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EFEITO DA TAURINA E DO AMBIENTE ENRIQUECIDO NO CEREBELO DE RATOS DIABÉTICOS – UMA ANÁLISE DE MARCADORES DE DANO CELULAR

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Orientadora: Profª Dra. Marilda da Cruz Fernandes

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Resumo da Dissertação

Introdução: O diabetes mellitus apresenta um grande aumento na prevalência a nível mundial, sendo uma das doenças crônicas de maior impacto nos gastos com saúde. O quadro de hiperglicemia leva a diversos distúrbios metabólicos que geram um estresse oxidativo, afetando muitos tecidos e sistemas incluindo o sistema nervoso central. A taurina demonstrou efeitos antioxidantes desempenhando um papel neuroprotetor juntamente com o enriquecimento ambiental. **Objetivos:** O objetivo deste trabalho foi estudar o efeito do aminoácido taurina e do enriquecimento ambiental, avaliando-os de forma isolada e combinada, sobre o cerebelo de ratos induzidos ao diabetes, verificando os resultados através de marcadores de danos celular para lesão e morte celular, e avaliação de células gliais e filamentos intermediários. **Material e Métodos:** No experimento foram utilizados 88 ratos, machos, Wistar; divididos em dois grupos de 44 animais cada, onde um grupo foi alocado em caixas de moradia padrão e o outro em gaiolas com ambiente enriquecido. Dos 44 animais de cada grupo, aproximadamente metade foi induzido ao diabetes *mellitus* tipo 1, sendo uma fração tratado com taurina por trinta dias consecutivos. Ao trigésimo dia foram eutanasiados após a perfusão transcardíaca e retirados os cerebelos que foram fixados, processados e seccionados para realização da técnica de imuno-histoquímica utilizando os marcadores caspase-3 clivada, S100, GFAP e vimentina. **Resultados:** Taurina e ambiente enriquecido reduziram a imunorreatividade para GFAP. Taurina reduziu o número de células positivas para caspase-3 clivada, enquanto o ambiente enriquecido aumentou a imunorreatividade para esta proteína. Nenhuma mudança foi observada para imunorreatividade de células positivas

para S100. O diabetes aumentou a imunorreatividade para vimentina e o ambiente enriquecido foi eficaz na proteção contra este efeito. **Conclusão:** Nossos resultados demostram o efeito benéfico da taurina e do enriquecimento ambiental frente a um quadro hiperglicêmico em diferentes tipos de células, representando um potencial efeito neuroprotetor.

Palavras-chave: Cerebelo, taurina, ambiente enriquecido, diabetes *mellitus,* imuno-histoquímica.

Abstract

Introduction: Diabetes mellitus shows a large increase in global prevalence, being one of the chronic diseases with the greatest impact on health spending. Hyperglycemia leads to various metabolic disorders that generate oxidative stress, affecting many tissues and systems including the central nervous system. Taurine demonstrated antioxidant effects playing a neuroprotective role together with environmental enrichment. **Aim of study**: The objective of this work was to study the effect of the amino acid taurine and environmental enrichment, evaluating them in an isolated and combined way, on the cerebellum of rats induced to diabetes, verifying the results through markers of cell damage for cell death and injury, and evaluation of glial cells and intermediate filaments. **Material and Methods**: In the experiment, 88 male Wistar rats were used; divided into two groups of 44 animals each, where one group was allocated in standard housing boxes and the other in enriched environment cages. Of the 44 animals in each group, approximately half were induced to type 1 diabetes mellitus, a fraction treated with taurine for thirty consecutive days. On the thirtieth day they underwent euthanasia, transcardiac perfusion and cerebellar removal, which were fixed, processed and sectioned for immunohistochemistry using the cleaved caspase-3, S100, GFAP and vimentin markers. **Results**: Taurine and enriched environment reduced immunoreactivity to GFAP. Taurine reduced the number of cells cleaved for caspase-3 cleaved, while the enriched environment increased the immunoreactivity for this protein. No change was observed for immunoreactivity of S100 positive cells. Diabetes increased immunoreactivity to vimentin and the enriched environment was effective in protecting against this effect.

Conclusion: Our results demonstrate the beneficial effect of taurine and environmental enrichment against a hyperglycemia in different cell types, representing a potential neuroprotective effect.

Keywords: Cerebellum, taurine, enriched environment, diabetes *mellitus,* immunohistochemistry.

Lista de abreviaturas

AE: Ambiente enriquecido
BDNF: Fator neurotrófico derivado do cérebro
BrdU: 5- bromo-2'-deoxiuridina
CAT: Catalases
DM: Diabetes *mellitus*
DM1: Diabetes *mellitus* tipo 1
DM2: Diabetes *mellitus* tipo 2
DA: Doença de Alzheimer
DP: Doença de Parkinson
EA: Enriquecimento ambiental
GFAP: Proteína ácida fibrilar glial
Gpx: Glutationa peroxidase
IHQ: Imuno-histoquímica
LPO: Peroxidação lipídica
LTP: Potenciais de longa duração
NCAM: Molécula de adesão celular neuronal
NeuN: Antígeno neuronal nuclear
NGF: Fator de crescimento neurotrófico
SN: Sistema nervoso
SNC: Sistema nervoso central
SNP: Sistema nervoso periférico
SOD: Superóxido dismutase
STZ: Estreptozotocina
TauT: Transportador de taurina
VIM: Vimentina

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SUMÁRIO

1. REFERENCIAL TEÓRICO

O diabetes *mellitus* (DM) é conhecido globalmente por ser uma doença metabólica crônica (Inam-u-llah e cols., 2018), sendo que os distúrbios metabólicos resultantes desta doença levam a complicações em diversos orgãos e sistemas (El-Akabawy e El-Kholy, 2014). O DM se caracteriza por um quadro de hiperglicemia, alterando o metabolismo de lipídios, carboidratos e proteínas, e esta condição leva ao estado de estresse oxidativo (Eltony, 2016; Sarkar e cols., 2017; Sherif, 2017).

Como resultado dos danos causados pelo aumento crônico da concentração de glicose intracelular temos diversas alterações estruturais, neuroquímicas e neurodegenerativas em diversas regiões do cérebro, incluindo o cerebelo (El-Akabawy e El-Kholy, 2014; Eltony, 2016; Calletti e cols., 2017; Guàrdia-Olmos e cols., 2017; Inam-u-llah e cols., 2018). Esses déficits são descritos nas áreas de velocidade psicomotora e de processamento, memória, habilidade verbal e funções relativas à atenção (Guàrdia-Olmos e cols., 2017).

O cerebelo é conhecido como principal centro motor do sistema nervoso central (SNC) (Eltony, 2016), sendo considerado uma estrutura cerebral crítica para que os movimentos voluntários sejam coordenados e controlados (Hami e cols., 2017). Os principais danos ao cerebelo se apresentam através de distúrbios motores na coordenação muscular, força e equilíbrio (Gelfo e cols., 2010), sendo ainda indicado, recentemente, por desempenhar um papel nas funções cognitivas, comportamentais e emocionais (Hami e cols., 2017).

A taurina é um aminoácido livre, ela é encontrada em grande abundância em mamíferos (Barakat e cols., 2002; Zhao e cols., 2018) e sua concentração provém de duas fontes, biossíntese e dieta (Ito e cols., 2015;

Suárez e cols., 2016). Estudos demonstram que ela pode apresentar algumas propriedades citoprotetoras através de ações como osmoregulador, neuromodulador (Barakat e cols., 2002; Liu e cols., 2013; Suárez e col., 2016; Calletti e cols., 2017; Zhao e cols., 2018), como reguladora de cálcio (Liu e cols., 2013; Adedara e cols., 2016; Zhao e cols., 2018) e também por seu efeito antioxidante (Patel e cols., 2016; Adedara e cols., 2016; Calletti e cols., 2017; Zhao e cols., 2018), termoregulador (Liu e cols., 2013), estabilizador de membranas (Liu e cols., 2013; Adedara e cols., 2016) e, ainda, como fator anti-inflamatório (Patel e cols., 2016; Adedara e cols., 2016; Chupel e cols., 2018; Zhao e cols., 2018).

O ambiente enriquecido (AE) é considerado como uma condição estratégica de forma natural que proporciona muitos estímulos sensoriais, motores, cognitivos e sociais quando comparado a um ambiente padrão (Vazquez-Sanroman e cols., 2013; Caporalli e cols., 2014; Pascual e Bustamante, 2013; Tomiga e cols., 2016). Tais estímulos dependem de uma interação espontânea entre o indivíduo e um ambiente físico mais complexo (Pascual e Bustamante, 2013).

A exposição ao ambiente enriquecido gera um grande número de mudanças a níveis tanto moleculares quanto celulares, que incluem diversas regiões do cérebro, incluindo o cerebelo (Stamenkovic e cols., 2016). Seus efeitos positivos no cérebro incluem aumentos da neurogênese e proliferação celular, dos níveis de fator neurotrófico derivado do cérebro (BDNF), do número de contatos sinápticos e uma redução nos efeitos ansiolíticos em roedores (Tomiga e cols., 2016).

Deste modo, nosso experimento utilizando ratos se propõe a unir e avaliar dois componentes, de forma isolada e combinada, suplementando os animais com taurina e expondo-os a um ambiente enriquecido, modelos esses que apresentam um quadro de hiperglicemia e se torna muito relevante no sentido de elucidar maiores resultados sobre o tema.

1.1 **Diabetes *mellitus***

A principal característica do DM é o quadro hiperglicêmico, que decorre da glicose em baixos níveis no meio intracelular e altos níveis no meio extracelular, em consequência da destruição ou perda da funcionalidade das células beta-pancreáticas, o que define o DM como uma condição metabólica crônica, caracterizada pela deficiência total ou parcial da secreção de insulina e/ou seu funcionamento (Kumar e cols., 2005).

Podemos classificar o DM de acordo com a ação das células beta pancreáticas produtoras de insulina. No DM1 ocorre uma reação auto-imune nas células beta-pancreáticas e sua consequente degeneração, gerando uma dependência à insulina. Já no DM2, quando o tecido periférico se torna resistente à insulina ou devido à deficiência de insulina, se caracteriza por dislipidemia e hiperglicemia crônica (Yi e cols., 2009; Inam-u-Ilah e cols., 2018).

Esta doença gera diversos distúrbios metabólicos que leva órgãos e sistemas a sofrerem múltiplas complicações (El-Akabawy e El-Kholy, 2014) e representa um fator de risco importante para doença arterial coronariana, acidente vascular cerebral, doença vascular periférica, cardiomiopatia, nefropatia diabética, retinopatia diabética e neuropatia periférica (Calletti e

cols., 2017; Athyros e cols., 2018). Ela também induz a alterações metabólicas e vasculares e alterações no sistema nervoso central (SNC), elevando o risco de declínio cognitivo, demência e transtornos do humor (Calletti e cols., 2017). Os indivíduos adultos diagnosticados com DM possuem um risco de duas a quatro vezes maior de desenvolverem doenças cardiovasculares, doença vascular periférica e acidente vascular cerebral em comparação com pessoas saudáveis (Greenwood e Winocur, 2005; Messier, 2005).

O DM pode ser induzido através de algumas substâncias químicas, como por exemplo a estreptozotocina (STZ). Esta substância é um antibiótico que através da grande formação de radicais livres destrói seletivamente as células beta-pancreáticas e se acumula na porção central das ilhotas suprimindo desta forma a liberação de insulina. Por este motivo é utilizada na indução de modelos animais de DM1 (Lebed e cols., 2008; Lenzen, 2008).

Esta substância reproduz nos modelos animais diversas alterações que são observadas nos seres humanos, como a hiperglicemia, hipoinsulinemia, hiperfagia, polidipsia, a perda de massa corpórea, neuropatia periférica, déficits cognitivos e disfunção do metabolismo energético, representando um modelo animal a ser amplamente utilizado para estudar as alterações periféricas e centrais consequentes da hiperglicemia crônica (Serino e cols., 1998; Beauquis e cols., 2010; Braga e cols., 2016).

Buscando um melhor entendimento dos efeitos do diabetes sobre o organismo, uma diversidade de pesquisas tem sido feita com modelos animais de diabetes induzido (Beauquis e cols., 2006; Lebed e cols., 2008; Revsin e cols., 2009).

1.2 **Sistema nervoso central**

O sistema nervoso (SN) é constituído pelo SNC, composto de encéfalo e medula espinhal, e pelo sistema nervoso periférico (SNP), composto por nervos e gânglios. O SNC é revestido por meninges, compostas por tecido conjuntivo, e protegido externamente por revestimento ósseo, denominado de crânio e vértebras. O encéfalo é dividido em cérebro, cerebelo e tronco encefálico. Ele representa a maior parte do encéfalo e possui dois hemisférios, direito e esquerdo, que são conectados por axônios do corpo caloso, cada um dividido em quatro lobos: frontal, parietal, occipital e temporal (Machado, 2000; Junqueira e Carneiro, 2013).

O córtex, também conhecido como substância cinzenta, fica situado na parte mais externa do cérebro, contém em sua maioria corpos celulares, na parte mais interna, temos a substância branca, formada principalmente por axônios, prolongamentos celulares e células da glia (Machado, 2000; Junqueira e Carneiro, 2013).

O SNC é composto de um tecido formado por duas classes principais de células, os neurônios e as células da glia, eles abrangem os astrócitos, oligodendrócitos, micróglia e células ependimárias (Junqueira e Carneiro, 2013).

Os neurônios se classificam como a unidade funcional do SNC e são constituídos por dendritos, corpo celular e axônio, revestidos ou não por bainha de mielina. Eles possuem a capacidade de responder a diversos estímulos a que são submetidos através de impulsos nervosos, que desencadeiam reações fisiológicas, motoras, emocionais, entre outras. Os astrócitos fazem a

sustentação do SNC, e se ligam aos capilares sanguíneos através dos pés vasculares, auxiliando os neurônios na transmissão sináptica e promovem sua excitabilidade, além de controlarem as substâncias a nível extracelular dos neurônios. Os oligodendrócitos têm a função de isolar eletricamente os axônios dos neurônios dentro do SNC e produzem em torno deste, a bainha de mielina. A micróglia representa as células fagocitárias do SNC, enquanto as células epiteliais ependimárias revestem as paredes dos ventrículos cerebrais (Junqueira e Carneiro, 2013).

1.2.1 Cerebelo

O cerebelo, palavra derivada do latim, que significa pequeno cérebro (Teive e Arruda, 2016), faz parte do sistema nervoso central (Kalanjati e cols., 2017). Fica situado na fossa posterior do crânio, acoplado posteriormente ao tronco encefálico (Teive e Arruda, 2016). Divide-se, filogeneticamente, em três partes denominadas de arquicerebelo, paleocerebelo e neocerebelo. Seu córtex é composto de substância cinzenta e possui três camadas que contornam uma região central composta de substância branca (Guedes, 2012).

As três camadas do seu córtex, da superficial à mais profunda, se denominam: camada molecular, camada de células de Purkinje e camada granular; o restante é a medula cerebelar onde predomina substância branca. As células de Purkinje demonstram desempenhar papéis importantes na conectividade do cerebelo com as outras áreas do cérebro (Kalanjati e cols., 2017).

Através de projeções organizadas topograficamente no seu córtex, o cerebelo conecta-se com uma grande variedade de áreas da medula espinhal, tronco encefálico e córtex cerebral (Stoodley e Schmahmann, 2010).

Existem dois sistemas de fibras aferentes que chegam ao córtex cerebelar: as fibras musgosas e as fibras trepadeiras (Ito, 2006). As musgosas tem origem em diferentes segmentos da medula espinhal e do córtex cerebral (Stoodley e Schmahmann, 2010; Guedes, 2012). Já as trepadeiras originam-se da oliva inferior (Guedes, 2012).

A superfície do cerebelo possui lâminas muito finas e transversais chamadas folhas, e um grande número de fissuras. Entre essas folhas penetra a substância branca (Guedes, 2012), nela estão fibras mielinizadas que provém de outras regiões do SNC e de onde se projeta a saída do córtex cerebelar (Balsters e cols., 2010). No interior da substância branca encontram-se os núcleos cerebelares profundos, aonde chegam as fibras eferentes e de onde saem as projeções cerebelares para outras áreas do SNC (Habas, 2010; Freeman e Steinmetz, 2011).

Sua camada molecular possui dois tipos de interneurônios inibitórios GABAérgicos, que são denominados células em cesto e células estreladas. Na parte superior da camada estão localizadas as células estreladas, e entre a metade e o terço inferior estão as células em cesto (Dizon e Khodakhah, 2011; Guedes, 2012).

Ao finalizarem dentro da camada molecular os axônios das células estreladas irão formar sinapses com os dendritos das células de Purkinje (Kenyon, 1997). Os axônios das células em cesto dão origem aos ramos paralelos à superfície ou descendentes (Weisheit e cols., 2006). Dos ramos

descendentes se formam colaterais que envolvem as células de Purkinje e o início do seu axônio (Guedes, 2012). Estas células, em cesto e estreladas, recebem aferências excitatórias das fibras paralelas e dos axônios das células granulares da camada granular (Weisheit e cols., 2006).

As células de Purkinje possuem um grande corpo celular conhecido pelo formato de pêra, que se enfileiram formando a camada de células de Purkinje, (Guedes, 2012). Do corpo celular destas células, geralmente, se origina apenas um dendrito espesso e de comprimento variável. Desse dendrito partem dendritos secundários, que variam em espessura e dos secundários irão se ramificar os terciários (Tanaka, 2009). O único axônio das células de Purkinje se orienta transversalmente na camada granular se dirigindo para a substância branca. Ramos colaterais são projetados deste axônio antes da sua saída do córtex cerebelar e podem terminar acima ou abaixo da camada de células de Purkinje (Guedes, 2012). A grande maioria dos axônios das células de Purkinje se dirige para os núcleos profundos e uma pequena parte deles se projeta para os núcleos vestibulares (Ito, 2006).

As células de Purkinje liberam o neurotransmissor GABA, inibindo suas células-alvo (Person e Raman, 2012). As fibras trepadeiras e as fibras paralelas emitem projeções excitatórias para as células de Purkinje, as fibras trepadeiras irão formar sinapses excitatórias com os dendritos primários e secundários e as fibras paralelas fazem seu contato sináptico com os espinhos dos dendritos terciários (Guedes, 2012). As células de Purkinje receberão aferências inibitórias dos interneurônios da camada molecular (Barmack e Yakhnitsa. 2008).

As células da glia de Bergmann são uma forma especializada de astrócito que está localizada entre as células de Purkinje e um pouco abaixo destas (Perea e Araque, 2005; Guedes, 2012). Estas células são muito mais numerosas que as de Purkinje e se envolvem em sinapses da camada molecular (Hoogland e Kuhn, 2010). Nesta camada se originam radialmente as fibras da glia de Bergmann que terminam na superfície cerebelar.

Na camada granular encontram-se numerosas células granulares. Estas células são pequenos neurônios com citoplasma escasso, que em preparações histológicas se destacam pelos seus núcleos fortemente corados (Guedes, 2012).

Cada célula granular possui um único axônio ascendente à superfície do cerebelo e de três a cinco dendritos que formam “galhos” na sua porção final. Os axônios das células granulares se bifurcam na camada molecular, assumindo uma projeção paralela à superfície (Yamazaki e Tanaka, 2009; Guedes, 2012). Assim, os axônios das células granulares são denominados de fibras paralelas. Estas fibras são glutamatérgicas apresentando varicosidades por toda sua extensão (Guedes, 2012).

No circuito cerebelar estão envolvidas as células de Purkinje, com capacidade de receber e enviar estímulos inibitórios e excitatórios, isto permite o controle da atividade cerebelar. As células da glia de Bergmann realizam a interação com as células de Punkinje nas sinapses excitatórias via glutamato. Para evitar possíveis danos neuronais, as células gliais por terem afinidade com os receptores de glutamato, são responsáveis por manter os níveis de glutamato extracelular em concentrações não excitotóxicas, realizando sua receptação da fenda sináptica, quando em excesso (Nagayach e cols., 2014).

1.3 **Complicações do diabetes no sistema nervoso central**

Uma das complicações que ocorre em virtude do desenvolvimento gradual de lesão nos órgãos-alvo do SNC é a encefalopatia diabética, este dano pode ser resultado do aumento crônico da concentração de glicose intracelular o que leva a várias alterações estruturais, neuroquímicas e neurodegenerativas em regiões do cérebro (El-Akabawy e El-Kholy, 2014).

A essas regiões incluem-se o córtex frontal, hipocampo e cerebelo (El-Akabawy e El-Kholy, 2014; Eltony, 2016). As alterações patológicas sustentam diferentes aspectos cognitivos, motores e neuroendócrinos (El-Akabawy e El-Kholy, 2014).

É de conhecimento que o estresse oxidativo está no centro do quadro estrutural de alterações associadas aos efeitos, a longo prazo, do diabetes no cérebro. Uma das explicações é que a redução das defesas antioxidantes associada ao aumento na produção de radicais livres causados pela hiperglicemia, tornam o cérebro vulnerável aos efeitos nocivos dos radicais livres, logo, se torna vulnerável também a danos celulares e a um comprometimento funcional (Clark e cols., 2017).

1.3.1 Danos no tecido nervoso

O cérebro em comparação com outros órgãos é mais suscetível aos efeitos danosos do estresse oxidativo como resultado de altas demandas das altas taxas de metabolismo oxidativo, metais redoxiativos como ferro e cobre

em abundância, altos níveis de ácidos graxos poli-insaturados peroxidáveis e uma relativa escassez das suas defesas antioxidantes. O desequilíbrio pode resultar em danos oxidativos, como espécies reativas de oxigênio (EROs) e enzimas, principalmente as de defesa antioxidante, situação que favorece o desenvolvimento do estresse oxidativo (Clark e cols., 2017).

Ocorre uma diminuição na atividade das catalases (CAT), glutationa peroxidase (Gpx), da superóxido dismutase (SOD), no estado antioxidante total. Ocorre também, um aumento na autoxidação da glicose, peroxidação lipídica (LPO) e da formação de óxido nítrico, além dos danos oxidativos de lipídeos cerebrais, proteínas e ácidos nucléicos (Clark e cols., 2017).

A hiperglicemia crônica, decorrente do diabetes, gera uma série de lesões no SNC, entre estas lesões estão: a perda e atrofia de espinhos dendríticos (Malone e cols., 2008), diminuição na neurogênese adulta (Kempermann e cols., 1998; Malberg e cols., 2000), interferência na proliferação celular (Beauquis e cols., 2006; Zhang e cols., 2008; Balu e Lucki, 2009), alterações de metabolismo mitocondrial e respiração celular, aumento da produção de EROs (Choi e cols., 2014) e aumento da apoptose neuronal (Hawkins e Davies, 2001). Todos esses fatores prejudicam o funcionamento normal do SNC.

No cerebelo, os danos podem ocasionar problemas motores, de equilíbrio e postura (Arantes e cols., 2013), aumento na apoptose de neurônios piramidais do córtex e também das células de Purkinje de ratos adultos (Eltony, 2016). As células gliais danificadas possuem sua função comprometida, não conseguindo realizar a recaptação de glutamato na fenda sináptica. Isto gera um efeito excitotóxico, que aumenta o influxo de Ca^{2+} para o interior do

neurônio, inicia-se assim a produção descontroladas de EROs. Esta elevada produção de EROs não consegue ser controlada pela atividade antioxidante normal, que se torna insuficiente, desta forma esse estresse oxidativo causa inúmeros prejuízos ao tecido nervoso (Nardin, 2006; Mello e cols., 2012; Reagan, 2012; Verkhratsky e cols., 2015).

1.4 **Taurina**

A taurina (ácido 2-aminoetanossulfônico) é um aminoácido livre, que contém enxofre e é abundantemente encontrada em mamíferos (Barakat e cols., 2002; Ito e cols., 2015; Adedara e cols., 2016; Suárez e col., 2016; Calletti e cols., 2017; Borck e cols., 2018; Schaffer e Kim, 2018; Zhao e cols., 2018) estando altamente expressa em uma variedade de órgãos como o cérebro (Barakat e cols., 2002; Liu e cols., 2013), coração e rins (Liu e cols., 2013). A concentração de taurina é alterada por dois meios, podendo ser através da síntese *in vivo* pela oxidação e transulfuração do aminoácido cisteína ou obtida a partir da dieta, através de ovos, carnes e frutos do mar (Ito e cols., 2015; Adedara e cols., 2016 ; Suárez e col., 2016; Zhao e cols., 2018).

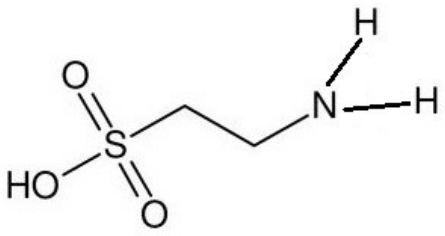

Figura 1: estrutura molecular da taurina (adaptado de Szymanski e Winiarska, 2008).

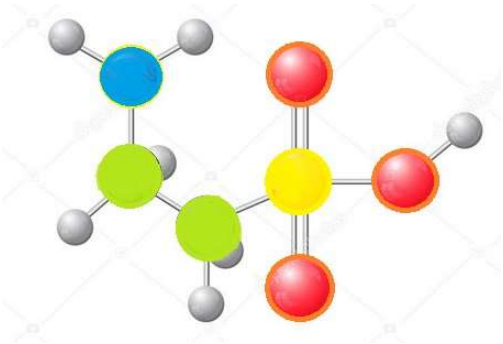

Figura 2: estrutura atômica da taurina (adaptado de Szymanski e Winiarska, 2008).

Sua síntese ocorre no fígado a partir de dois aminoácidos, metionina e cisteína, que sofrem ação de algumas enzimas, sendo estes aminoácidos transformados em hipotaurina e ao final em taurina (Vitvitsky e cols., 2011; Menzie e cols., 2013). A cisteína dioxigenase é a enzima chave para este processo sendo encontrada principalmente nas células da glia (Barakat e cols., 2002).

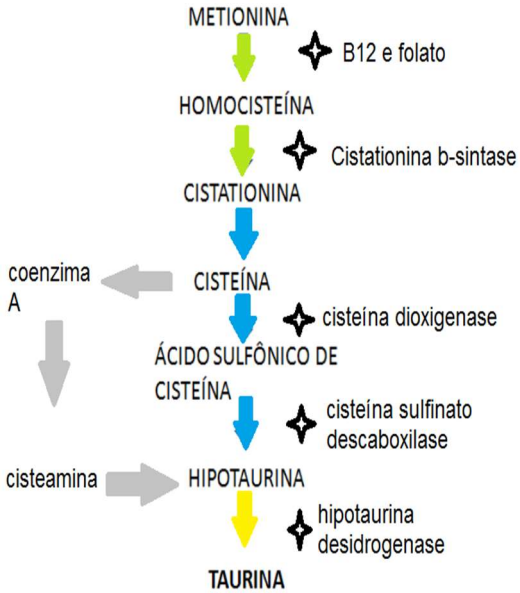

Figura 3: Rota de biossíntese da Taurina (adaptado de De Luca e cols., 2015).

Apesar da falta de um grupo funcional oxidável e demonstrando uma baixa ação de eliminação de radicais livres, a taurina é consistentemente relatada por proteger células e órgãos em espécies de mamíferos contra as consequências do estresse oxidativo promovido por uma diversidade de agentes químicos, bioquímicos, tóxicos e de estados patológicos (Patel e cols., 2016).

Algumas propriedades citoprotetoras vem sendo demonstradas através de suas ações: osmoregulador, neuromodulador (Barakat e cols., 2002; Liu e cols., 2013; Suárez e col., 2016; Calletti e cols., 2017; Zhao e cols., 2018), regulador de cálcio (Liu e cols., 2013; Adedara e cols., 2016; Zhao e cols., 2018) e também por seu efeito antioxidante (Patel e cols., 2016; Adedara e cols., 2016; Calletti e cols., 2017; Zhao e cols., 2018), termoregulador (Liu e cols., 2013), estabilizador de membranas (Liu e cols., 2013; Adedara e cols., 2016) e como fator anti-inflamatório (Patel e cols., 2016; Adedara e cols., 2016; Chupel e cols., 2018; Zhao e cols., 2018).

Existe um campo de dados clínicos relacionados ao efeito anti-inflamatório da taurina sobre as inflamações neural e sistêmica que vem crescendo, nele incluem-se a lesão medular, acidente vascular cerebral isquêmico, lesão cerebral traumática, isquemia, reperfusão hepática e lesão pulmonar (Zhao e cols., 2018).

A administração de taurina também exerce efeitos ansiolíticos explicados pela sua propriedade de melhorar a transmissão GABAérgica. Ela aumenta ainda a subunidade α-2 do receptor GABA e expressão de RNA

mensageiro (RNAm) do Fator neurotrófico derivado do cérebro (BDNF) no hipocampo de ratos diabéticos. Ela é relatada também por melhorar o controle da glicose em todo corpo, através do controle de níveis glicêmicos e de colesterol e no controle da pressão arterial (Calletti e cols., 2017). Além disso, melhora a secreção e/ou sensibilidade a insulina e o metabolismo de glicose e lipídios (Borck e cols., 2018).

Estudos clínicos sugerem que a suplementação de taurina exerce um efeito benéfico, inclusive sobre o diabetes, obesidade e hipercolesterolemia induzida pela obesidade, entre diversas outras doenças. O que indica a importância nutricional da taurina na prevenção de doenças relacionadas ao estilo de vida (Ito e cols., 2015).

Estudos ainda demonstram que a taurina está envolvida nos processos de plasticidade sináptica dos potenciais de longa duração (LTP), como fase tardia (LTP tardia), que é dependente da síntese proteica de LTP. Sendo a taurina participante neste processo de indução do LTP tardio, uma redução na sua concentração poderia contribuir para déficit de saúde durante o envelhecimento (Suárez e cols., 2016).

A taurina pode melhorar déficits relacionados à idade, como contração do músculo estriado, estresse oxidativo causado no músculo cardíaco (Suárez e cols., 2016), aquisição e melhora de aprendizagem e retenção de memórias, e diminuição de comportamentos depressivos, através de sua suplementação (Suárez e cols., 2016; Calletti e cols., 2017), sendo seus níveis influenciados por doenças, dieta e envelhecimento (Ito e cols., 2015; Chupel e cols., 2018).

1.5 **Enriquecimento ambiental**

O ambiente enriquecido (AE) é considerado uma condição estratégica natural que propicia grande estímulo sensorial, motor, cognitivo e social comparado a um ambiente padrão (Pascual e Bustamante, 2013; Vazquez-Sanroman e cols., 2013; Caporalli e cols., 2014; Tomiga e cols., 2016), esses estímulos dependem da interação espontânea entre o indivíduo e um ambiente físico complexo (Pascual e Bustamante, 2013).

Embora o enriquecimento cognitivo não impeça o aparecimento das doenças neurodegenerativas, ele pode fornecer uma forma de proteção contra a expressão dos sintomas clínicos. Assim, essa complexidade ambiental promove um desenvolvimento de propriedades neuroplásticas permitindo que o funcionamento motor e cognitivo seja normal mesmo na presença de patologias cerebrais (Foti e cols., 2011).

O AE consiste em abrigar grupos de animais em áreas mais espaçosas e gaiolas complexas que contém uma variedade de objetos como abrigos, brinquedos, rodas de corrida e túneis (figura 4b) com intenção de facilitar a estimulação cognitiva sensorial, motora e social em relação a outro ambiente com condições de habitação padrão (figura 4a), o que fornece aos animais uma oportunidade de realizar comportamentos específicos da espécie (Sztainberg e Chen, 2010).

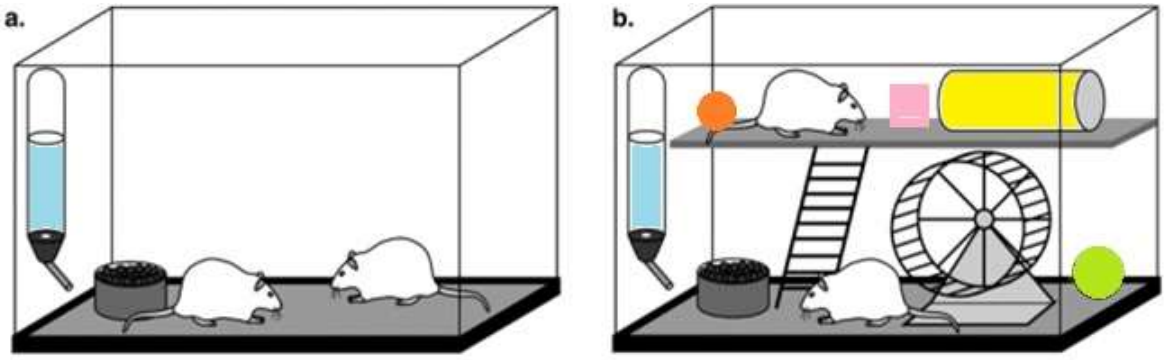

Figura 4: Caixa de moradia padrão (a) e moradia com enriquecimento ambiental (b) (adaptado de Ortuzar e cols., 2011).

Os objetos utilizados com o intuito de gerar estímulos diferentes geralmente variam conforme as características de forma, cor, textura e tamanho. Para cada estudo os itens selecionados para enriquecer o ambiente dependem do tipo de experiência que o pesquisador quer fornecer aos modelos animais, que podem ser de acordo com as questões científicas específicas do estudo, como social, cognitiva, motora ou sensorial. O grau de complexidade também varia, sendo simples, com um ou dois objetos para uma gaiola padrão, ou para o uso de gaiolas complexas contendo uma grande variedade de objetos (Sztainberg e Chen, 2010).

Também representam uma fonte de variação entre estudos, as espécies, sexo, idade e número de animais alojados juntos no ambiente. O protocolo para procedimento de enriquecimento ambiental (EA) inclui duração de exposição ao enriquecimento, frequência com a qual os objetos e suas posições são modificados e se os animais são expostos 24 horas/dia ao AE ou se, por ventura, são restritos a exposição por apenas alguns minutos/horas por dia (Sztainberg e Chen, 2010).

Decorrente da exposição ao AE, apresentam-se numerosas mudanças a níveis moleculares e celulares, relacionadas a várias regiões do cérebro, incluindo o cerebelo (Stamenkovic e cols., 2016). O EA foi demonstrado por modular a plasticidade no hipocampo (Vazquez-Sanroman e cols., 2013). Relacionado às mudanças na plasticidade temos diversos efeitos positivos no cérebro, como aumento da neurogênese (Vazquez-Sanroman e cols., 2013; Tomiga e cols., 2016), proliferação celular (Tomiga e cols., 2016) e sobrevida celular, alterações na morfologia glial (Vazquez-Sanroman e cols., 2013), supra-regulação de fatores de crescimento do fator neurotrófico derivado do cérebro (Vazquez-Sanroman e cols., 2013; Tomiga e cols., 2016).

O AE produz ainda diversas mudanças morfofuncionais no tecido cerebral (Pascual e Bustamante, 2013). Mudanças funcionais como melhorar habilidades motoras e cognitivas, alterar a reatividade ao estresse (Caporalli e cols., 2014) e redução dos efeitos ansiolíticos (Pascual e Bustamante, 2013; Leger e cols., 2015; Tomiga e cols., 2016); alterações morfológicas como aumento do peso do cérebro e da espessura cortical (Caporalli e cols., 2014; Leger e cols., 2015); micro estruturais como aumento da arborização dendrítica (Caporalli e cols., 2014; Pascual e Bustamante, 2013), densidade sináptica (Caporalli e cols., 2014; Pascual e Bustamante, 2013; Tomiga e cols., 2016), neurogênese; e moleculares como alterações na expressão gênica (Caporalli e cols., 2014), aumento da produção de neurotrofinas, como o fator de crescimento neurotrófico (NGF) e do BDNF (Gelfo e cols., 2010; Caporalli e cols., 2014), aumento da proliferação neuronal/glial, (Pascual e Bustamante, 2013) e modulação do sistema neurotransmissor (Caporalli e cols., 2014; Leger e cols., 2015).

Através da neuro-plasticidade induzida pelo AE nota-se a origem de importantes consequências funcionais, apresentando-se como um neuroprotetor para lesões resultantes de convulsões, isquemia e doença de Parkinson (DP). Diminui, também, o nível de estresse, melhorando o desempenho nas tarefas de memória e aprendizagem espacial e diminuindo os déficits emocionais e comportamentais observados, em modelos animais para estudo de autismo, esquizofrenia, lesão cerebral, doença de Alzheimer (DA), síndrome de Rett e doença de Parkinson (Vazquez-Sanroman e cols., 2013).

1.6 **A imuno-histoquímica para observação de danos**

Muitas técnicas são descritas pela sua capacidade de evidenciar alterações nas células do SNC e possíveis danos neurais. Frente as desordens metabólicas e doenças cerebrais, estudos vêm utilizando técnicas variadas para estudar o comportamento dos astrócitos (GFAP), a proliferação celular e neurogênese (5-bromo-2'-deoxyuridina (BrdU); o antígeno neuronal nuclear (NeuN); a molécula de adesão celular neuronal (NCAM) e apoptose neuronal (Caspase-3). Entre as técnicas utilizadas relatamos a imuno-histoquímica (IHQ), imunocitoquímica, imunofluorescência e *western blot*. Especificamente a técnica de IHQ detecta através de marcadores os componentes celulares nos tecidos de interesse, observada através de ligações antígeno-anticorpo que podem ser quantificadas (figura 5) (Kandratavicius e cols., 2007; Revsin e cols., 2009; Beauquis, 2010; Piazza, e cols., 2011; Hsiao e cols., 2014).

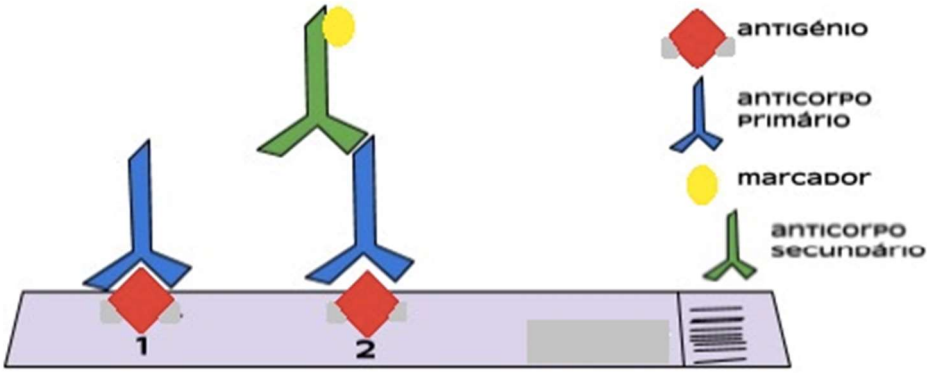

Figura 5: Esquema da técnica de imuno-histoquímica pelo método indireto. (adaptado de Lopes, 2018).

Podemos utilizar a Caspase-3 clivada como um marcador para detectar células em apoptose. Ela é uma protease da família das caspases e está intimamente ligada à cascata de eventos que termina em apoptose (Chen e cols., 2015). Nos quadros crônicos de hiperglicemia, ocorre a ativação de Caspase-3 em decorrência do dano mitocondrial e, assim, essas células em apoptose podem ser observadas pela técnica de IHQ (Hawkins e Davies, 2001; Zeng e cols., 2010; Menzie e cols., 2013; Rahmeier e cols., 2016).

A proteína ácida fibrilar glial (GFAP) é uma proteína de filamentos intermediários, caracterizada como principal componente estrutural dos astrócitos sendo encontrada no citoplasma de indivíduos adultos, já a vimentina (VIM), predomina no período embrionário, sendo perdida em decorrência do amadurecimento celular. É comum observar uma intensa expressão de GFAP e uma reexpressão da VIM, perdida anteriormente, em lesões no SNC (Saravia e cols., 2002; Revsin e cols., 2005; Orsini e cols., 2007; Lebed e cols., 2008).

Nos quadros estressores, como a hiperglicemia crônica, há o aumento da reatividade astrocitária, acarretando também num aumento da quantidade

de filamentos intermediários e de suas proteínas constituintes, como GFAP e VIM, representando a técnica de IHQ uma ferramenta útil para avaliar a dimensão dos danos neurais (Saravia e cols., 2002; Revsin e cols., 2005; Lebed e cols., 2008).

A S100 (solúveis em sulfato de amônia 100% saturado em pH neutro) pertencente a família das proteínas de ligação ao cálcio, como a calmodulina e a troponina C, encontra-se como S100A que é composta por uma cadeia alfa e beta e como S100B composta por duas cadeias beta. Os membros da família S100 demonstram-se úteis como marcadores para alguns tumores, na diferenciação epidérmica, e como marcadores para doenças inflamatórias, podendo mediar inflamação e atuar como agente microbiano (Nonaka e cols., 2008; Wolf e cols., 2010).

A S100B pode ser encontrada em astrócitos, predominando no citoplasma, nas células de Schwann, em algumas populações de neurônios, condrócitos, melanócitos, células de Langerhans, adipócitos, músculo esquelético e cardíaco. Ela pode ser associada à membrana plasmática, outras membranas intracelulares e ao citoesqueleto, quando solúveis no citoplasma. Esta fração beta da S100 reflete a extensão do dano tecidual sendo utilizada como marcador sérico de lesão cerebral, além disso, o aumento da liberação da S100B pelos astrócitos vem sendo atribuído como possível consequência da diminuição do fluxo de oxigênio para o cérebro (Costa e cols., 2013).

Por afetar grande parte da população mundial, o DM traz uma grande preocupação em relação à saúde e a qualidade de vida das pessoas diabéticas. A hiperglicemia constante causa danos celulares, sendo o estresse oxidativo um dos mecanismos principais destes danos, assim, suplementar o

organismo com substâncias antioxidantes pode demonstrar benefícios. O enriquecimento ambiental por proporcionar interação social, estímulos sensoriais, oportunidades de aprendizado e atividade física, pode apresentar melhora a qualidade de vida das pessoas.

Por ser um potente antioxidante a taurina se torna relevante ao funcionamento de alguns tecidos e por haver pouca produção endógena, a sua obtenção através da alimentação ou suplementação se torna importante.

Assim, este trabalho se propõe a unir e avaliar os dois componentes, tanto de forma isolada como de forma combinada, suplementando animais com taurina e expondo-os a um ambiente enriquecido, animais esses que apresentam um quadro de hiperglicemia. Tazendo relevância no sentido de elucidar maiores resultados sobre o tema.

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3. OBJETIVOS

3.1 Objetivo geral

Avaliar o efeito da taurina e do ambiente enriquecido sobre o cerebelo de ratos diabéticos e não diabéticos através dos marcadores caspase-3 clivada, GFAP, S100 e vimentina.

3.2 Objetivos específicos

a) Avaliar o efeito da taurina e do AE sobre a morte celular, através da quantidade de células marcadas com caspase-3 clivada no cerebelo de ratos diabéticos e não diabéticos;
b) Avaliar o efeito da taurina e do AE sobre a lesão celular, através da quantidade de células marcadas com S100 no cerebelo de ratos diabéticos e não diabéticos;
c) Avaliar o efeito da taurina e do AE sobre as células gliais, através da quantidade de células marcadas com GFAP no cerebelo de ratos diabéticos e não diabéticos;
d) Avaliar o efeito da taurina e do AE sobre os filamentos intermediários, através da quantidade de células marcadas com vimentina no cerebelo de ratos diabéticos e não diabéticos;
e) Através dos resultados das análises dos marcadores, verificar se a taurina e o ambiente enriquecido, isolados e/ou combinados, promovem uma neuroproteção sobre as células do cerebelo.

4. ARTIGO CIENTÍFICO REDIGIDO EM INGLÊS

“Taurine and environment enriched: two important neuroprotective tools against cerebellar alterations induced by stz-diabetic rats”

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TAURINE AND ENVIRONMENT ENRICHED: TWO IMPORTANT NEUROPROTECTIVE TOOLS AGAINST CEREBELLAR ALTERATIONS INDUCED BY STZ-DIABETIC RATS

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Abstract

The present study investigated whether treatment with TAU and the EE are able to protect the cerebellum of rats from damage caused by streptozotocin (STZ)-diabetes. Male *Wistar* rats were exposed to an EE for 30 days (for 24 hours). The diabetes induction occurred through an intraperitoneal injection of STZ (50 mg/kg, dissolved in citrate buffer, pH 4,5). After 30 days, the animals were submitted to euthanasia and the cerebellum was removed and processed for the immunohistochemical studies. Markers of apoptosis (cleaved caspase-3), inflammation (S100), astrogliosis (glial acid fibrillary protein, GFAP) and neuritic growth and proliferation (vimentin) were investigated in cerebellar leaflets in the molecular, granular and Purkinje cell layers. TAU and EE reduced the increase of the immunoreactivity to GFAP induced by diabetes. TAU reduced the number of positive cells for cleaved caspase-3. However, the EE increased the immunoreactivity for this protein. No changes were observed for immunoreactivity of S100 positive cells. STZ-diabetes increased immunoreactivity to vimentin and EE was effective in protecting against this effect. In conclusion, TAU and EE have a protective effect against the markers of astrogliosis and neuritic growth cerebellar leaflets of STZ-diabetic rats. This study also seeks to contribute to the investigation of new therapies that may serve as coadjuvants in the prevention or protection against the deleterious effects caused by this disease in situations of neuropathy. In conclusion, beneficial effect of TAU and EE against a hyperglycemia in different cell types, representing a potential neuroprotective effect.

Keywords: Cerebellum, taurine, enriched environment, STZ-diabetes, immunohistochemistry.

1. Introduction

Diabetes *mellitus* (DM) is known globally as a chronic metabolic disease [1] and the metabolic disturbances caused by this disease lead to complications in various organs and systems [2]. DM is characterized by hyperglycemia, altering the metabolism of lipids, carbohydrates and proteins, leading to oxidative stress [3, 4, 5].

As a result of the damage caused by the chronic increase of the intracellular glucose concentration, we have several structural, neurochemical and neurodegenerative alterations in regions of the brain including cerebellum [1, 2, 5, 6,]. These deficits are described in the areas of psychomotor speed and processing, memory, verbal ability, and functions related to attention and execution [6].

The cerebellum is known as the main motor center of the central nervous system (CNS) [5], and is considered a critical brain structure for voluntary movements to be coordinated and controlled [7], where the main damages occur through motor disturbances in coordination [8], and has recently been shown to play a role in cognitive, behavioral and emotional functions [7].

Taurine is a free amino acid found in great abundance in mammals [9, 10] and its concentration comes from two sources, through biosynthesis and diet [11]. It demonstrates a number of cytoprotective properties through actions in osmoregulation, neuromodulation, calcium regulation, antioxidant effect and anti-inflammatory factor [9, 12, 13]. Thus, it demonstrates its neuroprotective effects and its ability to exert beneficial effects on cognitive and behavioral

deficits in the conditions associated with oxidative stress and neuroinflammation [14, 15, 16].

Exposure to the enriched environment generates a large number of changes at both molecular and cellular levels, including various regions of the brain, including the cerebellum [17]. Studies, following these hypotheses, cite positive effects on the brain that include increases in neurogenesis and cell proliferation, brain-derived neurotrophic factor (BDNF) levels, synaptic contact numbers and a reduction in anxiolytic effects in rodents [18].

In this way, we verify if two different strategies using TAU and/or EE treatment are able to protect the cerebellum of animals exposed to STZ induced diabetes in rats.

2. Material and methods

2.1. Animals

Eighty - eight adult male Wistar rats weighing an average of 300g were obtained from the breeding colony of the Federal University of Health Sciences of Porto Alegre, Brazil (UFCSPA).

All procedures were approved by the Ethics Committee of the Federal University of Health Sciences of Porto Alegre - UFCSPA through Protocol 199/16 and all animals were handled in accordance with international and national laws for ethical care and handling of laboratory animals (European Communities Council Directive of 22 September 2010, 2010/63 / EU and Law 11,794 / 08).

2.2. Experimental groups

The animals were divided into two main groups, according to the environment in which they were allocated, one group housed in a standard box and the other group housed in cages with environmental enrichment. These two groups were divided into four subgroups related to diabetic and non-diabetic animals and the therapy used with each subgroup of animals (figure 1).

The enriched environment group had forty four of the animals exposed to a cage enriched environment measuring 56x56x56cm with three floors connected through ramps in order to stimulate the physical exercise. The animals were organized in six cages and were divided into eight animals per cage, thus promoting social interaction, and several objects such as balls, ladders and cubes were available to the animals, five of which were different at a time. Twice a week the shaving was done and in one of these exchanges a new object was introduced into the environment with the intention of stimulating sensory, motor and cognitive functions. The toys, too, were reorganized every day inside the cages, lasting this experiment thirty days.

The standard box group has the other forty-four animals housed in standard polypropylene housing boxes, measuring 41x34x16cm and organized into two animals per box, minimizing social interaction and without exposure to any type of stimulus through objects. The boxes were also sanitized twice a week. The experiment with this group lasted two months.

2.3. Environmental conditions

All animals in both groups were maintained under ideal conditions of temperature (22 ± 2 ° C) and humidity (55%), with light-dark cycles of 12h (7:00 - 19:00), with free access to water and feed.

2.4. Induction and confirmation of diabetes

Twenty-four animals from the standard box and twenty-four animals from the enriched environment were fasted for 16 hours and induced to diabetes by intraperitoneal administration of 50 mg/kg streptozotocin (Sigma®) dissolved in citrate buffer (pH = 4.5). Non-diabetic animals received only the buffer solution in the volume of 1 ml / kg. After 48 hours of induction, the glycoprotein was confirmed with a portable device (Glucotrend®, Boehringer Institute, Mannheim, Germany). Through a small incision in the distal portion of the mouse tail the blood was collected. After 72 hours of administration, animals that did not show glycemia greater than 200 mg / dL were excluded and glucose levels were measured weekly during the 30 days of the experiment until the day preceding euthanasia. On the day of diabetes induction, the animals were fasted for 16 hours.

2.5 Administration of taurine

Taurine (Pharmanostra®) was administered intraperitoneally at a dose of 100 mg / kg, dissolved in saline, where the first dose was given one day after

confirmation of diabetes, and was applied daily for 30 days in half of diabetic animals and half of non-diabetic animals from each environment group. Animals that did not receive taurine were given saline solution 1 mL / kg.

2.6. Transcardiac perfusion procedure

After the 30th day of the experiment all animals were euthanized by transcardiac perfusion. They were anesthetized with ketamine (80-100 mg / kg ip) and xylazine (5-10 mg / kg ip), 0.1 ml intraventricular heparin was injected and then the animals were perfused with 0.9% saline, followed by 4% paraformaldehyde diluted in 0.1M phosphate buffer (PBS), pH = 7.2-7.4; for thirty minutes. The cerebellum was then removed and maintained for 24 hours in 4% buffered paraformaldehyde. After the fabric was soaked in 70% ethanol until the diaphanization process. At this stage the samples were submitted to baths in absolute ethanol, xylol and liquid paraffin and finally included in paraffin blocks for later analysis through the immunohistochemical technique using the markers of interest evaluation of the tissue by means of stains.

2.7. Histological procedures

Sections were made in the paraffin blocks, where the organ is included, of 4μm in a rotating microtome, obtaining about 6 cuts per animal for each marker to be analyzed, using silanized slides, and after the immunohistochemistry for some markers and stains of choice for the tissue studied.

2.8. Immunohistochemistry

Sections of the cerebellum were assessed by the immunohistochemical technique for the markers: cleaved anti-Caspase-3, anti-GFAP, anti-Vimentin and anti-S100, and antibody analyzes and dilutions performed according to the manufacturer's recommendations.

In the dewaxing process the slides remained for 30 minutes in an oven at 70°C after slices of GFAP and S100 were allowed to stand in PBS (pH 7.4) with 0.5% Triton X-100 (PBS-tx) for fifteen minutes while the slices to be cleaved from caspase-3 and vimentin were allowed to stand in citrate buffer (pH 6.00) at 98°C for twenty minutes for antigen retrieval.

For endogenous peroxidase blockade in all markers, 5% hydrogen peroxide (30V) in methanol was used for 10 minutes for 3 times and for the blockade of non-specific proteins, 1% bovine serum albumin (BSA) (Sigma®) in PBS-tx for one hour at room temperature.

The slides were then incubated with primary monoclonal antibody (GFAP: Dako® 1: 500; caspase-3 cleaved: Cellular Technology® 1: 500 signaling; S100 Dako® 1: 2000 and Dako® vimentin 1: 200) overnight at 4 °C.

In the next step, sections of S100 and cleaved caspase-3 were incubated with the secondary antibody at room temperature, anti-rabbit Abcam®, for 1 hour and anti-rabbit Cell Signaling®, for 40 minutes; respectively, and those of GFAP and vimentin were incubated with the Invitrogen® secondary and tertiary antibody for 40 minutes each and Spring®, 30 minutes each, respectively.

For the development of the immunohistochemical reaction, 0.03% 3,3'-diaminobenzidine (DAB) (Dako®) was used in PBS-tx for 3 minutes and

contrasted with hematoxylin for cleaved caspase-3, in the final step the blades using Entellan® (Merck®).

All samples were photographed using an optical picker, GFAP and vimentin images being analyzed by optical densitometry using Image Pro Plus® 6.3 software (Media Cybernetics®). For cleaved caspase-3 and S100, the samples were analyzed by two investigators under the Olympus BX-40 (Olympus®) microscope.

Cells labeled for cleaved caspase-3 and S100 were quantified in the near, inner and outer line, the Purkinje cell layer; for GFAP in the center and intersection of the leaflets and for vimentin in the molecular layer.

2.9. Statistical analysis

Statistical analysis was performed by observing three-way variance (ANOVA) using SPSS software (IBM®) and followed by Bonferroni's post-hoc test. Statistical differences will be considered significant if $p < 0.05$ and the results were expressed as mean ± standard deviation.

3. Results

3.1. Environmental enrichment and taurine protect against increased immunoreactivity to GFAP protein in diabetic rats

Figure 2 shows the immunoreactivity for GFAP protein in the cerebellar leaflets of diabetic rats treated with TAU and exposed to EE (see scheme A and

image B). The results show that exposure to EE reduced the immunoreactivity for GFAP protein in the center of white matter of the cerebellum leaflets in all groups compared to the control group exposed to the standard box (VE) (environment variable, $F_{(7,64)}$=49,51, P<0,001, graph 1C). In parallel, both TAU treatment and EE protected against increased immunoreactivity for GFAP protein in diabetic rats (STZ) (environment variable vs diabetes and treatment vs diabetes, F(7,63)=8.14, P<0.01, graph 1C).

3.2. Taurine reduces the number of cleaved caspase-3 cells while exposure to environmental enrichment increases this immunoreactivity

Figure 3 shows the immunoreactivity for cleaved caspase-3 in the Purkinje cell layer of the cerebellar leaflets of diabetic rats treated with TAU and EE (see scheme A and image B). The results show that exposure to EE increased the number of caspase-3 cells cleaved in the cerebellar Purkinje cell layer in all groups compared to the control group exposed to standard box (ambient variable, $F_{(7,64)}$=21,82, P<0,001, graph 2C). In parallel, TAU treatment reduced the number of cleaved caspase-3 positive cells in non-diabetic and diabetic rats exposed to the standard box compared to the non-diabetic control group exposed to the standard box (treatment variable, $F_{(7,64)}$=5,12, P<0.05, graph 2C).

3.3. Environmental enrichment, taurine and diabetes do not alter the number of S100^{+} cells in the cerebellum of rats

Figure 4 shows the immunoreactivity for S100^{+} in the Purkinje cell layer of the cerebellar leaflets of diabetic rats treated with TAU and exposed to EE (see scheme A and image B). Graph 3C shows that both treatments did not modify the number of S100^{+} cells in the cerebellar rats ($F(7,64)=1,125$; $P>0,05$).

3.4. Environmental enrichment protects against increased immunoreactivity to vimentin induced by diabetes

Figure 5 shows the immunoreactivity for vimentin protein in the in the molecular layer of the cerebellar leaflets of diabetic rats treated with TAU and exposed to EE (see scheme A and image B). The results show that exposure to EE reduced the immunoreactivity to vimentin in the molecular layer of the cerebellum in all groups compared to the control group exposed to standard box (VE) (ambient variable, $F_{(7,64)}=99,78$, $P<0,001$, graph 4C). In parallel, diabetes increased the immunoreactivity for vimentin protein compared to the VE group. It was also observed that exposure to EE protected against increased immunoreactivity to vimentin induced by diabetes (varying diabetes vs environment, $F(7,64)=7,57$, $P<0.01$, graph 4C).

4. Discussion

The present study investigated the hypothesis that, using the TAU treatment and EE exposure strategies, under immunohistochemical parameters of astrogliosis, cell viability and inflammation in the cerebellar leaflets of STZ-induced diabetic rats, present neuroprotective effects

Here, we found that diabetic rats, during a chronic period of 30 days, had an increase in the immunoreactivity to GFAP and vimentin in the cerebellum. In pathological conditions, it is common to observe characteristics in the volume and shape of the astrocytes and these characteristics are visible after hypoxia, ischemia, trauma or convulsions. Therefore, modification of cytoskeletal proteins such as GFAP and vimentin are present in response to these cellular insults [19].

Nagayach and colleagues in 2014 observed that diabetes causes an increase in GFAP expression in both molecular and granular layers of the cerebellar leaflets. From the second week after induction, an increase in both immunoreactivity (optical density) and number of positive cells in diabetic rats can be seem. Our data are in agreement with Nagayach and colleagues, since we also found an increase in the GFAP immunoreactivity 30 days after induction. Astrocytes play an important role in the transport of glucose and its metabolites to neurons; and changes in glucose levels may be a possible reason for astrogliosis found in the cerebellum [20]. El-Akabady and El-Khaly (2014) also found an increase in GFAP immunoreactivity in all brain structures of STZ-diabetic mice 8 weeks after induction. In parallel, these authors point out that the astrogliosis observed in the cerebellum may be attributed due to

elevated glycemia culminating in an increase in astrocyte metabolism as well as a response to injury caused by the production of reactive oxygen species in the central nervous system [2].

One of the successful strategies recently carried out by our research group was to verify the protective effect of TAU and EE on learning, memory and immunohistochemical markers of apoptosis, astrogliosis and inflammation in different regions of the hippocampus of diabetic rats [16]. TAU is an abundant amino acid in the organism, has antioxidant and anti-inflammatory properties and oral supplementation has been associated in the literature with a successful protective protector for neuropathies and experimental models for neurodegenerative diseases [21, 22, 23].

According to these scientific evidence, for TAU vs GFAP, we found that supplementation with TAU was able to protect against increased immunoreactivity to GFAP caused by diabetes. In parallel, it has also been reported that TAU reduces the expression of inflammatory cytokines, astrocytic reactivity, and improves neurological function in rats submitted to traumatic brain injury [24].

Beyond of TAU treatment, neuroprotective effects were also seen when diabetic rats were exposed to EE. The second strategy to reduce the increase of immunoreactivity of GFAP induced by diabetes was the EE exposure. Besides, these data seem to be in line with Dorfman and collegues (2015) also found that EE protected against the increase of GFAP protein as well as a secretion of proinflammatory mediators in the optic nerve of diabetic rats [25].

Several evidences show an increase in the number of cleaved caspase-3 positive cells in the cerebellum of diabetic rats [2, 20, 26] and also highlights

changes in the morphology of Purkinje cells, including pyknosis, vacuolization, chromatolysis and cell ballooning [20]. In contrast, no alterations in the number of cleaved caspase-3 cells has been observed in diabetic rats in our findings.

Previously, our research group described that exposure to EE increased the immunoreactivity to cleaved caspase-3 in hippocampus of healthy rats [16]. A possible mechanism to explain this effect is due to increase in the cellular metabolism due to elevated consumption of ATP and oxygen by nerve cells. These events causing a physiological increase in the production of reactive oxygen species and pro-apoptotic factors [16, 27, 28].

Furthermore, the antioxidant and anti-apoptotic properties of TAU reduced the immunoreactivity to cleaved caspase-3 in both control and diabetic rats. These findings are in accordance with previous reports where TAU was able to reduce apoptosis in the nervous system of acrylamide-intoxicated mice [29], to protect cerebellar neurons from the external granular layer against ethanol-induced apoptosis in mice [30] and glutamate-induced apoptosis in cultured neurons [31].

The multigenic family of S100 proteins comprises 21 proteins that have Ca^{2+} binding motifs and are related to cell growth, differentiation and survival under various physiological and pathological conditions [32]. No significant differences were observed in S100 immunoreactivity among the groups treated in this study. In fact, the absence of an effect may be related to the antibody used since it recognizes all S100 protein isoforms. It is known that S100B is constitutively secreted by astrocytes under normal physiological conditions; and this protein has DAMP (damage associated molecular pattern) activity [33].

Evidence points to the role of S100B involved in immune responses and

associated inflammatory processes since it alterations in its homeostasis have been described under conditions of cerebral trauma, ischemia, neurodegeneration, autoimmunity and psychiatric disorders [34, 35]. Thus, a possible investigation on S100B would allow changes in its immunoreactivity. Moreover, an increase in immunoreactivity for S100B already reported in the cerebrospinal fluid and central nervous system of diabetic rats [36, 37].

Vimentin is a structural component of the cytoskeleton and compound intermediary filaments. This protein is found in cells of mesenchymal origin [38, 39] and in stages of cell differentiation [40, 41]. Since this protein is expressed by glial cells (specially astrocytes), the immunoreactivity for this protein has been employed in the investigation of the proliferation of these cells in the nervous tissue as well as evaluation of the development of neurites [40, 42].

In our study, it was seen that diabetes increased immunoreactivity to vimentin. In parallel, exposure to EE reduced the immunoreactivity of this protein and protected against changes induced by diabetes. Few studies have investigated the expression of vimentin in the brain of diabetic rats. Levin in 2009 verified the presence of vimentin in the brain of rats submitted to a model for Alzheimer's disease using Aβ-42 peptide. In this study, this protein was used as marker to evaluate the damage-response mechanism; and it was seen that in both mild and severe Alzheimer's disease, an increase in vimentin immunoreactivity occurs gradually [43].

Following this line, Singh and colleagues in 2018 found an increase in vimentin mRNA expression in the kidney of diabetic rats 2 and 4 months after induction. Here, the authors conclude that the increase in the vimentin

expression is associated with increase in the renal injury and inflammatory mediator production [44].

Finally, Ximena and colleagues (2012) found an increase in the expression of vimentin in response perilesional zone after placing a cortical stab wound in rats. In this same study, the role of EE was also investigated. No changes in immunoreactivity were observed for vimentin when the rats were exposed to EE. However, when the injured animals were pre-exposed to EE, a reduction in vimentin immunoreactivity was seen [45]. Different from our findings, the role of immunoreactivity for vimentin in healthy rats exposed to EE remains unknown. On the other hand, when a pathological condition is established, a protective effect is verified.

The present study showed that both TAU and EE have a beneficial effect on the investigated parameters. In contrast, the group associating both treatments (TAU *plus* EE) reproduced effects observed in the EE group in diabetic rats for vimentin and GFAP. Thus, for these markers, the TAU variable does not potentiate the effect induced by EE. No evidence has been found that TAU can act as a synergist along with exposure to EE, and so little that one can cut off the effect of the other. In conclusion, TAU and EE have a protective effect against the markers of astrogliosis and neuritic growth in cerebellar leaflets of STZ-diabetic rats. It is hoped that this evidence may contribute to a better understanding of the role of these agents in the neuroprotection found in diabetic rats. In addition, this study also seeks to contribute to the investigation of new therapies that may serve as coadjuvants in the prevention or protection against the deleterious effects caused by this disease in situations of neuropathy.

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6. List of figures

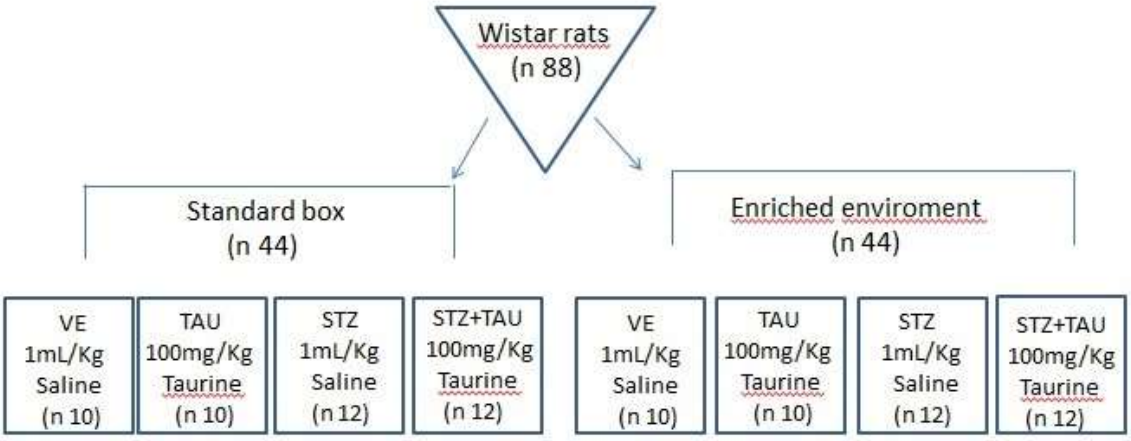

Figure 1: Diagram demonstrating experimental groups

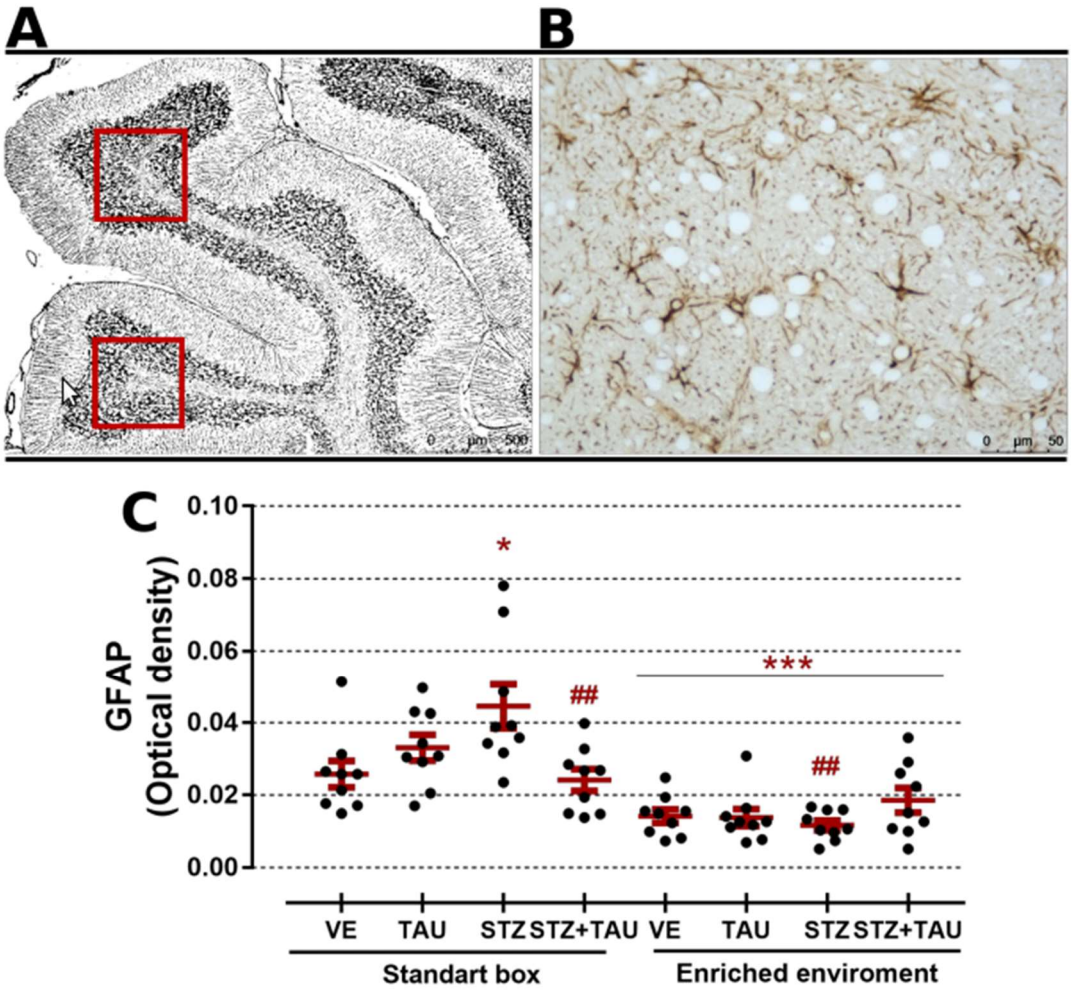

Figure 2: Immunoreactivity for glial acid fibrillary protein (GFAP) in the cerebellum of non-diabetic and diabetic rats (STZ injection, 50 mg / kg intraperitoneally) treated with taurine (TAU, 100 mg / kg intraperitoneally) or saline (VE, 1 ml / kg) and exposed to the enriched environment (for 30 days) or standard box. The red square represents the areas chosen for the analysis of optical density measurement for immunoreactivity (see image A). Representative image of positive immunoreaction for GFAP protein (image B, lens 40X). Grágico C shows the quantitative analysis for the optical density of the different groups. Data expressed as mean ± SEM. Anova three-way followed by Bonferroni's post hoc. * Denotes significant difference between groups vs. VE-standart box; # Denotes significant difference between groups vs STZ-standart box (*** P<0.001; # P<0.05, n of 9 animals per group).

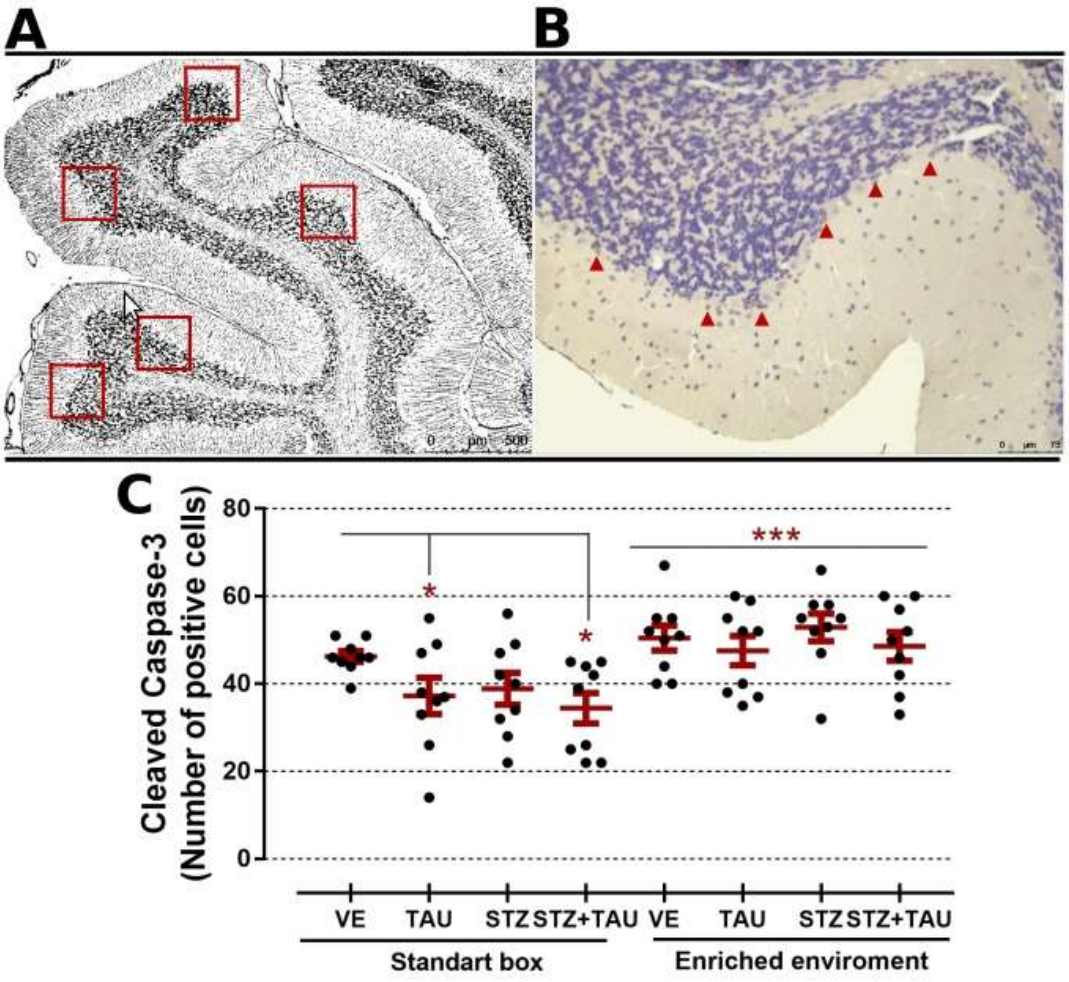

Figure 3: Immunoreactivity to caspase-3 cleaved in the cerebellum of non-diabetic and diabetic (STZ injection, 50 mg / kg intraperitoneally) treated with taurine (TAU, 100 mg / kg intraperitoneally) or saline (VE, 1 ml / kg) and exposed to the enriched environment (for 30 days) or standard box. The red square represents the areas chosen for the analysis of the measurement of the number of immunoreactive cells for cleaved caspase-3 (see image A). Representative image of positive immunoreaction for cleaved caspase-3 (see arrows, image B, lens 20X). Grágico C shows the quantitative analysis for the counting of the immunoreactive cells of the different groups. Data expressed as mean ± SEM. Anova three-way followed by Bonferroni's post hoc. * Denotes significant difference between groups vs. VE-standart box (* $P<0.05$, *** $P<0.001$, n of 9 animals per group).

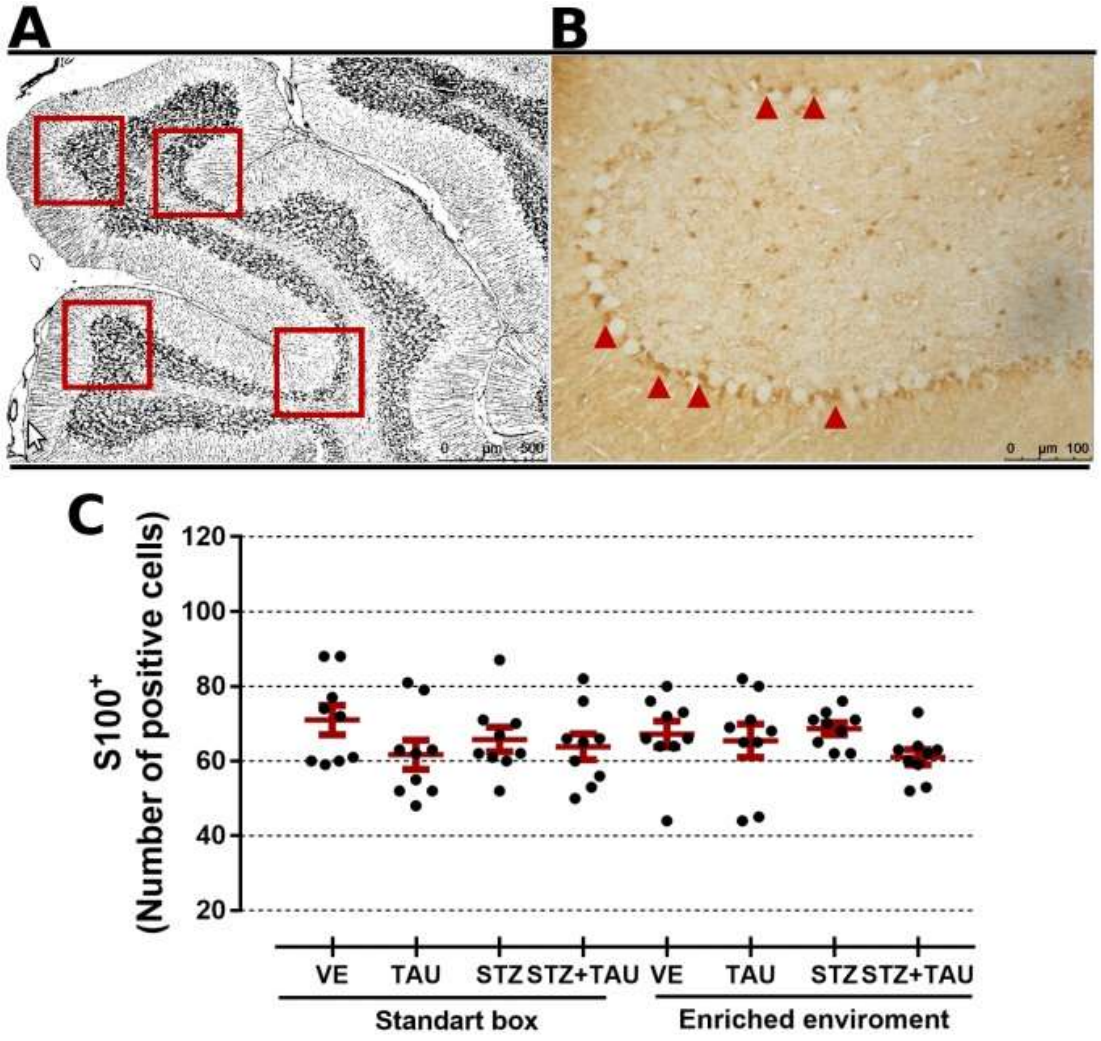

Figure 4: Immunoreactivity to S100 protein in the cerebellum of non-diabetic and diabetic (STZ injection, 50 mg / kg intraperitoneally) treated with taurine (TAU, 100 mg / kg intraperitoneally) or saline (VE, 1 ml / kg) and exposed to the enriched environment (for 30 days) or standard box. The red square represents the areas chosen for counting analysis for the number of immunoreactive cells (see image A). Representative image of positive immunoreaction for S100 protein (see arrows, image B, lens 20X). Grágico C shows the quantitative analysis of the S100 + cell count of the different groups. Data expressed as mean ± SEM. Anova three-way followed by Bonferroni's post hoc. * Denotes significant difference between groups ($P<0.05$, n of 9 animals per group).

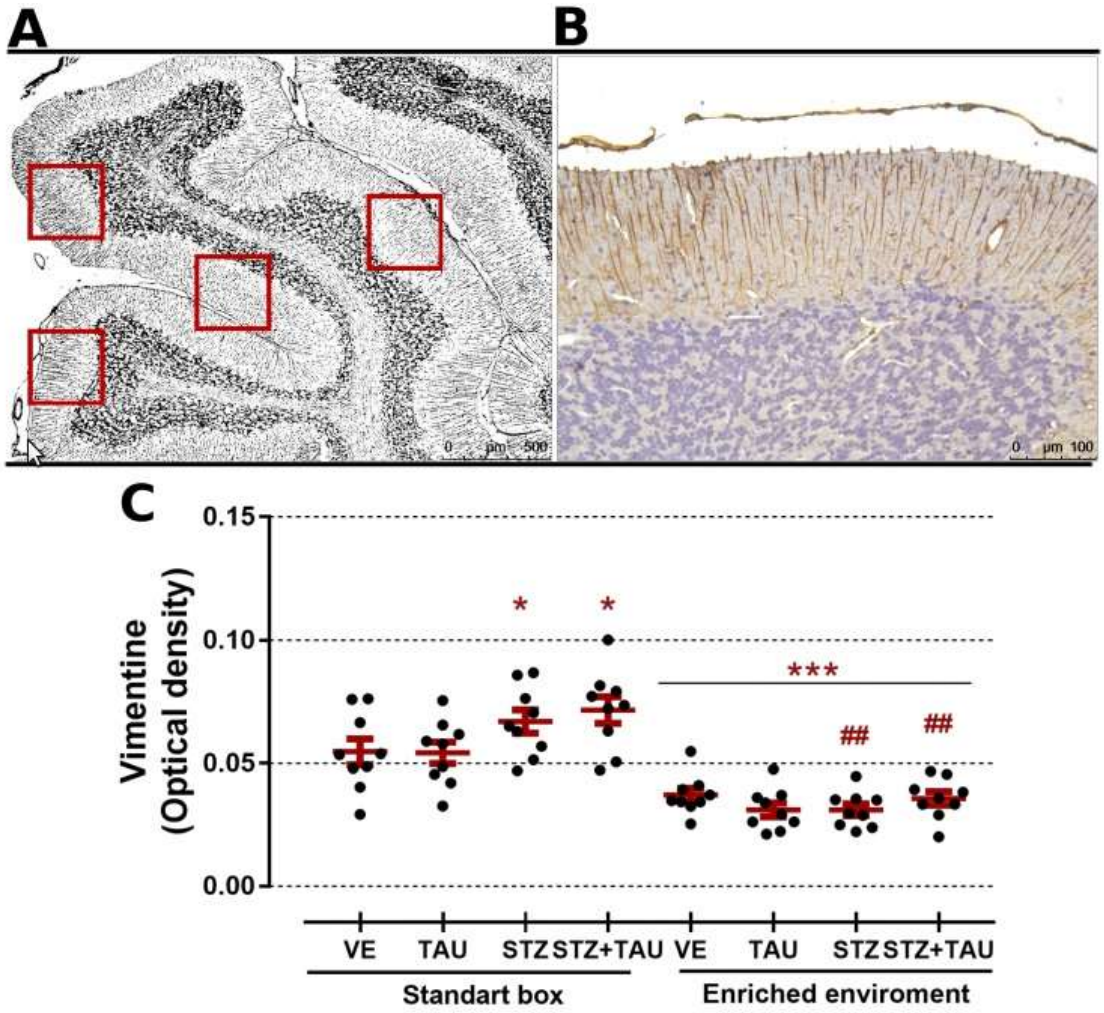

Figure 5: Immunoreactivity to vimentin protein in the cerebellum of non-diabetic and diabetic (STZ injection, 50 mg / kg intraperitoneally) treated with taurine (TAU, 100 mg / kg intraperitoneally) or saline (VE, 1 ml / kg) and exposed to the enriched environment (for 30 days) or standard box. The red square represents the areas chosen for the analysis of optical density measurement for immunoreactivity (see image A). Representative image of positive immunoreaction for GFAP protein (image B, lens 20X). Grágico C shows the quantitative analysis for the optical density of the different groups. Data expressed as mean ± SEM. Anova three-way followed by Bonferroni's post hoc. * Denotes significant difference between groups ($P<0.05$, n of 9 animals per group). # Denotes significant difference between groups vs STZ-standart box (*** $P<0.001$; # $P<0.05$, n of 9 animals per group).

5. CONCLUSÕES

Foi possível demonstrar, através deste estudo, que taurina e ambiente enriquecido apresentam uma proteção contra o aumento da reatividade astrocitária parecendo atuar na diminuição desses processos de degeneração em ratos diabéticos.

A taurina, parece atuar também de maneira protetiva contra o aumento de processos de apoptose neuronal. Sendo que o enriquecimento ambiental demonstrou uma proteção da estrutura citoesquelética do cerebelo.

Nossos resultados demostraram o efeito benéfico dos elementos taurina e enriquecimento ambiental, frente a um quadro hiperglicêmico, em diferentes tipos de células, representando um potencial efeito neuroprotetor, pois o efeito neurotóxico do DM foi significativamente reduzido.

6. ANEXOS

6.1 Normas para publicação da revista Neuroscience Letters

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CEUA –COMISSÃO DE ÉTICA NO USO DE ANIMAIS

PARECER CONSUBSTANCIADO DE PROJETO DE PESQUISA E ENSINO

1) PROTOCOLO Nº: 199/16 **Parecer 484/16**

2) DATA DO PARECER: 14/12/2016

3) TÍTULO DO PROJETO:

Efeito da taurina e ambiente enriquecido sobre os tecidos de ratos diabéticos.

4) PESQUISADOR RESPONSÁVEL:

Marilda da Cruz Fernandes

5) RESUMO DO PROJETO:

O presente projeto avalia o efeito da taurina e do ambiente enriquecido, como fatores isolados ou em conjunto, sobre ratos wistar em modelo de diabetes mellitus induzido por estreptozotocina.

6) OBJETIVOS DO PROJETO:

Avaliar pelo método de imuno-histoquímica, vários marcadores nos seguintes órgãos: cerebelo, fígado, rins, intestino, pâncreas e testículos. Serão avaliados marcadores de morte celular, inflamatórios e de proliferação celular. Além disso, será investigado pelo mesmo método, lesões de epitélio intestinal e danos cerebelares, observando as diferenças entre animais diabéticos e não diabéticos.

7) FINALIDADE DO PROJETO: ☐ Ensino ☒ Pesquisa

8) ITENS METODOLÓGICOS E ÉTICOS DO PROJETO:

Título	☒ Adequado	☐ Comentários
Introdução	☒ Adequada	☐ Comentários
Objetivos	☒ Adequados	☐ Comentários

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Relevância e Justificativa [X] Adequados [ ] Comentários

Materiais e Métodos [X] Adequados [ ] Comentários

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Orçamento e fonte financiadora [X] Adequados [ ] Comentários

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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: **Número Amostral:**

Redução Amostral: [ ] Sim [ ] Não
Justifique:

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Substituição de Metodologia: [ ] Sim [X] Não
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Acomodação e manutenção dos animais: ☐ Adequada ☐ Inadequada
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Não se aplica

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Não se aplica

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Não se aplica

Local de Realização (Biotério/Labotarório): Laboratório de Patologia da UFCSPA

Outra instituição. Qual?

11) CRONOGRAMA DE UTILIZAÇÃO DE ANIMAIS

Data	Espécie	Sexo	Quantidade

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

[X] Aprovado

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☐ Com Pendência

☐ Não aprovado

Data de início____/____/2018 Data de Término____/____/2022

Comentários gerais sobre o projeto:

Obs. Não consta a assinatura do responsável pelo Laboratório onde será realizada a pesquisa.