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Efeito da Suplementação de Zinco Associada com Dieta de Cafeteria sobre Marcadores de Neuroinflamação e Memória em Ratos Wistar

Porto Alegre
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Dissertação submetida ao Programa de Pós-Graduação em Ciências da Saúde da Universidade Federal de Ciências da Saúde de Porto Alegre como requisito para a obtenção do grau de Mestre.

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“A coisa mais bela que o homem pode experimentar é o mistério. É essa emoção fundamental que está na raiz de toda a ciência e toda a arte”.

Albert Einstein

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LISTA DE ABREVIATURAS

COX2: cicloxigenase 2

GFAP: proteína ácida fibrilar glial (do inglês, *glial fibrillary acidic protein*)

Iba-1: imunomarcador de micróglia (do inglês, *ionized calcium-binding adapter molecule-1*)

IL1-β: interleucina-1β

IL-6: interleucina-6

LPS: lipopolissacarídeo

NFκB: fator nuclear kappa B (do ingles, *nuclear factor kappa B*)

OMS: organização mundial de saúde

SIH-SUS: Sistema de Informações Hospitalares do Sistema Único de Saúde

SNC: sistema nervoso central

TLR: receptor do tipo *toll* (do inglês*, toll like receptor*)

TNF-α: fator de necrose tumoral-α (do inglês*, tumor necrosis factor alpha*)

ZAG: zinco-α2 glicoproteína

ZIP: proteína transportadora de zinco (para o citosol) (do inglês*, zinc importer protein*)

Zn: zinco

ZnT: proteína transportadora de zinco (para o meio extracelular ou para vesículas intracelulares) (do inglês*, zinc transporter*)

SUMÁRIO

RESUMO

A obesidade desencadeia um perfil pró-inflamatório sistêmico, que afeta o centro sistema nervoso central (SNC), predispondo ao desenvolvimento de doenças neurológicas. O Zn é um mineral que tem potente ação anti-inflamatória, portanto, pode desempenhar um papel como agente neuroprotetor nesse perfíl obesogênico. O objetivo deste estudo foi avaliar os efeitos da suplementação de Zn sobre parâmetros metabólicos, neuroinflamatórios e de memória em ratos Wistar machos que receberam dieta de cafeteria. Para isso, 28 ratos Wistar foram divididos em 4 grupos: dieta controle (CT); CT + Zn; dieta de cafeteria (CAF); CAF + Zn. A dieta foi administrada por 20 semanas e o tratamento com Zn (10mg/Kg/dia, por gavagem) começou a partir da 16ª semana até o final do protocolo da dieta. A memória foi avaliada pelo teste de reconhecimento de objetos e o índice de reconhecimento (IR) foi calculado. O ganho de peso e a gordura visceral foram quantificados. Os níveis de insulina, glicose e triglicerídeos foram avaliados no plasma, bem como os níveis de TNF-α e IL-6. Também foi calculado o índice de HOMA (HOMA-IR). No SNC, foi avaliado por Western blot a expressão proteica de GFAP, Iba-1 e TLR-4 no córtex cerebral e TLR-4 no hipocampo. Os dados foram avaliados por ANOVA de duas vias seguido pelo teste de Tukey considerando $p \geq 0,05$ como significante. Os animais que receberam CAF apresentaram maior ganho de peso e gordura visceral que o grupo CT, sendo que o Zn não interferiu nessas medidas. A CAF também causou um aumento nos níveis de glicose e triglicerídeos no plasma e a suplementação com Zn reverteu a hiperglicemia causada pela CAF. Os níveis de insulina foram semelhantes entre os grupos, mas o HOMA-IR foi maior nos grupos que receberam CAF, sem efeito do Zn. O TNF-α foi aumentado nos grupos CAF, e o Zn não modificou esse resultado. A IL-6 foi reduzida nos grupos que receberam Zn, tendo diferença significativa entre os grupos CAF e CAF+Zn. No córtex cerebral, a expressão de GFAP foi semelhante entre os grupos; o Iba-1 foi aumentado pela CAF, mas reduzido no grupo CAF+Zn; já o TLR-4 aumentou nos grupos CAF e CAF+Zn. No hipocampo, a expressão de TLR-4 foi aumentada no grupo CAF e reduzida no grupo CAF+Zn. No teste de reconhecimento de objetos, os animais obesos apresentaram pior desempenho e o tratamento com Zn foi capaz de reverter esse déficit. Assim, nossos resultados demonstram que a suplementação de zinco reduziu parcialmente a disfunção metabólica causada pela dieta da cafeteria. Por outro lado, o Zn foi efetivo para amenizar a neuroinflamação e o déficit de memória e neuroinflamação causados pela obesidade.

Palavras chave: obesidade, TNF-α, IL-6, GFAP, Iba-1, TLR-4, memória de reconhecimento.

ABSTRACT

Obesity triggers a systemic proinflammatory profile that affects the central nervous system (CNS), predisposing to the development of neurological diseases. Zn is a mineral that has potent anti-inflammatory action, so it can play a role as a neuroprotective agent in obesity. The aim of this study was to evaluate the effects of Zn supplementation on metabolic, neuroinflammatory and memory parameters in male Wistar rats following cafeteria diet. Twenty-eight Wistar rats were divided into 4 groups: control diet (CT); CT + Zn; cafeteria diet (CAF); CAF + Zn. The diet was administered for 20 weeks and treatment with Zn (10mg/kg/day, by gavage) started at week 16 and it was conducted until the end of the diet protocol. Memory was assessed by the object recognition test and the recognition index (IR) was calculated. Weight gain and visceral fat were quantified. Plasmatic levels of insulin, glucose and triglyceride levels were evaluated. HOMA index (HOMA-IR) was calculated TNF-α and IL-6 plasma levels were also analysed. In the CNS, protein expression of GFAP, Iba-1 and TLR-4 in the cerebral cortex and TLR-4 in the hippocampus were evaluated by Western blot. Data were analysed by two-way ANOVA followed by Tukey test considering $p \geq 0.05$ as significant. The animals that received CAF presented higher weight gain and visceral fat than the CT groups, and Zn did not interfere in these measurements. CAF also caused an increase in plasma glucose and triglyceride levels and supplementation with Zn reversed the hyperglycemia caused by CAF. Insulin levels were similar between groups, but HOMA-IR was higher in the groups receiving CAF, with no effect of Zn. TNF-α was increased in CAF groups, and Zn did not modify this result. IL-6 was reduced in the groups that received Zn, having significant difference between CAF and CAF + Zn groups. In the cerebral cortex, GFAP expression was similar between groups; Iba-1 was increased by CAF, but reduced in the CAF + Zn group; TLR-4 increased in the CAF and CAF + Zn groups. In the hippocampus, TLR-4 expression was increased in the CAF group and reduced in the CAF + Zn group. In the object recognition test, obese animals presented worse performance and Zn treatment was able to reverse this deficit. Thus, our results demonstrate that zinc supplementation partially reduced metabolic dysfunction caused by CAF. On the other hand, Zn was effective to reduce neuroinflammation and memory deficit caused by obesity.

Keywords: obesity, TNF-α, IL-6, GFAP, Iba-1, TLR-4, recognition memory.

1 INTRODUÇÃO

1.1 OBESIDADE

A obesidade é considerada uma doença crônica, multifatorial que vem se tornando uma epidemia mundial, o que afeta negativamente a saúde da população (Bahia *et al.*, 2012). Ela é caracterizada pelo acúmulo excessivo de gordura corporal, que está associada a uma maior prevalência de doenças cardiovasculares (Lotufo, 2000; Aghamohammadzadeh and Heagerty, 2012), alguns tipos de câncer (Demark-Wahnefried *et al.*, 2012), diabetes mellitus tipo 2 (Wannamethee and Shaper, 1999), predisposição a doenças intestinais (Jayarathne *et al.*, 2019; Luck *et al.*, 2019), aumento da desmineralização óssea (Neglia *et al.*, 2016), prejuízos cognitivos (Dye *et al.*, 2017) e doenças neurológicas (O'brien *et al.*, 2017).

Segundo a Organização Mundial da Saúde (OMS), a obesidade atualmente ocasiona mais mortes no mundo do que a desnutrição. Este perfil quase triplicou desde 1975. Em 2016, mais de 1,9 bilhão de adultos apresentavam sobrepeso e mais de 650 milhões eram obesos (Who, 2019).

No Brasil, a prevalência de obesidade também vem crescendo nos últimos anos. Entre 2006 e 2018, o percentual de obesos entre a população adulta aumentou de 11,8% para 19,8% (Vigitel, 2018). Esse aumento do número de indivíduos obesos reflete no aumento do custo de internações hospitalares. Somente em 2018, o Sistema de Informações Hospitalares do Sistema Único de Saúde (SIH-SUS) registrou um gasto em torno de 64,3 milhões de reais com internações devido à obesidade e suas complicações (Sih/Sus, 2018).

O acúmulo excessivo de tecido adiposo é considerado um dos maiores riscos à saúde humana, e está associado a diversos fatores, incluindo componentes genéticos, dieta inadequada e falta de atividade física (Engin, 2017). Esse excesso de gordura é caracterizado por inflamação crônica e de baixo grau que também acomete os demais tecidos metabólicos, sendo chamada de metainflamação (Hotamisligil, 2017). Nesse quadro de sinalização pró-inflamatória, ocorre ativação de receptores de membrana celular tais como

o receptor do fator de necrose tumoral alfa (TNF-α) e os receptores do tipo *toll* (TLR, do inglês *toll like receptor*), os quais induzem diversas alterações intracelulares que resultam em desequilíbrios metabólicos (Hotamisligil, 2017).

Os TLRs são importantes em sinalizar a identificação de invasores ou de qualquer injúria tecidual. Em mamíferos, já foram estudados 13 membros da família *toll-like* (Muzio *et al.*, 2000), sendo que o TLR-4 é responsável pelo reconhecimento do lipopolissacarídeo (LPS), o qual faz parte da parede celular das bactérias gram-negativas (Grootjans *et al.*, 2016; Ryu *et al.*, 2017). O LPS pode estar aumentado em quadros de infecção, porém, sabe-se que na obesidade ocorre alteração da microbiota intestinal, o que também pode levar ao aumento dos níveis sanguíneos de LPS, que, por sua vez, causa ativação do TLR-4 (Hersoug *et al.*, 2018). A cascata de sinalização iniciada pelo TLR-4 determina a ativação do fator de transcrição nuclear kappa B (NFκB) que estimula a transcrição de genes de citocinas pró-inflamatórias (Figura 1) (Rogero and Calder, 2018).

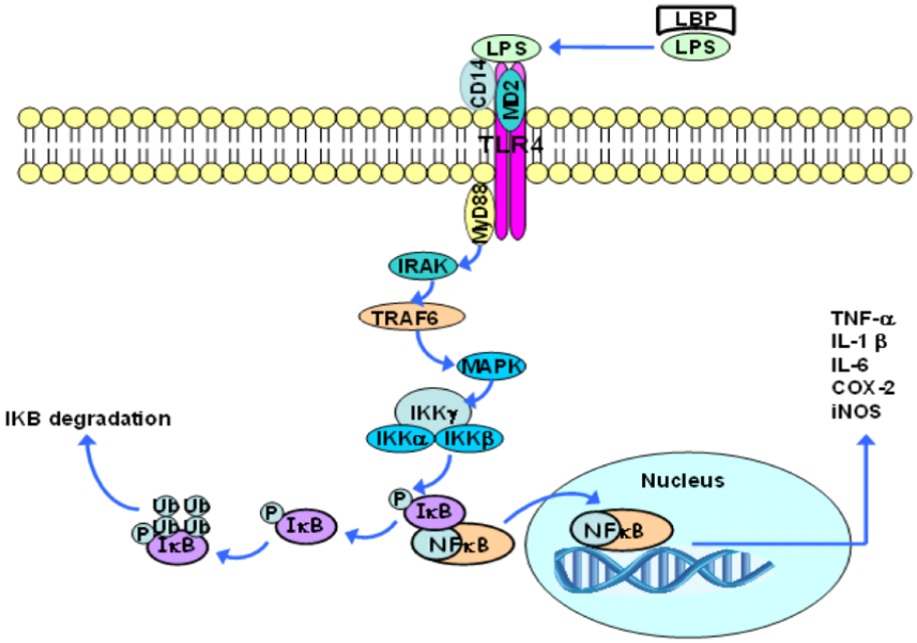

Figura 1. Cascata de sinalização ativada pelo TLR4. Estímulos como LPS ativam o TLR4 levando ao aumento da expressão de fatores pró-inflamatórios (Rogero and Calder, 2018).

Ainda, os ácidos graxos livres, que estão aumentados na obesidade, também podem ser agonistas do TLR-4 (Wang *et al.*, 2017). Além disso, a

liberação exacerbada desses ácidos graxos contribui para a resistência à insulina e acentua a inflamação do tecido adiposo. Com isso, observa-se o aumento da expressão de algumas citocinas pró-inflamatórias como a interleucina 6 (IL-6) e o TNF-α (Engin, 2017).

O TNF-α é uma citocina pró-inflamatória que pode desencadear a ativação de vias de necrose ou apoptose celular (Idriss and Naismith, 2000). A IL-6 também é uma citocina produzida e secretada por macrófagos ativos e linfócitos T como uma resposta a lesões teciduais, estimulando a resposta imune, porém quando sua síntese por algum motivo é desregulada, ela desempenha um efeito patológico em estados inflamatórios crônicos, como na obesidade (Tanaka *et al.*, 2014).

1.2 OBESIDADE E SISTEMA NERVOSO CENTRAL – ENVOLVIMENTO DA NEUROINFLAMAÇÃO

Hormônios metabólicos como a insulina, por exemplo, interferem na função cognitiva, modulando os processos de formação e consolidação da memória e também de neurogênese. Dessa forma, alteração da sinalização da insulina no sistema nervoso central (SNC) pode desencadear disfunções neurológicas (Spinelli *et al.*, 2019).

Em um estudo realizado em modelo animal foi demosntrado que a obesidade potencializa diversos mecanismos neurotóxicos, aumentando assim, a predisposição ao desenvolvimento de doenças neurológicas. Ainda não se sabe os mecanismos exatos relacionados, mas diversas evidências indicam que esta exacerbação das respostas inflamatórias no SNC pode ser uma característica patofisiológica importante destes quadros (Boini *et al.*, 2017).

Foi demonstrado que o consumo de dieta hiperlipídica por roedores causa déficits de memória, o que está associado com ativação da microglia e de astrócitos, e aumento da produção de mediadores pró-inflamatórios como cicloxigenase-2 (COX-2), TNF-α, interleucina-1β (IL-1β) e IL-6 (Hajiluian *et al.*, 2018; Duffy *et al.*, 2019). Recentemente tem se observado a relação entre

obesidade e doenças neurodegenerativas ocasionadas por este ambiente pró-inflamatório, como doença de Parkinson e de Alzheimer (Mazon *et al.*, 2017).

Dessa forma, o fenótipo pró-inflamatório sistêmico presente na obesidade também atinge o SNC. Assim como em outros tecidos, a neuroinflamação sustentada é caracterizada por um desequilíbrio na secreção de citocinas anti e pró-inflamatórias, com predomínio das últimas. Isso pode predispor a diversas situações patológicas, como por exemplo, a deposição de peptídeo β-amilóide, o que é característico da doença de Alzheimer (Pistollato *et al.*, 2016). No hipocampo, a exacerbação do perfil pró-inflamatório está associada com redução da capacidade de neurogênese (Ziv *et al.*, 2006). Este perfil também leva a mudanças comportamentais, déficit cognitivo, depressão e ansiedade (Almeida-Suhett *et al.*, 2019).

Sabe-se que na obesidade existe um desbalanço homeostático com predomínio de características pró-inflamatórias, e no SNC ocorre ativação microglial hipotalâmica, o que interfere na regulação dos circuitos de fome e saciedade bem como sobre a regulação do metabolismo energético corporal. Contudo, a resposta neuroinflamatória, decorrente da obesidade não se restringe ao hipotálamo (Valdearcos *et al.*, 2017).

O hipocampo também é uma região atingida pela neuroinflamação causada pela obesidade, gerando um risco aumentado para o declínio cognitivo e propensão para o desenvolvimento de doença de Alzheimer (Barron *et al.*, 2017).

Assim, a regulação dos mediadores inflamatórios parece ser uma boa estratégia de prevenção ou atenuação de doenças que estão associadas ao declínio cognitivo, como também parece ser o caso da obesidade (Mendiola and Cardona, 2018).

A resposta inflamatória do tecido nervoso envolve principalmente astrócitos e microglia. Essas células desempenham um papel importante na manutenção da homeostase do microambiente neuronal, protegendo o SNC de patógenos. A microglia é responsável pela vigilância imunológica do parênquima cerebral, sendo assim, são as células imunes residentes do SNC, responsáveis por realizar fagocitose. A microglia é o primeiro tipo celular a ser ativado na neuroinflamação, e promove liberação aguda de inúmeros mediadores pró-

inflamatórios em resposta à injúria tecidual. Porém, quando existe uma ativação crônica da microglia, a neuroinflamação exacerbada pode favorecer um ambiente neurotóxico (Chen and Qian, 2016; Shabab *et al.*, 2017).

Além disso, a característica mais comum entre algumas doenças neurodegenerativas é a neuroinflamação, que é iniciada com a ativação microglial. A microglia pode ser classificada em dois tipos com características divergentes e opostas, sendo eles o fenótipo M1 (pró-inflamatório) ou M2 (imunossupressor) (Figura 2). Além de alteração no padrão de secreção de moléculas, cada fenótipo também é caracterizado por morfologias distintas, sendo que no estado M1, as células assumem um formato mais ameboide, o que é diferente do M2, no qual a microglia apresenta-se mais ramificada (Tang and Le, 2016).

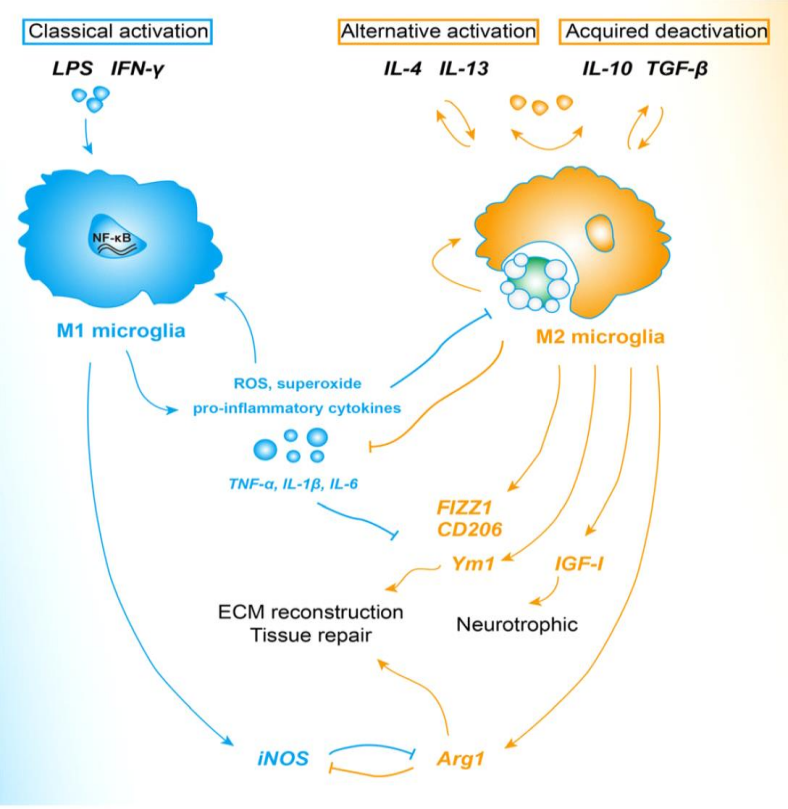

Figura 2. Características funcionais da microglia. No estado de ativação M1, ocorre produção de citocinas pró-inflamatórias e espécies reativas de oxigênio. No estado M2 a produção de mediadores anti-inflamatórios é estimulada (Tang and Le, 2016).

Dessa forma, a microglia influencia na manutenção da homeostase do SNC, servindo como macrófagos residentes neste tecido. Essas células reconhecem estímulos nocivos por expressar uma variedade de receptores associados ao sistema imune, como os TLRs. No SNC, os TLRs estão presentes em neurônios, astrócitos e microglia. Nas doenças neurodegenerativas ocorre ativação de TRL2, 4 e 9, indicando que sua expressão na microglia pode mediar a ligação entre o fenótipo pró-inflamatório associado a estas doenças (Orihuela *et al.*, 2016).

Para a avaliação da ativação microglial costuma-se utilizar como marcador o Iba-1 (do inglês, *ionized calcium-binding adapter molecule-1*), uma proteína que juntamente com os TLRs citados acima estão altamente expressos em doenças neurodegenerativas. Sua detecção costuma ser utilizada para avaliar o estado inflamatório do SNC (Norden *et al.*, 2016).

Além da microglia, os astrócitos também são células gliais ativadas em resposta a injúrias. Um dos marcadores mais utilizados para astrócitos é a proteína glial fibrilar ácida (GFAP). O GFAP está presente no citoplasma dos astrócitos e demarca o seu citoesqueleto. Os astrócitos representam células complexas e com grande diversidade funcional. Estão presentes em todo o SNC e participam ativamente na manutenção dos circuitos neuronais. (Norden *et al.*, 2016).

1.3 ZINCO

O zinco é o segundo micronutriente mais abundante no corpo, sendo encontrado em sua forma livre como um cátion divalente (Zn^{2+}). Cerca de 10% do total de proteínas do corpo estão ligadas a ele, e a partir desta ligação, o zinco afeta diversas funções no organismo, promovendo regulação do sistema imune, modulação do estresse oxidativo e apoptose celular (Baltaci *et al.*, 2018).

Seus níveis adequados podem ser obtidos através de ingestão dietética ou suplementação, contudo, ainda não são estabelecidos os valores ideais de consumo (De Carvalho *et al.*, 2017). A absorção intestinal de zinco pode ser afetada por fatores como fitato, oxalato, taninos e polifenóis, sendo facilitada na presença de alguns aminoácidos, tornando sua biodisponibilidade instável

(Lonnerdal, 2000). Assim, a biodisponibilidade do zinco é variável, mas de acordo com o *Dietary Reference Intake*, que apresenta os valores de referência nutricional para a ingesta de nutrientes para indivíduos saudáveis, o consumo do zinco deve ser de 7 a 9,4 g/dia em adultos (Institute of Medicine, 2001).

Em relação à distribuição do zinco no organismo, 60% encontra-se localizado nos músculos, 30% nos ossos, 5% no fígado e na pele e o restante em outros tecidos, conforme esquematizado na Figura 3.

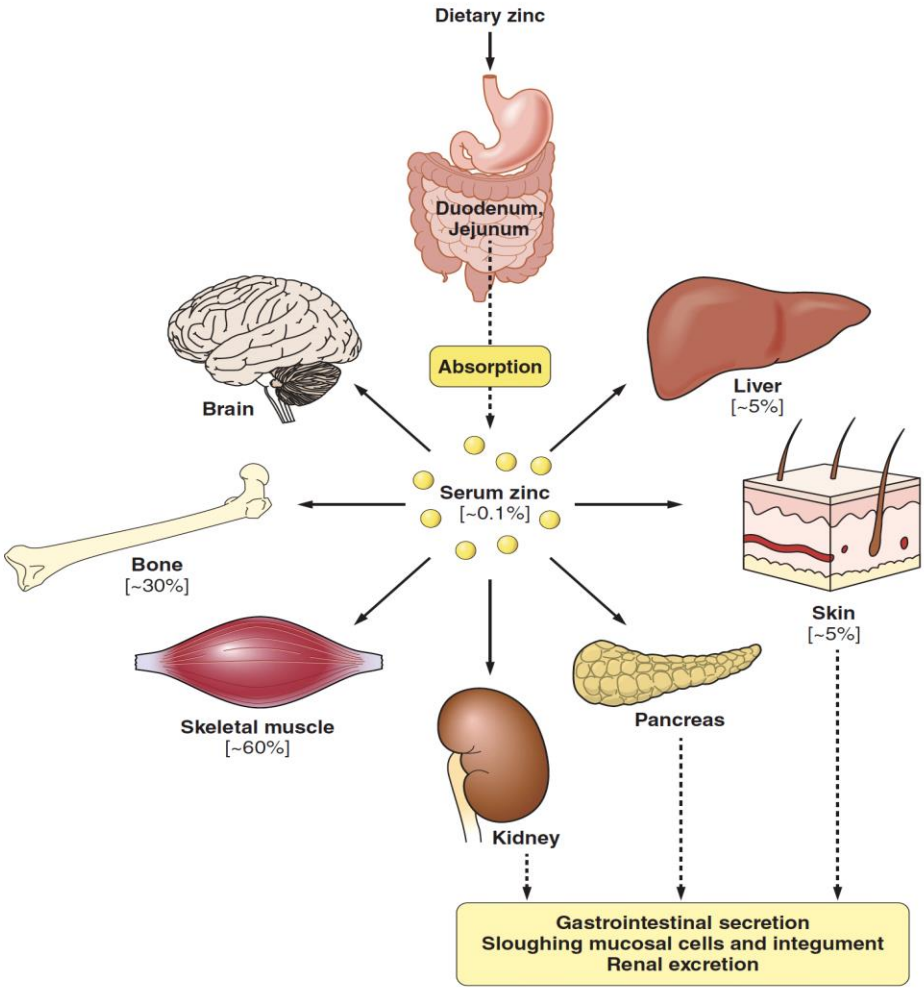

Figura 3. Distribuição do zinco no organismo (Kambe *et al.*, 2015).

A homeostase do zinco no organismo depende da ação das metalotioneínas e de seus transportadores (Fukunaka and Fujitani, 2018). O sistema imunológico pode ser prejudicado pela alteração dessa homeostase, o que ocorre nos processos inflamatórios que por sua vez modulam a expressão destes transportadores (Kambe *et al.*, 2015). As metalotioneínas são proteínas

de ligação ao zinco e são encontradas no citosol das células eucarióticas. Essas proteínas intracelulares tem função de ligação a diferentes metais, regulando a concentração citosólica dos mesmos. A partir disso, as metalotioneínas exercem papel de detoxificação de metais e também atuam impedindo o estresse oxidativo, embora possuam baixa afinidade com o Zn (Miles *et al.*, 2000). Já os transportadores medeiam a compartimentalização do mineral nas organelas e vesículas, regulando sua disponibilização para as proteínas que requerem zinco para exercerem suas funções (Fukunaka and Fujitani, 2018).

O transporte de zinco depende de duas famílias de proteínas, ZIP e ZnT. A família ZIP possui 14 transportadores que conduzem o zinco a partir do meio extracelular ou de organelas em direção ao citosol. Já a família ZnT é formada por 10 proteínas que transportam o zinco do citosol para o interior de organelas ou para o meio extracelular (Kambe *et al.*, 2015). Dessa forma, a manutenção da concentração do zinco no citoplasma é finamente controlada, como demostrado na Figura 4.

Esse controle rigoroso é de extrema importância para o organismo, pois deficiência de zinco predispõe, por exemplo, ao desenvolvimento de doenças cardiovasculares por meio da ativação de vias pró-inflamatórias e de morte celular (Juriol *et al.*, 2018). Também pode causar diminuição do paladar, caquexia e sarcopenia (Suzuki *et al.*, 2011). O zinco desempenha um papel importante regulando a expressão de citocinas, inibindo a inflamação e ativando enzimas antioxidantes para reduzir o estresse oxidativo. Ele também atua no metabolismo de ácidos graxos e de carboidratos (Miles *et al.*, 2001). Além disso, o zinco também exerce o papel de molécula de sinalização intracelular, agindo como um segundo mensageiro, inibindo ou estimulando a atividade enzimática de diversas rotas envolvidas no controle da apoptose e proliferação celular (Kambe *et al.*, 2015).

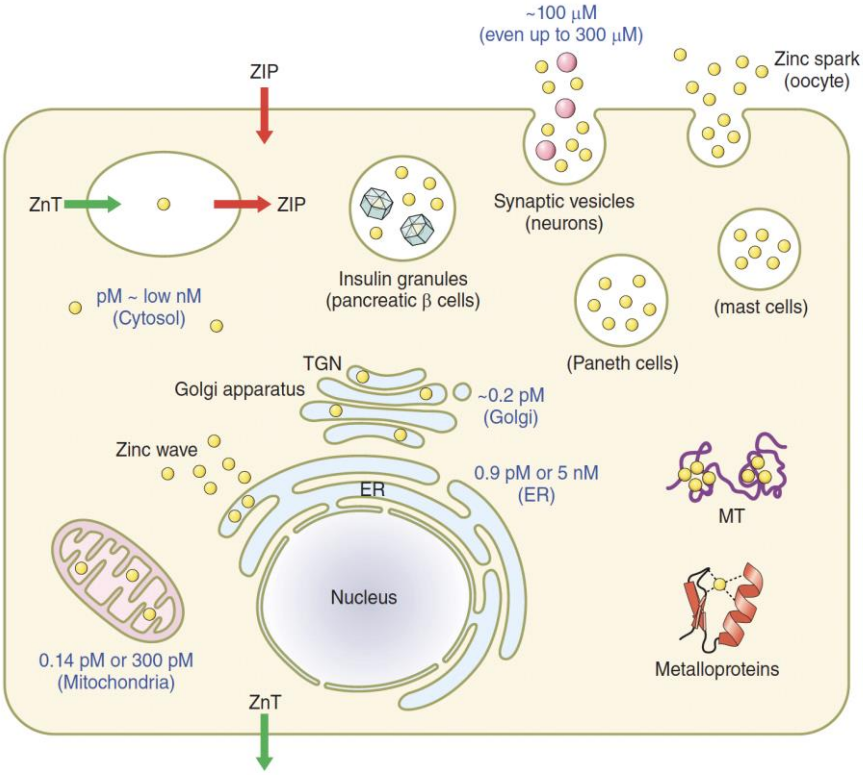

Figure 4. Distribuição de zinco nas células. Os transportadores ZnT (setas verdes) e ZIP (setas vermelhas) organizam-se para manter a homeostase celular do zinco. Os íons de zinco (Zn^{2+}) no citosol estão ligados às metaloproteínas e metalotioneínas (MTs), ou sequestrados ou liberados de organelas/vesículas intracelulares através de transportadores ZnT ou ZIP, respectivamente. Assim, a concentração "livre" de Zn^{2+} no citosol é muito baixa (Kambe *et al.*, 2015).

Durante uma infecção, a secreção aumentada de citocinas pró-inflamatórias, como IL-6, reduzem os níveis plasmáticos de zinco. Isso ocorre porque a IL-6 regula a expressão de ZIP14 nas células hepáticas, o que aumenta os níveis de zinco no fígado, o qual se liga à metalotioneína (Gammoh and Rink, 2017).

Não está claro de que forma a obesidade pode interferir na homeostase do zinco, contudo, já foi demonstrado que o zinco compõe a estrutura da zinco-α2 glicoproteína (ZAG, do inglês *zinc-α2-glycoprotein*), a qual está reduzida em obesos (Severo *et al.*, 2019). A ZAG é uma adipocina que estimula a lipólise e tem atividade anti-inflamatória (Severo *et al.*, 2019). Além disso, tem sido demonstrado que a suplementação de zinco melhora a sensibilidade à insulina em obesos (De Andrade *et al.*, 2017). Assim, o zinco pode ter um papel importante na regulação do metabolismo energético, o que também é

corroborado com o fato de que pessoas com diabetes mellitus dos tipos 1 e 2 apresentam deficiência de zinco (Samadi *et al.*, 2019).

Estudos com animais *knockout* para o gene do ZnT8, um transportador que insere zinco nos grânulos de secreção de insulina nas células β-pancreáticas, mostrou uma redução na tolerância à glicose nestes animais. Esse achado deve-se a uma falha na secreção de insulina, mostrando a importância do zinco no metabolismo da glicose (Fukunaka and Fujitani, 2018).

No SNC, o zinco também medeia diversas funções, incluindo divisão celular, apoptose e modulação da função sináptica. Fisiologicamente, o zinco permeia a barreira hematoencefálica quando ligado a aminoácidos, e estes complexos são transportados para as células gliais e neurônios. Porém, desequilíbrios na homeostase do zinco podem ser evidenciados em doenças neurodegenerativas. Na doença de Alzheimer ocorre uma alteração dos transportadores de zinco no SNC, o que está relacionado com a deposição de peptídeo β-amilóide, sugerindo que a deficiência no transporte de zinco tenha um papel na progressão da doença (Xu *et al.*, 2019),

A importância do zinco tem sido descrita em alguns circuitos neurais, relacionando-se ao funcionamento de redes sinápticas excitatórias glutamatérgicas (Vergnano *et al.*, 2014). Este micromineral está envolvido com o desenvolvimento cognitivo e, apesar de seu mecanismo de ação não ser claro, estudos demonstram que o zinco é essencial para a neurogênese, migração neuronal e modulação sináptica. Atuando como antioxidante, o zinco também interfere em funções neurológicas, tais como, aprendizagem e memória (Baltaci *et al.*, 2018).

Entretanto, modelos experimentais, como por exemplo, lesão traumática cerebral, apontam um papel controverso para o zinco. Enquanto alguns estudos destacam a toxicidade do zinco neste modelo (Hu *et al.*, 2017), outros indicam que sua suplementação pode ser benéfica, produzindo neuroproteção (Plum *et al.*, 2010; Vonder Haar *et al.*, 2016).

Assim, visto que a suplementação de Zn, embora ainda seja controversa, pode trazer benefícios tais como a atenuação do perfil inflamatório, já que a neuroinflamação está presente em diversas doenças neurológicas. O estudo

desses compostos torna-se relevante em condições que apresentam intensificação da resposta neuroinflamatória, como na obesidade.

2 OBJETIVOS

2.1 OBJETIVO GERAL

Avaliar se o tratamento com zinco interfere no metabolismo, memória e em marcadores de neuroinflamação em ratos Wistar que receberam dieta de cafeteria.

2.2 OBJETIVOS ESPECÍFICOS

- Determinar se a suplementação de zinco modifica aspectos metabólicos e inflamatórios dos animais estudados.
- Avaliar se a dieta de cafeteria e a suplementação com zinco interferem na memória declarativa de longa duração dos animais estudados.
- Verificar se a dieta e a suplementação com zinco interferem em parâmetros neuroinflamatórios no córtex cerebral e hipocampo por meio de avaliação de ativação glial e da expressão de TLR4.

3 ARTIGO CIENTÍFICO

Os resultados serao apresentados na forma de artigo científico. Assim, o artigo a seguir foi redigido conforme as normas de publicação da revista Obesity (fator de impacto: 3.742), na qual o trabalho já foi aceito para publicação, com o DOI: 10.1002/oby.23024. As normas para publicação podem ser obtidos no anexo 2 dessa dissertação.

Zinc supplementation decreases obesity-related neuroinflammation and improves metabolic function and memory in rats

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Keywords: obesity, TNF-α, IL-6, GFAP, Iba-1, TLR-4, recognition memory

Running title: Effects of zinc supplementation in obese rats

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Abstract

Objective: To evaluate the effects of Zn supplementation on the metabolic and neuroinflammatory parameters in cafeteria diet (CAF)-induced obesity in Wistar rats.
Methods: Animals were divided into 4 groups: control diet (CT); CT+Zn; cafeteria diet (CAF); CAF+Zn. The diet was administered for 20 weeks; Zn treatment (10mg/kg/day) started at week 16 and it was conducted until the end of the diet protocol. Weight gain and visceral fat, plasma levels of glucose, triglycerides, insulin, TNF-α, and IL-6, as well as HOMA-IR, were assessed. GFAP and Iba-1 expressions in the cerebral cortex and TLR-4 in the cerebral cortex and hippocampus were evaluated. Memory was assessed by the novel object recognition test. **Results**: CAF increased weight gain and visceral fat, plasma glucose, triglyceride, and TNF-α levels. Zn reversed the hyperglycemia caused by CAF and reduced IL-6 levels. In the cerebral cortex, GFAP was similar between groups; Iba-1 was increased by CAF, but reduced in the CAF+Zn group; Zn reduced CAF-dependent TLR-4 increase in the hippocampus, but not in the cerebral cortex. CAF-fed animals showed impaired recognition memory and Zn reversed it.
Conclusions: These findings demonstrate that zinc partially reverted obesity-related metabolic dysfunction and reduced neuroinflammation and memory deficit caused by CAF.

Introduction

The global epidemic of obesity has been mainly related to behavioral and environmental factors, such as the increasing consumption of a high-calorie diet and a sedentary lifestyle, characteristic of Western society (1, 2). Obesity is characterized by a body mass index (BMI) $\geq$ 30kg/m^2, and this abnormal fat accumulation can lead to the development of secondary diseases such as diabetes, hypertension, cancer, and neurological disorders (3, 4, 5).

Inflammation is one of the most relevant features of obesity. Different from the classic acute inflammation, which is essential for tissue recovery, in obesity occurs a chronic low-grade inflammatory state, known as metabolic inflammation that is mainly derived from the infiltrating macrophages in the adipose tissue (3,

6). Also, the activation of toll-like receptor 4 (TLR-4) pathway, triggers pro-inflammatory cytokines synthesis, such as interleukin 6 (IL-6) and tumor necrosis factor-α (TNF-α) that are capable of crossing the blood-brain barrier and reach the brain, causing neuroinflammation (5, 6). This neuroinflammatory state is responsible for the reduction in cognitive function associated with obesity, characterized by impaired memory, attention, and inhibitory control (7). Thus, the search for new strategies to revert the deleterious effects of obesity is primordial.

It is known that people with obesity and low dietary zinc (Zn) intake, show worse ability to respond to inflammation (8). On the other hand, Zn supplementation is capable of stimulating insulin secretion and increases the sensitivity to this hormone (8). Also, Zn can act as a neurotransmitter and second messenger in the central nervous system, regulating hippocampal long-term potentiation (LTP). Zn is transported into glutamatergic vesicles through zinc transporter 3 (ZnT3) and, after being released into the synaptic cleft, Zn modulates NMDA receptors. Zinc also binds to ZnR/GPR39, which signals through mitogen-activated protein kinase (MAPK) and calcium/calmodulin-dependent protein kinase (CaMKII) pathways, promoting neuronal survival and playing a role in learning and memory processes (9). Cafeteria diet (CAF) is a robust animal model of diet-induced obesity, which uses palatable foods with high sugar and fat content of to mimic the Western diet consumed by the human population (10, 11). Therefore, CAF is widely used as a preclinical model of obesity. Here we evaluate whether Zn supplementation could influence metabolic, neuroinflammatory, and cognitive parameters of CAF-fed rats.

Methods

Animals

Twenty-eight male Wistar rats from the animal facility of the Universidade Federal de Ciências da Saúde de Porto Alegre (UFCSPA) received standard chow and water ad libitum throughout the experiment. Only male rats were employed to avoid female hormonal fluctuations, which may impact the results of this study. Animals were kept in plastic cages (two rats per cage) under standardized conditions of room temperature (22-24ºC) and 12h of light and dark cycle. All experiments were approved by the Institutional Animal Care and Use Committee of UFCSPA with protocol No. 570/18. All research procedures were designed to minimize the number and the suffering of animals.

Experimental groups and diets

Three months-old rats were divided into four experimental groups (n=7 per group): Control diet (CT); CT+Zn; Cafeteria diet (CAF); and CAF+Zn. Control diet or cafeteria diet were administrated for 20 weeks with Zn supplementation starting at the 16th week. Animals allocated to CT and CAF groups received 0,5 mL of water (vehicle), while CT+Zn and CAF+Zn groups received Zn at the dose of 10mg/Kg diluted in water. All groups received either water or Zn by gavage every day, for four weeks.

Animals from CT and CT+Zn groups were fed *ad libitum* with Nuvilab® CR-1 standard rat chow (Nuvital®, Curitiba, PR, Brazil) providing a total energy content of 3.4 kcal/g (63% carbohydrates, 26% protein, 11% fat).

For CAF and CAF+Zn groups were given three different cafeteria diet menus (interchanged every two days to maintain novelty), each menu was

composed by a sweet and a savory human palatable food besides the normal chow (Nuvital®, Curitiba, PR, Brazil). Animals always received food in excess to ensure unlimited access. The menus were composed by selected snacks, cake, potato chips, biscuits, cream wafer, sausage, mortadella, and chocolate. Besides water *ad libitum*, CAF animals received an orange-based soft drink. The total energy content of the CAF was calculated based on the manufacturer's information (Table 1). CAF provided an average of 4.5 kcal/g distributed in 42% of carbohydrates, 16% of protein, and 42% of fat. The leftovers of each component of CT and CAF diets were weighed daily, included the measurement of soft-drink consumption, to determine food intake per cage. Likewise, animals were weighed weekly to determine weight gain.

Novel object recognition (NOR) test

NOR was performed to evaluate long-term memory retention at week 16th and at the end of the experiment (20th week). The habituation phase was performed on the first day of the test, in which animals were placed for ten minutes in an acrylic box (40 cm X 40 cm X 20 cm) only containing shavings. Twenty-four hours later, each animal was settled into the same box and was left to explore two equal objects (A and B) for five minutes (training session). On the third day, the test phase was conducted. Object B was replaced by another object with different size, color, and shape (object C). Two different plastic building blocks were used (rectangular and pyramid-shaped, size 7 cm x 4,5 cm x 4,5 cm approximately). Rats were left free to explore objects A and C for 5 minutes and time spent exploring each one was counted to calculate recognition index (RI) as demonstrated below. Exploration was interpreted as directing the nose toward

the object at a distance of no more than 2 cm and/or touching the object with the nose or mouth.

RI = Time spent exploring the new object C / [Time exploring the familiar object (A) + time exploring the new object (C)]

Tissue and blood collection

At the end of the 20th week of the diet, animals were euthanized by decapitation after 6 hours of fasting, tissues and blood were collected. Blood was centrifuged at 3500 rpm for 10 minutes and plasma obtained was stored at -80ºC for further analysis. Liver and visceral adipose tissue depots (abdominal and epididymal) were collected, weighed, and stored at -80ºC. The brain was removed and the cerebellum, hippocampus, and cerebral cortex were quickly dissected out and stored at -80ºC.

Zinc determination

Zinc content in the liver and cerebellum were determined by flame atomic absorption spectrometry (FAAS) using acid digestion. Nitric acid (65%, v:v) was added to samples, which were heated at 100ºC for 90 minutes. The determination of Zn was performed in a flame atomic absorption spectrometer (Shimadzu, model AA 7000F) equipped with a hollow cathode lamp and a deuterium lamp as a background corrector. The wavelength used was 213.9 nm. The current of the hollow cathode lamp was 8 mA for Zn, while the slit width of the monochromator was 0.7 nm. The mixture of gases was composed of air and acetylene. To carry out the analyzes, the analytical curve was performed with a standard Zn stock solution of 1000 mg/L (Merck) with a purity level of 99.9%.

Biochemical analyses

Fasting plasma levels of glucose and triglycerides were quantified using enzymatic colorimetric kits (Labtest, Brazil). Insulin levels in the plasma were determined by ELISA (Insulin ELISA kit, Sigma Cat# RAB 0904). Subsequently, HOMA index was calculated to determine insulin resistance. HOMA index was calculated as follows: glucose blood levels (mmol/L) x insulin (mU/L) blood levels / 22.5.

Cytokine measurements

Levels of inflammatory cytokines IL-6 (Thermo Fisher Scientific Cat# 88-7064-88, RRID:AB_2574990) and TNF-α (Thermo Fisher Scientific Cat# 88-7340-88, RRID:AB_2575092) were quantified in the plasma by ELISA kits following the manufacturer's instructions.

Western Blot Analysis

To evaluate the protein expression of GFAP (a marker of astrocytes), Iba-1 (a marker of microglia), and TLR-4 in the cerebral cortex and hippocampus, tissues were first homogenized in RIPA buffer with protease inhibitors cocktail. Samples were centrifuged for 10 minutes at 8,000 rpm and the concentration of proteins was determined using Bradford protein assay. Laemmli buffer were mixed with 30ug of proteins and heated 100^{o}C for 3 minutes. After that, proteins were loaded on a 10% SDS-PAGE gel at 140V for 2h. Proteins were transferred into nitrocellulose membranes and blocked overnight at 4^{o}C with 5% non-fat milk in Tris-buffered solution (TBS) with tween. Membranes were then washed with

distilled water and incubated overnight with primary antibody against GFAP (1:1000, Millipore Cat# MAB360, RRID:AB_11212597), Iba-1 (1:500, Milipore Cat# MABN92, RRID:AB_10917271), TLR-4 (1:200, Santa Cruz Biotechnology Cat# sc-293072, RRID:AB_10611320) and β-actin (Santa Cruz Biotechnology Cat# sc-47778 HRP, RRID:AB_2714189). The next day, membranes were washed with TBS, and then, incubated for two hours with an HRP-conjugated secondary antibody. A chemiluminescent signal was visualized in a Chemi-Doc MP Imaging System (Bio-rad, USA) after the exposition of membranes to electrochemiluminescence solution (Bio-rad, USA).

Statistical analysis

Data were analyzed by two-way ANOVA followed by Tukey post hoc test. The two factors evaluated were diet (CT or CAF) and treatment (vehicle or Zn). Weight gain per week was analyzed by repeated-measures ANOVA. NOR test results in the 16^{th} week of the diet were analyzed by unpaired Student's t-test. Results were expressed as mean ± standard error of the mean and considered significant at $p<0.05$. All analyses were performed using Graphpad Prism 8.

Results

We measured Zn content in the liver and cerebellum to confirm whether supplementation was efficient to elevate tissue Zn levels. The liver was chosen to provide a measurement in a metabolic organ, while the cerebellum was used to demonstrate if Zn would reach the brain. We indeed found increased concentrations in both liver (Figure 1A) and cerebellum (Figure 1B) after Zn treatment. Interestingly, besides the effect of Zn supplementation (liver:

$F_{1,20}$=282, p<0.0001; cerebellum: $F_{1,17}$=58.19, p<0.0001), we also found a diet effect on Zn levels (liver: $F_{1,20}$=10.87, p=0.0036; cerebellum: $F_{1,17}$=13.9, p=0.0017). These findings show that CAF can diminish hepatic and cerebral Zn concentrations.

CAF-fed rats showed an increased body weight compared to CT diet (diet effect, $F_{1, 23}$=69.28, p<0.0001) (Figure 2A). The difference in body weight of CAF and CT groups was already demonstrated in the 9th week of diet administration (Figure 2B). Visceral adipose tissue weight was also higher in CAF and CAF+Zn than in CT and CT+Zn groups ($F_{1, 23}$=53.82, p<0.0001) (Figure 2C). Thus, Zn supplementation did not decrease body weight and visceral adiposity. Figure 2D also shows an average of energy and macronutrient intake in CT and CAF groups.

Fasting glycemia was increased in CAF group, but in CAF+Zn, the glucose levels were similar to CT groups (Figure 3A), showing Zn treatment reversed the increased glucose levels found in obese rats (diet effect: $F_{1, 20}$=18.12, p<0.0004; Zn effect: $F_{1, 20}$=5.21, p<0.03; interaction: $F_{1, 20}$=15.66, p<0.0008). Triglycerides levels were also increased following CAF (diet effect: $F_{1, 17}$=21.70, p<0.0002) (Figure 3B). Zn treatment causes a slight decrease in the triglycerides levels, yet CAF+Zn group was not significantly different of CAF group. Plasma insulin was not different among groups (Figure 3C), but HOMA-IR was increased in CAF and CAF+Zn groups (diet effect: $F_{1, 16}$=6.19, p<0.02), however, post-hoc test did not show differences among groups (Figure 3D).

TNF-α plasma levels were increased following CAF (diet effect: $F_{1,20}$=26.49, p<0.0001), and Zn supplementation did not change it (Figure 4A). Interestingly, IL-6 plasma concentration did not increase in response to CAF, but

Zn supplementation reduced IL-6 levels in both CT+Zn and CAF+Zn groups, showing a Zn treatment effect on the IL-6 plasma levels (Zn effect: $F_{1, 22}$=26.49, p<0.0001) (Figure 4B). Also, post-hoc test showed CAF+Zn IL-6 levels were lower when compared to CAF and CT groups, while there is no significant difference between CT and CT+Zn, suggesting that Zn supplementation in obesity is differently regulated.

To evaluate the neuroinflammatory state, we analyzed GFAP, Iba-1, and TLR-4 protein expression in the cerebral cortex. GFAP did not change in response to diet or Zn supplementation (Figure 5A). However, for Iba-1 expression, two-way ANOVA showed Zn supplementation effect and an interaction between the type of diet and Zn supplementation (Zn effect: $F_{1,15}$=8.27, p<0.01; interaction: $F_{1,15}$=9.4, p<0.007). Also, in the post-hoc test, we found a higher Iba-1 expression in CAF-fed rats compared to CAF+Zn group (p<0.01), showing decreased microglial activation in obese rats following Zn treatment (Figure 5B). TLR-4 expression in the cerebral cortex increased following CAF (diet effect: $F_{1,16}$=6.39, p<0.02), with no effect of Zn supplementation (Figure 5C). In the hippocampus, we found an increase in TLR-4 in CAF-fed animals, which was significantly different from all the other groups, showing again that Zn supplementation in obese animals reduced neuroinflammation by decreasing TLR-4 expression (Figure 6). Two-way ANOVA showed diet and Zn treatment effects as well interaction between these factors (diet effect: $F_{1,14}$=12.68, p<0.003; Zn effect: $F_{1,14}$=6.8, p<0.02; interaction: $F_{1,14}$=5.34, p<0.03).

We also evaluated declarative long-term memory by the NOR test. We performed this behavioral task twice: at the 16th week of the diet, before Zn

treatment, and at the 20th week, following Zn supplementation. Sixteen weeks of CAF caused a decrease in the recognition index, showing that obese rats already had memory impairment at this time point (Figure 7A). At the 20th week, following zinc or vehicle administration, two-way ANOVA showed an interaction between diet and supplementation ($F_{1,17}$=13.14, p<0.003), and Zn reversed the memory deficit in obese animals (Figure 7B).

Discussion

It has already been shown that Zn plays a role in lipid and glucose metabolism besides regulates immune response (12). However, there is a lack of evidence on its potential as a therapeutic agent in the treatment of obesity. Here, we have shown that Zn supplementation did not decrease weight gain, visceral adiposity, and TNF-α plasma levels altered by CAF. However, we found a decrease in glucose and IL-6 plasma levels following Zn supplementation in obese rats. Also, Zn supplementation reduced microglial activation in the cerebral cortex and TLR-4 expression in the hippocampus, suggesting Zn diminished neuroinflammation caused by CAF. We also found an improvement of the declarative memory in CAF+Zn-fed rats. presumably related to all metabolic and anti-inflammatory benefits of Zn supplementation, which emphasizes its positive effects on the treatment of obesity.

We have previously shown that Zn supplementation decreased weight gain and visceral adiposity and improved insulin sensitivity in high-fat diet (HFD)-fed rats (13). In the present study, we used the cafeteria diet (CAF) to induce obesity, since it better simulates the Western diet usually consumed by the human population. The highly obesogenic features of CAF lead to a more

pronounced metabolic dysfunction in comparison to HFD (10). Likely, that is the reason we could not find a beneficial effect of Zn in the reduction of weight and adiposity in CAF-fed rats.

It is known that Zn is necessary for the synthesis of insulin and a higher Zn intake is associated with a lower risk of developing type 2 diabetes (14). Moreover, Zn levels are reduced in patients with type 1 and type 2 diabetes (15). Based on HOMA-IR, we did not find a protective effect of zinc on insulin sensitivity, since HOMA-IR was increased in both CAF and CAF+Zn groups. However, we only measured fasting glucose and insulin and our findings suggest that CAF-fed rats did not exhibit an impairment in insulin secretion, which is similar to certain stages of diabetes mellitus type 2, but they might show some insulin resistance, as demonstrated by HOMA-IR. On the other hand, glycemia was increased in CAF-fed animals, and Zn supplementation decreased glucose plasma levels in obese rats, showing that glucose uptake is improved by Zn treatment. Thus, despite the robust metabolic dysfunction induced by CAF, Zn was efficient to decrease plasma glucose in obese rats to the control levels.

In obesity, hypertrophy and hyperplasia of adipose tissue is accompanied by immune cells infiltration and increased production of pro-inflammatory cytokines, such as TNF-α (16). The increased TNF-α levels that we found following CAF is coherent with this feature, and in this case, Zn did not ameliorate this inflammatory parameter. This finding is in line with the insulin sensitivity, which was also impaired after CAF with no effect of Zn. It is known that increased TNF-α impairs insulin signaling (17). Despite that, we cannot discard a beneficial effect of Zn on this pathway because of the effect on glucose levels found in CAF-fed animals. It is worth to mention that our supplementation started at the 16th

week of CAF, and at this point, metabolic dysfunction was already established. In a previous study, we showed that 16 weeks of CAF induced hyperglycemia and decreased insulin levels, probably due to a pancreatic dysfunction (11), showing again the harmful effects of CAF. By starting the supplementation at week 16, our goal was to revert the pathological consequences of obesity. Thus, we can affirm that despite the short time of treatment (only 4 weeks), Zn partially reverted metabolic changes caused by obesity. This pattern was also seen in the triglycerides levels of CAF+Zn group, since there was a reduction in their levels but not statistically different from CAF group. In a clinical trial with pre-diabetes subjects, 20 mg/day of Zn for 4 weeks already decreased total and LDL-cholesterol, but no effects on triglycerides were shown (18). However, a meta-analysis study showed that Zn supplementation decreases triglycerides levels, but this study included clinical trials ranging from 1 month to 7 years of supplementation with different doses of Zn (19). Thus, we can assume that a longer period of Zn supplementation would be necessary to improve triglycerides levels.

Another important finding of the present study was the reduction of IL-6 plasma levels after Zn supplementation. The anti-inflammatory effect of Zn occurs through the inhibition of NF-κB activity, probably by upregulation of A20, a zinc-finger protein that inhibits NF-κB, and consequently, suppresses cytokines gene expression (20). It was already shown that zinc supplementation for 8 weeks in women with obesity decreases IL-6 plasma levels, but do not change TNF-α (21). Thus, since IL-6 and TNF-α are both regulated by NF-κB, IL-6 seems to be more sensitive to Zn supplementation. Jung et al (2015) showed an inverse relationship between serum Zn levels and inflammatory markers, which include TNF-α, IL-6,

and C-reactive protein for women, but in men they only found this association for IL-6, reinforcing that Zn may act mainly on IL-6 regulation (22). However, more studies are necessary to better understand this differential effect on the cytokines.

These partial beneficial effects of Zn on metabolic parameters shown here are noteworthy, since CAF is a very deleterious diet, as above-mentioned. However, the main findings of the present study were the beneficial effect of Zn supplementation on neuroinflammation and memory of obese rats. Glial cells activation is a central component of the neuroinflammatory responses, being astrocytes and microglia the main cellular players in such phenomenon. It is recognized that HFD is accompanied by astrocytic and microglial activation in the hypothalamus (23), but other brain regions not directly involved in the control of energy metabolism also exhibit glial morphological and functional responses. The cerebral cortex and hippocampus are crucial for several brain functions including cognition, and since obesity predisposes to cognitive impairment (24), these brain regions are also highly relevant in the study of obesity. Here, we did not find increased GFAP expression in cerebral cortex following CAF, as it would be expected, but we cannot exclude astrocyte participation in CAF driven-neuroinflammation. Astrocyte activation may involve proliferation and morphological changes (25), thus Western blot analysis perhaps was not sensitive enough to detect astrocytic remodeling in CAF. It was shown increased GFAP expression in the hypothalamus but no changes were found in cerebral cortex or hippocampus following HFD (26). On the other hand, GFAP immunoreactivity was increased in cerebral cortex of rats following CAF (27), thus, results regarding astrocytic activation in experimental models of obesity are conflicting.

Microglia are the immune resident cells in the CNS and they become activated under a variety of insults. In obesity, peripheral inflammatory factors and immune cells may reach the brain, activating microglial cells (28). Also, gut microbiota communicates to the brain via vagus nerve or through inflammatory or neuroendocrine mediators and may alter microglia state to a proinflammatory phenotype (M1) (29). In this condition, microglia produces and releases high amounts of neurotoxic molecules such as proinflammatory cytokines, reactive oxygen species, and nitric oxide that cause neuronal dysfunction (30). Here, we showed for the first time that Zn decreased Iba-1 expression in the cerebral cortex of CAF-fed animals, suggesting an effect of Zn in the blockade of neuroinflammation. In LPS-treated BV2 cells, a microglial cell line, there was a decrease in inflammatory cytokine secretion in response to zinc co-treatment. It was also showed an increase in A20 expression in BV2 cells followed by Zn treatment (31). As above-mentioned, A20 is a negative regulator of NF-kB, thus, A20 upregulation diminishes inflammation. It was previously shown that total or partial loss of A20 leads to spontaneous neuroinflammation (32), so, the underlying mechanism of Zn effects on the brain are probably linked to A20.

TLRs are pattern recognition receptors that sense potential harmful pathogens. Although it is extensively described that saturated fatty acids act as agonists for TLR-4 (33), recently, it was demonstrated that TLR-4 is not a receptor for long-chain saturated fatty acids but it does mediate obesity-induced inflammation (34). High-fat and high-sugar diets change gut microbiota and increase the permeability of the intestinal wall, which facilitates LPS extravasation and consequently endotoxemia. Thus, in obesity, TLR-4 is activated by LPS, which is its canonical ligand triggering proinflammatory signaling that culminates

with metabolic dysfunction (35). In the brain, TLR-4 is highly expressed in microglia (30) and it was shown that the treatment with a TLR-4 inhibitor decreases hippocampal microglial activation followed by HFD (36). Here, we showed increased TLR-4 expression in CAF-fed rats in both cerebral cortex and hippocampus, which is in agreement with previous studies employing HFD models (37, 38, 39). Furthermore, this neuroinflammatory feature is associated with cognitive alterations such as memory impairment, as we also demonstrated in the present study as well. Furthermore, we showed that Zn supplementation reverses memory deficit as demonstrated by the novel object recognition test. This behavioral outcome is probably associated with the reduction in microglial activation in the cerebral cortex and TLR-4 expression in the hippocampus. Interestingly, Zn treatment in the CAF group was associated with a decreased TLR-4 expression in the hippocampus, but not in the cerebral cortex. This might occur because, in the CNS, zinc is mainly present in the hippocampus. Also, it was previously shown the hippocampus is the only brain region that shows a reduction in zinc concentration following a zinc-deficient diet (40).

Zinc is essential for physiological processes in the brain. It modulates glutamatergic neurotransmission at being transported through Zn transporter 3 (ZnT3) into the presynaptic vesicles (41). It was demonstrated that ZnT3 knockout mice had impaired hippocampus-dependent memory due to a decrease in Erk1/2 MAP kinase pathways in hippocampal mossy fiber terminals, evidencing the role of Zn in memory formation (42). Pathological conditions such as Alzheimer's disease and type 2 diabetes also affect Zn homeostasis, which is a contributing factor for cognitive dysfunction (43). Besides, Zn administration improved spatial memory and exploratory activity in lean rats (44), and Zn

deficiency, together with other micronutrients, was associated with lower working memory performance in healthy aged individuals (45). Thus, brain function depends on zinc homeostasis but is uncertain whether Zn supplementation is beneficial for neurological function when Zn intake is already normal. However, in inflammatory conditions such as obesity, Zn supplementation provides systemic benefits, including the decrease in neuroinflammation, which improves synaptic transmission and consequently cognition.

In summary, we showed Zn ameliorates glucose homeostasis and acts as an important anti-inflammatory agent in CAF-induced obesity. As the neurological function is highly affected by metabolic disturbances, obesity treatments should also focus on substances that show beneficial effects in the brain. In the last decades, neuroinflammation has been arising as an important etiologic factor for neurodegenerative and psychiatric diseases. Our findings regarding the effects of Zn reducing neuroinflammation and improving memory are highly relevant. Therefore, we showed that Zn supplementation might be a strategy for the treatment of obesity driven-neuroinflammation.

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Figures Captions

Figure 1. Content of Zn in the liver (A) and cerebellum (B) of control (CT) and cafeteria diet (CAF)-fed rats. Rats received vehicle (water) or Zn treatment by gavage from 16th to 20th week of the experiment. Zn supplemented groups showed higher Zn content than vehicle-treated groups. * $p<0.05$ compared to CT and CAF. # $p<0.05$ compared to CT, CT+Zn and CAF+Zn. n=4-6/group.

Figure 2. Body weight of rats consuming cafeteria diet (CAF) or control diet (CT) during 20 weeks (A). CAF-fed animals started to weight more than CT groups at the 9th week of diet administration. At the 20th week, the weight gain of CAF and CAF+Zn groups were higher than CT and CT+Zn groups (B). At the end of experiment, visceral fat depot was also higher in CAF and CAF+Zn compared to CT and CT+Zn groups (C). Energy and macronutrients consumption of CT and CAF groups are shown in D. Arrow indicates the beginning of Zn supplementation. * $p<0.05$ comparing CAF and CAF+Zn to CT and CT+Zn groups. n=6-7 animals/group.

Figure 3. Cafeteria diet (CAF) increased glucose plasma levels and Zn supplementation returned glycemia to control levels (A). Triglycerides increased following CAF, but Zn supplementation partially reduced triglycerides levels (B). Plasma insulin was similar among groups (C). HOMA-IR was increased in CAF

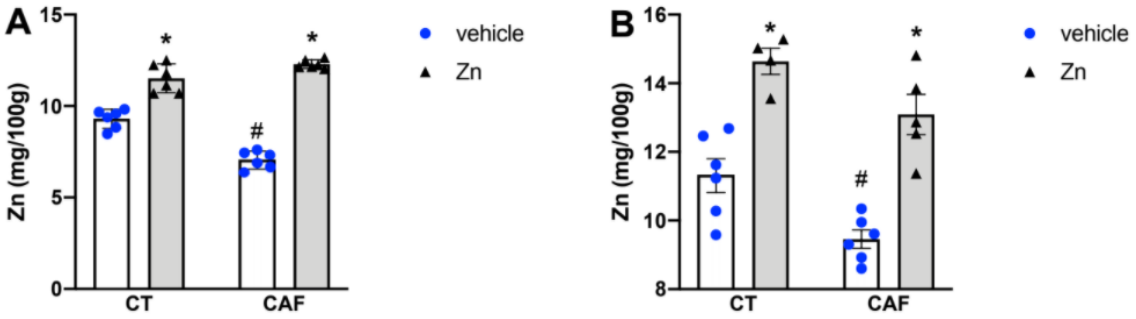
and CAF+Zn group. CT, control diet. * $p<0.05$ comparing CAF to all groups. # $p<0.05$ comparing CAF to CT and CT+Zn groups. n=4-7 animals/group.

Figure 4. TNF-alpha and IL-6 plasma levels. TNF-alpha plasma concentration increased following cafeteria diet (CAF) with no effect of Zn supplementation. IL-6 levels decreased in Zn-treated rats. CT, control diet. * $p<0.05$ comparing CAF and CAF+Zn to CT and CT+Zn groups. # $p<0.05$ comparing CAF+Zn to CAF and CT groups. n=6-7 animals/group.

Figure 5. Western blot analysis of GFAP (A), Iba-1 (B) and TLR4 (C) in the cerebral cortex. GFAP did not show changes among groups. Iba-1 increased following cafeteria diet (CAF), but Zn decreased Iba-1 expression in CAF+Zn group. TLR4 expression was increased in CAF-fed groups (diet effect) with no Zn effect. Upper images are representative bands for each group. CT, control diet. * $p<0.05$ comparing CAF to CAF+Zn. n=4-7 animals/group.

Figure 6. Western blot analysis of TLR4 in the hippocampus. TLR-4 protein expression was higher in CAF group and Zn treatment decreased TLR-4 expression in obese animals. Upper images are representative bands for each group. CAF, cafeteria diet. CT, control diet. * $p<0.05$ comparing CAF to all the other groups. n=4-5 animals/group.

Figure 7. Novel object recognition test at 16 (A) and 20 weeks (B) of diet administration. Cafeteria diet (CAF) induced a decrease in the recognition index which was reverted by Zn supplementation. CT, control diet. * $p<0.05$ comparing CAF to CAF+Zn and CT groups. n=4-14 animals/group.



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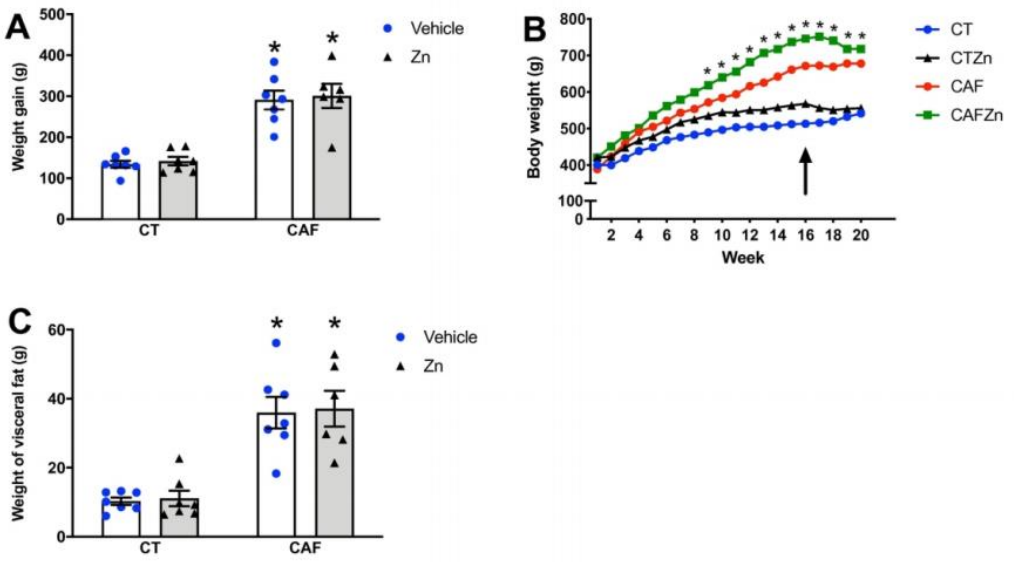

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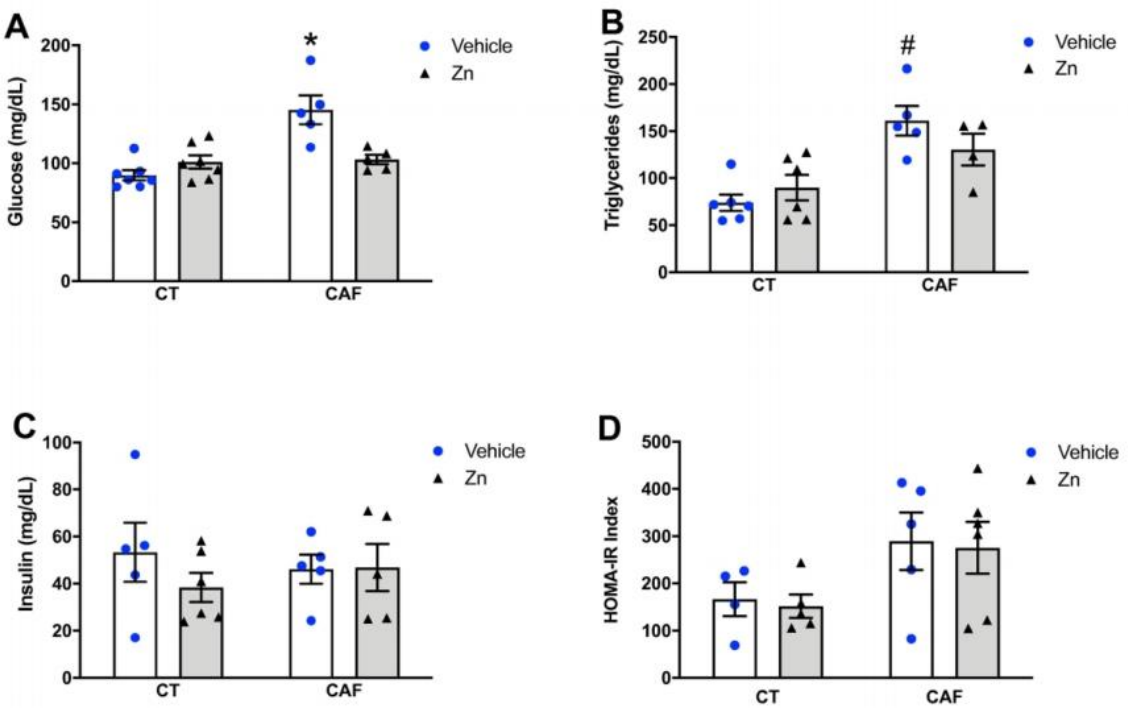

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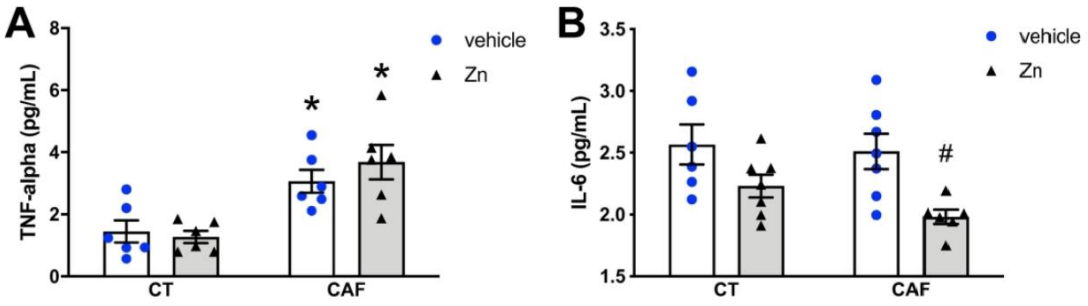

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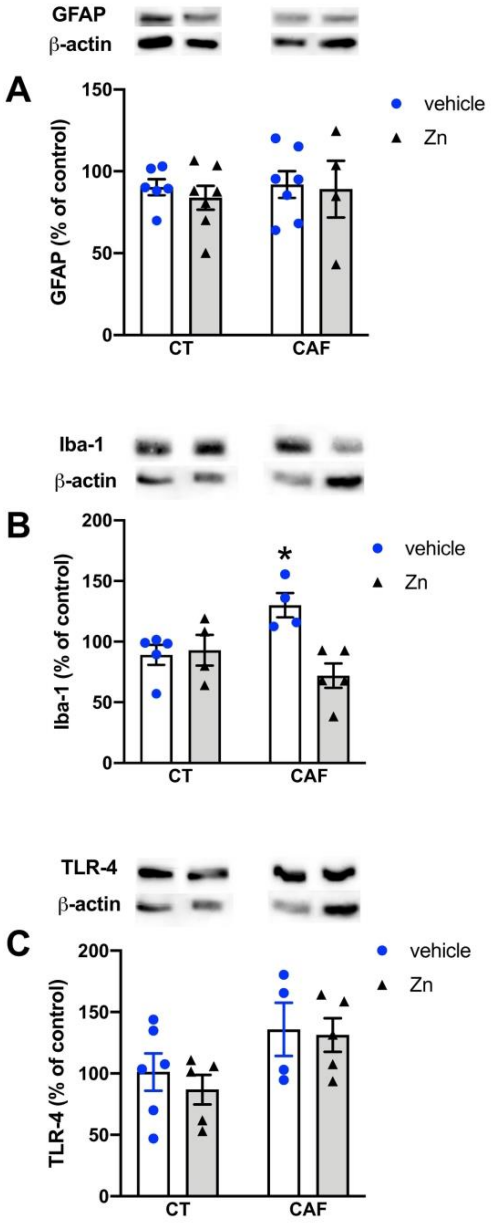

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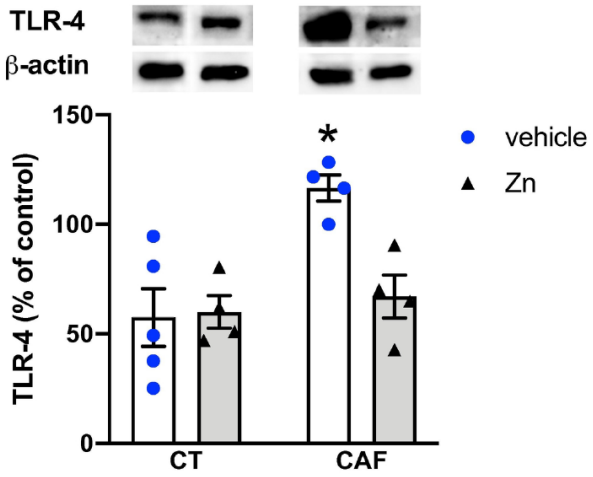

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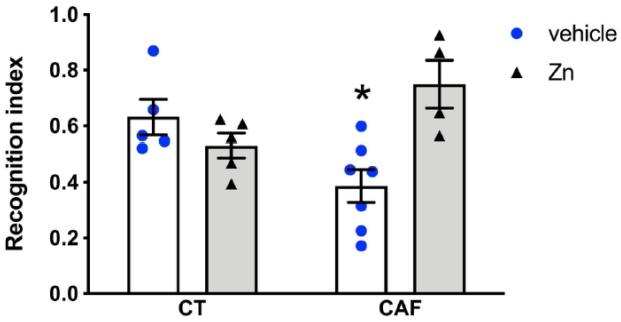

Figure 7. Recognition object test. Cafeteria diet (CAF) induced a decrease in the recognition index, which was reverted by Zn supplementation. CT, control diet. * $p<0.05$ comparing CAF to CAF+Zn and CT groups. n=4-7 animals/group.

115x60mm (300 x 300 DPI)

4 CONCLUSÕES

Sabe-se que o zinco tem papel importante sobre o metabolismo de lipídios e de glicose, além de também serem descritos seus efeitos anti-inflamatórios e antioxidantes. No presente trabalho foi demonstrado que a suplementação de Zn em animais obesos causa uma melhora parcial sobre os parâmetros metabólicos estudados. Cabe destacar que a dieta de cafeteria é reconhecidamente mais prejudicial do que outros tipos de dietas hipercalóricas usadas em estudos animais. Assim, o tempo prolongado de exposição à dieta de cafeteria empregado neste estudo promoveu uma grave disfunção metabólica. Além disso, a suplementação tardia de Zn, a qual foi iniciada na 16ª semana de dieta, não foi suficiente para reverter toda a disfunção gerada pela dieta de cafeteria. Contudo, apesar desses fatores, houve um efeito importante do Zn sobre os níveis plasmáticos de glicose e IL-6. Ainda, foi demonstrado que a suplementação de zinco foi capaz de diminuir a ativação microglial no córtex cerebral e de TLR-4 no hipocampo, demonstrando um efeito do Zn na diminuição da neuroinflamação. Esse efeito também foi evidenciado no desfecho comportamental, pois o Zn melhorou o desempenho dos animais obesos no teste de memória de reconhecimento. Assim, concluímos que o Zn pode ser utilizado como uma estratégia para o tratamento da obesidade e de suas repercussões neurológicas. Com base nesses resultados, acreditamos que sua utilização de forma preventiva ou por períodos mais prolongados pode ser mais efetiva para a normalização dos parâmetros metabólicos.

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ANEXO 1 – Comprovante de aprovação na CEUA

REPÚBLICA FEDERATIVA DO BRASIL
MINISTÉRIO DA EDUCAÇÃO

UFCSPA
UNIVERSIDADE FEDERAL DE CIÊNCIAS DA SAÚDE DE PORTO ALEGRE

CEUA – COMISSÃO DE ÉTICA NO USO DE ANIMAIS

PARECER CONSUBSTANCIADO DE PROJETO DE PESQUISA E ENSINO

1) PROTOCOLO Nº: 226/19

2) DATA DO PARECER: 13-05-18 **Parecer 570/18**

3) TÍTULO DO PROJETO:

Modelo experimental de suplementação de ômega-3 ou zinco como estratégias para o combate aos efeitos metabólicos e neuroinflamatórios da obesidade.

4) PESQUISADOR RESPONSÁVEL:

Renata Guedes

5) RESUMO DO PROJETO:

A obesidade ocasiona alterações metabólicas, causando secreção crônica de moléculas pró-inflamatórias. Este estado inflamatório desencadeia a ativação de vias de sinalização que favorecem a morte celular. Os órgãos metabólicos como intestino e fígado são diretamente afetados. O projeto pretende avaliar o efeito da suplementação de ômega-3 e zinco para verificar a melhora do perfil metabólico e neuroinflamatório em ratos obesos.

6) OBJETIVOS DO PROJETO:

O objetivo principal do projeto é avaliar o efeito da suplementação de ômega-3 ou zinco sobre parâmetros metabólicos e neutroinflamatorios em ratos submetidos a uma dieta hiperlipídica.

7) FINALIDADE DO PROJETO: [ ] Ensino [X] Pesquisa

8) ITENS METODOLÓGICOS E ÉTICOS DO PROJETO:

Título	[X] Adequado	[ ] Comentários
Introdução	[X] Adequada	[ ] Comentários
Objetivos	[X] Adequados	[ ] Comentários
Relevância e Justificativa	[X] Adequados	[ ] Comentários
Materiais e Métodos	[X] Adequados	[ ] Comentários
Cronograma para execução da pesquisa	[X] Adequado	[ ] Comentários
Orçamento e fonte financiadora	[X] Adequados	[ ] Comentários
Referências Bibliográficas	[X] Adequadas	[ ] Comentários

9) O PROJETO ESTÁ ADEQUADO À LEGISLAÇÃO VIGENTE:
[X] Sim [ ] Não

10) INFORMAÇÕES RELATIVAS AOS ANIMAIS:

Grau de dor/estresse: **B** | **C** [ ] **D** [ ] **E** [ ]

Justifique:

Espécie: Ratos Wistar **Número Amostral:** 98

Redução Amostral: [ ] Sim [X] Não

Justifique:

Substituição de Metodologia: [ ] Sim [X] Não

Se achar necessário, justifique e sugira uma nova metodologia:

Aprimoramento da Metodologia: [ ] Sim [X] Não

Se achar necessário, justifique e sugira aprimoramentos da metodologia:

Acomodação e manutenção dos animais: [X] Adequada [ ] Inadequada

Se achar inadequada cite abaixo as melhorias necessárias:

Manipulação dos animais: [X] Adequada [ ] Inadequada

Se achar inadequada cite abaixo as melhorias necessárias:

Analgesia dos animais (se aplicável): [x] Adequada [ ] Inadequada

Se achar inadequada cite abaixo as melhorias necessárias com analgésico substituto:

Anestesia dos animais (se aplicável): [x] Adequada [ ] Inadequada

Se achar inadequada cite abaixo as melhorias necessárias com anestésico substituto:

Eutanásia dos animais (se aplicável): [x] Adequada [ ] Inadequada

Se achar inadequada cite abaixo as melhorias necessárias com metodologia substituta:

Local de Realização (Biotério/Labotarório):

Outra instituição. Qual? Biotério/UFCSPA

11) CRONOGRAMA DE UTILIZAÇÃO DE ANIMAIS

Data	Espécie	Sexo	Quantidade

12) RECOMENDAÇÃO: As pendências deverão ser respondidas em uma carta, indicando as páginas do projeto que foram alteradas (nova versão), assinadas pelo pesquisador responsável.

[X] Aprovado

[ ] Com Pendência

[ ] Não aprovado

Data de início 06/2018.

Data de Término 01/2021.

Comentários gerais sobre o projeto:
Todas as considerações foram atendidas desta forma o projeto foi aprovado.

ANEXO 2 – Normas para submissão do artigo

Submission Checklist Obesity requests that all corresponding/submitting authors work through this checklist during submission of their manuscript to our online submission and peer-review system. The checklist is for authors' use only—please do not upload with your manuscript.

- All manuscript files are in the correct formats (i.e, Main Document, including any tables, is a Word file; each figure is in a separate tiff, eps, png, or pdf file)
- The Main Document file does not contain any figures
- The abstract is structured (as pasted into the "Abstract" area of the submission system and in the Main Document) and does not exceed 200 words; unstructured abstracts are acceptable for narrative Reviews and Perspectives
- Answers to the three study importance questions have been included in the Main Document
- The corresponding author has collected ICMJE Forms for Disclosure of Potential Conflicts of Interest from every author, and a full and accurate Disclosure statement that includes all of the information from each author's form has been compiled and included on the Title Page of the Main Document
- A complete Title Page is included in the Main Document (including all funding information, conflict of interest disclosure, and clinical trial registration number)
- The maximum number of words has not been exceeded for the article type:
  - Original Article: 4000
  - Review: 6000
  - Brief Cutting Edge Report: 1500
  - Perspective: 1000
  - Commentary: 500
  - Letter to the Editor: 500
- The maximum number of references has not been exceeded for the article type:
  - Original Article: 45
  - Review: 100
  - Brief Cutting Edge Report: 20
  - Perspective: 10
  - Commentary: 5
  - Letter to the Editor: 5
- The maximum number of tables and/or figures has not been exceeded for the article type:
  - Original Article: 8
  - Review: 8
  - Brief Cutting Edge Report: 3

  - Perspective: 2
  - Commentary: 1
  - Letter to the Editor: 1 October 2019
- The appropriate checklist referenced at the EQUATOR Network Network (http://www.equatornetwork.org/reporting-guidelines/) is included as a supplementary file, if none of the checklists apply to your study, please explain in your cover letter why none is needed
- If the study is a clinical trial, the CONSORT diagram and checklist have been included with the submission, and a data sharing plan is included in the Acknowledgments section
- If the study uses mathematical modeling, all model assumptions in list form are provided, (b) all variables are defined, with units, and (c) and the actual model and model code are submitted.
- For basic science and preclinical research, the manuscript includes a description of all reagents (antibodies, primers, cell lines, etc.) as well as animal models (strain, sex, age of animals)
- If this study is a secondary analysis, the reference list cites the data source using its unique, persistent identifier
- The tables and/or figures are clearly and correctly labeled on the table or figure; a separate list of captions for figures is provided
- The submitting author certifies that he/she has read the journal's Ethical Policies, including the criteria for authorship
- The article does not use potentially pejorative adjectives or adverbs when describing individuals with overweight or obesity, and it does not directly or indirectly attribute moral judgments or character flaws to this population; importantly, authors should not use "obese" as an adjective or noun to describe an individual person or a group of people, but instead use terms such as "people with obesity" and "populations with obesity" (not "obese people" or "people who are obese")
- If a figure, table, or text is being reproduced from a previously published work, written permission has been obtained from the copyright holder, and this is included with the submission
- All coauthors have been listed on the web submission in Step 4 (Authors & Institutions), along with complete and correct contact details
- The submitting author has identified at least three preferred reviewers; the email addresses and institutions plus a reason for each recommendation are a requirement of the web submission in Step 5 (Reviewers & Editors)
- The submitting author certifies that he/she has read and understood the journal's page charges policy and confirms that funds are available to pay any charges
- If a color figure is included, the submitting author certifies that he/she has read and understood the journal's color charges policy and confirms that funds are available to pay any charges

- If a Supporting Information file has been included, verify that all figure captions and any other supporting detail are in the supplementary file instead of in the Main Document, as this file will be published “as is.” In addition, label all supplemental figures and tables as Table S1, Figure S1, etc. (not Table 1, Figure 1)
- The PDF proof generated by the submission system has been carefully reviewed before clicking “Submit” (checked for correct margins, presence of all files, correct appearance of figures, etc.)