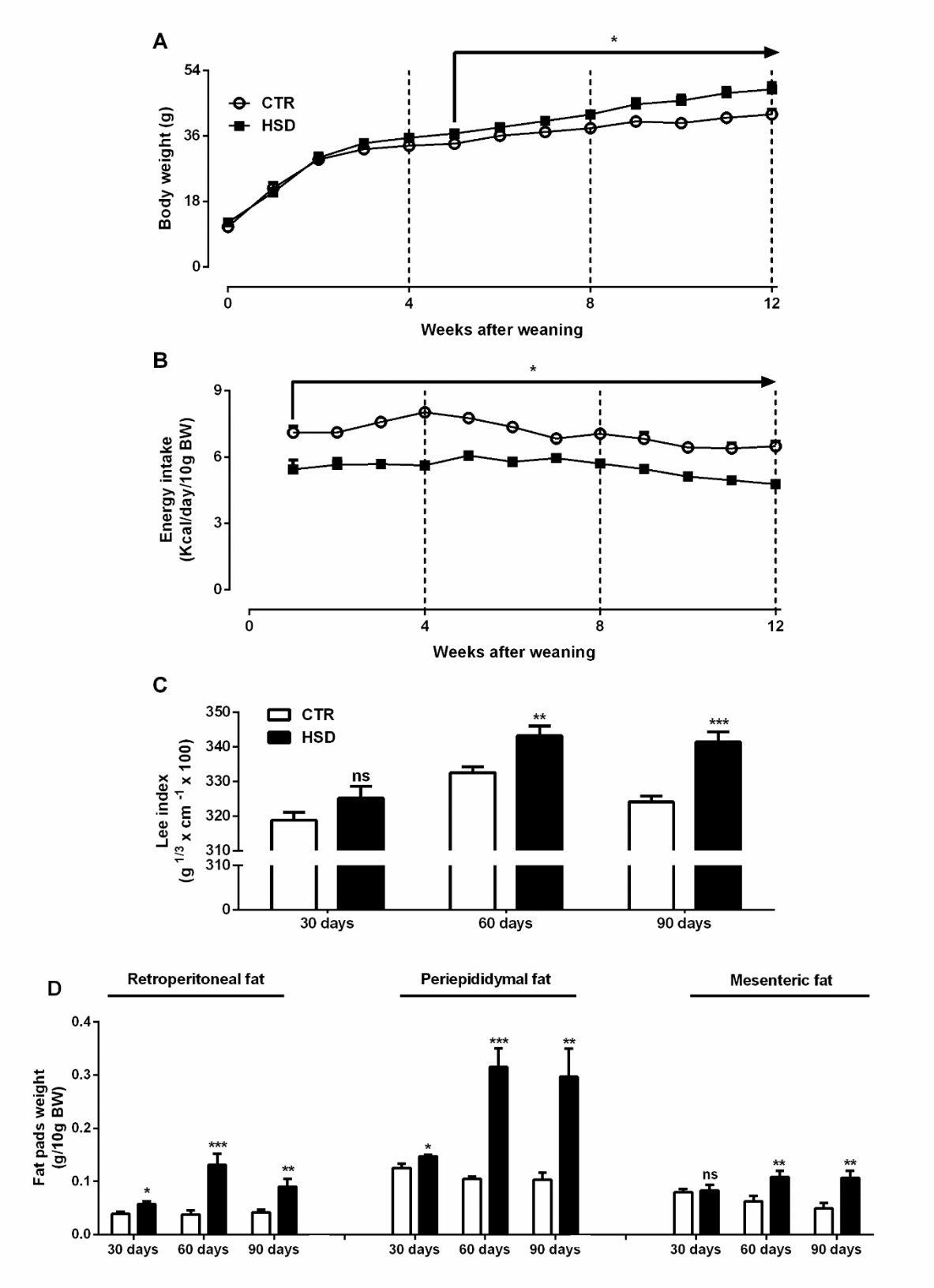

A
Body weight (g)
CTR
HSD
Weeks after weaning
B
Energy intake (Kcal/day/10g BW)
Weeks after weaning
C
Lee index (g 1/3 x cm -1 x 100)
CTR
HSD
ns
30 days
60 days
90 days
D
Retroperitoneal fat
Periepididymal fat
Mesenteric fat
Fat pads weight (g/10g BW)
30 days
60 days
90 days

Figure 1

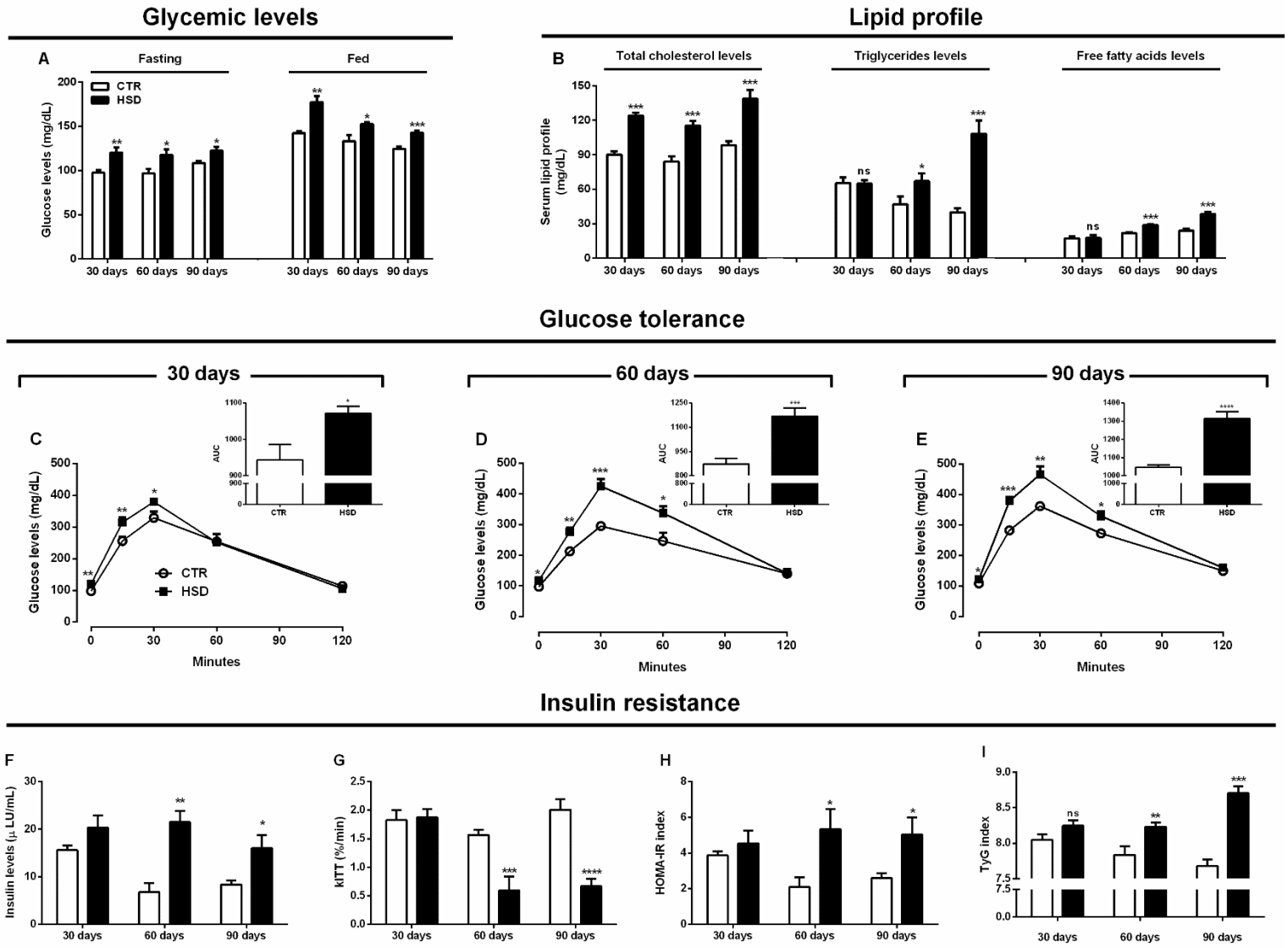

Glycemic levels
A
Fasting
Fed
CTR
HSD
Glucose levels (mg/dL)
30 days
60 days
90 days
Lipid profile
B
Total cholesterol levels
Triglycerides levels
Free fatty acids levels
Serum lipid profile (mg/dL)
ns
Glucose tolerance
30 days
60 days
90 days
C
D
E
AUC
Glucose levels (mg/dL)
Minutes
Insulin resistance
F
G
H
I
Insulin levels (µ LU/mL)
kITT (%/min)
HOMA-IR index
TyG index

Figure 2

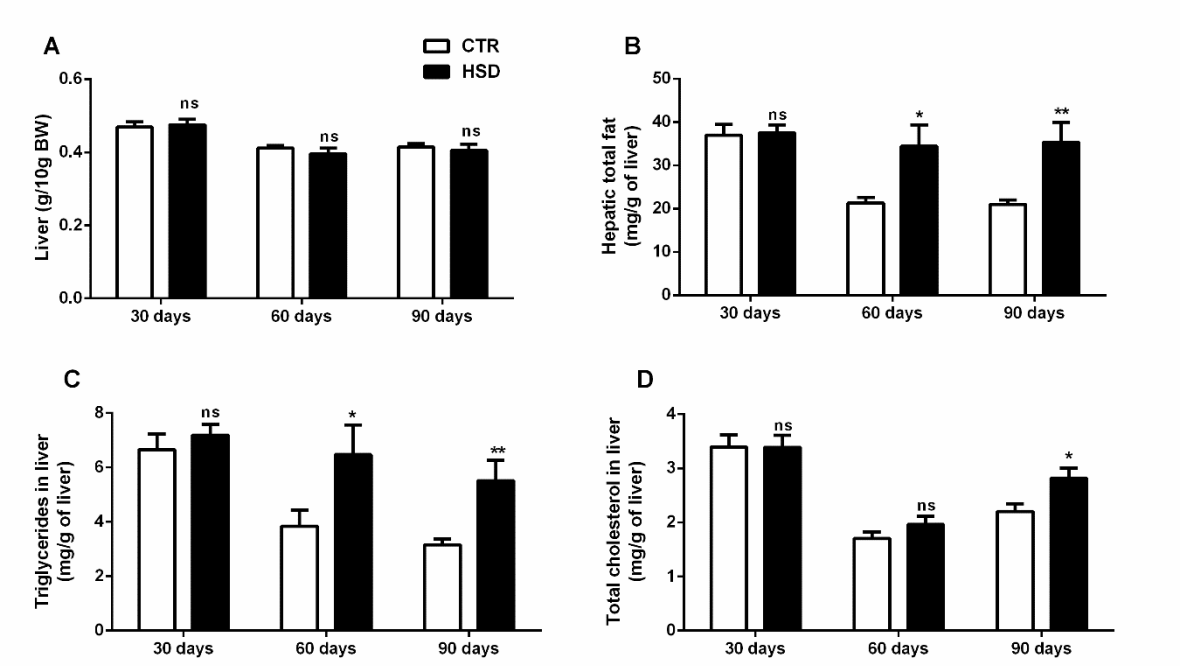
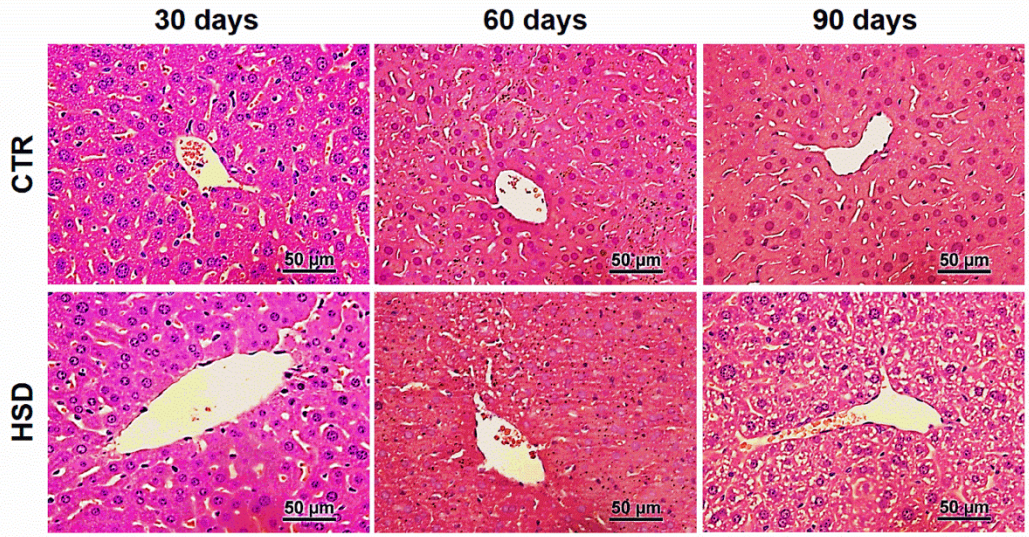

A
CTR
HSD
Liver (g/10g BW)
0.6
0.4
0.2
0.0
ns
ns
ns
30 days
60 days
90 days
B
Hepatic total fat (mg/g of liver)
50
40
30
20
10
0
ns
*
**
30 days
60 days
90 days
C
Triglycerides in liver (mg/g of liver)
8
6
4
2
0
ns
*
**
30 days
60 days
90 days
D
Total cholesterol in liver (mg/g of liver)
4
3
2
1
0
ns
ns
*
30 days
60 days
90 days

Figure 3



	30 days		60 days		90 days	
	CTR	HSD	CTR	HSD	CTR	HSD
Steatosis	0.17 ± 0.17	0.33 ± 0.21	0.00 ± 0.00	**0.67 ± 0.33 ***	0.00 ± 0.00	**0.83 ± 0.40 ***
Ballooning	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.17 ± 0.17	0.00 ± 0.00	0.17 ± 0.17
Inflammation	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Total	0.14 ± 0.14	0.29 ± 0.18	0.00 ± 0.00	**0.86 ± 0.46 ***	0.00 ± 0.00	**0.86 ± 0.46 ***

Figure 4

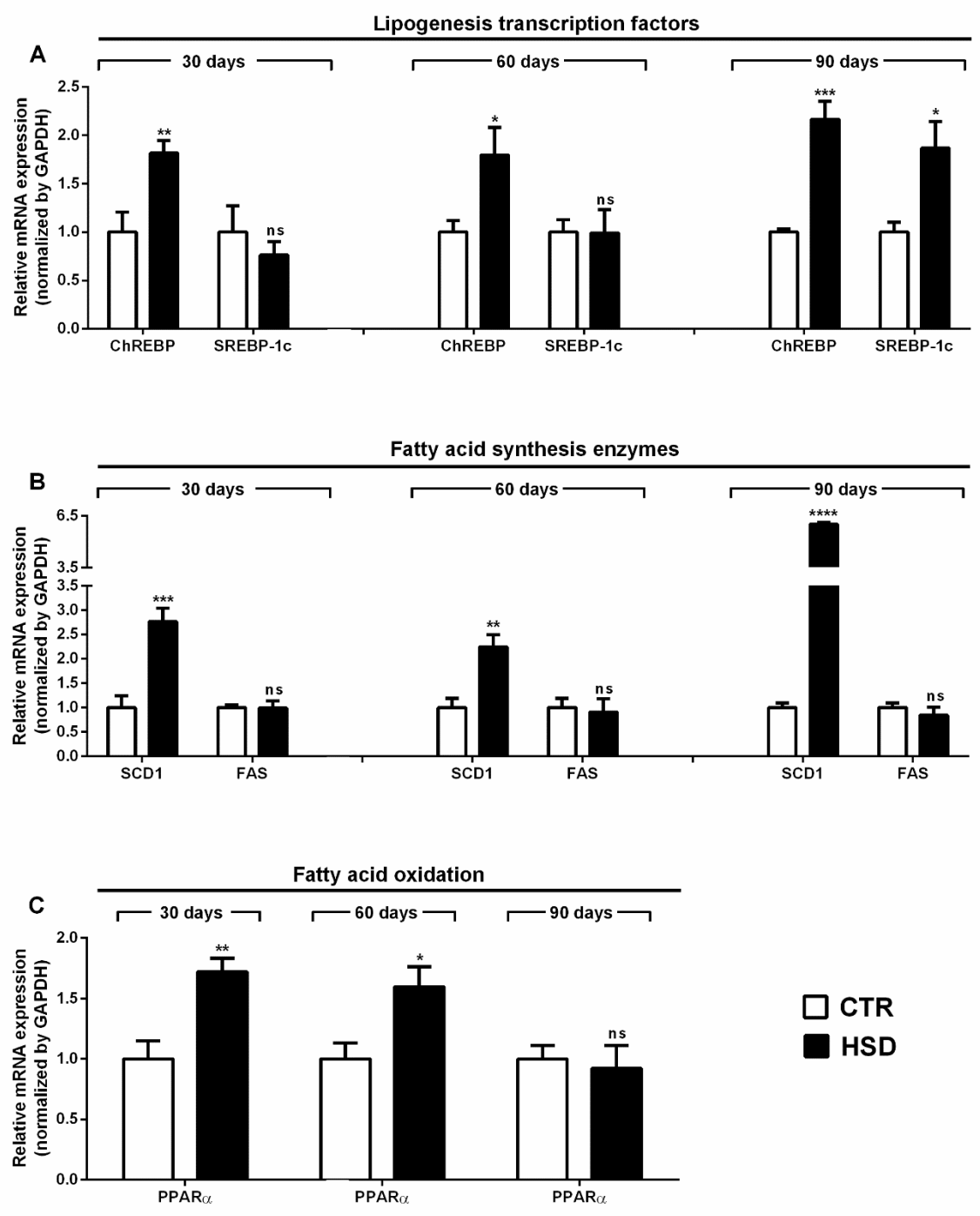

Lipogenesis transcription factors
A
30 days
60 days
90 days
Relative mRNA expression (normalized by GAPDH)
2.5
2.0
1.5
1.0
0.5
0.0
**
ns
*
ns
***
*
ChREBP
SREBP-1c
ChREBP
SREBP-1c
ChREBP
SREBP-1c
Fatty acid synthesis enzymes
B
30 days
60 days
90 days
Relative mRNA expression (normalized by GAPDH)
6.5
3.5
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
***
ns
**
ns
****
ns
SCD1
FAS
SCD1
FAS
SCD1
FAS
Fatty acid oxidation
C
30 days
60 days
90 days
Relative mRNA expression (normalized by GAPDH)
2.0
1.5
1.0
0.5
0.0
**
*
ns
PPARα
PPARα
PPARα
CTR
HSD

Figure 5

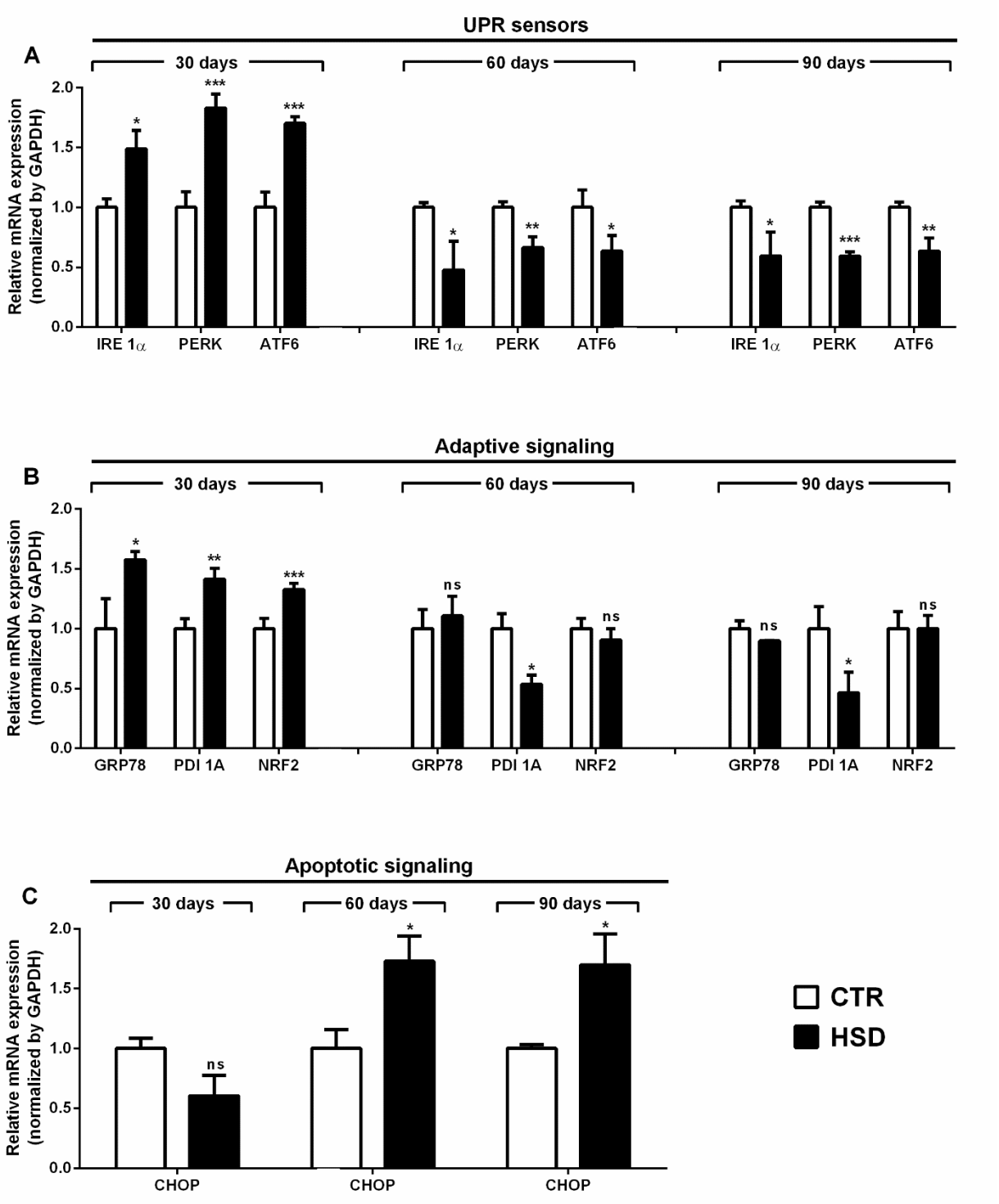

UPR sensors
A
30 days
60 days
90 days
Relative mRNA expression (normalized by GAPDH)
IRE 1α
PERK
ATF6
Adaptive signaling
B
GRP78
PDI 1A
NRF2
Apoptotic signaling
C
CHOP
ns
CTR
HSD

Figure 6

Supplementary Table 1. Primers sequences

Genes*	Foward primer	Reverse primer	Amplicon	GenBank nº
GAPDH	5'-TAACATCAAATGGGGTGAGG-3'	5'-GTGAAGACACCAGTAGACTC-3'	60	NM_001289726.1
ChREBP	5'-TTAGTTCCTGAGCAGAGAGG-3'	5'-ACTAGTGCAAAGGCAAAGAG-3'	58	NM_021455.4
SREBP-1c	5'-GGCTGTTGTCTACCATAAGC-3'	5'-ATGTCCTCCTGTGTACTTGC-3'	61	NM_011480.4
SCD1	5'-AAAACTGGACATGTCTGACC-3'	5'-TACCTCCTCTGGAACATCAC-3'	57	NM_009127.4
FAS	5'-TGGAAAGATAACTGGGTGAC-3'	5'-AGAACCCAGAATGGATACCT-3'	60	NM_007988.3
PPARα	5'-ATGCCAGTACTGCCGTTTTC-3'	5'-GGCCTTGACCTTGTTCATGT-3'	40	NM_001113418.1
IRE1α	5'-AAGGTATGTTTGACAACCGA-3'	5'-AAGCTAAAACACTCAGGGAG-3'	61	NM_023913.2
PERK	5'-CGAGAATGATGGGAAAAAGC-3'	5'-GGCTAGATGAAACCAAGGAA-3'	65	NM_010121.2
ATF6	5'-CTCTCAGGTTGAACCATGAA-3'	5'-GTCCTTTCCACTTCATGTCT 3'	58	NM_001081304.1
GRP78	5'-ACACTTGGTATTGAAACTGTGG-3'	5'-GATCTGAGACTTCTTGGTGG-3'	71	NM_001163434.1
PDI A1	5'-AGCTGCCGCAAAACTGAA-3'	5'-TGGCATCCACCTTTGCTAGT-3'	59	NM_011032.2
NRF2	5'-CGTGAGTCCTGGTCATCAAA-3'	5'-AGCCTCTAAGCGGCTTGAAT-3'	64	NM_010902.3
CHOP	5'-TCACACGCACATCCCAAA-3'	5'-GGCCATAGAACTCTGACTGGAA-3'	56	NM_001290183.1

* GAPDH: Glyceraldehyde-3-phosphate dehydrogenase; ChREBP: Carbohydrate-responsive element-binding protein; SREBP-1c: Sterol regulatory element-binding protein 1c; SCD1: Stearoyl-CoA desaturase-1; FAS: Fatty acid synthase; PPARα: Peroxisome proliferator-activated receptor alpha; IRE1α: Inositol-requiring enzyme 1 alpha; PERK: Protein kinase RNA-like ER kinase; ATF6: Activating transcription factor 6; GRP78: Glucose regulated protein 78; PDI A1: Protein disulfide isomerase family A member 1; NRF2: Nuclear factor, erythroid derived 2, like 2; CHOP: C/EBP-homologous protein.

Supplementary Table 2. Two-way ANOVA analysis for morphometric parameters

Morphometric parameters	30 days		60 days		90 days		*p*-values		
	CTR	HSD	CTR	HSD	CTR	HSD	Effect of diet	Effect of period	Interaction
Body weight (g)	33.4 ± 0.4	35.5 ± 1.1	38.14 ± 0.7	41.9 ± 0.6	41.9 ± 1.4	48.8 ± 1.8	0,1233	***p* < 0.0001**	***p* < 0.0001**
Energy intake (Kcal/day/10g BW)	8.03 ± 0.09	5.62 ± 0.2	7.05 ± 0.15	5.71 ± 0.17	6.4 ± 0.2	4.77 ± 0.17	**0.0198**	***p* < 0.0001**	***p* < 0.0001**
Lee index ($g^{1/3}$/cm x 100)	318.9 ± 2.2	325.2 ± 3.4	332.6 ± 1.7	324.1 ± 1.6	324.1 ± 1.5	341.4 ± 2.9	***p* < 0.0001**	***p* < 0.0001**	0.1035
Retroperitoneal fat (g/10g BW)	0.039 ± 0.004	0.057 ± 0.005	0.038 ± 0.008	0.13 ± 0.02	0.042 ± 0.005	0.089 ± 0.015	***p* < 0.0001**	***p* < 0.0205**	**0.0151**
Periepididymal fat (g/10g BW)	0.12 ± 0.008	0.15 ± 0.003	0.1 ± 0.005	0.1 ± 0.29	0.1 ± 0.01	0.29 ± 0.05	***p* < 0.0001**	**0.0270**	**0.0031**
Mesenteric fat (g/10g BW)	0.08 ± 0.006	0.083 ± 0.01	0.063 ± 0.009	0.11 ± 0.01	0.049 ± 0.009	0.11 ± 0.01	**0.0002**	0.7725	**0.0356**

Mice fed a standard chow (CTR, n = 7) and high-sucrose diet (HSD, n = 7) for 30, 60 and 90-days from weaning. Data are expressed as mean ± SEM (two-way ANOVA) to examine the effects of HSD and period, followed by Bonferroni post hoc analyses $p \leq 0.05$.

Supplementary Table 3. Two-way ANOVA analysis for biochemical parameters

Biochemical parameters	30 days		60 days		90 days		*p*-values		
	CTR	HSD	CTR	HSD	CTR	HSD	Effect of diet	Effect of period	Interaction
Fasting Glucose (mg/dL)	97.5 ± 3.2	120.3 ± 5.6	97 ± 4.7	117.4 ± 6.5	108.3 ± 2.4	122.3 ± 4.3	***p* < 0.0001**	0.2411	0.6568
Fed glucose (mg/dL)	142.1 ± 2.2	177.3 ± 6.9	133 ± 7.1	152.4 ± 2	124.4 ± 2.7	142.7 ± 2.3	***p* < 0.0001**	***p* < 0.0001**	0.1235
Total cholesterol (mg/dL)	90.1 ± 2.9	124.1 ± 2.5	84.2 ± 4.4	115.3 ± 4.1	98.3 ± 3.4	138.7 ± 7.5	***p* < 0.0001**	**0.0015**	0.6068
Triglycerides (mg/dL)	65.3 ± 4.9	64.9 ± 2.9	46.8 ± 6.9	66.9 ± 6.9	39.7 ± 3.5	108.3 ± 11.5	***p* < 0.0001**	0.0727	**0.0001**
Free fatty acids (mg/dL)	17.3 ± 1.6	17.48 ± 2.6	21.7 ± 0.8	28.7 ± 0.8	23.9 ± 1.6	38.4 ± 1.7	***p* < 0.0001**	***p* < 0.0001**	**0.0006**
GTT (AUC)	943.7 ± 42.9	1072 ± 19.3	872.1 ± 35.3	1168 ± 52.9	1046 ± 14.6	1314 ± 39.5	***p* < 0.0001**	***p* < 0.0001**	0.0779
Insulin (µLU/ml)	15.6 ± 0.9	20.3 ± 2.6	8.5 ± 2.3	21.5 ± 2.3	8.3 ± 0.9	15.9 ± 2.8	***p* < 0.0001**	**0.0362**	0.1578
kITT (%/min)	1.8 ± 0.2	1.9 ± 0.1	1.6 ± 0.1	0.6 ± 0.2	2.01 ± 0.2	0.7 ± 0.1	***p* < 0.0001**	**0.0003**	**0.0007**
HOMA IR	3.9 ± 0.2	4.5 ± 0.7	2.1± 0.6	5.3 ± 1.1	2.6 ± 0.3	5.02 ± 0.1	**0.0015**	0.7880	0.2286
TyG index	8.0 ± 0.1	8.2 ± 0.1	7.8 ± 0.1	8.2 ± 0.1	7.7 ± 0.1	8.7 ± 0.1	***p* < 0.0001**	0.2081	**0.0002**

Mice fed a standard chow (CTR, n = 7) and high-sucrose diet (HSD, n = 7) for 30, 60 and 90-days from weaning. Data are expressed as mean ± SEM (two-way ANOVA) to examine the effects of HSD and period, followed by Bonferroni post hoc analyses $p \leq 0.05$.

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Supplementary Table 4.Morphometric and lipid profile of livers of mice fed for 30, 60 and 90-days from weaning

Morphometric and lipid profile of livers	30 days		60 days		90 days		*p*-values		
	CTR	HSD	CTR	HSD	CTR	HSD	Effect of diet	Effect of period	Interaction
Liver (g/10g BW)	0.47 ± 0.01	0.47 ± 0.02	0.41 ± 0.01	0.39 ± 0.02	0.41 ± 0.01	0.40± 0.01	0.71	***p* < 0.0001**	0.58
Hepatic total fat (mg/g of liver)	36.9 ± 2.5	37.5 ± 1.9	21.3 ± 1.3	34.4 ± 5	20.9± 1.1	35.3 ± 4.6	**0.0103**	**0.0014**	0.3083
Triglycerides in liver (mg/g of liver)	6.6 ± 0.6	7.2 ± 0.4	3.8 ± 0.6	6.5 ± 1.1	3.17± 0.2	5.51 ± 0.7	**0.044**	**0.0044**	0.31
Total cholesterol in liver(mg/g of liver)	3.4 ± 0.2	3.4 ± 0.2	1.7 ± 0.1	1.9 ± 0.2	2.19 ± 0.15	2.82 ± 0.18	0.0672	***p* < 0.0001**	0.259

Mice fed a standard chow (CTR, n = 7) and high-sucrose diet (HSD, n = 7) for 30, 60 and 90-days from weaning. Data are expressed as mean ± SEM (two-way ANOVA) to examine the effects of HSD and period, followed by Bonferroni post hoc analyses $p \leq 0.05$.

5. CONSIDERAÇÕES FINAIS

Mesmo com o crescente número de estudos destacando o papel de dietas ricas em carboidratos sobre o desenvolvimento de DHGNA, as vias de sinalização envolvidas ainda não são totalmente conhecidas. Neste contexto, a investigação do estresse do retículo endoplasmático como um modulador das vias lipogênicas responsáveis pelo agravamento da DHGNA ganha um importante destaque.

Em nosso estudo demonstramos que a exposição a dieta rica em sacarose após o desmame e por períodos curtos de acompanhamento (30 dias), inicia discretas alterações metabólicas acompanhadas de uma sinalização adaptativa ao estresse mediada pelo retículo endoplasmático e um equilíbrio entre a síntese e oxidação de ácidos graxos no fígado, mantendo sob controle o surgimento de esteatose.

Entretanto, com a continuidade da exposição à sacarose por períodos mais crônicos (a partir de 60 dias) observa-se a instauração de um perfil obesogênico marcado por dislipidemias e resistência à insulina. Associado às alterações metabólicas, observou-se uma sinalização lipogênica marcada por expressão de genes relacionados a síntese de ácidos graxos e estresse do retículo endoplasmático, caracterizado por falência de qualquer resposta adaptativa e um padrão apoptótico. A lipogênese descontrolada associada ao estresse do retículo endoplasmático são cruciais para o desenvolvimento de esteatose microvesicular, uma etapa precursora para o surgimento de doenças hepáticas mais severas.

Considerando-se o elevado consumo de açucares de adição pela população, principalmente entre as crianças (Bray e Popkin, 2014; Mosca *et al.*, 2016) os dados apresentados em nosso estudo também reforçam os conceitos de programação metabólica, que estabelecem que insultos em fases iniciais da vida (pós-desmame, por exemplo) impactam sobre o genótipo do indivíduo, aumentando a predisposição para o desenvolvimento e/ou agravamento tardio de doenças crônicas não-transmissíveis, como a SM e a DHGNA (Fuente-Martin *et al.*, 2012; Tavares *et al.*, 2012; Zheng *et al.*, 2016).

Em suma, nosso estudo, de forma inovadora, teve sucesso em estabelecer a relação existente entre o desenvolvimento de síndrome metabólica induzida por sacarose, ativação desregulada da síntese *de novo* de lipídeos e estresse do retículo

endoplasmático, que em conjunto promoveram o surgimento de DHGNA. O maior entendimento das vias de sinalização envolvidas na patogênese destas doenças são relevantes para nortear possíveis abordagens terapêuticas e medidas preventivas para reduzir sua incidência.

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ANEXOS

- **ANEXO I - Artigo já publicado no periódico *Metabolic Brain Disease***

- **ANEXO II - Parecer do Comitê de Ética**

- **ANEXO III - Normas de publicação do periódico escolhido para submissão do artigo**

ANEXO I - Artigo já publicado no periódico *Metabolic Brain Disease*

- Artigo publicado no periódico ***Metabolic Brain Disease***

- Qualis Capes 2015 – Medicina I: **Classificação B1**

- Fator de impacto: **2.297**

- Título: **"Early and sustained exposure to high-sucrose diet triggers hippocampal ER stress in young rats" - DOI 10.1007/s11011-016-9830-1**

- Autores: **Bruno Araújo Serra Pinto, Thamys Marinho Melo, Karla Frida Torres Flister, Lucas Martins França, Daniela Kajihara, Leonardo Yuji Tanaka, Francisco Rafael Martins Laurindo, Antonio Marcus de Andrade Paes**

Metab Brain Dis
DOI 10.1007/s11011-016-9830-1

ORIGINAL ARTICLE

Early and sustained exposure to high-sucrose diet triggers hippocampal ER stress in young rats

Bruno Araújo Serra Pinto[1] · Thamys Marinho Melo[1] · Karla Frida Torres Flister[1] · Lucas Martins França[1] · Daniela Kajihara[2] · Leonardo Yuji Tanaka[2] · Francisco Rafael Martins Laurindo[2] · Antonio Marcus de Andrade Paes[1]

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Abstract Early-life environmental insults have been shown to promote long-term development of chronic non-communicable diseases, including metabolic disturbances and mental illnesses. As such, premature consumption of high-sugar foods has been associated to early onset of detrimental outcomes, whereas underlying mechanisms are still poorly understood. In the present study, we sought to investigate whether early and sustained exposure to high-sucrose diet promotes metabolic disturbances that ultimately might anticipate neurological injuries. At postnatal day 21, weaned male rats started to be fed a standard chow (10 % sucrose, CTR) or a high-sucrose diet (25 % sucrose, HSD) for 9 weeks prior to euthanasia at postnatal day 90. HSD did not alter weight gain and feed efficiency between groups, but increased visceral, non-visceral and brown adipose tissue accumulation. HSD rats demonstrated elevated blood glucose levels in both fasting and fed states, which were associated to impaired glucose tolerance. Peripheral insulin sensitivity did not change, whereas hepatic insulin resistance was supported by increased serum triglyceride levels, as well as higher TyG index values. Assessment of hippocampal gene expression showed endoplasmic reticulum (ER) stress pathways were activated in HSD rats, as compared to CTR. HSD rats had overexpression of unfolded protein response sensors, PERK and ATF6; ER chaperone, PDIA2 and apoptosis-related genes, CHOP and Caspase 3; but decreased expression of chaperone GRP78. Finally, HSD rats demonstrated impaired neuromuscular function and anxious behavior, but preserved cognitive parameters. In conclusion, our data indicate that early exposure to HSD promote metabolic disturbances, which disrupt hippocampus homeostasis and might precociously affect its neurobehavioral functions.

Keywords High-sucrose diet · Metabolic syndrome · Hippocampus · Unfolded protein response · Neurological impairment · Developmental origins of health and disease

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Introduction

Metabolic syndrome (MetS) is conceptually defined as the co-occurrence of at least three out of the following metabolic disturbances: central/visceral obesity, hypertriglyceridemia, hyperglycemia, insulin resistance, hyperinsulinemia and hypertension (Alberti et al. 2009). While precise mechanisms underlying the development of its clustered comorbidities are not completely understood, MetS has rapidly reached epidemic proportions worldwide in a straight relationship with the access to sugar-rich foods and beverages, as well as less physical activity (Bray et al. 2004). Additionally, accumulating data have suggested that metabolic (re)programming processes subsequent to early-life insults, i.e. during pregnancy, infancy or childhood, may lead to long-term development of chronic non-communicable diseases, such as MetS, cancer, mental illness, among others (Chan et al. 2015; Gluckman et al. 2007; Inadera 2013). This hypothesis for the developmental origins of health and disease (DOHaD) was first proposed in the 1990's by Dr. David Barker, and attracted increasing attention of scientists, medical doctors, epidemiologists and policymakers around the world (Cooper 2013).

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ANEXO II - Parecer do Comitê de Ética

UNIVERSIDADE FEDERAL DO MARANHÃO
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Certificamos que a proposta intitulada "**Caracterização do impacto da obesidade sobre o surgimento e progressão de marcadores moleculares envolvidos no desenvolvimento de esteatose hepática não alcoólica**" registrada com o nº **23115.002832/2017-25**, sob a responsabilidade de **Karla Frida Torres Flister**, 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 de Experimentação Animal (CONCEA), e foi considerado **APROVADO** pela Comissão de Ética no Uso de Animais (CEUA - UFMA) da Universidade Federal do Maranhão em reunião de 22/05/2017.

FINALIDADE	()Ensino (**X**)Pesquisa ( ) Extensão
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Profa. Dra. Lucilene Amorim Silva
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ANEXO III - Normas de publicação do periódico escolhido para submissão do artigo

THE JOURNAL OF NUTRITIONAL BIOCHEMISTRY

AUTHOR INFORMATION PACK

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