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Efeitos da suplementação de aminoácidos de cadeia ramificada ou zinco nos parâmetros neuroinflamatórios e de memória de ratos obesos

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LISTA DE ABREVIATURAS

BCAA: aminoácidos de cadeia ramificada (do inglês, *branched chain amino acids*)

BCATm: aminotransferase de cadeia ramificada mitocondrial

BCKD: desidrogenase dos alfa-cetoácidos de cadeia ramificada.

CA1: região 1 do Corno de Ammon

CPF: córtex pré-frontal

GFAP: proteína fibrilar glial ácida

HFD: dieta hiperlipídica e hipercalórica (do inglês, *high-fat diet*)

IL-10: Interleucina-10

IL-18: Interleucina-18

IL1-β: Interleucina-1β

IL-4: Interleucina-4

IL-6: Interleucina-6

IMC: índice de massa corporal

IR: índice de reconhecimento de objetos

mTOR: proteína alvo da rapamicina

NMDA: N-metil-D-aspartato.

NO: óxido nítrico

OMS: Organização Mundial de Saúde

SD: dieta padrão (do inglês, *standard diet*)

SNC: Sistema nervoso central

TGF-β: fator de transformação de crescimento beta

TNF-α: fator de necrose tumoral alfa

ZAG: zinco-α2-glicoproteína

Zn: zinco

ZnT3: transportador de zinco-3

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RESUMO

A obesidade é uma doença caracterizada pela produção excessiva de mediadores pró-inflamatórios, o que afeta o encéfalo e gera neuroinflamação. Entre uma série de suplementos comerciais que possuem propriedades imunomoduladoras conhecidas, podemos destacar o zinco (Zn) e os aminoácidos de cadeia ramificada (BCAA). Dessa forma, o objetivo do presente estudo é avaliar se 4 semanas de suplementação com Zn ou BCAA podem alterar os parâmetros neurofuncionais e neuroinflamatórios em ratos obesos. Para tanto, 36 ratos Wistar machos foram divididos nos seguintes grupos: dieta padrão (SD, do inglês *standard diet*); SD + BCAA; SD + Zn; dieta hiperlipídica e hipercalórica (HFD, do inglês *high-fat diet*); HFD + BCAA; HFD + Zn (n = 6/grupo). A dieta foi administrada por 20 semanas e a suplementação com BCAA (750mg/kg/dia) ou Zn (1,2 mg/kg) foi realizada por gavagem a partir da 16ª semana. O projeto foi aprovado pela CEUA/UFCSPA (536/17). A memória declarativa de longa duração foi avaliada por meio do teste de reconhecimento de objetos (IR). Os parâmetros neuroinflamatórios foram analisados pela expressão gênica das citocinas IL-6 e TNF-α e também por imuno-histoquímica para GFAP, a partir da qual foram mensurados o número de astrócitos e sua morfologia (método de Sholl). Os resultados (média ± EPM) foram analisados por ANOVA de duas vias seguida por teste de Bonferroni, (valores de $p<0,05$ considerados como significantes). Os resultados indicam que houve interação entre dieta e suplementação (Zn e BCAA) no índice de reconhecimento de objetos, porém, apenas a suplementação de Zn foi capaz de reverter o comprometimento de memória causado pela obesidade. Não foram encontradas diferenças na expressão do gene da IL-6 no córtex cerebral e no hipocampo. A expressão gênica do TNF-α no hipocampo aumentou em ratos obesos e nenhum dos tratamentos modificou esse parâmetro. Houve também uma redução do número de células GFAP+ no córtex cerebral após a HFD, sem efeito significativo de nenhuma das suplementações. No hipocampo, o giro denteado e a área CA1 mostraram uma diminuição nas células GFAP+ em ratos alimentados com HFD após suplementação com Zn ou BCAA. A análise de Sholl mostrou que a HFD diminuiu o número de ramificações primárias, o comprimento total das ramificações

mais longas e o número total de interseções de processos astrocitários no córtex cerebral. Além disso, no giro denteado houve interação no número de interseções onde o Zn impediu o aumento do número de interseções após a HFD. Em CA1, houve interação no comprimento total das ramificações e o BCAA preveniu a diminuição do comprimento das ramificações após a HFD. Sendo assim, podemos concluir que a obesidade afeta a memória de reconhecimento de longa duração e a suplementação de Zn é capaz de reverter esse efeito. Por outro lado, nenhuma das suplementações foi capaz de afetar a expressão de IL-6 e TNF-α no córtex cerebral e hipocampo dos animais. Além disso, a reatividade dos astrócitos causada pela HFD é área-dependente, sendo o córtex cerebral o mais suscetível.

Palavras-chave: Obesidade; Neuroinflamação; Astrócitos; Aminoácidos de cadeia ramificada; BCAA; Zinco.

ABSTRACT

Obesity is characterized by an excessive production of pro-inflammatory mediators. In the brain it leads to neuroinflammation. Among a number of commercial supplements which have known immunomodulatory properties, we can highlight zinc (Zn) and the branched chain amino acids (BCAAs). Therefore, the aim of the present study is to evaluate if 4 weeks of Zn or BCAA supplementation can affect neurofunctional and neuroinflammatory parameters of obese rats. For this, 36 male Wistar rats were divided into the following groups: standard diet (SD); SD + BCAA; SD + Zn; hyperlipidic and hypercaloric diet (HFD); HFD + BCAA; HFD + Zn (n = 6 / group). The diet was administered for 20 weeks and the supplementation with BCAA (750mg / kg / day) or Zn (1.2 mg / kg) was performed by gavage from the 16th week. The project was approved by CEUA / UFCSPA (536/17). The long-term declarative memory was evaluated using the object recognition test (RI). The neuroinflammatory parameters were analyzed through IL-6 and TNF-α cytokines gene expression and also by immunohistochemistry for GFAP, from which the number of astrocytes and their morphology (Sholl method) were measured. The results (mean ± SEM) were analyzed by two-way ANOVA followed by Bonferroni test ($p<0.05$ was considered significant). The results indicate that there was interaction between diet and supplementation (Zn and BCAA) in the object recognition index, and only Zn supplementation was able to reverse the memory impairment caused by obesity. No differences were found in IL-6 gene expression in the cerebral cortex and hippocampus. On the other hand, TNF-α gene expression was increased in the hippocampus following HFD, and none of the treatments were able to reverse it. There was also a decrease in the number of GFAP + cells in the cerebral cortex of obese rats, with no significant effect of any of the supplementations. In the hippocampus, the dentate gyrus and CA1 area showed a decrease in GFAP+ cells in HFD-fed rats after supplementation with Zn or BCAA. The Sholl analysis showed that HFD decreased the number of primary ramifications, total length of the longest ramifications, and the total number of astrocyte processes intersections in the cerebral cortex. In addition, in the dentate gyrus there was an interaction in the number of intersections, where Zn prevented the increase of the

number of intersections after HFD. In CA1 area, there was interaction in the total length of the longest ramifications, where BCAA prevented the length reduction following the HFD. Thus, we can conclude that obesity affects long-term recognition memory and Zn supplementation is capable of reversing it. None of the supplementations were able to affect the expression of IL-6 and TNF-α in the cortex and hippocampus of the animals. Also, the reactivity of astrocytes caused by HFD is area-dependent, being the cerebral cortex most susceptible.

Palavras-chave: Obesity; Neuroinflammation; Astrocytes; Branched-chain amino acids; BCAA; Zinc

1. INTRODUÇÃO

1.1 Obesidade

A obesidade pode ser definida como um índice de massa corporal (IMC) maior ou igual a 30kg/m^2 ($\mathrm{IMC} = \frac{Peso}{Altura^2}$), o que caracteriza um acúmulo anormal ou excessivo de gordura corporal (Kopelman, 2000). Essa doença é apontada pela Organização Mundial da Saúde como um dos maiores problemas de saúde pública no mundo (James, 2008). No Brasil, dados da Vigitel indicam que entre 2006 e 2016 o número de indivíduos obesos cresceu 60%. Em 2016, 18,9% dos brasileiros já eram considerados obesos e mais de 50% da população apresentava excesso de peso (IMC ≥ 25kg/m^2) (Vigitel, 2016). A epidemia global de obesidade resulta de fatores genéticos e ambientais. Dos fatores ambientais, as mudanças na dieta e no estilo de vida na sociedade moderna causam um aumento do acesso a alimentos com baixo teor nutricional e alto teor energético, além da diminuição dos níveis de atividade física (Kopelman, 2000; Cordain *et al.*, 2005; Malik *et al.*, 2013). Dessa forma, o alto consumo e baixo gasto energético levam a uma excessiva oferta de nutrientes que acabam sendo estocados no tecido adiposo (Reilly e Saltiel, 2017).

Sabe-se que o tecido adiposo é fundamental no controle da homeostase energética. Os adipócitos são células especializadas no armazenamento de lipídeos e possuem todo a maquinaria celular necessária para sintetizar ácidos graxos (lipogênese) quando existe grande oferta de energia, e para mobilizá-los quando existe déficit calórico (lipólise) (Fonseca-Alaniz *et al.*, 2006). Além desses, outros tipos de células estão presentes no tecido adiposo, como fibroblastos, células vasculares e células do sistema imunológico.

Mais do que armazenar energia, o tecido adiposo funciona também como um órgão metabólico que é capaz de secretar hormônios e outras proteínas, e com isso regular a ação de diversos tecidos (Ouchi *et al.*, 2011). As substâncias secretadas nessa região são conhecidas como adipocinas e sua liberação é profundamente alterada quando a obesidade se instala. São exemplos de adipocinas relacionadas com a homeostase energética e

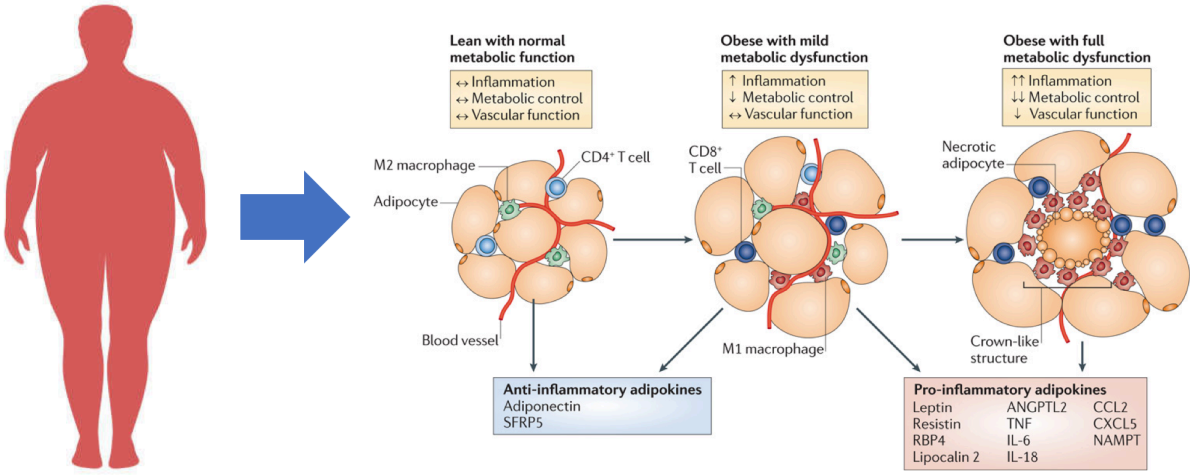
inflamatória a leptina, a adiponectina, a Interleucina 6 (IL-6) e o fator de necrose tumoral (TNF-α) (Ouchi *et al.*, 2011; Reilly e Saltiel, 2017).

Na obesidade, devido a crescente oferta de nutrientes, ocorre um aumento na necessidade de estocar lipídeos e consequentemente um aumento no número e na quantidade de adipócitos. Essa expansão acelerada dos adipócitos gera estresse celular, o que acaba desencadeando o processo inflamatório (Reilly e Saltiel, 2017). O estopim para o início do processo inflamatório não está bem estabelecido, porém alguns estudos tem indicado que o mecanismo pode estar relacionado à sinalização de origem intestinal, como os lipopolissacarídeos derivados das bactérias gram-negativas; os componentes da dieta, como os ácidos graxos livres que são conhecidos por promoverem inflamação; ou ainda por outros metabólitos (Reilly e Saltiel, 2017).

O tecido adiposo naturalmente contém células do sistema imunológico, que são responsáveis pela vigilância, coordenação e manutenção do sistema. No entanto, ao contrário do que ocorre no tecido saudável, o tecido obeso apresenta um infiltrado de células imunológicas com perfil pró-inflamatório (Ouchi *et al.*, 2011). Dessas, os macrófagos passam por uma mudança fenotípica importante, saindo do seu estado M2 (anti-inflamatório) e se tornando macrófagos pró-inflamatórios (M1) que secretam citocinas como a Interleucina 1β (IL1β), IL-6 e o TNF-α. A combinação do aumento do número de macrófagos, causado pela infiltração do tecido, e sua mudança para o estado M1 é uma característica importante da inflamação do tecido adiposo que acompanha a obesidade (Figura 1) (Ouchi *et al.*, 2011; Ellulu *et al.*, 2017).

Classicamente, a inflamação é caracterizada por calor, rubor, edema, dor e perda de função e é um processo natural que auxilia na resolução de processos de agressão tecidual. No entanto, as alterações na liberação de fatores pró-inflamatórios causada pela obesidade leva a um estado inflamatório crônico e de baixo grau conhecido como meta-inflamação ou inflamação metabólica (Gregor e Hotamisligil, 2011; Ouchi *et al.*, 2011). As alterações metabólicas e inflamatórias causadas pela obesidade estão relacionadas com um aumento no risco de desenvolvimento de outras doenças sistêmicas como o diabetes mellitus, dislipidemias e hipertensão (Gregor e Hotamisligil, 2011; Ellulu

et al., 2017). Além disso, o sistema nervoso central (SNC) também sofre com as consequências da obesidade. Os mediadores pró-inflamatórios liberados pelo tecido adiposo são capazes de atravessar a barreira hematoencefálica e desencadear um estado de neuroinflamação, que predispõe ao aparecimento de outras doenças como depressão, Parkinson e Alzheimer (Tucsek *et al.*, 2014).



Adaptado de Ouichi et al. 2011, Nat Rev Immunol. 2011

Figura 1. A meta-inflamação derivada da obesidade. A inflamação metabólica relacionada à obesidade tem início com a hipertrofia e hiperplasia do tecido adiposo devido ao excesso de nutrientes e a crescente necessidade de estocar lipídeos, o que acaba gerando estresse celular. Dessa forma, o tecido perde a capacidade de manutenção da homeostase e começa a apresentar características pró-inflamatórias, como a infiltração de macrófagos M1 e a secreção de IL-6 e TNF-α. Essas citocinas entram na corrente sanguínea e são capazes de alcançar diversos tecidos, entre eles o sistema nervoso central (FONTE: Ouchi *et al.*, 2011).

1.2 Neuroinflamação

Um dos primeiros eventos ocasionados pela obesidade no SNC é a alteração da integridade da barreira hematoencefálica (Rhea *et al.*, 2017). As citocinas pró-inflamatórias secretadas pelo tecido adiposo periférico se difundem através do sangue e chegam à microcirculação cerebral, onde irão causar, entre outros, estresse oxidativo, disfunções dos pericitos e alterações nas junções oclusivas, e, dessa forma, alterar a função das células endoteliais cerebrais (Tucsek *et al.*, 2014; Van Dyken e Lacoste, 2018). Essas alterações estruturais aumentam a permeabilidade da barreira ocasionando extravasamento de leucócitos, a expressão gênica dos astrócitos se modifica e a microglia se torna

ativada, levando a um aumento da liberação de fatores pró-inflamatórios locais, o que configura o início do quadro de neuroinflamação (Van Dyken e Lacoste, 2018).

Estudos utilizando ressonância magnética demonstram que o córtex cerebral de indivíduos obesos apresenta um volume total menor do que o de indivíduos saudáveis, e mais, que algumas áreas são mais acometidas do que outras pelos efeitos da doença (Raji *et al.*, 2010). Essas alterações estruturais indicam que a obesidade causa atrofia de diversas estruturas cerebrais, entre as áreas mais afetadas podemos destacar o hipotálamo, o hipocampo e o córtex pré-frontal (CPF).

O hipotálamo tem sido bastante estudado por ser uma das primeiras regiões afetadas pela obesidade e por sua função no controle da fome, saciedade e homeostase energética (Buckman *et al.*, 2013). Essa região apresenta neurônios capazes de reconhecer hormônios provenientes do plasma, como a insulina e a leptina, e responder de forma a estimular ou inibir o apetite. Nesse sentido, uma vez que o hipotálamo é afetado pelos efeitos deletérios da obesidade, essa estrutura perde a capacidade de manutenção da sua função e acaba contribuindo para a perpetuação da doença. Nos últimos anos, no entanto, o efeito da obesidade vem sendo estudado também em outras áreas do SNC. Entre elas, as principais áreas afetadas pela obesidade no que tange as funções cognitivas são o CPF e o hipocampo (Francis e Stevenson, 2013; Hargrave *et al.*, 2016; Guillemot-Legris e Muccioli, 2017; Lowe *et al.*, 2019).

Os processos cognitivos afetados por dietas hipercalóricas são principalmente a memória, a atenção e o controle inibitório, esses processos são predominantemente orquestrados pelo CPF e pelo hipocampo (Francis e Stevenson, 2013). O CPF está relacionado, entre outros, na tomada de decisões, planejamento e regulação de comportamentos. Sendo assim, o CPF desempenha um papel importante na vulnerabilidade de um individuo à obesidade, visto que a preferência natural dos seres humanos é por alimentos ricos em açúcar e gordura e a capacidade de ignorar ou inibir os estímulos alimentares resulta de uma regulação comportamental proveniente dessa região (Lowe *et al.*, 2019). Já o hipocampo, que pode ser dividido em CA1, CA2, CA3 e

giro denteado, também apresenta receptores para leptina e insulina, dessa forma, também tem sido relacionado com o controle de ingesta alimentar (Hargrave *et al.*, 2016). Além disso, essa região é primordial no processamento das memórias de curta e longa duração, na memoria espacial, e ainda diferentes memórias não espaciais, além do processo de tomada de decisão (Hargrave *et al.*, 2016; Guillemot-Legris e Muccioli, 2017).

O hipocampo desempenha um papel crítico na formação da memória, constituindo uma estrutura espaço-temporal na qual os vários componentes sensoriais, emocionais e cognitivos de uma experiência estão unidos. Essa estrutura permite que a experiência seja armazenada de modo que possa ser posteriormente recuperada como uma lembrança consciente (Broadbent *et al.*, 2010; Hargrave *et al.*, 2016; Guillemot-Legris e Muccioli, 2017).

A neuroinflamação causada pela obesidade afeta a capacidades de certas regiões corticais de realizar suas funções (Nguyen *et al.*, 2014; Guillemot-Legris *et al.*, 2016). Entre as células da glia que participam do processo de neuroinflamação podemos ressaltar o papel dos astrócitos e da microglia (Figura 2) (Martin-Jimenez *et al.*, 2017; Cope *et al.*, 2018).

A microglia é considerada a primeira e principal forma de defesa imunológica do SNC (Guillemot-Legris *et al.*, 2016; Gzielo *et al.*, 2017; Cope *et al.*, 2018). Em condições fisiológicas, a microglia usualmente permanece em um "estado de repouso" e apresenta uma morfologia ramificada, onde suas arborizações se movem, e são frequentemente reconstruídas (Davalos *et al.*, 2005; Nimmerjahn *et al.*, 2005), esta característica permite que as células atuem em todo o ambiente do SNC – estima-se que todo o parênquima cerebral poderia ser monitorado em poucas horas (Davalos *et al.*, 2005; Hanisch e Kettenmann, 2007). Quando uma lesão é identificada, as células microgliais respondem tornando-se ativadas e adotam uma morfologia ameboide (Hanisch e Kettenmann, 2007). Entre o estado de repouso (ou ramificado) até a ativação da microglia (ou estado ameboide), as células passam por uma profunda transformação morfológica e funcional, onde a microglia se modifica de uma célula "vigilante" para uma célula fagocítica (Hanisch e Kettenmann, 2007; Aguzzi *et al.*, 2013; Cope *et al.*, 2018). A fagocitose desempenha um papel

importante na resposta imunológica, sendo a principal forma de destruição e remoção de patógenos invasores. De uma forma geral, as células da microglia reconhecem e se tornam ativadas graças a dois principais aspectos, são eles: a manifestação inesperada de fatores estranhos ao ambiente do SNC; e a manifestação de fatores típicos do SNC em condições ou quantidades anormais (Hanisch e Kettenmann, 2007; Aguzzi *et al.*, 2013). Em outras palavras, a microglia pode identificar e responder a sinais "conhecidos" ou "desconhecidos" de desequilíbrio. É valido salientar que as características de ativação da microglia/inflamação podem contrastar em diferentes locais do SNC. A importância da ativação microglial em condições fisiológicas é clara, uma vez que é fundamental para a preservação da homeostase no SNC, no entanto, a ativação exagerada da microglia pode levar a consequências neurotóxicas, como a liberação anormal de espécies reativas de oxigênio e óxido nítrico (NO), que podem agravar o dano neuronal (Kim e De Vellis, 2005).

Para a compreensão do papel da microglia em condições patológicas do SNC é importante caracterizar os fenótipos de células microgliais, os dois principais são denominados M1 e M2 (Mosser, 2003; Mantovani *et al.*, 2004). Morfologicamente, o fenótipo M1 apresenta uma forma arredondada sem processos aparentes, enquanto o fenótipo M2 são mais alongados e apresentam processos curtos (Zhang *et al.*, 2014; Kalakh e Mouihate, 2017). Os fenótipos apresentam características bastante distintas, as células M1 expressam moléculas pró-inflamatórias, como interleucina-1 (IL-1), interleucina-6 (IL-6) e fator de necrose tumoral (TNF-α), causando neurotoxicidade e podendo levar a morte neuronal. Por outro lado, o fenótipo M2 apresenta potencial anti-inflamatório, sendo caracterizado pela síntese e liberação de citocinas como interleucina-4 (IL-4), interleucina-10 (IL-10) e fator de crescimento transformante (TGF-β), promovendo o crescimento axonal sem causar a morte neuronal (Kigerl *et al.*, 2009; Shechter e Schwartz, 2013; Zhang *et al.*, 2014; Kalakh e Mouihate, 2017).

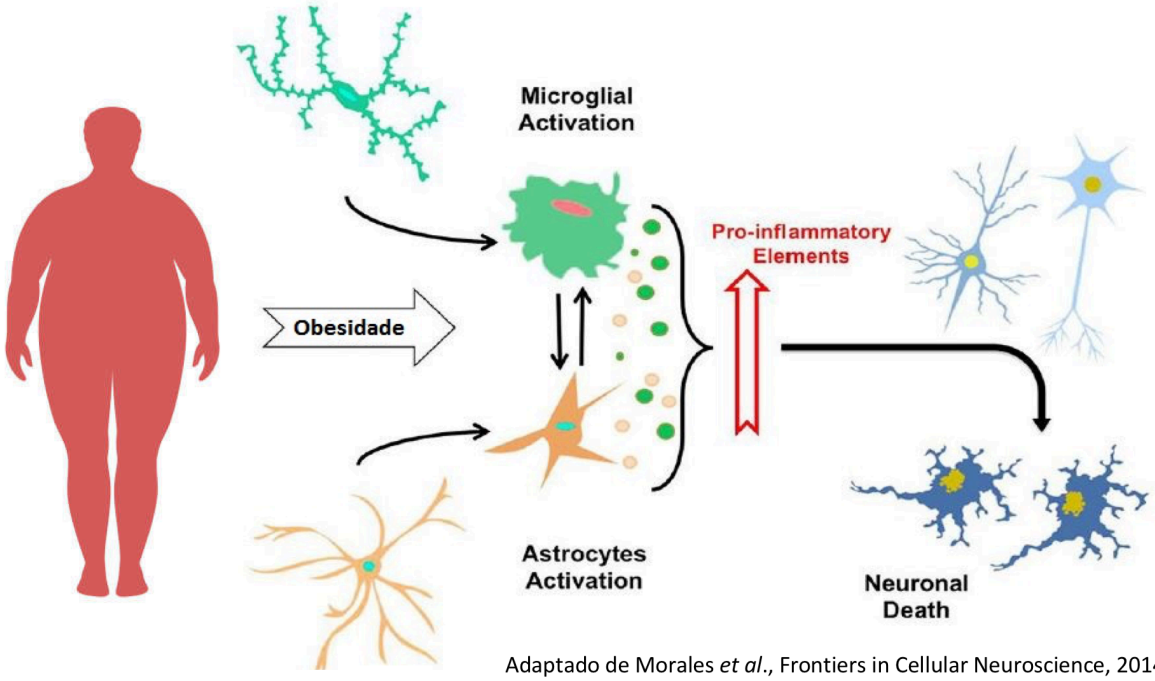
Os astrócitos são as células gliais mais abundantes e constituem aproximadamente 30% das células do sistema nervoso central (Liddelow e Barres, 2017). Os astrócitos são fundamentais para o fornecimento de energia para os

neurônios (Belanger *et al.*, 2011); regulação do fluxo de sangue pela barreira hematoencefálica (Quaegebeur *et al.*, 2011); controle dos níveis de íons extracelulares e neurotransmissores (Danbolt, 2001; Kofuji e Newman, 2004; Simard e Nedergaard, 2004) e modulação da atividade sináptica (Perea *et al.*, 2009; Halassa e Haydon, 2010; Araque *et al.*, 2014). Além dessas funções, os astrócitos também são ativados na resposta inflamatória no SNC.

Apesar de não serem consideradas células do sistema imunológico, é de conhecimento geral que em resposta a situações patológicas, as células astrocitárias sofrem uma serie de alterações, como hipertrofia, hiperplasia, e aumento da expressão de proteína fibrilar glial ácida (GFAP) (Rossi, 2015). A GFAP é comumente utilizada como marcador imuno-histoquímico para identificação de astrócitos maduros (Catalani *et al.*, 2002). Além disso, semelhante ao descrito para a microglia, quando ativadas, as células astrocitárias também podem apresentar dois fenótipos independentes conhecidos como A1 e A2 (Liddelow e Barres, 2017; Lee *et al.*, 2019). Os astrócitos A1 apresentam perfil pró-inflamatório e regulam positivamente genes que já foram relacionados a destruição de sinapses, sugerindo que os astrócitos A1 podem ser nocivos aos neurônios. Por outro lado, os astrócitos A2 regulam fatores neurotróficos e promovem a sobrevivência e o crescimento neuronal. Dessa forma, os astrócitos A2 teriam funções reparadoras no SNC (Liddelow e Barres, 2017). Tanto células da microglia como astrocitárias são capazes de responder a mudanças no ambiente do SNC de diversas formas, entre elas alterando sua estrutura. Dito isso, o número de células e a morfologia glial tem sido estudadas como forma de avaliar as mudanças de função (Davalos *et al.*, 2005; Buckman *et al.*, 2013; Tomassoni *et al.*, 2013; Gzielo *et al.*, 2017). Além disso, estudos recentes sugerem que as células da microglia, e outras células do sistema imune infiltradas no SNC, poderiam modificar a resposta astrocitária à lesão (Liddelow e Barres, 2017; Liddelow *et al.*, 2017). Sendo assim, a interação coordenada dessas células é necessária para a resolução e regulação da resposta inflamatória.

Tendo em vista os efeitos deletérios da obesidade, principalmente no SNC, existe uma crescente necessidade de encontrar alterativas que possam tratar ou prevenir essas perdas sem os efeitos adversos observados em outras terapêuticas.

Sendo a obesidade uma doença com perfil inflamatório, a busca por tratamentos que visem modular esse aspecto é essencial. Dito isso, os aminoácidos de cadeia ramificada (BCAA, do inglês *branched chain amino acids*) e o zinco (Zn) surgem como opções viáveis devido as suas capacidades imunomoduladoras.



Adaptado de Morales *et al*., Frontiers in Cellular Neuroscience, 2014

Figura 2. Neuroinflamação, obesidade e o papel das células da glia. Os elementos pró-inflamatórios originados no tecido adiposo obeso são capazes de atravessar a barreira hematoencefálica e atingir o sistema nervosos central. No sistema nervoso central, diversas células serão ativadas, nesse sentido, podemos enfatizar o papel da microglia e dos astrócitos, principais células responsáveis pela resposta imunológica cortical. A ativação dessas células acabam gerando um aumento local de elementos pró-inflamatórios que em excesso irão causar dano neuronal e predispor o aparecimento de diversas doenças neurológicas como a depressão, Parkinson e o Alzheimer (FONTE: Morales *et al.*, 2014)

1.3 Aminoácidos de cadeia ramificada

Aminoácidos são moléculas orgânicas fundamentais para a síntese de proteínas. Essas moléculas apresentam um carbono saturado que apresenta uma ligação com um átomo de hidrogênio, um grupamento amino, um grupamento ácido e um radical orgânico variável (que determina a identidade de um aminoácido especifico). O corpo humano utiliza 20 diferentes aminoácidos, que podem ser divididos de forma simplificada entre aminoácidos essenciais, que precisam ser adquiridos através da dieta, e aminoácidos não essenciais, que

são sintetizados pelo organismo. Entre os aminoácidos essenciais, podemos chamar a atenção para os aminoácidos de cadeia ramificada: valina, leucina e isoleucina. O BCAA é substratos para a síntese de compostos nitrogenados, além de servir como moléculas sinalizadoras capazes de regular o metabolismo da síntese de glicose, lipídios e proteínas (Nie *et al.*, 2018). Além disso, o BCAA também tem sido estudado por seus efeitos na modulação do sistema imunológico (Rosa *et al.*, 2016; Batatinha *et al.*, 2019).

Ao contrário da maioria dos aminoácidos, apenas uma pequena fração de BCAA é metabolizado pelo fígado, e a maior parte alcança estruturas importantes como músculo, tecido adiposo e o encéfalo através do sistema circulatório. Cerca de 10% do BCAA ingerido atravessa a barreira hematoencefálica (De Simone *et al.*, 2013). O transporte do BCAA pela barreira hematoencefálica ocorre através de um transportador de aminoácidos (Hawkins *et al.*, 2006) isso significa que quando as taxas de BCAA aumentam, esses aminoácidos passam a competir com outros aminoácidos como o triptofano, (precursor de serotonina), tirosina e fenilalanina (precursores de catecolaminas), causando uma diminuição no transporte dessas substâncias. Dessa forma, podemos inferir que o aumento do BCAA no sangue causa uma diminuição da síntese e liberação de serotonina e catecolaminas no encéfalo (Fernstrom e Fernstrom, 2007). No entanto, uma vez no encéfalo, o BCAA irá participar direta ou indiretamente na síntese de neurotransmissores e na manutenção do balanço de nitrogênio no ciclo do glutamato-glutamina entre astrócitos e neurônios (De Simone *et al.*, 2013). Assim, sabe-se que o BCAA é essencial para a manutenção da homeostase no sistema nervoso central.

A suplementação do BCAA tem sido utilizada com diversos objetivos, entre eles como tratamento para doenças com perfil pró-inflamatório. Evidências sugerem que a falta de BCAA na dieta prejudica a resposta imunológica e aumenta a vulnerabilidade do organismo a certas doenças (Zhang *et al.*, 2017) e que a suplementação oral de BCAA melhora a resposta fagocitária dos neutrófilos e a atividade dos linfócitos em pacientes com cirrose hepática (Nakamura, 2014). Entre os aminoácidos que compõe o BCAA, a isoleucina é incorporada majoritariamente pelos linfócitos, seguidos pelos eosinófilos e

neutrófilos, sendo capaz de regular a imunidade inata e adaptativa (Gu *et al.*, 2019); Já a leucina, regula o sistema imunológico através da via de sinalização mTOR, promovendo a diferenciação e ativação de linfócitos e células apresentadoras de antígeno (Powell *et al.*, 2012; Soliman, 2013). Finalmente, alguns estudos apontam que a valina é capaz de melhorar a função das células dendríticas de pacientes cirróticos (Kakazu *et al.*, 2007). Outros benefícios da suplementação de BCAA incluem a regulação do peso corporal, melhora na síntese de proteínas musculares e auxilio na manutenção da homeostase da glicose (Bifari e Nisoli, 2017).

Uma alteração característica causada pela obesidade é a elevação dos níveis sanguíneos de leucina, valina e isoleucina (Adams, 2011). Estudos têm associado o aumento dos níveis de BCAA sanguíneo com o aumento do risco de desenvolvimento de diabetes e doenças cardiovasculares (Wang *et al.*, 2011; Yang *et al.*, 2014). O mecanismo gerador do aumento nos níveis de BCAA em indivíduos obesos não é completamente compreendido. Pietiläinen *et al*. (2008), verificaram que gêmeos homozigóticos, discordantes para obesidade, apresentam diferentes níveis de catabolismo mitocondrial do BCAA no tecido adiposo, indicando redução no mecanismo de catabolismo desses aminoácidos em indivíduos obesos (Pietilainen *et al.*, 2008). Um dos fatores que poderia explicar essa diminuição do catabolismo é a queda na concentração das proteínas BCATm e BCKD E1α (envolvidas nas duas primeiras etapas enzimáticas do catabolismo do BCAA) que ocorre em situações de sobrepeso (She *et al.*, 2007). Em contraste com essa informação, um estudo de 2008 utilizou a suplementação de BCAA por 10 semanas em ratos obesos com diabetes tipo II e verificou um aumento nos níveis de enzimas do complexo α-cetoácido de cadeia ramificada (BCKDH) (importante na regulação no catabolismo do BCAA), indicando que a suplementação do BCAA auxilia no aumento do catabolismo do próprio BCAA pelo fígado (Kuzuya *et al.*, 2008).

Em relação ao sistema nervoso central, camundongos alimentados com dieta enriquecida com BCAA apresentam hiperexcitabilidade dos neurônios corticais. O excesso de BCAA poderia causar um excesso de glutamato, causando uma estimulação excessiva do receptor NMDA, principal receptor do

sistema glutamatérgico, e iniciar um processo neurotóxico, relacionado com a morte de células neuronais (Contrusciere *et al.*, 2010). Curiosamente, a neurotoxicidade é área dependente, não sendo detectada em neurônios do hipocampo (Contrusciere *et al.*, 2010). O aumento dos níveis de BCAA também tem sido associado com alterações da expressão fenotípica da microglia, que apresenta uma inclinação parcial em direção ao estado M2 (anti-inflamatório), com expressão de IL-10 e atividade fagocítica aumentada, mas também com um aumento na geração de radicais livres e diminuição das funções neuroprotetoras (De Simone *et al.*, 2013).

Essa dualidade encontrada na literatura sobre os efeitos do BCAA no organismo, principalmente no SNC, evidenciam a necessidade de novas pesquisas sobre os potenciais efeitos desses aminoácidos.

1.4 Zinco

O zinco (Zn) é um micronutriente essencial e o segundo elemento traço mais abundante no corpo humano, ficando atrás apenas do ferro. O Zn dietético é absorvido nos enterócitos do intestino delgado proximal e posteriormente distribuído para os tecidos, o transporte desse micronutriente é feito principalmente ligado à albumina (proteína sérica com grande afinidade por metais). Visto que o corpo humano não apresenta uma reserva especializada de Zn, a ingesta diária desse metal é necessária (Bonaventura *et al.*, 2015). Sua deficiência pode causar alterações fisiológicas como danos oxidativos, alterações do sistema imunológico e danos neurológicos (Frederickson *et al.*, 2005; Hagmeyer *et al.*, 2014; Livingstone, 2015).

A maior parte do Zn se encontra no ambiente intracelular, onde ele se apresenta como componente estrutural de diversas metaloenzimas e metaloproteínas, incluindo anidrase carbônica, fosfatase alcalina, álcool desidrogenase, DNA polimerase, fator de alongamento da cadeia proteica e cobre-zinco superóxido dismutase. O Zn está envolvido no mecanismo de transcrição de genes, sinalização celular, liberação de hormônios e apoptose celular. Cerca de 3.000 fatores de transcrição contêm Zn e várias das enzimas relacionadas ao Zn

são necessárias para o metabolismo de carboidratos, gordura e proteínas, além da remoção de espécies reativas de oxigênio (Livingstone, 2015). Dessa forma, podemos afirmar que o Zn é essencial para a manutenção da homeostase corporal.

A deficiência de Zn pode atingir diferentes células do sistema imunológico, afetando tanto o sistema imunológico inato como o adaptativo (Bonaventura *et al.*, 2015). Na falta desse micronutriente, ocorre uma perda da capacidade de fagocitose das células polimorfonucleares, uma redução da produção de NAPDH oxidase e consequente redução da produção de espécies reativas de oxigênio, o que diminui a capacidade de neutralização de patógenos. Além disso, a privação de Zn causa diminuição da adesão de monócitos, bem como redução da maturação de macrófagos, das células *natural killers* e das células dendríticas. A deficiência de Zn causa atrofia tímica com subsequente diminuição do número e da diferenciação de células T. A produção de citocinas também é modificada pela deficiência de Zn (Bonaventura *et al.*, 2015).

Na obesidade, ocorre uma diminuição das concentrações séricas de Zn. Indivíduos obesos com baixo consumo de Zn na dieta apresentam um pior estado inflamatório, além de perfil lipídico alterado e aumento da produção de insulina, também apresentam menor capacidade de responder ao dano oxidativo e a inflamação, levando a uma piora dos sintomas já existentes na obesidade (Costarelli *et al.*, 2010). Nesse sentido, estudos têm utilizado o Zn como suplemento por sua capacidade de estimular a secreção de insulina e aumentar a sensibilidade dos indivíduos a esse hormônio (Cruz *et al.*, 2017). Um dos possíveis mecanismos que explicam essa relação entre Zn e obesidade é que o excesso de tecido adiposo pode causar uma redução da expressão gênica e da secreção de zinco-α2-glicoproteína (ZAG), uma adipocina com atividade anti-inflamatória e mobilizadora de lipídios. O Zn é capaz de estimular a função da ZAG, visto que essa adipocina possui sítios de ligação para o metal (Severo *et al.*, 2019). Por outro lado, poucos são os estudos que avaliam o papel no Zn no SNC de obesos.

Como o cálcio, o Zn pode agir no SNC tanto como um neuromodulador essencial quanto como uma potente neurotoxina, dependendo da sua concentração intracelular. De forma geral, o Zn atua de duas formas diferentes, como um neurotransmissor ou como segundo mensageiro (Yamasaki *et al.*, 2007;

Fukada *et al.*, 2011). Como neurotransmissor, o Zn é armazenado em vesículas sinápticas e liberado por exocitose após estimulação pré-sináptica, e então acessa as células vizinhas ao se ligar a receptores de membrana, através de canais proteicos ou pela ação de transportadores (Bonaventura *et al.*, 2015). Como segundo mensageiro, a sinalização do Zn é ativada quando estímulos extracelulares, como citocinas ou fatores de crescimento, aumentam os níveis de óxido nítrico (NO), estimulando a liberação de Zn^{2+}. Além disso, o Zn também é capaz de afetar a excitabilidade do cérebro através da modulação do glutamato, e é importante na plasticidade sináptica (Frederickson *et al.*, 2005). Neurônios que contêm Zn podem ser encontrados em diversas regiões do encéfalo, no entanto, os neurônios do hipocampo são aqueles que apresentam uma maior concentração desse metal (Frederickson *et al.*, 2005). No hipocampo, o Zn, através da ação do transportador de zinco-3 (ZnT3), regula a formação de memória, atuando pela via da cinase regulada por sinais extracelulares (ERK)1/2 (Sindreu *et al.*, 2011). Animais *knock-out* para ZnT3 apresentaram memória de medo prejudicada, além de declínio na memória espacial. Tendo em vista os efeitos deletérios da obesidade e o potencial terapêutico do Zn, mais estudos que analisem os efeitos desse metal no SNC são necessários.

2. JUSTIFICATIVA

A obesidade caracteriza-se por um estado inflamatório crônico de baixo grau que repercute no SNC, gerando um quadro de neuroinflamação. Sabe-se que este perfil pró-inflamatório está relacionado com um aumento na suscetibilidade a doenças como depressão, Parkinson e Alzheimer. Com o crescente número de obesos na população brasileira e mundial, é de grande interesse a busca por possibilidades terapêuticas que minimizem os impactos da neuroinflamação associada à obesidade. Assim, o estudo da suplementação de agentes com potencial imunomodulador torna-se altamente relevante. Sabe-se dos efeitos positivos da administração de Zn e BCAA em relação a parâmetros imunológicos, contudo os estudos que envolvem o SNC ainda são limitados e evidenciam a necessidade de novas pesquisas na área.

3. OBJETIVOS

3.1 Objetivo geral

Avaliar os efeitos do BCAA e do Zn sobre marcadores neurofuncionais e neuroinflamatórios em ratos Wistar.

3.2 Objetivos específicos

I. Avaliar se a dieta hiperlipídica e hipercalórica associada à suplementação de BCAA ou Zn interfere na memória dos animais estudados;

II. Determinar se a dieta hiperlipídica e hipercalórica associada à suplementação de BCAA ou Zn modifica a expressão gênica das citocinas IL-6 e TNF-α no córtex cerebral e no hipocampo dos animais estudados;

III. Verificar se se a dieta hiperlipídica e hipercalórica associada à suplementação de BCAA ou Zn afeta o número de células GFAP+ no córtex cerebral e no hipocampo dos animais estudados;

IV. Avaliar se a dieta hiperlipídica e hipercalórica associada à suplementação de BCAA ou Zn modifica a morfologia de células GFAP+ no córtex cerebral e no hipocampo dos animais estudados.

4. ARTIGO CIENTÍFICO

Os resultados serão apresentados na forma de artigo cientifico. Assim, o artigo a seguir foi redigido conforme as normas de publicação da revista *Neuroscience* (fator de impacto: 3.382), na qual o trabalho será submetido para publicação. As normas para publicação podem ser obtidos no endereço eletrônico: https://www.journals.elsevier.com/neuroscience

Neurofunctional and neuroinflammatory responses following zinc and branched chain aminoacids supplementation in obese rats

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Abstract

The excessive production of pro-inflammatory mediators is one of the main characteristics of obesity, which affects the brain leading to neuroinflammation. Zinc (Zn) and the branched chain amino acids (BCAA) are commercial supplements known by their immunomodulatory properties. Thus, the purpose of this study was to evaluate if 4 weeks of Zn (1,2 mg/kg/day) or BCAA (750mg/kg/day) supplementation can affect neurofunctional and neuroinflammatory parameters of obese rats by a high fat diet (HFD). Our results demonstrated that obese rats had a worst performance in the object recognition test, which was reversed only by Zn supplementation. No differences were found in IL-6 gene expression in cerebral cortex and hippocampus; however, TNF-α gene expression in hippocampus was increased following HFD. None of the treatments affected the cytokines gene expression. The cell counting and the morphological analysis showed that astrocyte reactivity caused by HFD is area dependent, being the cerebral cortex more susceptible to the diet. In summary, obesity caused neuroinflammation and affected the recognition memory. And even though BCAA can affect some morphological parameters, only Zn improved memory. Future studies are needed to clarify the pathways by which Zn causes memory improvement in obesity. Also, Zn supplementation may be a potential target for therapeutic intervention to reduce the impact of obesity on the brain.

Key-words: high-fat diet; astrocytes; object recognition test; interleukin-6, tumor necrosis factor-α; Sholl circles.

INTRODUCTION

Micronutrients are required by organisms in small quantities to maintain a variety of physiological functions and most of them are essential, that is, they need to be obtained from the diet (Statovci *et al.*, 2017). Therefore, recently the supplementation of minerals and amino acids have been widely used as treatment and/or prevention of a range of diseases and conditions, such as non-alcoholic fatty liver disease (Eslamparast *et al.*, 2015) and even schizophrenia (Firth *et al.*, 2017).

The Western diet (WD) is a modern human dietary pattern that is characterized by a high intake of fats and sucrose, and an imbalanced consumption of micronutrients and fibers (Cordain *et al.*, 2005). WD intake growth in westernized societies, and its nutritional characteristics, has been linked with an increase in body weight (Bjorntorp, 1996; Caballero, 2007; Statovci *et al.*, 2017). Obesity is a disease characterized by an excessive body fat accumulation, and it can leads to a low-level, chronic and generalized inflammatory state known as metabolic inflammation (Beydoun *et al.*, 2008; Gregor e Hotamisligil, 2011; Ulla *et al.*, 2017). It is caused mainly by the overproduction of pro-inflammatory mediators by the adipose tissue. The peripheral metabolic changes caused by obesity are related to an increased risk of developing other systemic disorders such as diabetes, hypertension and cancer (Gregor e Hotamisligil, 2011; Malik *et al.*, 2013).

The pro-inflammatory cytokines, such as interleukin-6 (IL-6) and tumor necrosis factor (TNF-α), can cross the blood brain barrier (BBB) and reach the brain by a saturable transport mechanism (Banks *et al.*, 1994; Pan e Kastin, 2002; Rhea *et al.*, 2017). In the brain the glial cells, mainly microglia and astrocytes, respond to high levels of cytokines by changing its morphology and becoming activated (Martin-Jimenez *et al.*, 2017; Cope *et al.*, 2018). Astrocytes are the most abundant type of glial cells in the central nervous system (CNS) (Pekny e Pekna, 2014). They are essential for the homeostasis of the CNS, being involved in the modulation of neuron synaptic activity and in the regulation of the BBB (Rossi, 2015). Also, hypothalamic astrocytes have already been linked to the control of energy homeostasis in the whole body (Rossi, 2015; Camandola, 2018). The way astrocytes respond to injury

is changing their morphological and molecular profile at different levels; these changes spectrum can alter the cell activities through gain and/or loss of function (Pekny e Pekna, 2014; Sofroniew, 2014). When glial cells become chronically activated, it results in local increase of pro-inflammatory elements which triggers neuronal death, which may explain why obesity also have been recently related with neurological diseases such as Parkinson's, Alzheimer's and depression (Tucsek *et al.*, 2014)

Among a range of commercial supplements that have known immune properties, we can highlight Zinc (Zn) and the branched chain amino acids (BCAA). Zn is a mineral of nutritional importance. Its deficiency can cause a series of physiological changes such as increase in oxidative stress and NF-kB activation, which can result, for instance, in cognitive impairment (Frederickson *et al.*, 2005; Prasad, 2013). Zn supplementation has been found to improve blood pressure, glucose, and LDL cholesterol serum levels (Olechnowicz *et al.*, 2018). Zn is also involved in the regulation of immune cells (Bonaventura *et al.*, 2015). Additionally, high body mass index significantly reduces the neuronal expression of zinc transporter protein-1 (ZNT1), showing that obesity can alter intracellular Zn homeostasis (Olesen *et al.*, 2016).

BCAA (valine, leucine and isoleucine) are essential amino acids. Evidence suggests that low BCAA dietary intake impairs the immune response and increases the vulnerability to certain diseases (Zhang *et al.*, 2017). Oral supplementation of BCAA improves phagocytic neutrophil response and lymphocyte activity in patients with hepatic cirrhosis (Nakamura, 2014). Isoleucine has been used as an option for immunotherapy in cases of tuberculosis, since it can increase in the excretion of β-defensin, a peptide that has antimicrobial activity (Mao *et al.*, 2013). Leucine regulates the system through mTOR signalling, promoting the differentiation and activation of lymphocytes and antigen cells (Powell *et al.*, 2012; Soliman, 2013) Finally, recent studies indicate that valine may be related to the improvement of dendritic cells in cirrhotic patients (Kakazu *et al.*, 2007). Other advantages of BCAA supplementation include the ability to control body weight, to improve muscular protein synthesis and to maintain glucose homeostasis (Bifari e Nisoli, 2017).

Considering that obesity causes a chronic inflammatory state which can affect peripheral and CNS structures, and that Zn and BCAA can improve metabolic and inflammatory parameters, the present study aimed to evaluate whether 4 weeks of Zn or BCAA supplementation can impact on recognition memory and neuroinflammatory parameters of obese rats.

EXPERIMENTAL PROCEDURES

Animals

Male Wistar rats (n=36) were used. The animals were from the animal facility of the Federal University of Health Sciences of Porto Alegre (UFCSPA), and had free access to standard rat chow and water until the age of 3 months. They were kept in plastic cages (2-3 rats per cage) under controlled temperature (22-24°C) and light (12 h light/12 h dark cycle) conditions.

All procedures were approved by the Institutional Animal Care and Use Committee (UFCSPA, Brazil, protocol No. 359/16). All efforts were made to minimize animal suffering and to reduce the number of animals used in the experiments, which were performed in accordance with the international laws for the care of laboratory animals, following the 3Rs guidelines.

Diet and supplementation

Rats were randomly allocated in one of the 6 experimental groups (n=6/group), which were: Standard diet + vehicle (SD); SD+Zn; SD+BCAA; High fat diet + vehicle (HFD); HFD+Zn; and HFD+BCAA. Diets were administrated for 19 weeks. The standard diet groups were feed with Nuvilab® CR-1 standard rat chow, with a caloric content of 3.4 kcal/g (63% carbohydrates, 11% lipids, and 26% proteins, NUVITAB, Brazil). Animals from the HFD groups were feed with Pragsoluções® hight-fat diet, with a caloric content of 4.5 kcal/g (35.7%

carbohydrates, 45.1% lipids, and 19.2% proteins, Pragsoluções Biociências, Brazil), and also had free access to a solution containing 42g/L of fructose (55% fructose and 45% sucrose) (Synth, Brazil). All animals had access to water *ad libidum*.

The supplementation of Zn or BCAA was administered by gavage. Zn and BCAA were diluted in water, at the doses of 1,2 mg/kg and 750mg/kg, respectively. Animals received Zn or BCAA supplementation daily from the 15th week until the end of the experiment. SD and HFD groups received water by gavage for the same period.

Recognition memory

The object recognition test was performed in the last week of diet administration. The test is composed of 3 phases, divided in 3 days. First, rats were habituated in an acrylic box (40 cm x 40 cm x 20 cm) containing only shavings. Twenty-four hours after habituation, the training session was conducted. Training session consists on placing the animals individually in the left rear quadrant of the box containing two identical objects (A and B), and allowing the animals to freely explore the objects for 5 minutes. Finally, the long-term memory retention test was performed twenty-four hours after the training session. For the test, one of the objects presented during the training session was replaced by a new object ©, and each rat was reintroduced into the box having 5 minutes to explore them. The exploration time of the familiar and new object was measured. Exploration of an object was defined as directing the nose towards the object at a distance less than 2 cm and/or when the animal touched the object with its nose (Ennaceur e Delacour, 1988). Finally, the recognition index (RI) was calculated.

$$RI = \frac{\text{Time spent exploring the new object ©}}{\text{Time exploring the familiar object (A) + time exploring the new object (C)}}$$

Tissue samples

After 19 weeks of diet, the animals were fasted for 6 hours and then euthanatized using 50 mg/kg of xilasin hydrochloride (Rompum®) and 100 mg/kg of ketamine hydrochloride (Ketalar®) for tissue collection. The brain was quickly removed and the right and left hemispheres were separated. The right hemisphere was put in a fixative solution for histological analyses. The cerebral cortex and the hippocampus was dissected from the left hemisphere and quickly frozen in liquid nitrogen, as previously described in de Moura et al. (2015) (De Moura *et al.*, 2015).

Imunohistochemistry

For immunohistochemistry, the right hemisphere of the brain was fixed in a zinc buffer solution (pH 7.4) for 48 h at room temperature. After this period, tissues were dehydrated, embedded in paraffin and sectioned (8 μm thick) using a microtome. Sections were treated with 3% hydrogen peroxide in 10% methanol for 30 min, washed in PBS for 30 min and incubated for 30 min in 3% normal goat serum in PBS containing 0.4% Triton X-100 (PBS-T). Then, sections were incubated for 48 h at 4 °C with a monoclonal anti-GFAP anti-body (Millipore), diluted 1:750. Sections were incubated with anti-mouse IgG peroxidase-conjugated secondary antibody (Sigma), diluted 1:500 in PBS-T, for 90 min at room temperature. Immunoreaction was developed using a solution of 0.06% 3,3′-diaminobenzidine tetrahydrochloride (Sigma) and 0.005% hydrogen peroxide in PBS. Sections were counterstained with hematoxylin, dehydrated, and covered with Entellan (Merck) and coverslips.

Astrocytes counting and morphological analyses

The images were analyzed using the ImageJ software. Digital images were acquired using a digital camera coupled to an Olympus BX-41 microscope using a 20x objective lens. Three animals per group were analyzed. In nonadjacent sections, three randomly selected fields were analyzed in the cortex and two in each

hippocampus regions (CA1 and Dentate Gyrus). All the GFAP + cells of the fields were counted.

Morphologic analysis was performed on 10-15 astrocytes of each animal. Three animals per group were analyzed. Images were analyzed using Image Pro Plus software. For a general analysis of astrocytic ramification, we used an adaptation of Sholl's concentric circles technique (Figure 1) (Sholl, 1953; Dall'oglio *et al.*, 2008). Shortly, the area around each astrocyte was divided in four quadrants, two laterals (i.e. right/left) and two central (i.e. superior/inferior). Following, around ten virtual circles with 2 µm intervals were drawn around each cell, and the number of intersections of astrocytic processes with each virtual circle was quantified in all quadrants. To quantify the number of primary ramifications, all the processes extending directly from the soma were counted. The longest primary process of the central and lateral quadrants were measured by tracing the process with an automated measurement tool, then the results were sum to find the total length of the longest primary processes.

Molecular analyses

RNA extraction

RNA was extracted from the cerebral cortex and the hippocampus with Trizol (Invitrogen), which was used according to the manufacturer's recommendations. The structures of the brain were homogenized in the presence of Trizol (1:5, v/v); aqueous phase was obtained by centrifugation (12000 x g, 15 min). The RNA was precipitated with isopropanol, for 15 min at room temperature, followed by centrifugation at 12000 x g for 10 min. The pellets were resuspended in 0.1% DEPC-treated water. The concentration of total RNA was determined by measuring the optical density at 260 nm and purity of the RNA was evaluated on the basis of the 280 nm/260 nm ratio and electrophoresis on agarose gel (Langnaese *et al.*, 2008).

cDNA synthesis

Total RNA (1 µg) was used as a template to synthesize cDNA. The RNA was incubated for 5 min at 60 °C with 1 µl oligo (dT) (0.5 µg/µl, Invitrogen), 1 µl 10 mM dNTP and DEPC- water to a final volume of 12 µl, and then for 1 min on ice. The following reagents were then added to a final volume of 19 µl: 4 µl of RT buffer (50 mM Tris-HCl, pH 8.3, 75 mM KCl, 3 mM $MgCl_2$), 2 µl 0.1 M DTT, and 1 µl RNaseOUT (40 U/ µl, Invitrogen). After 2 min incubation at 37 °C, 1 µl M-MLV- RT (200 U/µl; Invitrogen) was added and cDNA synthesis was carried out at 50 °C for 1 h. The reaction was inactivated by incubation at 70 °C for 15 min.

Real-time PCR (qPCR)

The expression of TNF-α and IL-6 and was measured. The housekeeping gene beta-actin (ActB), which has been shown to be stable in each brain area, was used as control (Moura *et al.*, 2014). Primer sequences are presented in Table 1. Amplification was carried out using 7.5 µL of SYBR Green PCR Master Mix (Applied Biosystems), 0.5 µL of forward and reverse primers (0.33 M each), 100 ng of cDNA and nuclease-free water, in a total volume of 15 µL. Reactions were performed in an optical 96-well plate, using a StepOnePlus™ thermocycler (Applied Biosystems). After an initial denaturation step at 95 °C for 10 min, amplification was performed in 40 cycles of denaturation at 95 °C for 30 s, annealing at 60 °C for 40 s and extension at 72 °C for 40 s. Amplification was followed by a melt curve analysis to confirm PCR product specificity. No signals were detected in the no-template controls. The experimental Ct (cycle threshold) was calculated using the algorithm enhancements provided by the equipment. All samples were run in duplicate and the mean value of each duplicate was used for all further calculations (Langnaese *et al.*, 2008; Cook *et al.*, 2010; Nelissen *et al.*, 2010). The Ct value of each reaction was used to calculate the level of mRNA expression of each specific gene, after normalizing it to the expression of the control housekeeping gene (Zimmermann-Peruzatto *et al.*, 2017).

Gene	Primer F	Primer R
TNF-α	5′ TGGCGTGTTCATCCGTTCTCTACC3’	5′ CCCGCAATCCAGGCCACTACTT3’
IL-6	5′ GACCAAGACCAT CCAACTCATC3’	5′ GCTTAGGCATAGCACACTAGG3’
ActB	5’TATGCCAACACAGTGCTGTCTGG3’	5′ TACTCCTGCTTGCTGATCCACAT3’

Table 1: Primer sequences used. Abbreviations: TNF-α tumor necrosis factor alpha, IL-6 interleukin-6, ActB beta-actin.

Statistical analyses

Treatment and diet were used as factors for all ANOVA analyses, except for the object recognition test, in which experimental group and object (familiar and new) were used as factors. The data are expressed as mean ± SEM, Data were analyzed by two-way ANOVA with Bonferroni post-hoc. GraphPad Prism 5.0 was used for the statistical analyses. The significance level was set at $p < 0.05$.

RESULTS

In a previous study, we showed that HFD-fed rats had higher weight gain and visceral adiposity than SD group, and Zn treatment was able to revert these results in obese rats. On the other hand, BCAA did not exert any effect on these parameters (Thoen *et al.*, 2018). Taking these results, the present study evaluated the effect of Zn and BCAA supplementation associated with HFD on memory and neuroinflammatory markers.

Long-term memory was evaluated by the object recognition test (Figure 2). It was found an interaction between diet and supplementation (Zn and BCAA) in the object recognition index ($F_{2,24}$=6.165, p=0.0069, Figure 2B). There was a significant increase in the RI of HFD+Zn rats compared to HFD group, showing that Zn treatment is capable of revert the memory impairment elicited by obesity. However, BCAA supplementation did show this effect, since there was a decrease in the RI of HFD+BCAA compared to SD+BCAA group. The total exploration time was also

evaluated, no differences were found among the groups independently of the diet and/or supplementation (Figure 2A).

In order to investigate the neuroinflammatory state in the brain following HFD, we evaluated the gene expression of the pro-inflammatory cytokines TNF-α and IL-6 (Figure 3). No differences were found in IL6 gene expression in the cerebral cortex and in the hippocampus (Figure 3B; Figure 3C). However, there was an effect of the diet in TNF-α gene expression in the hippocampus ($F_{1,21}$=5.425, p=0.0069, Figure 3A), which was increased by the HFD. None of the treatments modified TNF-α expression in the hippocampus of obese rats.

We also analyzed GFAP immunoreactivity in the cerebral cortex and hippocampus as another parameter of neuroinflammation. The number of astrocytes in the cerebral cortex was reduced following HFD (diet effect, $F_{1,77}$=16.73, p=0.0001, Figure 4A) without an effect of Zn or BCAA supplementation. In the hippocampus, we evaluated dentate gyrus and CA1 areas. Contrarily to cerebral cortex, both dentate gyrus and CA1 showed a decrease in GFAP-positive cells in HFD-fed rats that received Zn and BCAA supplementation (treatment effect, dentate gyrus: $F_{2,72}$=6.720, p=0.0021, Figure 4B; CA1: $F_{2,75}$=4.447, p=0.015, Figure 4C).

Morphology of astrocytes was evaluated by Sholl circles analysis measuring the number of primary ramifications from the soma, total length of the longest ramifications and the total number of intersections of astrocytic processes with the concentric circles. In the cerebral cortex, there was an effect of the diet in all morphological parameters evaluated. The number of ramifications was decreased following HFD ($F_{1,169}$=18.39, p<0.0001, Figure 5A). Total length was also reduced by HFD ($F_{1,167}$=18.17, p<0.0001, Figure 5B) and SD groups treated with Zn and BCAA were significantly different from HFD-supplemented groups . The results concerning the number of intersections followed the same pattern ($F_{1,167}$=47, p<0.0001, Figure 5C), being SD+Zn and SD+BCAA different from HFD+Zn and HFD+BCAA, respectively.

In the dentate gyrus, we did not find any effect of diet and/or supplementations in the number of primary ramifications and in the total length. However, there was an interaction in the number of intersections ($F_{2,180}$=3.357, p=0.037, Figure 5F). Post-hoc analysis showed that Zn prevented the increase in the number of intersections after HFD, which was not observed by BCAA supplementation.

The number of primary ramifications and the number of intersections in CA1 area of the hippocampus were not affected by diet and/or Zn and BCAA treatments. Different from what we found in the dentate gyrus, we found an interaction in the total length of the ramifications ($F_{2,237}$=10.37, p<0.0001, Figure 5H). HFD caused a decrease in the dimension of the ramifications, while BCAA supplementation affected it differently depending on the diet, which were not found after Zn supplementation. A summary of all morphological findings is shown in Table 2.

DISCUSSION

It is well established in the literature that obesity causes memory impairment (Cordner e Tamashiro, 2015; Heyward *et al.*, 2016; De Andrade *et al.*, 2017). Molecular features associated to obesity-related memory decline include deficits in synaptic plasticity, reduced hippocampal neurogenesis, and alterations in the expression of memory-associated genes and epigenetic mechanisms (Fischer *et al.*, 2007; Heyward *et al.*, 2016). In the present study, we show for the first time that 4 weeks of Zn supplementation were capable of improving the recognition memory of obese rats. It is known that Zn deficiency can trigger behavioral changes associated with neurological disorders (Hagmeyer *et al.*, 2014). In obesity, Zn has already been shown to have important effects on thermoregulation mechanisms and adipose tissue metabolism (Di Martino *et al.*, 1993; Fukunaka e Fujitani, 2018). In addition, obesity causes a decrease in the expression of genes that regulate intracellular Zn homeostasis in the brain (Olesen *et al.*, 2016). Cope et al., 2011 demonstrated that 4 weeks of Zn supplementation, the same protocol used in the present study, improved spatial memory and learning after traumatic brain injury

(Cope *et al.*, 2011). The mechanism by which Zn affects memory is still poorly understood. However, intracellular $Zn^{(2+)}$ signaling in the hippocampus is required for cognition and related with the object recognition test performance, possibly acting through the long-term potentiation modulation (Tamano *et al.*, 2015; Tamano *et al.*, 2016).

On the other hand, the supplementation of BCAA was not able to reverse the detrimental effects of HFD on recognition memory. In a model of Alzheimer's disease, Tournissac et al., 2018, also found no benefits on the object recognition test performance after supplementation with BCAA (Tournissac *et al.*, 2018). BCAA reaches the CNS through an amino acid transporter in the BBB, which is also responsible for the transport of other amino acids such as tryptophan (a precursor of serotonin) and threonine, thus, the supplementation of BCAA can reduce the brain uptake of those amino acids (Coppola *et al.*, 2013). A high level of threonine in the brain is related with better results in the object recognition test, and serotonin is related with behavioral changes (Coppola *et al.*, 2013; Scaini *et al.*, 2014; Tournissac *et al.*, 2018). Hence, this may be the reason why we did not find an improvement of the object recognition index after 4 weeks of BCAA supplementation.

It is known that the metabolic inflammation caused by obesity increases the levels of pro-inflammatory cytokines such as IL-6 and TNF-α (Ellulu *et al.*, 2017). Here we found an increase in TNF-α gene expression in the hippocampus. Surprisingly, IL-6 gene expression did not change neither in the hippocampus nor in the cerebral cortex. Hippocampus is highly susceptible to obesogenic diets. It is described that markers of inflammation are augmented in the hippocampus earlier than in hypothalamus or perirhinal cortex (Beilharz *et al.*, 2016). Yet, it was previously reported that 12 or 24 weeks of HFD increased the TNF-α levels in the hippocampus (Spagnuolo *et al.*, 2015) and even a high-energy diet for a short-term (1 to 4 weeks) is already able to enhance the TNF-α gene expression (Beilharz *et al.*, 2016). In our study, the increased TNF-α gene expression corroborates with the recognition memory impairment following HFD. These findings are in line with the

neuroinflammatory hypothesis of obesity-driven cognitive deficit (Guillemot-Legris e Muccioli, 2017).

Zn supplementation improved the performance in the recognition object test, but did not diminish TNF-α expression in hippocampus, suggesting that benefits provided by Zn are not limited to the restraint of inflammation. Furthermore, we have previously demonstrated that Zn treatment reverted metabolic dysfunction caused by HFD (Thoen *et al.*, 2018), but here we show that it was not sufficient to prevent the hippocampal increase in TNF-α gene expression. We hypothesize that Zn supplementation may be providing its beneficial effect on memory through a mechanism involving the brain-derived neurotrophic factor (BDNF) and metalloproteinase-9, both important modulators for learning and memory, since Zn can activate this pathway enhancing synaptic plasticity and thus, neuronal functioning (Frazzini *et al.*, 2018). On the other hand, BCAA administration did not alter the memory outcome and the obesity-related increase of TNF-α gene expression in the hippocampus. High doses of BCAA can alter the inflammatory profile in the cortex and hippocampus of rats and in mixed glial cell cultures (De Simone *et al.*, 2013; Rosa *et al.*, 2016), however, those changes seem not to be sufficient to revert the detrimental effects of obesity.

Astrocyte reactivity is another feature of neuroinflammation caused by obesity (Buckman *et al.*, 2013). Despite the changes in glial cells following obesity are more prominent in the hypothalamus, HFD may affect different brain areas (Lizarbe *et al.*, 2018). Corroborating with our results, previous studies have found an increase in the number of GFAP+ cells in the cerebral cortex after a HFD (Tomassoni *et al.*, 2013; De Andrade *et al.*, 2017). This feature may be related to astrogliosis, which is widely associated with neurodegenerative disorders (Sofroniew, 2014). In the present study, we showed that HFD did not change the number of GFAP+ cells in the hippocampus, whereas it caused a decrease in the number of astrocytes in the cerebral cortex. Based on our previous study, it seems a divergent finding in the cerebral cortex(De Andrade *et al.*, 2017). However, it is worth to mention that the composition of the diet used here was different, since rats received fructose and sucrose solution along with HFD. It is well known that obesity

leads to cerebral atrophy, which can be related with a disruption in cerebral vascular function and inflammation (Raji *et al.*, 2010). Nevertheless, the underlying mechanisms are not completely understood (Gunstad *et al.*, 2008; Raji *et al.*, 2010; Nguyen *et al.*, 2014). It is reasonable to suppose the effects of HFD administrated in combination with a high fructose and sucrose solution are more harmful than fed animals exclusively with a HFD. Thus, a process of cerebral atrophy promoted for that diet could explain the reduction observed in the number of cortical astrocytes. Curiously, both BCAA and Zn supplementation decreased the number of astrocytes in the hippocampus without interfere in the cortical number of these cells. Zn plays crucial roles in the CNS, but shows toxic effects on cultured astrocytes through a mechanism involving generation of reactive oxygen species (Bishop *et al.*, 2007; Moriyama *et al.*, 2018). Similar outcomes can be caused by accumulation of intracellular BCAA, leading to increased oxidative stress and consequently cytotoxicity (Zhenyukh *et al.*, 2017).

When using Sholl's method to analyze the astrocytes, we found that even though HFD was not able to significantly affect the number of GFAP+ cells in the hippocampus, it can alter their morphology. In the CA1 region, BCAA treatment in lean animals caused a decrease in the total length of astrocytic processes, but in obese animals, it increased. Gzielo et al (2017), also found that obesity affects the morphology of the astrocytes in the CA1 area (Gzielo *et al.*, 2017). Therefore, our findings indicate that following HFD, BCAA supplementation was capable to avoid the decline in astrocyte arborization. On the other hand, in the dentate gyrus, HFD increased the number of processes intersections through the circles, indicating a rise in astrocytes arborization. In this case, only Zn supplementation was able to reverse this effect. Thus, both treatments had some effect on reversing the HFD-elicited morphological changes in the hippocampus. In the cerebral cortex, all morphological parameters analyzed decreased after HFD, and none of the supplementations affected it. These results are in agreement with the reduced number of astrocytes also described here and it provides additional support to the hypothesis of cortical atrophy. Recent studies suggest that obesity-related astrocyte reactivity is area dependent (Tsai *et al.*, 2018) and our findings corroborate with it.

Therefore, we provide additional evidence on obesity-driven neuroinflammation, which is known to increase the risk of neurological diseases. Thus, the search for treatments capable of minimize or reverse the impact of obesity in the CNS is paramount. Here we show that BCAA affects some astrocyte morphological parameters, and Zn supplementation was able to ameliorate recognition memory. In addition, future studies are needed to clarify the mechanisms by which Zn promotes memory improvement in obesity. However, we can presume that Zn supplementation may be a potential target for human therapeutic intervention.

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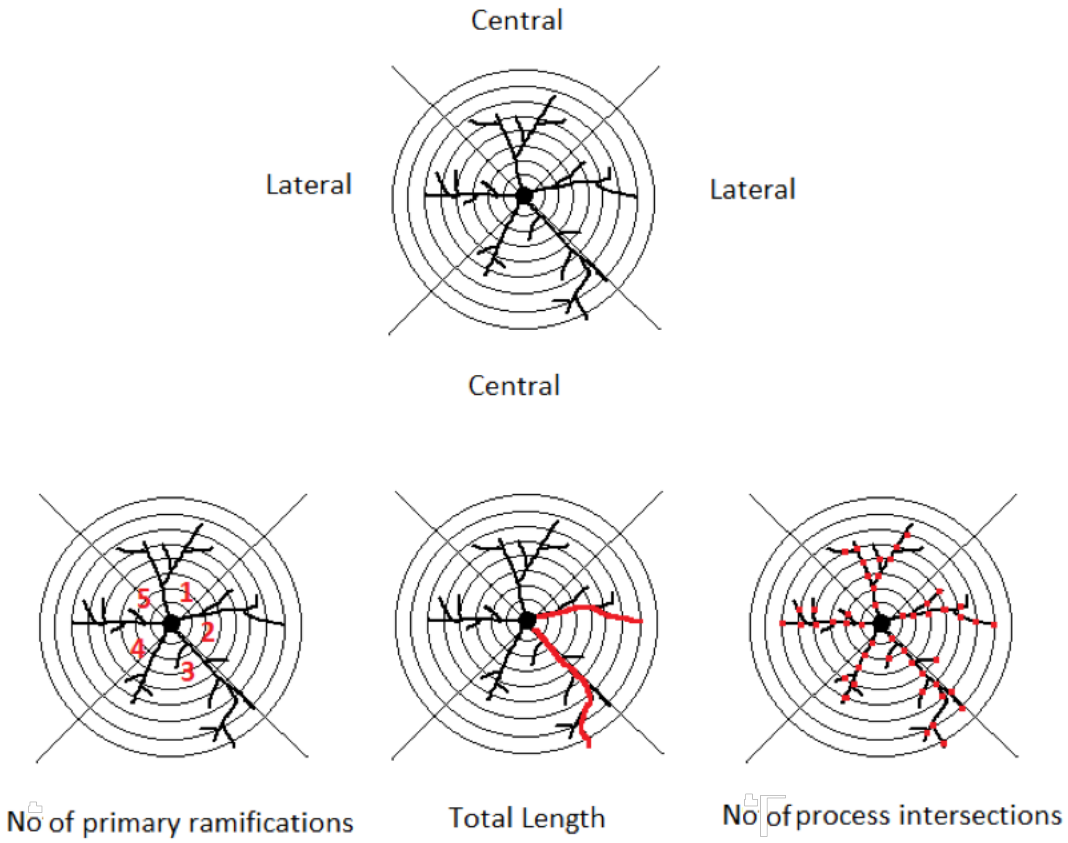

Figure 1. Schematic representation of Sholl circles analysis. Morphology of GFAP+ cells were evaluated considering the following parameters: number of primary ramifications; total length of the longest process of the central and lateral quadrants; and the number of process intersections.

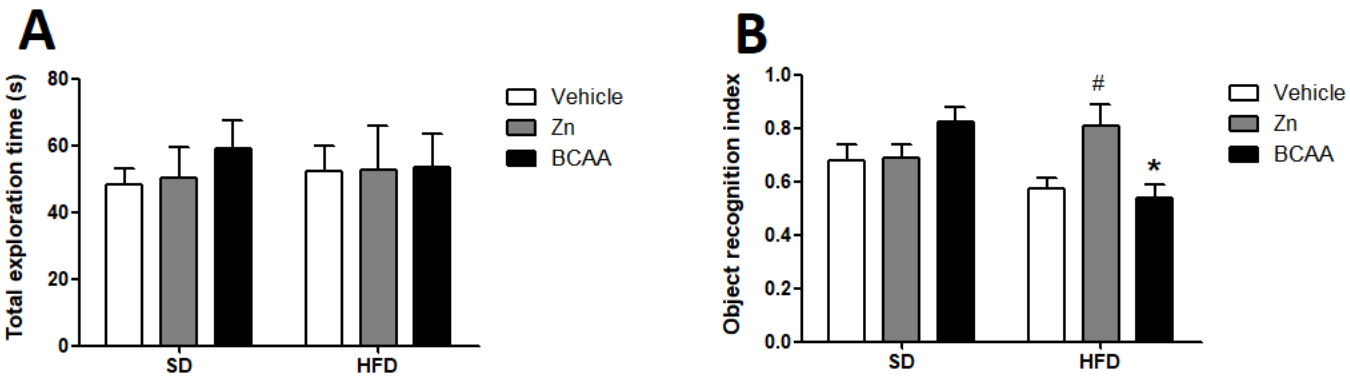

Figure 2. HFD impairs object recognition memory and Zn supplementation reverses it. (A) There is no difference in the total exploration time (novel and familiar objects) among the experimental groups. **(B)** Object recognition index was decreased following HFD and Zn, but not BCAA, reversed this effect. Data are expressed as mean ± SEM. * (p< 0,05) when compared SD and HFD with the same treatment. **#** (p< 0,05) when compared with HFD vehicle. SD, standard diet. HFD, high-fat diet.

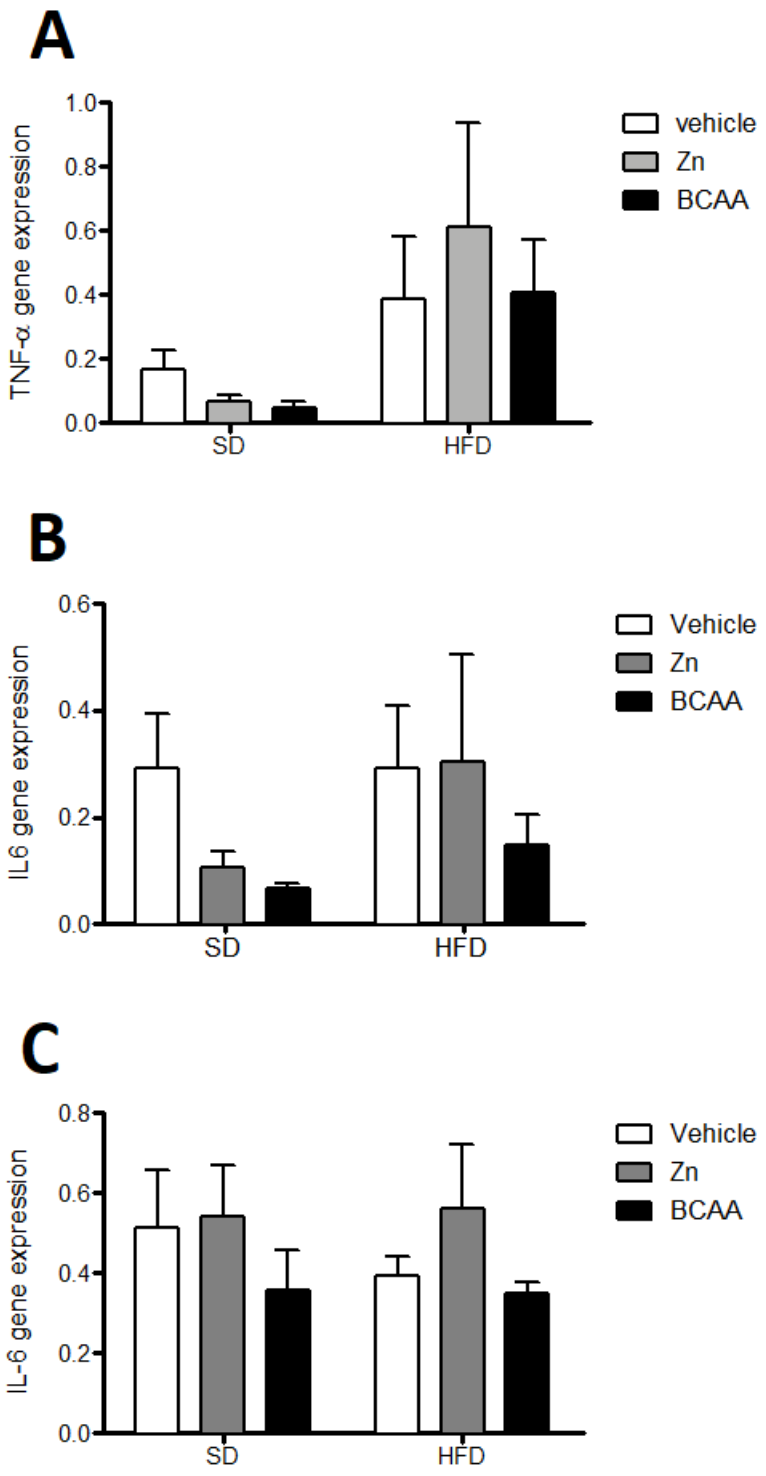

Figure 3. HFD increased TNF-α expression on the hippocampus, however it did not affect the IL-6 gene expression in the cerebral cortex and hippocampus. (A) There was an increase in the gene expression of TNF-α in the hippocampus in HFD-fed rats (diet effect), none of the supplementations significantly affected it. **(B)** IL-6 gene expression in the hippocampus, there was no significant difference among the experimental groups. **(C)** IL-6 expression on the cerebral cortex, there was no significant difference among the experimental groups. Data are expressed as mean ± SEM. SD, standard diet. HFD, high-fat diet.

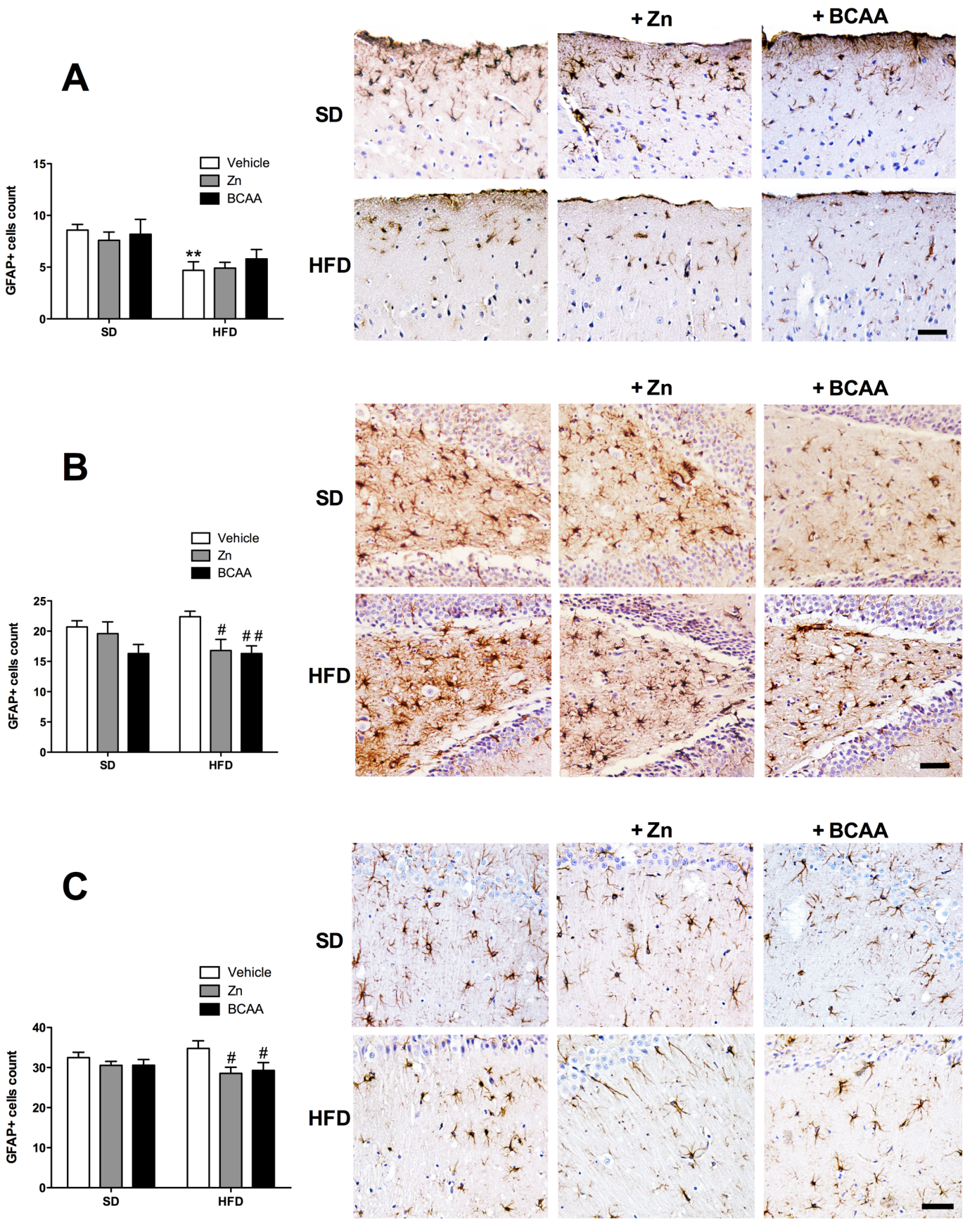
A
B
C
+ Zn
+ BCAA
SD
HFD
Vehicle
Zn
BCAA
GFAP+ cells count
**
#
##
SD
HFD

Figure 4. Immunohistochemistry for GFAP (number of cells per region). **(A)** HFD reduced the number of GFAP+ cells in the cerebral cortex regardless of the Zn or BCAA supplementation. In the hippocampus, both supplementations decreased the number of GFAP+ cells in dentate gyrus **(B)** and CA1 **(C)** when compared with HFD vehicle**.** Data are expressed as mean ± SEM. ** (p< 0,01) when compared SD and HFD with the same treatment. **#** (p< 0,05) **# #** (p< 0,01) when compared with HFD vehicle. Scale bar = 50µm. SD, standard diet. HFD, high-fat diet.

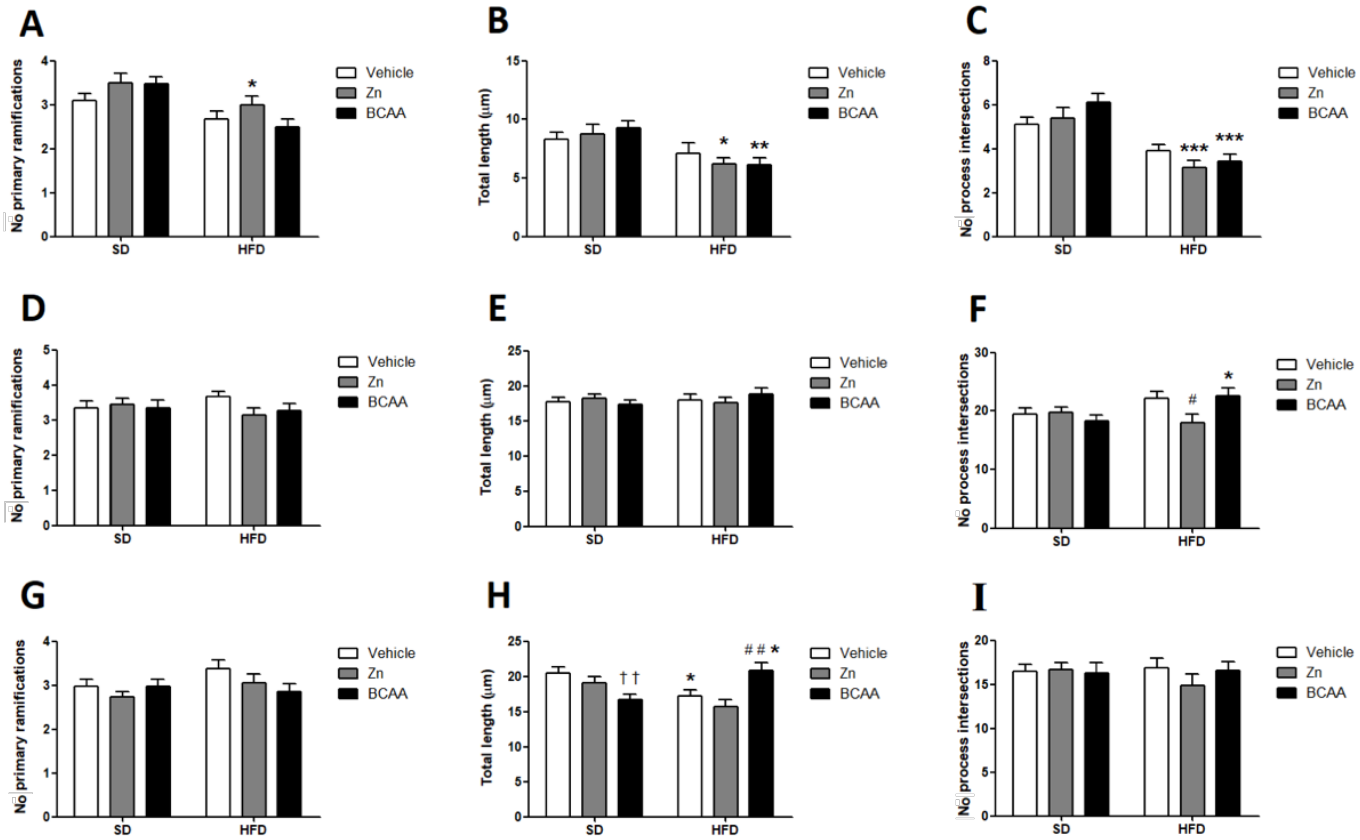

Figure 5. Sholl circles analysis of astrocytes. (A-C) Morphological analysis of the astrocytes in the cerebral cortex. HFD decreased astrocyte arborization as showed by all parameters evaluated. **(D-E)** Morphological analysis of the astrocytes in the dentate gyrus. HFD increased the number of process intersections, only Zn supplementations was capable of reversing it. **(G-I)** Morphological analysis of the astrocytes in the CA1 area of the hippocampus. HFD decreased the total length of the longest ramifications. Only BCAA supplementation was capable of reversing it. Data are expressed as mean ± SEM. * (p< 0,05) ** (p< 0,01) *** (p< 0,001) when compared SD and HFD with the same treatment. **#** (p< 0,05) **# #** (p< 0,01) when compared with HFD vehicle. **† †** (p< 0,01) when compared with SD vehicle. SD, standard diet. HFD, high-fat diet.

		Astrocyte number	Morphology		
			No primary ramifications	Total Length	No intersections
Cerebral cortex		Diet Effect HFD ↓	Diet Effect HFD ↓	Diet Effect HFD ↓	Diet Effect HFD ↓
Hippocampus	**Dentate gyrus**	Treatment Effect Zn ↓ BCAA ↓	No effect	No effect	Interaction HFD ↑ Zn ↓
	CA1	Treatment Effect Zn ↓ BCAA ↓	No effect	Interaction HFD ↓ BCAA ↑	No effect

Table 2. Summary of the immunohistological analysis for GFAP+ cells.

5. CONSIDERAÇÕES FINAIS

O projeto de mestrado inicialmente apresentado ao Programa de Pós-graduação em Biociências envolvia experimentos *in vitro* para o estudo dos mecanismos celulares e moleculares das células gliais de ratos obesos, além do estudo *in vivo* apresentado nesta dissertação. Para os experimentos *in vitro*, induzimos obesidade em ratos Wistar por meio de administração de dieta de cafeteria, a qual consiste na oferta de alimentos palatáveis com alto teor energético. Ao final do período da dieta, realizamos o teste de reconhecimento de objetos nesses ratos e após eutanásia os encéfalos desses animais obesos e também de controles foram removidos e realizamos o isolamento das células microgliais. A partir disso, seriam realizados experimentos com as culturas celulares para a compreensão de mecanismos neuroinflamatórios ativados pela obesidade. Contudo, não obtivemos sucesso com o isolamento das células e não foi possível dar seguimento a esta parte do projeto. Dessa forma, neste momento do trabalho, tínhamos apenas os resultados do teste comportamental e dados metabólicos dos ratos que receberam dieta de cafeteria. A partir disso, observamos que o resultado do teste comportamental realizado parecia controverso com o que é descrito na literatura, pois os ratos obesos mostraram melhor desempenho no teste de memória do que os ratos controle. Esse resultado foi intrigante e passamos a considerar a composição da dieta empregada, que além dos alimentos ofertados, também incluía refrigerante do tipo cola, que contém cafeína. Assim, discutimos que a cafeína poderia ser o motivo da melhora na performance no teste de memória. Para esclarecer essa questão, repetimos o experimento e trocamos o refrigerante por outro, a base de suco de laranja, e neste caso, o resultado foi o prejuízo de memória decorrente da dieta de cafeteria que era esperado. Entendemos que esses achados seriam insuficientes para compor a dissertação de mestrado, no entanto, esses resultados geraram um artigo, o qual foi recentemente publicado na revista *Neurochemical Research* e está anexado na dissertação (Anexo A).

Com base no relato indicado acima, o objetivo principal deste estudo ficou limitado ao estudo *in vivo* dos efeito da suplementação de Zn ou BCAA sobre

parâmetros neuroinflamatórios de ratos obesos. Para esses experimentos, a indução da obesidade foi realizada por meio da oferta de ração hiperlipídica e solução de frutose e sacarose. Nossos resultados principais indicaram que apesar das propriedades imunomoduladoras de ambos os compostos, apenas o Zn foi capaz de reverter a perda de memória de reconhecimento observada nos ratos obesos. Também avaliamos a expressão gênica de duas citocinas pró-inflamatórias (IL-6 e TNF-α) no córtex cerebral e no hipocampo dos animais. A IL-6 permaneceu inalterada, independente da dieta ou da suplementação ofertada. Já o TNF-α aumentou no hipocampo após a HFD, o que corrobora com a hipótese de déficit cognitivo causado pela neuroinflamação. Curiosamente, o Zn foi capaz de melhorar a performance dos animais no teste de reconhecimento de objetos, mas, assim como o BCAA, não foi capaz de reverter o aumento do TNF-α no hipocampo, indicando que os benefícios do Zn não estão limitados à modulação do sistema imunológico e podem interferir em outros mecanismos neuroprotetores.

Outra característica da neuroinflamação é a ativação astrocitária ou astrogliose. Nosso trabalho demonstrou que a obesidade não afetou o número de células GFAP+ no hipocampo, mas diminuiu significativamente o número de células no córtex cerebral, o que pode estar caracterizando um quadro de atrofia cortical. Além disso, embora a HFD não tenha sido capaz de afetar significativamente o número de células GFAP+ no hipocampo, ela alterou sua morfologia. Na região CA1, o tratamento com BCAA interagiu com a dieta e foi capaz de evitar o declínio na arborização de astrócitos causado pela obesidade. Já no giro dentado, a HFD aumentou o número de intersecções dos processos astrocitários, indicando um aumento na arborização dessas células. Neste caso, apenas a suplementação de Zn foi capaz de reverter esse efeito. No córtex cerebral, todos os parâmetros morfológicos analisados diminuíram após a HFD e nenhuma das suplementações foi capaz de reverter esse efeito. Estes resultados estão de acordo com a diminuição do número de astrócitos nessa região, e fornece suporte à hipótese de atrofia cortical.

6. CONCLUSÕES

Nesse trabalho, tanto a dieta de cafeteria, ofertada através de alimentos palatáveis com alto teor energético, como a dieta hiperlipídica e hipercalórica, ofertada por meio de ração, foram capazes de causar as alterações metabólicas características da obesidade em ratos Wistar. No primeiro estudo, os desfechos de memória foram diferentes entre os grupos que divergiam no tipo de refrigerante ofertado, nesse sentido, concluímos que o conteúdo da dieta de cafeteria é um aspecto altamente relevante no desenvolvimento das metodologias de estudo e deve ser cuidadosamente planejado. Além disso, constatamos que a cafeína, presente nos refrigerantes do tipo cola, pode ter afetado o desempenho no teste de memória, mesmo estando presente em baixas doses. No segundo estudo, a memória foi prejudicada após a exposição à dieta hiperlipídica e hipercalórica e apenas a suplementação com Zn mostrou-se capaz de reverter esta alteração. Além disso, a análise morfológica dos astrócitos trouxe informações importantes sobre as adaptações celulares decorrentes da neuroinflamação na obesidade, mostrando que certas áreas cerebrais são mais suscetíveis que outras, o que corrobora com achados recentes da literatura.

Assim, nossos estudos sugerem dois potenciais tratamentos (cafeína e Zn) para prevenir ou reverter os efeitos deletérios da obesidade sobre a cognição. Ainda, nosso estudo demonstrou mais uma vez o papel fundamental das células da glia no processo de neuroinflamação, e que os astrócitos podem também ser utilizados como alvo de novas abordagens terapêuticas.

7. PERSPECTIVAS

Com base nos resultados obtidos neste estudo, as principais perspectivas para a continuidade deste trabalho são:

- Obtenção de culturas primárias de células gliais de ratos obesos para avaliar a expressão de mediadores inflamatórios e outras moléculas envolvidas na sinalização da neuroinflamação;

- Realizar experimentos *in vitro* testando o papel da cafeína, do Zn e do BCAA em astrócitos e microglia;

- Avaliar outros marcadores neuroinflamatórios (outras citocinas, permeabilidade da barreira hematoencefálica, infiltração linfocitária, etc) para um estudo mais abrangente dos efeitos das suplementações com cafeína, Zn e BCAA em animais obesos;

- Investigar outras vias pelas quais o Zn possa estar promovendo papel neuroprotetor na obesidade, tais como ativação de fatores de crescimento neuronal, modulação sobre o sistema glutamatérgico, mecanismos epigenéticos, etc.

- Avaliar se a suplementação de cafeína ou Zn em humanos também minimiza a perda cognitiva provocada pela obesidade.

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ANEXOS

Anexo A - Artigo

Neurochemical Research
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ORIGINAL PAPER

Food Selection of Cafeteria Diet Affects Memory Dysfunction Related to Obesity

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Abstract
Cafeteria diet (CAF) mimics human Western diet and has been used in animal models to study obesity. The purpose of this study is to demonstrate that our CAF model induces metabolic disorder related to obesity and affects recognition memory in Wistar rats. We also compared the intake of two different soft drinks, as part of the CAF, on recognition memory. Our results demonstrate that CAF-fed rats increased weight gain and visceral adiposity, and exhibited hyperglycemia, hypertriglyceridemia, high leptin and low insulin plasma levels. Moreover, CAF animals showed higher lipid peroxidation in the liver and developed non-alcoholic fatty liver disease. Surprisingly, the group fed with cola-based soft drinks presented an improvement in recognition memory, whereas animals fed with orange-based soft drinks showed worse performance in this task. Our data indicates that CAF induces obesity and affects recognition memory, but the composition of the diet interfere when the neurological function is evaluated.

Keywords Diet-induced obesity · Object recognition test · Cola-based soft drink · Metabolic dysfunction · Oxidative stress · Non-alcoholic fatty liver disease

Introduction

Obesity prevalence has increased worldwide at an alarming rate. This metabolic disorder is deeply associated with conditions such as insulin resistance, diabetes, hepatic steatosis, and chronic low-grade inflammation, as well as with neurodegenerative diseases such as Alzheimer and Parkinson [1–3]. In the central nervous system (CNS), obesity-induced neuroinflammation is characterized by the activation of glial cells (microglia and astrocytes) and it can affect different CNS functions [2]. In addition, the blood brain barrier (BBB) is altered, causing an increase in its permeability that can facilitate the entry of peripheral immune cells such as lymphocytes enhancing neuroinflammation [2, 4]. The hypothalamus is a brain region involved in body weight regulation and energy balance and there is strong evidence of neuroinflammation driven by obesity in this region. However, other structures, like the hippocampus, which is crucial for learning and memory, are also affected [2, 4, 5]. The susceptibility of different brain regions can explain the worst cognitive performance, memory decline and high predisposition to neurodegenerative diseases found in obese individuals [3, 6].

Thus, obesity is a major challenge for basic science and clinical research, and different scientific models have been created trying to mimic human obesity to better understand this disease and to develop new treatment possibilities. There is a range of commonly utilized animal models of obesity. In rodents, we can highlight transgenic animals, chemical and diet-induced models of obesity [7].

Cafeteria diet (CAF) has been widely used as a diet-induced obesity model due to its capacity to cause metabolic

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dysfunction and related disorders, including a significant shift on gut microbiota and decrease of its diversity [8]. To simulate the Western diet, CAF has a variety of highly palatable energy-dense foods commonly used in human diet that contain substantial amounts of salt, sugar and fat. However, there is no consensus about the components of the CAF, and we can find a diversity of food combinations in the literature, which challenges the comparison of data obtained from this diet-induced obesity model [9].

In the past few decades, the use of high sugar beverages, such as soft drinks, has increased significantly [10]. Among the commonly consumed sweet beverages, we can highlight cola-based soft drinks that are also a source of other substances like caffeine. Caffeine is a psychoactive and its use is correlated with a variety of risks and benefits. Low to moderate doses of caffeine has been associated with alertness, vigilance and attention. On the other hand, high doses of caffeine increase tension and symptoms of anxiety. Regarding memory, the results are still controversial [11].

In the present study, we aimed to demonstrate the CAF ability to induce obesity and its associated metabolic and cognitive dysfunctions. Since there is no consensus about the effects of soft drinks on memory, we also sought to elucidate the effects of differential composition of CAF, by using two different soft drinks (a cola-based vs. an orange-based soft drink) on the long-term recognition memory of Wistar rats.

Materials and Methods

Animals

Male Wistar rats from the animal facility of the Universidade Federal de Ciências da Saúde de Porto Alegre (UFCSPA) were used. Animals had free access to standard rat chow and water until the age of 3 months. They were kept in plastic cages (2–3 rats per cage) under controlled temperature (22–24 °C) and light (12 h light/12 h dark cycle) conditions.

All efforts were made to minimize animal suffering and to reduce the number of animals used in the experiments, which were performed in accordance with the international laws for the care of laboratory animals, following the 3Rs guidelines. All procedures were approved by the Institutional Animal Care and Use Committee (UFCSPA, Brazil, protocol No.536/17).

Diet and Experimental Groups

Three months-old rats were randomly divided into two experimental groups: Control diet (CT, n = 16) and cafeteria diet (CAF, n = 20). After that, only for the object recognition (OR) test, a third group was added to the study. This group also received the cafeteria diet, however the soft drink offered was different. Thus, animals tested in the OR received: cafeteria diet + cola-based soft drink (n = 10), or cafeteria diet + orange-based soft drink (n = 10). The special diets for the groups CT, and CAF-cola or CAF-orange soft drinks, were administered for 16 weeks. After that, only animals from CT and CAF-cola were euthanized to collect the tissues used in all further assays.

CT group was fed 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). Animals from CAF group were fed, besides the standard chow, with eight palatable human food items with high energy content (Table 1): Salty snacks (Pastelina®), cake, potato chips (Ruffles®), biscuits (Tortinhas®, Isabela), cream wafer (Wafer Strawberry®, Isabela), sausage (Alibem®), mortadella (Perdigão®) and chocolate (Top®). CAF-fed rats also had free access to standard rat chow (Nuvital®, Curitiba, PR, Brazil), a soft drink (cola-based soft drink (Coca-Cola®) or orange-based soft drink (Fanta Orange®) and water ad libitum. On average, total CAF energy content included 47,5% carbohydrates, 13,5%

Table 1 Composition of CAF and energy content

	Serving size	Calories (kcal)	Total carbohydrate (g)	Protein	Total fat
Cake	60 g	221	34	3.6	8.3
Salty snacks	25 g	129	13	1.6	8
Biscuits	30 g	152	20	2	7.1
Potato chips	25 g	141	12	1.6	9.7
Cream wafer	30 g	162	19	1	9.1
Sausage	50 g	120	3	6	9.5
Mortadella	40 g	113	2.4	4.8	9.4
Chocolate	25 g	137	15	0.9	8.4
Orange-soft drink	200 ml	99	24	0	0
Cola-soft drink	200 ml	85	21	0	0

protein and 39% fat, providing a total energy content of around 4.4 kcal/g, plus 0.45 kcal/ml of soft-drink. Every day, a variety of two high-energy content items, one sweat and one salty, were mixed together and was provided in excess quantities, items were changed daily to maintain novelty (Table 2). CAF rats were fed immediately before the beginning of the dark period. The leftovers of each food item were weighed daily to determine food intake per cage. The animals were weighed in the beginning of the experiment and every week to determine weight gain.

Object Recognition Test

In the last week of diet administration, animals were submitted to the object recognition test. The test was composed of three phases divided in 3 days. On the first day, rats were habituated in an acrylic box (40 cm × 40 cm × 20 cm) containing only shavings. Twenty-four hours after habituation, the training session was conducted. Animals were placed individually in the left rear quadrant of the box containing two objects (A and B), and were allowed to freely explore them for 5 min. The long-term memory retention test was performed twenty-four hours after the training session. For the test, each rat was reintroduced into the box where one of the objects presented during training was replaced by a new object (C). The exploration time of the familiar and new object was measured. Exploration of an object was defined as directing the nose towards the object at a distance < 2 cm and/or when the animal touched the object with its nose [12]. Finally, the recognition index (RI) was calculated.

$$RI = \frac{\text{Time spent exploring the new object (C)}}{\text{Time exploring the familiar object (A)} + \text{time exploring the new object (C)}}$$

Tissue and Blood Collection

After 16 weeks of diet, animals from the groups CT and CAF-cola were anesthetized with ketamine (100 mg/kg, intraperitoneal) and xylazine (10 mg/kg, intraperitoneal), the retro-orbital sinus blood was collected and the rats were euthanized for tissue collection. The serum was obtained after blood centrifugation (2500 RPM for 10 min at 4 °C) and stored at − 20 °C for later analysis. The liver and visceral fat were quickly removed and weighted. The right upper lobe was used for histology and the remaining liver was frozen for oxidative stress analyses.

Table 2 Diet options

	Diet composition
Diet 1	Chow + salty snacks + cake + soft-drink + water
Diet 2	Chow + potato chips + biscuits + soft-drink + water
Diet 3	Chow + cream wafer + sausage + soft-drink + water
Diet 4	Chow + chocolate + mortadella + soft-drink + water

Items were weighted before and after consume to calculate the food intake per cage. Items were mixed together and offered ad libitum

Blood Analyses

To analyze serum levels of insulin and leptin, a commercial kit (Millipore, USA) of an enzyme-linked immunosorbent assay (ELISA) was used following the manufacturer's instructions. Serum levels of glucose, triglycerides and cholesterol were also analyzed using colorimetric kits (Labtest, Brazil), according to the manufacturer's instructions.

Analyses of Oxidative Stress

To evaluate oxidative stress in the liver, the thiobarbituric acid reactive substances (TBARS) method was used. This assay quantifies low molecular weight compounds, predominantly malondialdehyde (MDA), which complex with thiobarbituric acid (TBA). The amount of aldehyde products generated by lipid peroxidation was quantified by the TBA reaction, using 3 mg of protein/sample added to 1 mL of thiobarbituric acid (0.67%) dissolved in 0.026 M Tris–HC1 buffer (pH 7.0). The absorbance of the supernatant was determined at 535 nm using a spectrophotometer (SpectraMax M2e Microplate Reader, Molecular Devices, MDS Analytical Technologies, USA). The results were expressed as nmol of malondialdehyde (MD) per milligrams of protein [13]. The protein concentrations in homogenates were quantified using the Bradford method [14]. We also used the Superoxide Dismutase Assay Kit (Cayman Chemical, USA) in order to determine superoxide dismutase (SOD) activity. SOD activity is represented by U/mL (one unit is defined as the amount of enzyme needed to exhibit 50% dismutation of the superoxide radical).

Histological Analyses

Liver tissues were fixed in Bouin's solution and embedded in paraffin. Serial sections of 3 µm were obtained and stained in hematoxylin–eosin. Slides were analyzed using a light microscopy (× 200 magnification) by two pathologists which were blinded to experimental design. Evaluation was performed based on the score system described by Kleiner et al. which considers the presence of steatosis (< 5% = 0; 5–33% = 1; 33–66% = 2; > 66% = 3), cellular

ballooning (none = 0; few balloon cells = 1; many cells/prominent ballooning = 2) and lobular inflammatory infiltrate (none = 0; < 2 infiltrates = 1; 2–4 infiltrates = 2; > 4 infiltrates = 3) [15].

Statistical Analyses

Data were expressed as mean ± SEM and analyzed by Student's t test. GraphPad Prism 5.0 was used for the statistical analyses. The significance level was set at $p < 0.05$.

Results

Both CAF groups ingested 27% more kcal than the CT group, irrespective they received cola or orange-based soft drinks. Each rat drank, on average, 47 mL of cola soft drink, and the group that received orange beverage had on average 58 mL daily (data not shown).

Animals from CAF group showed a higher weight gain than CT animals (t = 2.971, df = 15, p = 0.009, Fig. 1a). The amount of visceral adipose tissue was also higher in CAF-fed animals (t = 3.137, df = 18, p = 0.005, Fig. 1b). As shown in Fig. 2, CAF induced hyperglycemia (t = 3.008, df = 12, p = 0.01, Fig. 2a) and hypertriglyceridemia (t = 2.291, df = 11, p = 0.04, Fig. 2b). Metabolic features of obesity were also noticed by the increase in leptin levels in the CAF group (t = 2.366, df = 10, p = 0.03, Fig. 2d). However, insulin plasma levels were lower following CAF in comparison to CT group (t = 3.002, df = 12, p = 0.01, Fig. 2c), which may indicate deterioration of the pancreatic function caused by obesity. Altogether, these results confirm the development of obesity in CAF-fed rats.

Oxidative stress parameters were evaluated in the liver, since it is an important metabolic organ (Fig. 3). We found an increase in malondialdehyde formation, which is a marker of lipid peroxidation, in CAF-fed rats (t = 2.730, df = 10, p = 0.02, Fig. 3a). On the other hand, SOD activity was similar between CT and CAF-fed animals (t = 0.4754, df = 11, p = 0.64, Fig. 3b). Thus, CAF induced an increase in lipid peroxidation but the antioxidant defenses did not counteract the oxidative stress. These findings reinforce that obesogenic diet leads to an imbalance in oxidative status in the liver.

Pathological analyses of liver tissue showed intense hepatocyte steatosis (score = 3) and cellular ballooning (score = 2) following CAF as presented in Fig. 4. No inflammatory infiltrate was seen in the liver sections. These findings are compatible with non-alcoholic fatty liver disease (NAFLD) and a high susceptibility to the development of non-alcoholic steatohepatitis (NASH).

The object recognition test was chosen to assess long-term memory. Surprisingly, we found a higher recognition index in CAF group (t = 2.861, df = 11, p = 0.01, Fig. 5a) that received cola-based soft drink, showing that CAF + cola-based soft drink improved recognition memory. In a different cohort of rats, the soft drink offered to the animals was replaced by an orange-based soft drink. In this group, the results were the opposite, CAF-fed animals showed impairment in the recognition memory (t = 2.783, df = 18, p = 0.01, Fig. 5b).

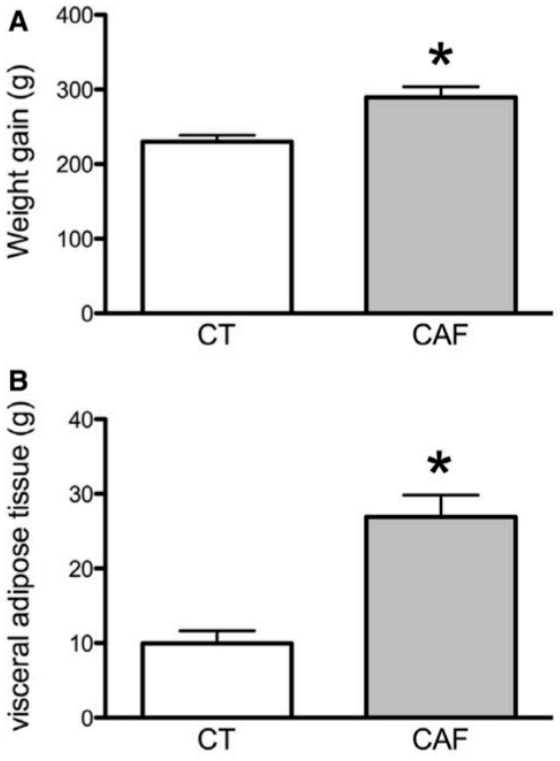

Fig. 1 Effects of CAF on weight gain and visceral adipose tissue. **a** CAF-fed rats presented significantly higher weight gain when compared with control group. **b** The total weight of visceral adipose tissue is increased in CAF-fed rats when compared with control group. Data are expressed as mean ± SEM. CAF, cafeteria diet + cola-based soft drink (n = 20); CT, control diet (n = 16); *p < 0.05

Discussion

Obesity is a public health issue characterized by a low-grade inflammation that affects numerous tissues and leads to higher risks of developing disorders such as insulin resistance, hyperlipidemia, non-alcoholic fatty liver diseases, hypertension, cardiovascular dysfunction, as well as neurodegenerative disorders [1, 3]. The increased prevalence of obesity is related to lifestyle changes, including the consumption of hypercaloric diets rich in fats and carbohydrates. To study the effects of this dietary pattern on health,

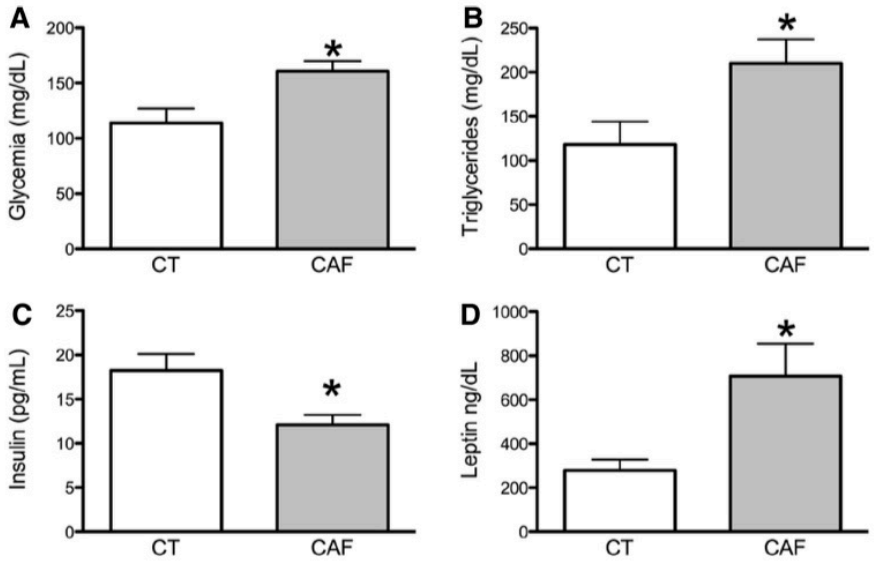

Fig. 2 Metabolic profile following 16 weeks of CAF. **a** Glycemia is increased in CAF-fed rats. **b** Triglycerides levels are significantly higher in CAF when compared with control group. **c** CAF group present reduced insulin plasma levels. **d** CAF-group presented increased leptin plasma levels. Data are expressed as mean ± SEM. *CAF* cafeteria diet + cola-based soft drink (n = 20); *CT* control diet (n = 16); *p < 0.05

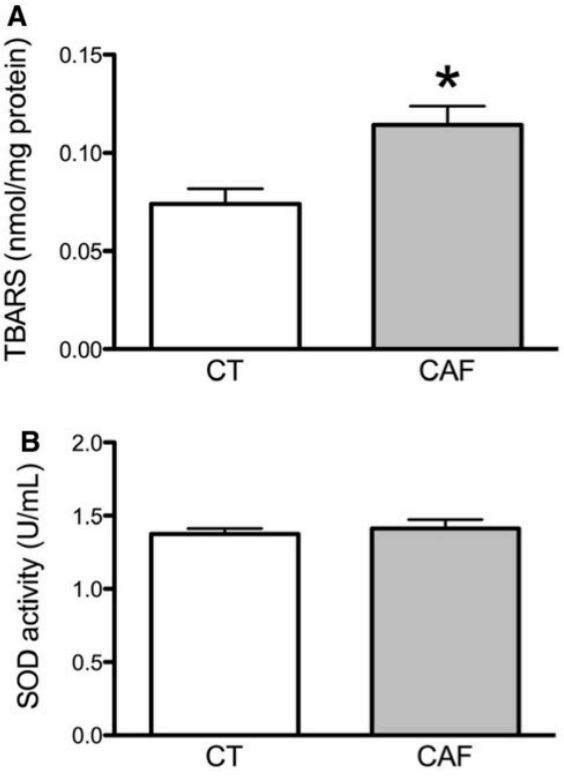

Fig. 3 Liver oxidative stress markers following CAF. **a** CAF-group showed an increase in TBARS, which indicates lipid peroxidation in the liver of obese rats. **a** Superoxide dismutase activity did not change between CT and CAF groups. Data are expressed as mean ± SEM. *CAF* cafeteria diet + cola-based soft drink (n = 20); *CT* control diet (n = 16); *SOD* superoxide dismutase, *TBARS* thiobarbituric acid reactive substances; *p < 0.05

the administration of CAF is a consolidated experimental model, representing the food intake of modern societies capable of induce obesity, glucose intolerance and inflammation in rats [16]. Here, we have demonstrated that our CAF model is effective in inducing obesity and metabolic dysfunction. We have also showed the impact of CAF on cognitive function of the rats. Furthermore, it is worth to mention the cognitive outcome was different depending on the soft drink used in CAF diet, as seen by the object recognition test.

It is well established that a dietary pattern rich in saturated fat and sugar cause detrimental effects on the brain and cognitive function [3]. Even before the development of metabolic syndrome, male rats fed with CAF have shown worst cognitive performance than control group [17]. Also, there is evidence that obesity causes an increase in adipocyte-secreted inflammatory cytokines. This inflammatory state will lead to an impairment of the cognitive behavior, and, thereby, triggers the process of dementia [6].

Despite the role of the hypothalamus in the body weight regulation and energy balance, the neuroinflammation derived from obesity is not restricted to this area [2]. Indeed, diet-induced obesity leads to an increase in BBB permeability in the hippocampus, while both prefrontal cortex and striatal BBB are unaffected [4]. The hippocampus is a crucial brain area for learning and memory, and it is highly susceptible to dietary insults [2, 5]. For instance, after only 10 days of exposure to Western diet, there was a decrease in the hippocampal GLUT1 that supports glucose transport into the CNS and this was associated with learning and memory deficits [18].

Different mechanisms were identified as being able to alter cognitive function in obesity, including reduced levels

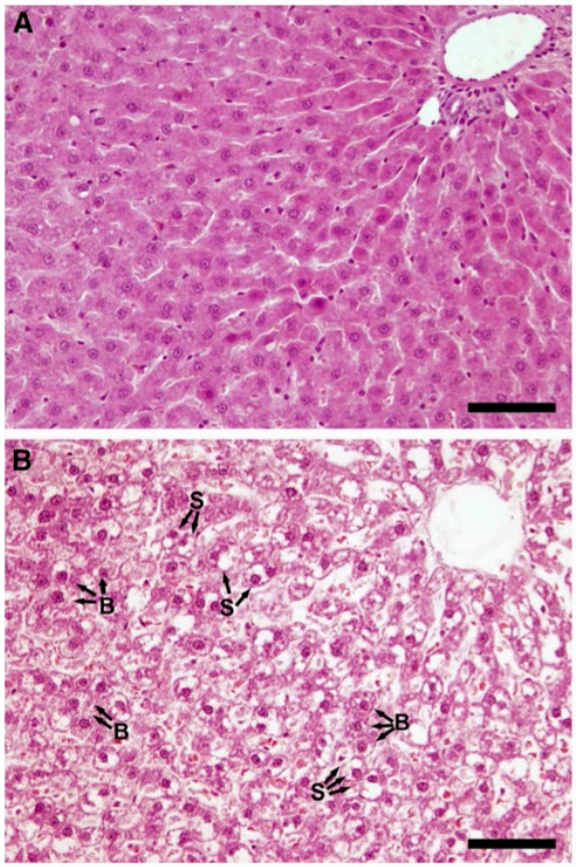

Fig. 4 Histological analysis of the liver. Hematoxylin–eosin staining of CT **a** and CAF-fed rats **b**. Hepatocyte steatosis (score = 3) and cellular ballooning (score = 2) are evident in the liver of CAF group. Arrows indicate steatosis (S) and ballooning (B). *CAF* cafeteria diet + cola-based soft drink (n = 20); *CT* control diet (n = 16); calibration bars = 100 µm

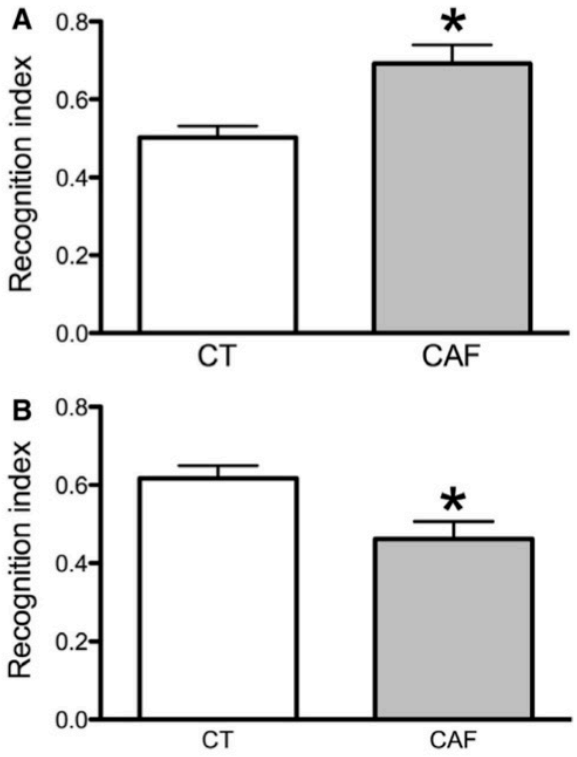

Fig. 5 Effects of different soft-drinks on long-term recognition memory. **a** CAF-fed rats that consumed a cola-based soft drink showed significantly higher recognition index when compared to CT group. **b** CAF-fed rats that consumed an orange-based soft drink showed significantly lower recognition index when compared to CT group. Data are expressed as mean ± SEM. n = 10 per group. *CAF* cafeteria diet, *CT* control diet; *p < 0.05

of brain-derived neurotrophic factor, altered glutamatergic signaling, impaired insulin regulation and TNF-α overexpression [5]. Insulin administration can improve memory, as demonstrated by an enhancement in the passive avoidance task in rats. However, the induction of hippocampal-specific insulin resistance can compromise the synaptic plasticity, which impairs spatial learning and memory [19]. Another important factor that can influence the object recognition (OR) test is the action of leptin, which was elevated in the CAF group in the present study. It has been demonstrated that increased levels of leptin in the brain can impact learning and memory, and the hippocampus has the highest synthesis of its receptors in the CNS. Studies propose that leptin modulates plasticity in learning and memory based behavioral tasks, so that obese rodents with deficiency of its receptors show impairments in hippocampal long-term potentiation (LTP), which is responsible for memory formation [20]. Our results in the OR test show that CAF-fed rats, that received different soft drinks, presented distinct outcomes. Surprisingly, the rats who had free access to cola-based beverage had a better performance on the object recognition test, indicating that cola beverage improved CAF-fed rats recognition memory.

One important component of cola based soft drinks is caffeine, which is one of the most widely consumed supplements in the world [21]. Caffeine is able to cross the BBB [22] and exerts its effects acting as an antagonist of A1 and A2 adenosine receptors. A1 receptors are located in all parts of the brain with higher concentrations in the hippocampus, cerebral and cerebellar cortices, and certain thalamic nuclei, whereas A2 subtypes are located in the dopamine-rich regions of the brain [23]. As an adenosine receptor antagonist caffeine can promote excitation of striatonigral neuronal projections and reduce inhibitory signals to striatopallidal neurons [11].

Recent studies indicate the risks related to the ingestion of soft drinks. For instance, soft drinks containing sodium

benzoate (SB), a commonly used food preservative, can impair memory and motor coordination [24]. Pase et al. also linked the cumulative intake of artificially sweetened soft drinks with a higher risk of stroke and dementia in humans [25]. Furthermore, the consumption of soft drinks containing sugar and caffeine is capable of causing worse sleep pattern and quality, especially in students [26]. On the other hand, in healthy human subjects, caffeine has shown to possess positive effects on long-term retention by enhancing memory consolidation [27]. In obese individuals, the apparent volume of distribution of caffeine is increased by 60%, but obesity has no effect on caffeine clearance and seems to prolong its half-life by 31–69% [28]. Hameleers et al. also observed that habitual caffeine consumption was associated with better long-term verbal memory [29]. In addition, it was shown that sucrose ingestion caused memory impairment, while rats receiving sucrose combined with caffeine exhibited a cognitive performance similar to control group [30]. These findings indicate that caffeine may protect against sucrose-evoked cognitive impairments, which corroborate with our results. Also, Alzoubi et al. found that caffeine prevents short and long-term memory impairments induced by chronic l-methionine administration. Authors infer that caffeine would be able to preventing reduction in antioxidant protection mechanisms in the hippocampus, during chronic l-methionine administration [31]. Here, CAF-fed rats had higher levels of lipid peroxidation in the liver and it was not affected by the cola-based soft drink consumption.

It is well established plasma levels of leptin are proportional to body fat content. Accordingly, we showed the amount of visceral adipose tissue as well as the plasma levels of leptin are higher in CAF-fed animals. Leptin has an inhibitory effect on insulin synthesis. Insulin pancreatic secretion leads to lipid storage and leptin production in the adipocytes, generating a regulatory process between β-cells and adipocytes. The administration of leptin reduces circulating insulin levels in inheritable obese phenotype mouse, and simultaneously leads to acute increase in blood glucose levels [32]. In our study we verified CAF induced hyperglycemia and decreased insulin plasma levels. Thus, the lower insulin levels may be caused by increased leptin, but it also might be a result of pancreatic dysfunction, since CAF model is highly obesogenic [33]. Inflammatory processes are usually supposed to be a consequence of obesity. In fact, chronic inflammation in adiposity can lead to insulin resistance and other obesity-related issues, denominated metabolic syndrome. Although, the source of this inflammatory event is not known, studies have evidenced that oxidative stress could be involved in pro-inflammatory status [34].

Oxidative stress is the most important event leading to complications in diet-induced obesity in rats. It has been demonstrated that oxidative stress caused by a high fat diet increases glucose intolerance and insulin resistance, and also rises plasma and tissues levels of oxidative stress markers [1]. Hypercaloric diets can lead to oxidative stress by increasing lipid peroxidation. Moreover, the fat consumption may modify cellular levels of pro and antioxidant substances, making cell membranes more susceptible to oxidation reactions [35]. It was previously demonstrated that CAF increased lipid peroxidation in the liver, testicles and kidney [16]. Ulla et al. also showed that obesity rises lipid peroxidation in hepatic tissues, as expressed by increased levels of MDA [1]. These studies are in accordance to our results, in which CAF-fed rats had higher levels of lipid peroxidation in the liver.

As mentioned before, obesity is an important risk factor for increasing lipid peroxidation and decreasing the activity of cytoprotective enzymes. Thus, CAF can induce inflammation, steatosis and fibrosis in the liver. Ulla et al. demonstrated, by histological analysis, that high carbohydrate and fat diet induced obesity in rats, and the animals presented inflammatory cells infiltration in the liver and lipid accumulation in hepatocytes [1]. Parafati et al. demonstrated the presence of liver fibrosis in CAF treated rats [36]. It corroborates with our histological evaluation of the liver, showing the presence of low lobular inflammatory infiltrate, hepatocellular ballooning and intense steatosis in CAF-fed rats, characterizing a nonalcoholic steatohepatitis (NASH). Thus, our results showed that 16 weeks of CAF is able to induce NASH which corroborates with previous studies, indicating that CAF is an important model to study fatty liver diseases.

Milagro et al. showed a correlation between hepatic lipid peroxidation and serum leptin levels, suggesting a possible link between adipose tissue and liver [34]. Leptin leads to insulin resistance and hepatic disease in both, cell cultures and animal models, by activating the transforming growth factor β axis and stellate cells. Serum leptin is related to liver steatosis, but not fibrosis, in NASH [37]. Our results show an increase in serum leptin levels in the CAF group, confirming its possible correlation to our histological findings.

In summary, CAF induced an increase in weight gain and visceral adiposity, as well as, hyperglycemia, hypertriglyceridemia, and increased leptin levels, proving that this model is consistent to induce obesity in rats. We also found increased lipid peroxidation in the liver and pathological characteristics of NAFLD. Based on our findings, cola-based soft drink could provide an improvement in recognition memory of obese animals. Conversely, an orange-based soft drink used as part of CAF showed opposite results. It suggests caffeine may protect the CNS from the declarative memory decline caused by obesity. However, more studies are necessary to assess the benefits of caffeine supplementation in obese subjects. Thus, besides being a widely used model for the study of brain function in obesity, the composition of the CAF should be taken into account when analyzing the neurological impact of this hypercaloric diet.

Acknowledgments This study was supported by the Brazilian National Council for Scientific and Technological Development (CNPq), the Coordination for the Improvement of Higher Education Personnel (CAPES) and the Foundation for Research Support of the State of Rio Grande do Sul (FAPERGS).

Author Contributions GSF, ACM performed experiments, analyzed data, and wrote the manuscript. SO, RT, EES and FF performed experiments. MG and MP contributed to data analysis and editing of the manuscript. RPG designed the study, interpreted the results and wrote the manuscript. All authors read and approved the final manuscript.

Funding This study was supported by the Brazilian National Council for Scientific and Technological Development (CNPq), the Coordination for the Improvement of Higher Education Personnel (CAPES) and the Foundation for Research Support of the State of Rio Grande do Sul (FAPERGS).

Compliance with Ethical Standards

Conflict of interest The authors declare that they have no conflicts of interest regarding this study.

Ethical Approval All efforts were made to minimize animal suffering and to reduce the number of animals used in the experiments, which were performed in accordance with the international laws for the care of laboratory animals, following the 3Rs guidelines. All procedures were approved by the Institutional Animal Care and Use Committee (UFCSPA, Brazil, protocol No.536/17). This article does not contain any studies with human participants performed by any of the authors.

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Anexo B – Aprovação da Comissão de Ética no Uso de Animais (CEUA).

CEUA –COMISSÃO DE ÉTICA NO USO DE ANIMAIS

PARECER CONSUBSTANCIADO DE PROJETO DE PESQUISA E ENSINO

1) PROTOCOLO Nº: 217/17 – segunda versão

2) DATA DO PARECER:08 de novembro de 2017 Parecer 536/17

3) TÍTULO DO PROJETO:

Análise dos efeitos do BCAA sobre marcadores de neuroinflamação em ratos Wistar.

4) PESQUISADOR RESPONSÁVEL:

Renata Guedes

5) RESUMO DO PROJETO:

A obesidade cria um ambiente inflamatório crônico que repercuta no SNC como neuroinflamação. A micróglia e astrócitos respondem a situações adversas com um fenótipo pró-inflamatório ou anti-inflamatório. A suplementação com BCAA é fundamental para a regulação do sistema imunológico, do peso corporal, da síntese de proteínas musculares e manutenção da homeostase da glicose. Aumento dos níveis de BCAA aumenta o risco para desenvolver diabetes e doenças cardiovasculares. Em relação ao SNC os efeitos da BCAA ainda não são completamente compreendidos.
Células microgliais e astrócitos de ratos Wistar submetidos a dieta padrão e hiperlipídica serão isoladas. As culturas celulares serão tratadas com BCAA e o meio será coletado para dosagem das citocinas. As células serão coletadas para a realização de Western blot, imunocitoquimica para micróglia e GFAP para astrócitos.

6) OBJETIVOS DO PROJETO:

O objetivo deste projeto é estudar os efeitos do BCAA sobre marcadores neuroinflamatórios em culturas primárias de micróglia e astrócitos.

7) FINALIDADE DO PROJETO: ☐ Ensino ☒ Pesquisa

8) ITENS METODOLÓGICOS E ÉTICOS DO PROJETO:

Título ☒ 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: Rato wistar **Número Amostral:** 30

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): [ ] 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): Biotério da UFCSPA

Outra instituição. Qual?

11) CRONOGRAMA DE UTILIZAÇÃO DE ANIMAIS

Data	Espécie	Sexo	Quantidade
	Rato wistar	macho	30

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 16/11/2017 Data de Término 01/12/2019

Comentários gerais sobre o projeto:

CURRÍCULO LATTES

Grace dos Santos Feijó

Endereço para acessar este CV: http://lattes.cnpq.br/0577859115786296
ID Lattes: **0577859115786296**
Última atualização do currículo em 25/06/2019

Possui graduação em Fisioterapia pela Universidade Federal do Rio Grande do Sul (2017). Participou do programa Ciência sem Fronteiras entre 2014-2015 na Queen Mary University of London. Tem atuado em pesquisa nas áreas de Fisioterapia e Neurociências, principalmente nos seguintes temas: Neuroinflamação, Biomecânica e Traumato-ortopedia. **(Texto informado pelo autor)**

Identificação

Nome Grace dos Santos Feijó
Nome em citações bibliográficas FEIJÓ, G. S.;FEIJÓ, GRACE DOS SANTOS

Endereço

Endereço Profissional
Universidade Federal do Rio Grande do Sul, Escola Superior de Educação Física.
Rua Felizardo
Jardim Botânico
90690200 - Porto Alegre, RS - Brasil
Telefone: (51) 33085804

Formação acadêmica/titulação

2017
Mestrado em andamento em BIOCIÊNCIAS (Conceito CAPES 4).
Fundação Universidade Federal de Ciências da Saúde de Porto Alegre, UFCSPA, Brasil.
Título: Análise dos efeitos do BCAA sobre marcadores de neuroinflamação em ratos Wistar,Orientador: Renata Padilha Guedes.
Palavras-chave: Neuroinflamação; Fisiologia; Metabolismo.
Grande área: Ciências Biológicas

2011 - 2017
Graduação em Fisioterapia.
Universidade Federal do Rio Grande do Sul, UFRGS, Brasil.
Título: Uma questão não resolvida em neuroinflamação: células da microglia do cérebro e da medula espinhal diferem em sua resposta morfológica à lesão?.
Orientador: Dr. Ping Yip; Dra. Flávia Gomes Martinez.

2006 - 2008
Ensino Médio (2º grau).
Instituto Federal Sul-Rio-Grandense, IFSUL, Brasil.

Formação Complementar

2019 - 2019
CURSO DE EXTENSÃO EM NEUROIMAGEM AVANÇADA. (Carga horária: 15h).
Hospital São Lucas da PUCRS, HSL, Brasil.

2014 - 2015
Extensão universitária em Biomedical Sciences.
Queen Mary - University of London, QMUL, Inglaterra.

2013 - 2014
Extensão universitária em Liga de Geriatria e Gerontologia.
Pontifícia Universidade Católica do Rio Grande do Sul, PUCRS, Brasil.

Atuação Profissional

Universidade Federal do Rio Grande do Sul, UFRGS, Brasil.

Vínculo institucional
2016 - 2016 Vínculo: Bolsista, Enquadramento Funcional: Estagiário Fisioterapia, Carga horária: 20
Outras informações Atribuições: Traçar metas com base na avaliação física e análise de exames complementares. Aplicação do tratamento em solo de pacientes com lesões musculoesqueléticas e relacionadas ao esporte. Discussão de casos e evolução de pacientes.

Vínculo institucional
2015 - 2015 Vínculo: Bolsista, Enquadramento Funcional: Bolsita Extensão, Carga horária: 20
Outras informações Atribuições: Avaliação física, análise de exames complementares, discussão de casos clínicos e de tratamentos no meio aquático.

Vínculo institucional
2014 - 2014 Vínculo: Bolsista, Enquadramento Funcional: Bolsista Extensão, Carga horária: 20
Outras informações Atribuições: Participação na implementação do projeto de extensão de fisioterapia traumato-ortopédica no ambiente hospitalar. Foco principalmente em pós-operatório de artroplastia total de joelho (ATJ), artoplastia total de quadril (ATQ) e politrauma. Discussão de casos e evolução de pacientes. Participação e apresentação em eventos científicos.

Vínculo institucional
2013 - 2013 Vínculo: Bolsista, Enquadramento Funcional: Bolsista CENESP - LAPEX/ESEF/UFRGS, Carga horária: 20
Outras informações Atribuições: Avaliação postural estática, manejo de equipamentos, utilização do software DIPA (Digital Image-based Postural Assessment), elaboração de laudos posturais, atividades de avaliação relacionadas à avaliação de flexibilidade e força.

Vínculo institucional
2011 - 2013 Vínculo: Bolsista, Enquadramento Funcional: Bolsista de iniciação científica, Carga horária: 20
Outras informações Atribuições: Foco na análise do movimento de bailarinas. Revisão de literatura, treinamento para operação de equipamentos, coleta e analise de dados, elaboração de artigos e relatórios, participação e apresentação em eventos científicos.

Áreas de atuação

1. Grande área: Ciências Biológicas / Área: Fisiologia / Subárea: Neurociências.
2. Grande área: Ciências Biológicas / Área: Fisiologia / Subárea: Fisioterapia e Terapia Ocupacional.

Idiomas

Inglês Compreende Bem, Fala Bem, Lê Bem, Escreve Bem.
Espanhol Compreende Razoavelmente, Fala Pouco, Lê Bem, Escreve Pouco.
Português Compreende Bem, Fala Bem, Lê Bem, Escreve Bem.

Produções

Produção bibliográfica

Artigos completos publicados em periódicos

Ordenar por

Ordem Cronológica

1. **FEIJÓ, GRACE DOS SANTOS**; DE OLIVEIRA, SIMONE ; THOEN, RUTIANE ; SCHAAB, ESTER ELENA ; DE MOURA, ANA CAROLINA ; FRANCO, FELIPE ; GIOVENARDI, MÁRCIA ; PORAWSKI, MARILENE ; GUEDES, RENATA PADILHA . Food Selection of Cafeteria Diet Affects Memory Dysfunction Related to Obesity. NEUROCHEMICAL RESEARCH JCR, v. -, p. 1-9, 2019.
2. GONTIJO, K. N. S. ; **FEIJÓ, G. S.** ; PAIXAO, L. R. ; LOSS, J. F. ; CANDOTTI, Cláudia Tarragô . ASSESSMENT METHOD OF DYNAMIC JOINT ALIGNMENT OF LOWER LIMB (MADAAMI) FOR DANCERS DURING THE PLIÉ. REVISTA BRASILEIRA DE CIÊNCIAS DO ESPORTE, v. XX, p. 1-12, 2016.
3. GONTIJO, KAANDA NABILLA SOUZA ; CANDOTTI, Cláudia Tarragô ; **FEIJÓ, GRACE DOS SANTOS** ; RIBEIRO, LAIS PAIXÃO ; LOSS, JEFFERSON FAGUNDES . Kinematic Evaluation of the Classical Ballet Step -Plié-. JOURNAL OF DANCE MEDICINE & SCIENCE, v. 19, p. 70-76, 2015.
4. **FEIJÓ, G. S.**; Silva T ; PAIXAO, L. R. ; GONTIJO, K. N. S. ; NOLL, M. ; CANDOTTI, Cláudia Tarragô . A influência do Método Pilates na flexibilidade da articulação coxofemoral, na força dos músculos abdominais e no equilíbrio postural ântero-posterior. Lecturas Educación Física y Deportes (Buenos Aires), v. 19, p. 1, 2014.
5. **FEIJÓ, G. S.**; SILVA, Tatiana da. ; RIBEIRO, LAIS PAIXÃO ; GONTIJO, K. N. S. ; NOLL, M. ; CANDOTTI, C. T. . A influência do Método Pilates na flexibilidade da articulação coxofemoral, na força dos músculos abdominais e no equilíbrio postural ântero-posterior. Lecturas Educación Física y Deportes (Buenos Aires), v. 19, p. 1, 2014.
6. **FEIJÓ, G. S.**; GONTIJO, K. N. S. ; PAIXAO, L. R. ; MAZETTO, L. ; NOLL, M. ; CANDOTTI, Cláudia Tarragô . A INFLUÊNCIA DA DANÇA DE SALÃO SOBRE A POSTURA CORPORAL DE ALUNOS DE UMA ESCOLA DE DANÇA EM BENTO GONÇALVES-RS. CINERGIS (UNISC), v. 14, p. 45-51, 2013.

Livros publicados/organizados ou edições

1. GONTIJO, K. N. S. ; **FEIJÓ, G. S.** ; PAIXAO, L. R. ; CANDOTTI, Cláudia Tarragô . E por falar em... CORPO PERFORMÁTICO - Fazeres e dizeres da dança. 6. ed. Joinville - SC: Nova Letra, 2013. v. 1. 283p .

Resumos expandidos publicados em anais de congressos

1. **FEIJÓ, G. S.**; GONTIJO, K. N. S. ; PAIXAO, L. R. ; CANDOTTI, Cláudia Tarragô ; LOSS, J. F. . Kinematics Evaluation of Pelvis During the Step Plié of Classical Ballet. In: XXIV Congress of the International Society of Biomechanics, 2013, Natal - RN. Poster Presentation of XXIV Congress of the International Society of Biomechanics, 2013.

Resumos publicados em anais de congressos

1. **FEIJÓ, G. S.**; DE OLIVEIRA, SIMONE ; Boranga, D.G ; JANTSCH, J. ; ARENA, R.M. ; BRAGA, M. F. ; CASTRO, L.F. ; PORAWSKI, MARILENE ; GUEDES, RENATA PADILHA . ANÁLISE DOS EFEITOS DO BCAA SOBRE MARCADORES METABÓLICOS E NEUROINFLAMATÓRIOS EM RATOS WISTAR. In: III Encontro do Programa de Pós-Graduação em Biociências, 2018, Porto Alegre. III Encontro do Programa de Pós-Graduação em Biociências, 2018.
2. **FEIJÓ, G. S.**; DE OLIVEIRA, SIMONE ; JANTSCH, J. ; ARENA, R.M. ; BRAGA, M. F. ; FRANCO, FELIPE ; PORAWSKI, MARILENE ; GUEDES, RENATA PADILHA . Análise dos efeitos do BCAA sobre marcadores metabólicos e neuroinflamatórios em ratos Wistar. In: XLI Reunião Anual da SBNeC, 2018, Santos. XLI Reunião Anual da SBNeC, 2018.
3. **FEIJÓ, G. S.**; MINOTTO, B. B. ; CAMARATTA, B. ; MARCHISIO, Ângela ; UMPIERRES, Carolina ; ROCHA, Clarice Sperotto dos Santos . Fisioterapia aplicada a traumato-ortopedia hospitalar: um estudo de caso. In: II Encontro de Traumatologia e Ortopedia: Reabilitação em foco, 2014, Pelotas/RS. Anais do II Encontro de Traumatologia e Ortopedia: Reabilitação em foco, 2014, Pelotas. II Encontro de Traumatologia e Ortopedia: Reabilitação em foco, 2014., 2014.
4. DOMANSKI, B. F. ; CANDOTTI, Cláudia Tarragô ; CAMARATTA, B. ; **FEIJÓ, G. S.** ; NOLL, M. . INSTRUMENTO FLEXICURVA: VARIABILIDADE DAS MENSURAÇÕES DA COLUNA VERTEBRAL. In: V Congresso Internacional de Fisioterapia Manual, 2012, Fortaleza. Revista Eletrônica Inspirar. Curitiba: Faculdade Inspirar, 2012. v. 4. p. 105-105.
5. CAMARATTA, B. ; CANDOTTI, Cláudia Tarragô ; DOMANSKI, B. F. ; **FEIJÓ, G. S.** ; NOLL, M. . PREVALÊNCIA DE ALTERAÇÕES POSTURAIS EM USUÁRIOS DO SUS DA CIDADE DE PORTO ALEGRE. In: V Congresso Internacional de Fisioterapia Manual, 2012, Fortaleza. Revista Eletrônica Inspirar. Curitiba: Faculdade Inspirar, 2012. v. 4. p. 127-128.

6. **FEIJÓ, G. S.**; CANDOTTI, Cláudia Tarragô ; CAMARATTA, B. ; DOMANSKI, B. F. ; NOLL, M. . PREVALÊNCIA DE DOR E DE INCAPACIDADE FUNCIONAL EM USUÁRIOS DO SUS. In: V Congresso Internacional de Fisioterapia Manual, 2012, Fortaleza. Revista Eletrônica Inspirar. Curitiba: Faculdade Inspirar, 2012. v. 4. p. 128-129.
7. DOMANSKI, B. F. ; CANDOTTI, Cláudia Tarragô ; NOLL, M. ; CAMARATTA, B. ; **FEIJÓ, G. S.** . INSTRUMENTOS FLEXICURVA E ARCÔMETRO: PERCEPÇÃO DE AVALIA- DORES E AVALIADOS.. In: V Congresso Internacional de Fisioterapia Manual, 2012, Fortaleza. Revista Eletrônica Inspirar. Curitiba: Faculdade Inspirar, 2012. v. 4. p. 105-106.

Apresentações de Trabalho

1. **FEIJÓ, G. S.**. Postural ideal, existe?. 2018. (Apresentação de Trabalho/Conferência ou palestra).

Bancas

Participação em bancas de comissões julgadoras

Outras participações

1. **FEIJÓ, G. S.**. 3a Experiência Beta - Encontro Nacional do Cientista Beta. 2018. Universidade do Vale do Rio dos Sinos.

Eventos

Participação em eventos, congressos, exposições e feiras

1. 16º Salão de Extensão UFRGS.FISIOTERAPIA AQUÁTICA 2015. 2015. (Outra).
2. 15º Salão de Extensão da UFRGS.FISIOTERAPIA TRAUMATO-ORTOPÉDICA EM AMBIENTE HOSPITALAR. 2014. (Outra).
3. II Encontro de Traumatologia e Ortopedia: reabilitação em foco.Fisioterapia aplicada a traumato-ortopedia hospitalar: um estudo de caso.. 2014. (Encontro).
4. I International Symposium on Cardiovascular Epidemiology. 2013. (Simpósio).
5. XXV Salão de Iniciação Científica.Avaliação cinemática da pelve durante o passo plié do ballet clássico. 2013. (Outra).
6. E por falar em... CORPO PERFORMÁTICO - Fazeres e dizeres da dança. Seminários de Dança.Análise cinemática dos passos elevé e relevé em primeira e segunda posição de pés. 2012. (Seminário).
7. Simpósio de Pesquisa do Exercício- SIPE. 2012. (Simpósio).
8. V Congresso Internacional de Fisioterapia Manual. PREVALÊNCIA DE DOR E DE INCAPACIDADE FUNCIONAL EM USUÁRIOS DO SUS. 2012. (Congresso).
9. XXIV Salão de Iniciação Científica.PREVALÊNCIA DE DOR NAS COSTAS E INCAPACIDADE FUNCIONAL EM USUÁRIOS DO. 2012. (Outra).
10. 9º Congresso da Rede Unida. 2010. (Congresso).

Organização de eventos, congressos, exposições e feiras

1. **FEIJÓ, G. S.**. III Encontro do Programa de Pós-Graduação em Biociências. 2018. (Outro).
2. **FEIJÓ, G. S.**. 9º Congresso da Rede Unida. 2010. (Congresso).

Página gerada pelo Sistema Currículo Lattes em 02/07/2019 às 14:21:00