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AVALIAÇÃO DOS EFEITOS DA METFORMINA SOBRE A NEURODEGENERAÇÃO NO MODELO DE ENCEFALOPATIA DIABÉTICA EM CAMUNDONGOS C57BL/6

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" AVALIAÇÃO DOS EFEITOS DA METFORMINA SOBRE A NEURODEGENERAÇÃO NO MODELO DE ENCEFALOPATIA DIABÉTICA EM CAMUNDONGOS C57BL/6"

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RESUMO

A hiperglicemia está relacionada com o desenvolvimento gradual de injúria e inflamação no encéfalo e é caracterizada por prejuízo das funções cognitivas, mudanças eletrofisiológicas e alterações estruturais principalmente no hipocampo. Essas alterações no SNC causadas pela hiperglicemia são consideradas como um quadro de encefalopatia diabética. A metformina é um fármaco aprovado para consumo humano para desordens metabólicas. Seu mecanismo de ação é através da ativação de AMPK, que regula negativamente os mediadores inflamatórios *in vitro* e *in vivo* em diferentes modelos animais, através da inibição do NF-κB. O objetivo deste estudo foi avaliar a ação da metformina sobre o processo de neurodegeneração induzido pela diabetes em camundongos machos C57BL/6. Foram usados camundongos machos C57BL/6, com 12-14 semanas de idade e peso entre 25-30 g distribuídos em cinco grupos experimentais: controle, estreptozotocina (STZ), STZ tratado com metformina na dose 100 mg/Kg (STZ+M100), STZ tratado com metformina na dose de 200 mg/Kg (STZ+M200) e apenas tratados com metformina na dose de 200 mg/Kg (M200). A diabetes foi induzida com STZ via intraperitoneal por dois dias consecutivos e a metformina foi administrada por gavagem durante 21 dias após a confirmação da diabetes. O peso e glicemia foram acompanhados semanalmente. No 21º dia foi realizado o T-maze e no 22º dia os animais foram eutanasiados, os cérebros dissecados para realização das análises de imunohistoquímica e western blot. Os animais controle e do grupo M200 não tiveram variação dos níveis normais de glicose e do peso corporal. Os animais do grupo STZ, STZ+M100 e STZ+M200 apresentaram perda de peso e elevada glicemia. Na análise da memória espacial o grupo STZ apresentou deficiência de memória e quando tratados com 200 mg/Kg de metformina apresentaram melhora significativa. Na avaliação da perda neuronal, os animais do grupo STZ apresentaram diminuição da marcação do FOX-1, do fator de crescimento neuronal e aumento da expressão da proteína apoptótica Bax, tais alterações foram revertidas pela ação da metformina na dose 200 mg/Kg (STZ+M200). Foi observada ativação dos astrócitos e micróglia no grupo STZ e houve redução de maneira dose-dependente, sendo a dose 200 mg/Kg mais efetiva. Nos animais controle e STZ não houve aumento da expressão de eNOS, enquanto que os grupos tratados com metformina apresentaram aumento da expressão dessa enzima. A expressão de VEGF foi aumentada no grupo STZ e reduzida significativamente no grupo STZ+M200. Os animais diabéticos também apresentam aumento da expressão de IL-1β, Iκβα e NF-κB. O tratamento dos animais diabéticos com 200 mg/Kg de metformina reduziu a inflamação pela redução significativa a expressão de IL-1β e p-Iκβα, entretanto não houve redução significativa de NF-κB. Em conclusão, o tratamento com metformina promoveu neuroproteção, reduziu a neuroinflamação e melhorou a formação da memória espacial em camundongos diabéticos.

Palavra-chave: encefalopatia diabética, dano da memória, metformina, diabetes tipo 1

ABSTRACT

Hyperglicaemia is associated with gradually developing injury and inflammation in brain and characterized by impairment cognitive, structural and electrophysiological changes, mainly in hippocampus. Changes caused by hyperglicaemia are called diabetic encephalopathy. Metformin is a molecule used to treatment metabolic disorders. Its mechanism action is through phosphorilation of AMPK, that downregulates mediators of inflammation *in vitro* and *in vivo* in several animals models, by inhibiting NF-κB. Thus, probably metformin actives AMPK and decreases inflammatory effects induced by NF-κB in glial cells on diabetic encephalopathy, avoiding loss neuronal and limiting neuroinflammation. The aim of this study was to evaluate effects of metformin on neurodegeneration process induced by diabetes in male C57BL / 6 mice. Fifty male C57BL / 6 mice, 12-14 weeks-old and 25-30g weight were distributed into five experimental groups: control, streptozotocin (STZ), STZ treated with metformin at dose 100 mg / kg (STZ + M100), STZ treated with metformin at dose of 200 mg / kg (STZ + M200) and only metformin at dose of 200 mg / kg (M200). Diabetes was induced with streptozotocin given intraperitoneally for two consecutive days and metformin was administered by gavage for 21 days after confirmation of diabetes. Weight and blood glucose levels were monitored weekly. At day 21 the animals were availed by T-maze and at 22 days the animals were euthanized, the brains dissected to perform the analysis of immunohistochemistry and western blot. The control animals and M200 had no change of glucose levels and body weight. The STZ group, STZ + M100 and M200 + STZ showed weight loss and high blood glucose. The STZ group showed impairment of memory and when treated with 200 mg / kg metformin showed significant improvement. The neuronal loss evaluation of the STZ group showed a decrease of FOX-1 labeling, a reduction of NGF, as well as an increased expression of the apoptotic protein Bax, such changes were reversed by the action of metformin in the dose 200 mg / kg (STZ + M200). The activation of astrocytes and microglial cells in STZ group was reduced in a dose-dependent manner and the dose 200 mg / kg more effective. The control and STZ groups showed no alteration in eNOS expression, while groups treated with metformin showed elevated expression of this enzyme. Expression of VEGF was increased in STZ group and significantly reduced in STZ + M200 group. Also diabetic animals showed increased IL-1β, Iκβα and NF-kB expressions. Conversely, diabetic mice treated with 200 mg / kg metformin reduced inflammation availed by IL-1β and p-Iκβα, however there was a significant reduction in NF-κB. In conclusion, treatment with metformin promoted neuroprotection, reduced the neuroinflammation and improved spatial memory in diabetic mice.

Key-word: diabetic encephalopathy, impairment memory, metformin, type 1diabetes

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LISTA DE ABREVIAÇÕES

ROS - espécies reativas de oxigênio

RNS - espécies reativas de nitrogênio

SNC - sistema nervoso central

AMPK - proteína quinase ativada por AMP

AGE - Glicação avançada final

ACC - acetil-CoA carboxilase

AMPKK - quinases de AMPK

Ter-172 - treonina-172

IGF1 - Fator de crescimento semelhante à insulina 1

IR - Receptor de insulina

IRS - Substratos do receptor de insulina

PI3K - Fosfatidilinositol-3 quinase

GSK3 - glicogênio sintase 3-quinase

mRNA – RNA mensageiro

AICAR - 5-amino-1-β-D-ribofuranosil-imidazol-4-carboxamida

eNOS - Óxido nítrico sintase endotelial

iNOS - Óxido nítrico sintase induzível

nNOS - Óxido nítrico sintase neuronal

TNFα – Fator de necrose tumoral

NF-κB- Fator nuclear kappa beta

RAGE - Receptores de produtos de glicação avançada final

VEGF - Fator de crescimento endotelial vascular

PKC - Proteína quinase C

IL-6 – Interleucina 6

MAPK – Proteína quinase ativada por mitógeno

STZ – estreptozotocina

ATP – Adenosina trifosfato

AMP – adenosina monofosfato

AICAR - 5-aminoimidazole-4-carboxamida-1-β-D-ribofuranosida

SUMÁRIO

1. INTRODUÇÃO

Diabetes mellitus é uma desordem metabólica caracterizada por hiperglicemia e insuficiência da secreção da insulina ou insensibilidade do receptor à insulina endógena (ROLO; PALMEIRA, 2006). Dados da organização mundial de saúde (WHO) mostram que em 2000 havia 171 milhões de pessoas diabéticas e a estimativa para 2030 era de 366 milhões (WORLD HEALTH ORGANIZATION, 2006). Entretanto, em 2011 ainda de acordo com a WHO, 347 milhões de pessoas já estavam diabéticas (WORLD HEALTH ORGANIZATION, 2011) e em 2014 a sociedade brasileira de diabetes apontou 387 milhões de diabéticos em todo o mundo. Quase metade do total de pessoas com diabetes ainda não foi diagnosticada e possui maior risco de desenvolver complicações com maiores custos de tratamento (SOCIEDADE BRASILEIRA DE DIABETES, 2014).

A patologia primária da diabetes, a hiperglicemia, causa estresse oxidativo e formação de produtos de glicação avançada final (AGE) levando a complicações secundárias como alterações endoteliais (CHILELLI; BURLINA; LAPOLLA, 2013), aumento do fluxo sanguíneo e distúrbios hemodinâmicos na retina (KOWLURU; KENNEDY, 2001; KUNISAKI et al., 1995), rins (JERUMS et al., 2003), bem como diminuição da condutibilidade dos nervos periféricos (VINCENT et al., 2004).

Acreditava-se que o sistema nervoso central (SNC) fosse relativamente poupado na diabetes (LI; SIMA, 2004), contudo a hiperglicemia está relacionada com o desenvolvimento gradual de danos e inflamação no encéfalo e é caracterizada por prejuízo das funções cognitivas e mudanças eletrofisiológicas. Essas mudanças no SNC causada pela hiperglicemia são chamadas de encefalopatia diabética (HERNÁNDEZ-FONSECA et al., 2009). As ações neurodegenerativas decorrentes de quadros hiperglicêmicos são associadas geralmente com as respostas inflamatórias crônicas, as quais poderão ser geradas a partir de espécies reativas de oxigênio (ROS) e espécies reativas de nitrogênio (RNS) o que pode implicar em morte celular (MELLO, 2012).

Estudos epidemiológicos mostram que pacientes diabéticos possuem uma incidência de duas a quatro vezes maior de sofrer um acidente vascular. Além disso, após a isquemia o prognóstico é pior do que em pacientes não diabéticos, pois a diabetes aumenta o dano neuronal e a área isquêmica (LI et al., 2004; MURANYI et al., 2003). Estudos epidemiológicos também mostram uma maior taxa de convulsão em diabéticos (MCCORRY et al., 2006; VERROTTI et al., 2012). Recentemente, a diabetes tem sido fortemente

correlacionado com a doença de Alzheimer, onde a deficiência de insulina agrava essa patologia (JOLIVALT et al., 2010; TAKEDA et al., 2011).

A encefalopatia diabética, caracterizada por demência e distúrbios cognitivos, tem sido pouco explorada. Portanto, faz-se necessário investigar a neurodegeneração provocada por hiperglicemia.

Metformina é um fármaco aprovado para o consumo humano e é administrado oralmente principalmente para desordens metabólicas (AUGUSTO CÉZAR SANTOMAURO JUNIOR, MICHELLE REMIÃO UGOLINI, ANA TEREZA SANTOMAURO, 2008). Além dessa atividade biológica central, a metformina reduz a produção de espécies reativas de oxigênio em células endoteliais (KUKIDOME et al., 2006), restaura a disfunção endotelial, reduzindo as complicações vasculares (CORREIA et al., 2008; MAJITHIYA; BALARAMAN, 2006), diminui os efeitos deletérios do envelhecimento em camundongos, pois reduz o estresse oxidativo e aumenta as defesas antioxidantes, diminuindo o acúmulo de danos oxidativos, reduz a resposta inflamatória (MARTIN-MONTALVO et al., 2013) independente da ativação do AMPK e aumenta a fagocitose microglial (ŁABUZEK et al., 2010b).

Drogas moduladoras da inflamação são potencialmente úteis e podem ser identificadas como novos medicamentos para adicionar efeitos benéficos aos tratamentos padrões em doenças causadas por inflamação crônica. Devido ao potencial anti-inflamatório e efetivo no controle da hiperglicemia, é possível que a Metformina reduza a neurodegeneração causada pela diabetes.

Portanto, este estudo se propõe a analisar a ação da Metformina sobre os neurônios, na modulação da ativação das células gliais e neuroinflamação em modelo experimental de encefalopatia diabética em camundongos C57BL/6.

2. OBJETIVOS

2.1 OBJETIVO GERAL

Avaliar a ação da Metformina sobre o processo de neurodegeneração induzido pela diabetes em camundongos machos C57BL/6.

2.2 OBJETIVOS ESPECÍFICOS

Caracterizar o efeito do tratamento *in vivo* da Metformina nas concentrações de 100 mg/Kg e 200 mg/Kg

Avaliar o quadro neurodegenerativo induzida pela diabetes no hipocampo.

Avaliar a ação da metformina na manutenção dos neurônios adultos através dos marcadores FOX-1, NeuN, Bax e Bcl-2.

Verificar a ação da metformina sobre a memória através do teste T-maze.

Avaliar os efeitos da Metformina sobre as células gliais através dos marcadores GFAP e Iba-1.

Avaliar os efeitos anti-inflamatórios da Metformina através dos marcadores Iκβα, NF-κB e IL-1β bem como a conservação do endotélio pela enzima sintase de óxido nítrico endotelial (eNOS) e pelo fator de crescimento vascular (VEGF).

Analisar o papel da Metformina sobre o metabolismo energético, através da concentração de glicose e ativação de AMPK.

Caracterizar os efeitos da Metformina na recuperação tecidual avaliando o Fator de Crescimento Neuronal (NGF).

3. JUSTIFICATIVA

Hiperglicemia causa prejuízos ao sistema nervoso central, como danos à formação da memória, perda de neurônios e inflamação. Danos no sistema nervoso central, provocados por diabetes, têm sido pouco explorados. Faz-se necessário, portanto, investigar as alterações no cérebro provocadas por hiperglicemia em modelos experimentais.

Estudos indicam que a metformina possui ação anti-inflamatória e promove a neurogênese. Drogas moduladoras da inflamação são potencialmente úteis e podem ser identificadas como novos medicamentos para adicionar efeitos benéficos aos tratamentos de eleição utilizados em doenças causadas por inflamação crônica.

Portanto, este estudo se propõe a analisar a ação da Metformina na modulação da ativação das células gliais, neuroinflamação e danos de memória espacial em modelo experimental de encefalopatia diabética em camundongos C57BL/6.

4. REVISÃO BIBLIOGRÁFICA

4.1 CAPTAÇÃO DE GLICOSE NO CÉREBRO

O cérebro é o órgão que consome metade do total do suprimento de glicose devido à intensa atividade metabólica dos neurônios e ainda por ser a única fonte energética dessas células. Sugere-se que no cérebro a captação de glicose ocorre pela sinalização da insulina e pelo fator de crescimento semelhante à insulina 1 (IGF1) (VILCHEZ et al., 2007).

A insulina é um hormônio anabólico produzido pelas células β nas ilhotas pancreáticas. Sua principal função é aumentar a taxa de captação de glicose nas células fornecendo uma fonte aumentada de energia (KUMAR et al., 2010) A supressão da sinalização da insulina endógena em humanos resulta na diminuição da captação de glicose no cérebro e em cultura de neurônios do hipocampo, por sua vez, o tratamento com insulina facilita a translocação do transportador de glicose GLUT3 (UEMURA; GREENLEE, 2006).

O IGF1 é um polipeptídeo produzido pelo cérebro e principalmente pelo fígado (DUAN, 2002) e tem facilidade de ultrapassar a barreira hematoencefálica (BONDY, 1991). Em camundongos IGF1-null, foi observada uma redução da captação de glicose no cérebro, mostrando que o IGF1 é importante para a captação de glicose neste tecido (CHENG et al., 1998).

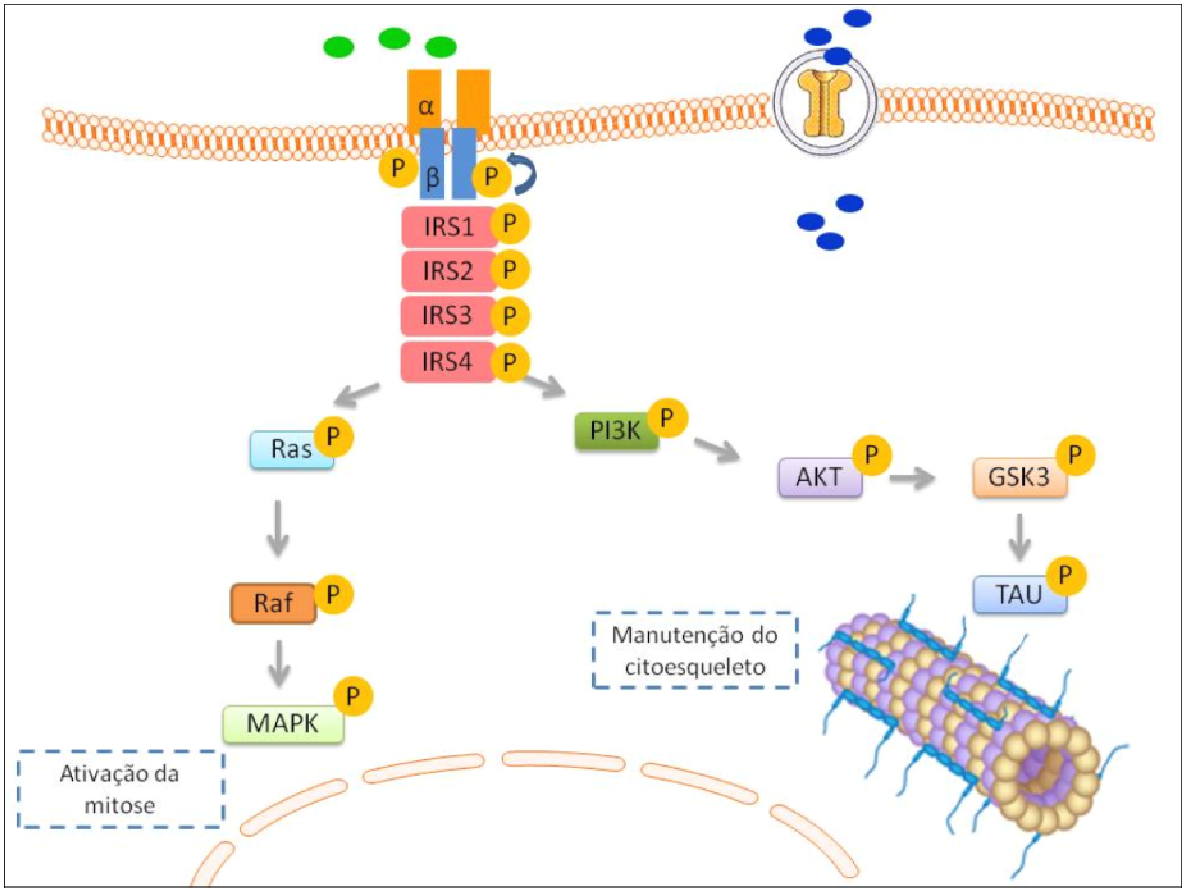
A insulina e IGF1 são polipeptídeos com estruturas homólogas e a mesma sequência de aminoácidos. Seus receptores também são homólogos, expressos na membrana plasmática e tem atividade tirosina quinase (LEROITH; ROBERTS JR., 1993), podendo ocorrer reações cruzadas entre polipeptídeos e seus receptores (BONDY; CHENG, 2004).

A transdução de sinal da insulina envolve a ativação da via do 3-fosfatidil inositol quinase (PI3K) (UNGER; LIVINGSTON; MOSS, 1991). O receptor de insulina (IR) é uma proteína tetramérica com duas subunidades (α e β). As subunidades α estão voltadas para o meio extracelular. A insulina, ao se ligar na subunidade α, promove a ativação da tirosina quinase das subunidades β, localizadas na parte interna da membrana plasmática, resultando na autofosforilação do IR. Após a autofosforilação segue-se então uma cascata de fosforilação dos substratos de receptor de insulina (IRS) de 1 a 4 e o PI3K é recrutado para a membrana para induzir a inserção do transportador de glicose GLUT-4 na membrana plasmática via fosforilação da Akt/PKB (proteína quinase B) (KUMAR et al., 2010). Além disso, a Akt fosforila a proteína glicogênio sintase 3-quinase (GSK3) inativando-a. Esta inativação diminui

a fosforilação da Tau permitindo a estabilização dos microtúbulos para o transporte de vesículas sinápticas e outros componentes celulares dos neurônios (figura 1) (CHO; JOHNSON, 2004).

Por outro lado, quando os IRS são fosforilados ocorre a fosforilação da proteína Ras, que por sua vez fosforila a Raf, recrutanto-a para a membrana (AVRUCH, 1998; SRIVASTAVA; PANDEY, 1998). A Raf é uma proteína quinase serina/treonina do tipo MAP3K que ao ser ativada, fosforila a proteína quinase ativada por mitógeno (MAPK) nos resíduos de serina/treonina, ativando a cascata de crescimento celular (figura 1) (ARTHUR; LEY, 2013; SRIVASTAVA; PANDEY, 1998).

Figura 1. Cascata de sinalização da insulina. Círculos verdes representam insulina e círculos azuis glicose.



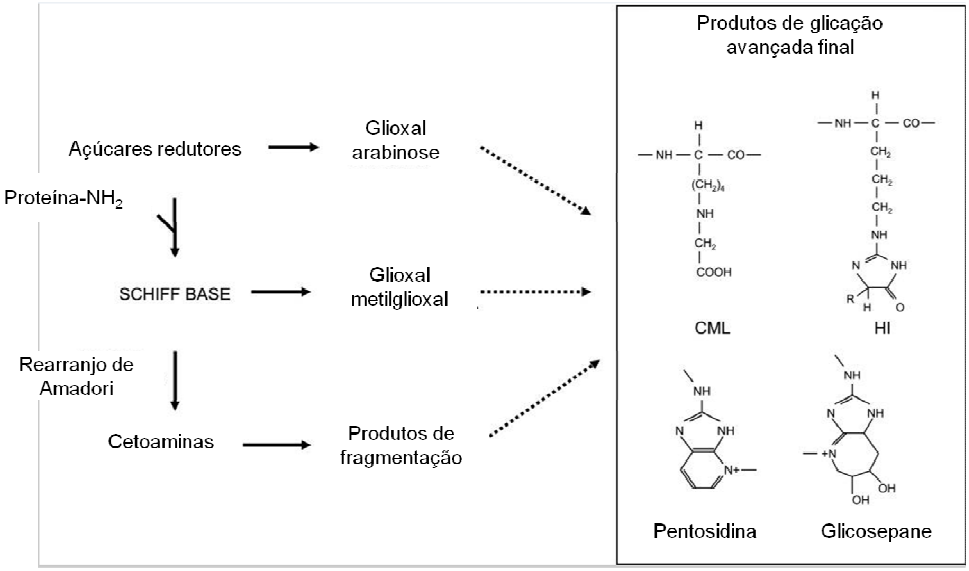
4.2 ALTERAÇÕES PATOLÓGICAS NO SNC CAUSADAS PELA HIPERGLICEMIA

4.2.1 Alterações endoteliais

As alterações iniciais causadas pela hiperglicemia são as complicações vasculares nos vasos periféricos e do SNC, mediadas principalmente pela formação de produtos de glicação avançada final (AGEs) (CHILELLI; BURLINA; LAPOLLA, 2013; NIIYA et al., 2006, 2012). AGEs constituem grande quantidade de substâncias formadas a partir de interações amino carbonilo, de natureza não enzimática, entre açúcares redutores ou lipídeos oxidados e proteínas, aminofosfolipídeos ou ácidos nucleicos (figura 2) (MONNIER, 2003). Essas substâncias são produzidas vagarosamente sob condições fisiológicas de várias maneiras e afetam principalmente moléculas de meia-vida longa, como o colágeno (FORBES; SOLDATOS; THOMAS, 2005). Durante algumas reações que levam a formação de AGEs, são geradas ROS que concorrem paralelamente no estresse oxidativo e com os danos estruturais e funcionais às macromoléculas (JAY; HITOMI; GRIENDLING, 2006). Fisiologicamente, também existem mecanismos de remoção das AGEs e ROS para evitar prejuízos à célula. Entretanto, sob condições hiperglicêmicas a produção e remoção de AGE e ROS é desequilibrada (GOLDIN et al., 2006; JAY; HITOMI; GRIENDLING, 2006).

Figura 2. Formação de produtos de glicação avançada final (AGEs) a partir de interações amino carbonilo (base de Schiff), de natureza não enzimática, entre açúcares redutores e proteínas, os quais sofrem rearranjo de Amadori para formar cetoaminas e os produtos finais de glicação avançada.

Figura 2. Formação de produtos de glicação avançada final (AGEs) a partir de interações amino carbonilo (base de Schiff), de natureza não enzimática, entre açúcares redutores e proteínas, os quais sofrem rearranjo de Amadori para formar cetoaminas e os produtos finais de glicação avançada.



Fonte: adaptação (SEMBA; NICKLETT; FERRUCCI, 2010)

Os AGEs podem danificar as células por três mecanismos. Primeiro, modificam as proteínas intracelulares incluindo as envolvidas na transcrição gênica. Segundo, a interação dos AGEs com os componentes da matriz extracelular modifica a sinalização entre as moléculas da matriz e interação da matriz com a célula, levando à diminuição da adesão celular. Terceiro, as proteínas e lipídeos circulantes modificadas por AGEs podem se ligar a receptores da AGE (RAGE) dos endotélios (e também de micróglia) (BROWNLEE, 2001). O RAGE tem uma expressão fisiológica mínima nos tecidos e vasculatura, contudo sua expressão é aumentada em alguns tipos celulares, como os endotélios e astrócitos, quando há excesso de AGE (GOLDIN et al., 2006; TOTH; MARTINEZ; ZOCHODNE, 2007). O reconhecimento e ligação da AGE com seu receptor RAGE aumenta o estresse oxidativo e o processo inflamatório mediado pelo fator de transcrição nuclear (NF-κB) (HASLBECK et al., 2004). O NF-κB regula a expressão de VEGF e RAGE e esses ativam o NF-κB levando a um ciclo vicioso (EVANS et al., 2003).

Em cultura de células endoteliais, a hiperglicemia induz a produção de espécies reativas de oxigênio (ROS), ativação do NF-κB e aumento dos níveis da proteína quinase C (PKC) e AGE. O bloqueio da geração de ROS suprimiu a ativação do NF-κB, PKC e AGE indicando que a formação de ROS é o evento primário seguido da ativação de outros processos (EVANS et al., 2003). Em paralelo, em camundongos diabéticos RAGE-null, não houve ativação do

NF-κB e as alterações endoteliais foram abolidas (MYINT et al., 2006; SHOJI et al., 2006). Assim, a formação de ROS e a ligação AGE-RAGE são importantes fatores nas complicações endoteliais causadas por hiperglicemia.

4.2.2 Danos neuronais e à glia

Astrócitos e neurônios apresentam uma comunicação recíproca na regulação e liberação de neurotransmissores, excitabilidade neuronal e transmissão sináptica. Os astrócitos estão envolvidos na regulação de água, íons, neurotransmissores e pH no ambiente neural, assim a sobrevivência neuronal depende da interação com os astrócitos (SIMARD; NEDERGAARD, 2004). Insultos físicos e metabólicos provocam alterações rápidas nas células gliais e este fenômeno é denominado gliose reativa, caracterizado, dentre outras alterações, pelo aumento nos níveis da proteína fibrilar ácida glial (GFAP) e S100B. Elevadas concentrações da S100B no soro ou no fluido cérebro espinhal tem consequências deletérias como ativação da resposta imune inata no cérebro levando à inflamação e morte celular (NARDIN et al., 2007).

Estudos *in vivo* e *in vitro* mostram que a hiperglicemia aumenta a atividade dos astrócitos e elevam os níveis de GFAP e S100B. Diabetes induzida por estreptozotocina (STZ) levou a um quadro de astrogliose no hipocampo de ratos diabéticos com aumento da expressão de GFAP e S100B, o qual possivelmente influenciou ou exacerbou o processo de morte celular com ativação da caspase-3, ativação microglial e danos aos astrócitos (NAGAYACH; PATRO; PATRO, 2014; NARDIN et al., 2007).

Astrócitos e micróglia mantêm baixo o nível extracelular do principal neurotransmissor excitatório do SNC, o glutamato. Em condições hiperglicêmicas a captação de glutamato pelos astrócitos é prejudicada (NARDIN et al., 2007) e a exposição dos neurônios a altas concentrações de glutamato e a ação deste sobre seus receptores leva ao aumento do influxo de Ca^{2+} intracelular, o que contribui para a excitotoxicidade neuronal. A ativação excessiva dos receptores de glutamato está envolvida em várias desordens neurodegenerativas e pode levar à injúria e morte celular (MELLO, 2012).

A ativação dos astrócitos e micróglia implica em reação inflamatória durante a encefalopatia diabética (LU et al., 2010). Nos astrócitos do hipocampo, o RAGE também parece ativar NF-κB (TOTH et al., 2006). O aumento da expressão do NF-κB também ocorre como consequência do dano da sinalização de insulina via fosforilação do Iκβ. O NF-κB reside no citosol sob a forma inativa, como um dímero das subunidades RelA e p65. Devido à sua ligação com a proteína inibitória Iκβα (ou Iκβ), o NF-κB é incapaz de se translocar para o

núcleo. Na sua via clássica de ativação, o Iκβα é fosforilado e subsequentemente degradado no proteossomo. Desta forma, o NF-κB é liberado para migração ao núcleo, onde ativará a transcrição de vários genes pró-inflamatórios (HAYDEN; GHOSH, 2008).

A translocação do NF-κB para o núcleo ativa a transcrição e aumento da expressão de RAGE e também aumenta a expressão dos genes do TNF-α, IL-6, IL-1β e IL-2 produzindo a inflamação (HASLBECK et al., 2004), enquanto que a interleucina anti-inflamatória IL-10 é regulada negativamente. A inflamação no SNC, com aumento de RAGE e GFAP, promove estresse apoptótico pelo aumento das proteínas pró-apoptótica Bax e Fas, ativação das caspases-3 e 9 e redução da expressão das proteínas antiapoptóticas Bcl-2 e Bcl-x. A ativação desses marcadores é acompanhada pelo aumento da coloração de TUNEL dos neurônios do hipocampo (SIMA; LI, 2005).

O estresse oxidativo e a apoptose resultam na morte das células neuronais, evento bem demonstrado no hipocampo. Além disso, ocorre perda de oligodendrócitos com consequente dano à substância branca (FRANCIS et al., 2008; TOTH et al., 2006). Inevitavelmente, essas mudanças levam ao distúrbio da comunicação sináptica. De fato, muitas pesquisas apontam a redução das proteínas sinápticas (como sinaptofisina e sinapsina 1), do fator de crescimento neuronal (NGF) e danos de aprendizado e memória como consequência da hiperglicemia crônica (ALVAREZ et al., 2009; JOLIVALT et al., 2008; LI; ZHANG; SIMA, 2005; LI et al., 2002; SIMA et al., 2009a; ZHAO et al., 2012).

4.3 METFORMINA: ATIVADOR DE AMPK

O fármaco metformina (1,1-dimetilbiguanida) tem sido prescrito como hipoglicemiante no tratamento da diabetes mellitus tipo 2 e no tratamento de síndrome metabólica desde a década de 60 (AUGUSTO CÉZAR SANTOMAURO JUNIOR, MICHELLE REMIÃO UGOLINI, ANA TEREZA SANTOMAURO, 2008; CORREIA et al., 2008). Sua ação anti-hiperglicêmica deve-se à inibição da gliconeogênese e glicogenólise no fígado e aumento da captação de glicose no músculo esquelético e adipócitos (KIRPICHNIKOV; MCFARLANE; SOWERS, 2002). Nos últimos anos, essa molécula começou a ser usada no tratamento de ovário policístico (KIRPICHNIKOV; MCFARLANE; SOWERS, 2002) e como coadjuvante da insulina em pacientes com diabetes tipo 1 com dificuldade de controlar a glicemia, promovendo redução das doses de insulina e do peso corporal dos pacientes pela inibição do apetite (LUND et al., 2008; VELLA et al., 2011).

O cloridrato de metformina não interage com as proteínas do plasma e seu tempo de meia vida é seis horas e meia. Após 12 horas, restam 20% do fármaco e após 24 a 48 horas há quantidades mínimas circulantes (LEE et al., 2014).

A terapia com metformina é contraindicada quando há insuficiência hepática, alcoolismo e infecções de moderada à severa. Essas condições predispõem ao desenvolvimento de acidose lática, pelo aumento da produção de ácido lático ou diminuição do seu metabolismo. Outro risco a ser considerado é quando há comprometimento da função renal. A metformina é excretada na urina sem alterações na sua molécula e a falha da eliminação provoca o acúmulo de metformina no organismo podendo causar a acidose lática (KIRPICHNIKOV; MCFARLANE; SOWERS, 2002) ou encefalopatia (JUNG et al., 2009).

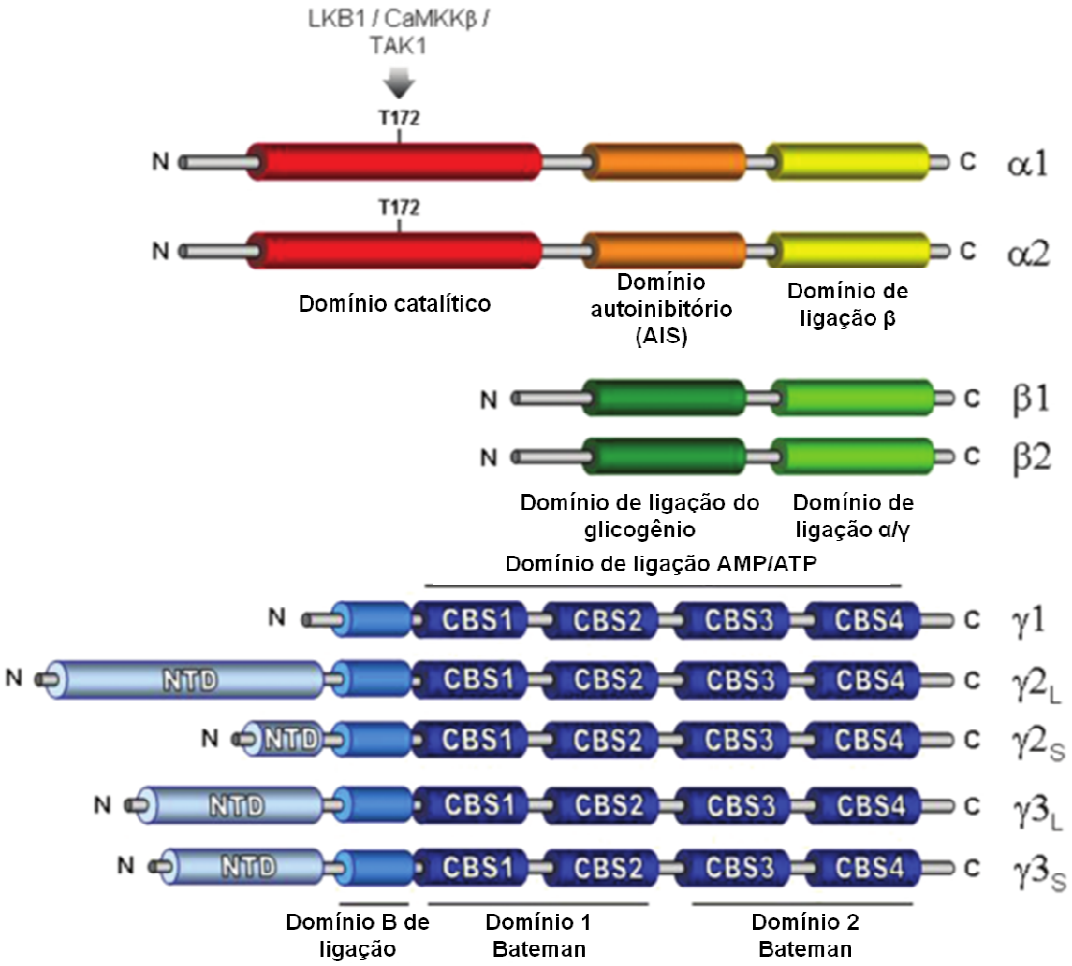
O principal alvo farmacológico da metformina é a ativação da proteína quinase ativada por AMP (AMPK) a qual desempenha o papel de regular o metabolismo energético. A AMPK ativada inibe a acetil-CoA carboxilase (ACC) e 3-hidroxi-3-metil-glutaril-coenzima A (HMG CoA redutase), limitando a síntese de colesterol e ácidos graxos quando há déficit energético (WINDER; HARDIE, 1996). O catabolismo também é estimulado, pois há um aumento da captação de glicose (translocação do receptor de glicose GLUT-4) (FUJII; JESSEN; GOODYEAR, 2006), ativação da glicólise e oxidação de glicose e ácidos graxos (CARLING; ZAMMIT; HARDIE, 1987). Portanto, a AMPK é ativada quando a demanda de energia é maior do que a fornecida e inibida quando o suprimento energético é maior que a demanda. Uma vez ativada, a AMPK utiliza a atividade da quinase serina/treonina para aumentar a taxa do catabolismo celular e diminuir o anabolismo simultaneamente, resultando no aumento da produção de ATP (AMATO; MAN, 2011).

AMPK é uma proteína heterotrimérica a qual possui três subunidades, α, β e γ. A primeira subunidade confere a atividade catalítica quinase e possui duas isoformas a α1 e α2. As subunidades β e γ (duas e três isoformas respectivamente) têm função regulatória. O domínio catalítico α é localizado na região N-terminal, enquanto a porção C-terminal promove a interação com as subunidades regulatórias (figura 3) (HUDSON et al., 2003).

A variação da subunidade α nos mamíferos é limitada a região não catalítica, visto que a porção catalítica N-teminal de α1 e α2 possuem 90% da sequência de aminoácidos iguais, enquanto que o restante da proteína tem 61% de homologia (STAPLETON et al., 1996). Também conservado dentro da subunidade α, há uma sequência autoinibitória (AIS) C-terminal, a qual tem função de inibir a atividade quinase da proteína (PANG et al., 2007). A

conservação dentro da subunidade β é localizada na parte central no domínio de ligação ao glicogênio (GBD) e a região C-terminal que funciona como o domínio de ligação, permitindo a associação com as subunidades α e γ. Nos mamíferos as subunidades γ contêm quatro domínios juntos chamados cristationina β-sintase (CBS) na região N-terminal. Estes domínios funcionam em pares, referentes ao domínio Bateman, que por sua vez se ligam a uma molécula de AMP ou ATP de maneira mutuamente exclusiva (figura 3) (AMATO; MAN, 2011).

Figura 3. Estrutura do AMPK. Duas isoformas α e β. Há três diferentes isoformas para a subunidade γ (γ1, γ2 e γ3). Cada subunidade γ2 e γ3 possui duas variações do processamento transcricional, havendo a forma curta e longa.



Fonte: adaptação (AMATO; MAN, 2011).

Todas as três subunidades de AMPK existem no SNC, no entanto a expressão varia de acordo com o subtipo celular e o estágio de desenvolvimento. A subunidade α2 é mais expressa nos neurônios que a α1. Ambas não são detectadas nas células gliais em condições basais, exceto nos astrócitos ativados. A subunidade γ1 é a isoforma dominante expressa nos

neurônios e ausente nos astrócitos, enquanto os níveis de expressão das subunidades β1 e β2 variam entre os diferentes tipos celulares (TURNLEY et al., 1999).

A AMPK é conhecida como uma proteína sensível às variações energéticas. A capacidade do domínio Bateman de se ligar exclusivamente a ATP ou AMP confere a sensibilidade de AMPK à taxa de AMP:ATP celular. A ligação do AMP ao domínio Bateman da subunidade γ promove a ativação do AMPK de três maneiras distintas (AMATO; MAN, 2011).

Primeiro, o AMP se liga ao primeiro domínio Bateman e diminui a afinidade do AMP de se ligar ao segundo domínio. Segundo, quando o AMP se liga à subunidade γ produz uma mudança conformacional no complexo da AMPK, expondo a treonina-172 (Tre-172) da subunidade α para ser fosforilada pelas quinases de AMPK (AMPKKs), serina treonina quinase 11 (LKB1), proteína quinase dependente de Ca^{2+}/calmodulina (CaMKK) e quinase 1 ativada por fator de transformação de crescimento β (TAK1β) (HAWLEY et al., 1996). Por último, para tornar a AMPK um melhor substrato para as AMPKKs, o AMP ligado também inibe a desfosforilação da Treonina-172 (Ter-172) (DAVIES et al., 1995). A combinação dos três mecanismos de ativação impede que o AMPK responda a níveis baixos de AMP. Uma vez que os níveis energéticos são restaurados, o ATP se liga ao domínio Bateman de maneira cooperativa, embora com menos afinidade que o AMP, para produzir o efeito antagônico sobre a ativação da AMPK (AMATO; MAN, 2011).

4.4 REGULAÇÃO PELO AMPK DOS TRANSPORTADORES DE GLICOSE NA ATIVIDADE NEURONAL

Os receptores de insulina são altamente enriquecidos nos sinaptossomas (WERTHER et al., 1989), tem sido colocalizados com os marcadores de axônios terminais (MIELKE; TAGHIBIGLOU; WANG, 2006) e encontrados na densidade pós-sináptica (ABBOTT; WELLS; FALLON, 1999; BOCKMANN et al., 2002). Os eventos ocorridos na transmissão sináptica, como ativação da bomba de sódio, rearranjo do citoesqueleto, sinalização e processo metabólico requerem um grande gasto de energia, portanto é conveniente a ligação entre a transmissão sináptica e a produção de energia (AMATO; MAN, 2011). Nos neurônios granulares do cerebelo foi observado o aumento dos níveis de AMPK fosforilado em resposta a estimulação de glutamato, acompanhada da diminuição da concentração de ATP celular, o que explica a ativação do AMPK. Além disso, a excitação pelo glutamato aumenta a expressão dos transportadores de glicose GLUT3 (WEISOVÁ et al., 2009). No músculo esquelético e cardíaco a ativação do AMPK também promove a translocação para a

membrana plasmática dos transportadores de glicose, mostrando o importante papel da ativação do AMPK para a captação de glicose (LONG; ZIERATH, 2006; STEINBERG; JØRGENSEN, 2007).

A ativação farmacológica do AMPK pelo 5-amino-1-β-D-ribofuranosil-imidazol-4-carboxamida (AICAR) aumenta a fosforilação da via PI3K-Akt. Enquanto que durante inibição da AMPK, a cascata PI3K-Akt também teve a atividade bloqueada, sugerindo então que o AMPK transloca os transportadores de glicose para facilitar a produção de energia na célula (AMATO; MAN, 2011). O AMPK é capaz de fosforilar o IRS-1 e a maior parte da cascata de sinalização da insulina/IGF1 (JAKOBSEN et al., 2001). Assim, sugere-se que pela ativação da cascata PI3K-Akt, provavelmente via fosforilação do IRS-1, o AMPK facilita a entrada de glicose e a produção ATP dentro da célula (AMATO; MAN, 2011).

4.5 AMPK E O ÓXIDO NÍTRICO

O NO é um gás solúvel produzido por células endoteliais, macrófagos/microglia e alguns neurônios do cérebro. O NO é sintetizado intracelularmente por três isoformas da enzima óxido nítrico sintase (NOS): endotelial (eNOS), neuronal (nNOS) e induzível (iNOS), a partir da L-arginina (KUMAR et al., 2010). O monofosfato cíclico de guanosina (GMPc) age como um mediador das ações do NO e modula vários processos celulares e fisiológicos, como a glicogenólise, relaxamento dos vasos musculares, vasodilatação e fluxo sanguíneo (MONTOLIU et al., 2010).

O tratamento com metformina diminui a mortalidade vascular independente do controle glicêmico (TURNER, 1998). Muitos estudos mostram o efeito benéfico da metformina em proteger a vasculatura periférica pela redução da inflamação induzida pela ativação do NF-κB, melhora da disfunção endotelial e proteção do endotélio via AMPK (CORREIA et al., 2008; DAVIS et al., 2006; MAJITHIYA; BALARAMAN, 2006). Muitos desses efeitos benéficos do AMPK na vasculatura periférica são mediados pela ativação da eNOS, pois o AMPK pode fosforilar a eNOS na serina 633 e 1177 no endotélio (MORROW et al., 2003).

A metformina ativa a eNOS e a bioatividade do NO de modo dependente de AMPK (DAVIS et al., 2006) e o NO também pode atuar como um ativador endógeno da AMPK, sugerindo uma relação de reciprocidade entre AMPK e eNOS. A inibição de NO em células endoteliais bovinas diminui a atividade do AMPK induzida pela metformina mostrando a dependência de NO para a metformina atuar no endotélio (ZOU et al., 2003).

Existem muitas diferenças entre os vasos sanguíneos periféricos e do cérebro quanto à organização e função do endotélio o que dificulta a extrapolação dos achados periféricos para o cérebro (GE; SONG; PACHTER, 2005). Contudo, a ativação da AMPK pode ativar a eNOS nos vasos do cérebro e também contribuir para diminuição dos efeitos da hipóxia, particularmente melhorando a produção do fator de crescimento endotelial vascular (VEGF) (LI; MCCULLOUGH, 2010). Já na diabetes, a privação de glicose no cérebro leva ao aumento da expressão de VEGF com consequente aumento da permeabilidade da barreira hematoencefálica (BHE) e alteração nos vasos sanguíneos. A ativação do AMPK pelo resveratrol restaura função da BHE e estrutura dos vasos (JING et al., 2013).

Existem poucos estudos sobre os efeitos da metformina sobre o hipocampo, sendo seus achados contraditórios. Não há estudos aprofundados sobre os efeitos da metformina no cérebro em condições de altos níveis de glicose e baixos níveis de insulina. A descoberta de que a metformina prolonga a vida e saúde celular, além de ter ação anti-inflamatória aumenta a importância de avaliar a ação dessa molécula em doenças que promovem neurodegeneração e inflamação. Desta forma, este trabalho contribui para a avaliação da metformina no processo neuroinflamatório e memória espacial da encefalopatia diabética em camundongos.

4.6 MODELO EXPERIMENTAL

A Estreptozotocina (STZ) [2-deoxi-(3-(metil-3-nitrosoureido)-D-glicopiranose] é um antibiótico sintetizado pelo *Streptomycetes achromogenes* e tem sido empregado como um agente quimioterápico alquilante. Em ratos e camundongos a STZ é usada para induzir diabetes (LENZEN, 2008).

Em ratos a dose usual é 65 mg/Kg, a qual pode ser administrada pela veia caudal ou via intraperitoneal (i.p). Os camundongos são mais resistentes à ação da STZ, sendo necessária, a administração de altas doses e apenas via i.p. Uma única dose de 200 mg/Kg produz a ação diabetogênica em camundongos (KING, 2012), contudo a padronização no nosso Laboratório de Ultraestrutura (CPqAM/Fiocruz) indicou uma alta taxa de mortalidade. A indução da diabetes em duas doses de 90 mg/Kg em dias consecutivos apresenta uma baixa taxa de mortalidade (10%) em experimentos com duração de oito semanas (JOLIVALT et al., 2010).

Dentre os tipos de indução química da diabetes, a STZ é mais usada principalmente na avaliação de alterações no SNC. Além de causar a hiperglicemia, essa substância também

exerce uma ação tóxica para o cérebro, e quando injetada no ventrículo do cérebro produz um dos modelos da doença de Alzheimer (CORREIA et al., 2012).

A STZ promove a destruição das células β do pâncreas de maneira irreversível e a sua toxicidade pode ocorrer principalmente por formar danos pela adição de radicais metil à estrutura do DNA (BENNET; PEGG, 1981) e secundariamente pela formação de ROS e NO (TURK et al., 1993). A depleção das células β pancreáticas, então eleva a glicose e reduz a insulina circulantes, características da diabetes tipo 1 (KUMAR et al., 2010). A STZ é captada pelas células β pancreáticas pelo receptor de glicose GLUT2, portanto é necessário um prévio jejum overnight para diminuir a competição com a glicose pelo receptor e a indução ser bem sucedida. A baixa expressão do GLUT2 confere resistência à STZ. Do mesmo modo, devido à baixa expressão do receptor GLUT2 em humanos, não é observada ação diabetogênica da STZ durante o tratamento com essa substância (SCHNEDL et al., 1994).

Após a administração da STZ, ocorre variação nos níveis de glicose plasmática até o estabelecimento da hiperglicemia. A primeira fase é o aumento da glicemia após uma hora da injeção e redução da insulina plasmática. Na segunda fase, há hipoglicemia pelo aumento dos níveis de insulina depois de 4-8 horas e esse quadro pode permanecer por várias horas. Por último, é estabelecida a hiperglicemia e hipoinsulinemia permanentemente após 48 horas, e morfologicamente as células secretoras de insulina encontram-se alteradas (LENZEN, 2008).

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EFFECTS OF METFORMIN ON INFLAMMATION AND SHORT-TERM MEMORY IN STREPTOZOTOCIN-INDUCED DIABETIC MICE

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ABSTRACT

The aim of the present study was to analyze the action of metformin on short-term memory, the activation of glial cells and neuroinflammation caused by experimental diabetic encephalopathy in C57BL/6 mice. Diabetes was induced by the intraperitoneal injection administration of a dose of 90 mg/kg of streptozotocin on two successive days. Mice with blood glucose levels ≥ 200 dl/mL were considered diabetic and were given metformin hydrochloride at doses of 100 mg/kg and 200 mg/kg (gavage twice daily) for 21 days. On the last day of treatment the mice were subjected to a T-maze test. On the 22nd day of treatment all animals were anesthetized and euthanized. Diabetic animals treated with metformin had a higher spatial memory score. The hippocampus of diabetic animals presented reactive gliosis, neuronal loss, NF-kB signaling activation, and high levels of IL-1 and VEGF. In addition, the T-maze test scores of these animals were low. Treatment with metformin reduced GFAP and Iba-1 expression (astrocyte and microglial markers), and inflammation markers (p-IKB, IL-1 and VEGF), while enhancing p-AMPK and eNOS levels and neuronal survival (Fox-1 and NeuN). Treatment with metformin also improved the spatial memory score of diabetic animals. In conclusion, the present study showed that metformin is able to significantly reduce neuroinflammation and can decrease the loss of neurons in the hippocampus of diabetic animals, which can subsequently promote improvements in spatial memory.

Key-words: Diabetic brain; Impairment cognitive; Diabetic encephalopathy; Type 1 diabetes

1. INTRODUCTION

Diabetes mellitus is a metabolic disorder characterized by hyperglycemia and the impaired secretion of endogenous insulin or insulin receptor insensitivity (ROLL, PALM, 2006). According to the World Health Organization (WHO), there were 171 million people with diabetes in the year 2000, and the estimate for 2030 was 366 million (WORLD HEALTH ORGANIZATION, 2006). Despite this forecast, in 2011 there were already 347 million people with diabetes (WORLD HEALTH ORGANIZATION, 2011).

This estimate is important because diabetes is related to secondary complications in various organs, related to angiopathy complications (KOWLURU; KENNEDY, 2001; Kunisaki et al., 1995). Additionally, hyperglycemia causes electrophysiological changes (ALLEN; FRIER; STRACHAN, 2004) and learning and memory impairment (JOLIVALT et al., 2008) in the central nervous system (CNS), which is accompanied by neuronal apoptosis (SIMA; LI, 2005). Neurodegeneration resulting from hyperglycemia is usually associated with chronic inflammatory responses, generated from reactive oxygen species (ROS) and reactive nitrogen species (RNS) (MELLO, 2012).

Hyperglycemia in a culture of endothelial cells induces ROS production, NF-κB activation, increased levels of protein kinase C (PKC) and advanced glycation end products (AGEs). The blockade of ROS generation suppresses the activation of nuclear factor κB (NF-κB), after decreased levels of PKC and AGE (NISHIKAWA et al., 2000). Therefore, the formation of ROS precedes the activation of other systems (Evans et al., 2003).

Vascular complications within the CNS are primarily mediated by AGE and its ability to bind to receptor advanced glycation end products (RAGE) (CHILELLI; Burlina; LAPOLLA, 2013; Niiya et al, 2006, 2012, Brownlee, 2001). While the physiological expression of RAGE is minimal in tissues and vasculature, it is greater in certain cell types such as endothelium and astrocytes when there is an excess of AGE (GOLDIN et al., 2006; TOTH; MARTINEZ; ZOCHODNE, 2007). AGE-RAGE binding increases oxidative stress and inflammation mediated by NF-kB (Haslbeck et al., 2004). Activation of NF-κB, which can be activated by both RAGE and VEGF, also increases the expression of VEGF, leading to a vicious cycle (Evans et al., 2003). In diabetic RAGE-null mice, no activation of NF-κβ or endothelial changes have been detected (Myint et al., 2006; Shoji et al., 2006).

In the hippocampus of diabetic mice, an inflammatory response occurs alongside astrocyte and microglial activation and increased expression of GFAP and S100B, which possibly

influences or exaggerates the process of neuronal death (NAGAYACH; Patro; Patro, 2014; NARDIN et al, 2007). Oxidative stress causes hippocampal neuronal death, as well as the loss of oligodendrocytes, with a consequent loss of white matter (Francis et al, 2008; TOTH et al., 2006). Many studies have shown a reduction in synaptic proteins such as synaptophysin and synapsin 1 (ARNOLD et al., 2014; DUARTE et al., 2012), nerve growth factor (NGF) (SIMA; LI, 2005) and learning and memory damage as a result of chronic hyperglycemia (ALVAREZ et al., 2009; JOLIVALT et al., 2008; SIMA et al., 2009b). Inevitably, these changes lead to synaptic communication disorders.

A number of studies have shown the beneficial effects of metformin, which protects the peripheral vasculature by reducing inflammation induced by the activation of NF-κβ, as well as benefiting endothelial dysfunction and protection of the peripheral endothelium via the activation of adenosine monophosphate-activated protein kinase AMPK (Correia et al, 2008;.. Davis et al, 2006; MAJITHIYA; Balaraman, 2006). However, it is not possible to generalize the data obtained from peripheral vessels to the CNS, due to the many differences between such vessels and and brain blood vessels (GE; SONG; Pachter, 2005).

The present study aims to analyze the action of metformin on short-term memory, the activation of glial cells and neuroinflammation in experimental diabetic encephalopathy in C57BL/6 mice.

2. METODOLOGY

2.1 Experimental design

Fifty C57BL/6 male mice aged 12-14 weeks and weighing 25-30 g were used in all experiments. All animals received standard food and water and were kept at a temperature of 22 °C and a light/dark cycle of 12 hours. They were distributed into five experiment groups: I. Control, II. Metformin 200 mg/Kg, III, Streptozotocin, IV. Streptozotocin+metformin 100 mg/Kg and V. Streptozotocin+Metformin 200 mg/Kg. Classical metabolic studies describe the total metabolic rate of a 30g mouse as 961 kJ per kg of body weight, which is approximately seven times the total metabolic rate of a 70kg human (138 kJ per kg) (Terpstra 2001). In the present study, the doses chosen (100 mg/Kg and 200 mg/Kg) were based on equivalent doses (700mg/Kg and 1,400 mg/Kg) given to humans.

Diabetes was induced as per the protocol described previously by Jolivalt et al., 2010, with modifications. Briefly, Streptozotocin (STZ) was administered by intraperitoneal injection at a dose of 90 mg/kg dissolved in citrate buffer pH 4.5 on two successive days, after overnight

fasting. Control and metformin 200 mg/kg groups received only the vehicle (citrate buffer). On the fourth day after induction, a tail puncture was performed to confirm diabetes induction using a One Touch Ultra Lifescan (J & J) glucometer. Mice with blood glucose levels ≥ 200 dl/mL were considered diabetic and were given metformin hydrochloride dissolved in distilled water at doses of 100 mg/kg and 200 mg/kg (gavage twice daily) for 21 days. On the last day of treatment, the mice were subjected to a T-maze test. All animals were anesthetized and euthanized on the 22nd day. Blood glucose and weight were monitored weekly. The experiment was approved by the Research Center Aggeu Magalhães ethics committee/Oswaldo Cruz Foundation (46/2013- CEUA/FIOCRUZ).

2.2 Behavioral testing (T-maze)

Hippocampal damage to short-term memory or working memory was assessed by subjecting mice to a spontaneous T-maze test. Briefly, the mice undergo a maze challenge, with their natural tendency being to enter the arm where they have previously spent most time. Therefore, the animals need to remember which was the last arm they visited (SHARMA; RAKOCZY; BROWN-BORG, 2010). The animals were placed in the T-maze one day before performing the task in order to allow them to become accustomed to the environment. During the test each mouse was placed in the start arm of the T-maze and allowed to select an arm to enter. Once the mouse had chosen an arm, it was confined to this arm for 30 seconds before returning to the start arm. The mouse was then allowed to explore the maze for 2 minutes.

2.3 Immunohistochemistry

Following anesthesia, the animals were transcardially perfused with physiological saline (20 ml), followed by 4% paraformaldehyde (Sigma–Aldrich) (40 ml) in 0.1M phosphate (sodium phosphate monobasic and dibasic heptahydrated – Sigma–Aldrich) buffered saline (PBS), pH 7.2. The cerebrum were immediately removed and post-fixed overnight in the same fixative. The samples were dehydrated in a series of ethanol rinses (Isofar Chemical Co., RJ, Brazil), cleared in xylene and embedded in paraffin (Merck, USA). Sections with a thickness of 5 µm were cut on an RM 2035 microtome (Reichert S, Leica), re-hydrated, and treated with 20mM citrate buffer, pH 6.0, at 100 ºC, for 30 min. Endogenous peroxidase was blocked with 3% hydrogen peroxide H_2O_2 and the sections were blocked with 1% bovine serum albumin (BSA, fraction V) (Miles, Naperville, IL, USA) for 1 h at room temperature. All groups were incubated with the following mouse polyclonal primary antibodies: VEGF

(Abcam, catalog number ab1316, USA), rabbit polyclonal primary antibodies NGF (Abcam, catalog number ab6199, USA), FOX1 (Santa Cruz Biotechnology, catalog number 135476, CA, USA,) and GFAP (DakoCytomation, catalog number: ZO 334, CA, USA). All antibodies were added at 1:100 dilution, overnight at 4 ºC. After washing, the sections were overlaid with a biotin-conjugated secondary antibody for 1 h (DakoCytomation, Biotinylated Link Universal HRP; catalog number: K0690, CA, USA), and visualized with 30-3-diaminobenzidine (DAB) as the chromogen. The slices were counter-stained with Harris' hematoxylin and mounted in entellan (Merck, catalog number: 1079610100, USA). Stained areas (pixels) were measured using the GIMP 2.6.11 software program (GNU Image Manipulation Program software, CA, USA).

2.4 Immunofluorescence

Following anesthesia, the animals were transcardially perfused with physiological saline (20 ml), followed by 4% paraformaldehyde (Sigma–Aldrich) (40 ml) in 0.1M phosphate (sodium phosphate monobasic and dibasic heptahydrated – Sigma–Aldrich) buffered saline (PBS), pH 7.2. The cerebrum were immediately removed and post-fixed in the same fixative overnight. The samples were dehydrated in a series of ethanol rinses (Isofar Chemical Co., RJ, Brazil), cleared in xylene and embedded in paraffin (Merck, USA). Sections with a thickness of 5 µm were cut on an RM 2035 microtome (Reichert S, Leica), re-hydrated, and treated with 20mM citrate buffer, pH 6.0, at 100 ºC, for 30 min. After, they were permeabilized with 0.5% Triton X-100 and incubated for 1 h with blocking solution (3% BSA plus 0.2% Tween 20 in Tris buffered saline). Subsequently, the sections were incubated with anti-NeuN antibodies (Abcam, catalog number: 104225) at a dilution of 1:100. Primary antibody were incubated overnight and then incubated with polyclonal fluor 546-conjugated secondary antibody (Alexa, catalog number A10040) against rabbit immunoglobulin for 1 h. The slices were washed and mounted in fluorescent Prolong Gold Antifade medium (Life Technologies, catalog number: P36930) for observation under an inverted fluorescence microscope (Zeiss MicroImaging GmbH) equipped with a camera (Zeiss AxioCam MRM) and the Re-lease 4.7.2 image analysis software program. Stained areas were measured using the GIMP 2.6.11 software (GNU Image Manipulation Program software, CNET Networks, Inc. Australia).

2.5 Western blot

The hippocampus were rapidly dissected and homogenized in an extraction containing protease inhibitor cocktail (10 mM EDTA, Amresco, Solon, USA; 2 mM phenylmethane sulfonyl-fluoride, 100 mM NaF, 10mM sodium pyrophosphate, 10mM $NaVO_4$, 10 µg of aprotinin/mL and 100 mM Tris, pH 7.4 – Sigma–Aldrich). The samples were mixed and homogenized to form a pool from each group. Homogenates were centrifuged and frozen at -80^{o}C. A quantity of 40 µg of total protein was loaded into each well of an electrophoresis gel and separated, before being electrophoretically transferred onto nitrocellulose membranes (BioRad, catalog number 162-0115). After blocking with 5% skim milk, the membranes were incubated for 2 h with rabbit polyclonal antibody from Abcam (CA, USA) against BAX (catalog number ab7977), BLC-2 (catalog number ab7973), NGF (catalog number ab6199), eNOS (catalog number ab66127) and p-NF-κB (catalog number ab97726), together with rabbit monoclonal antibody against AMPK (ab32047), mouse polyclonal antibody against VEGF (catalog number ab1316), and p-IKb (Cell Signaling, catalog number 9246L, MA, USA), p-AMPK (Cell Signaling, catalog number 2535s, MA, USA), TNF-α (Peprotech, catalog number: 500-P64, NJ, USA), IL-1β (GenWay Biotech, catalog number: 18-732-292194I, CA, USA), and IκB (Santa Cruz Biotechnology, CA, USA, catalog number sc-371). All the primary antibodies were diluted in blocking solution (1% bovine serum albumin, 0.02%phosphate buffered saline and 0.01% Tween) at a 1:1,000 dilution factor. Following washing, the membranes were incubated with horseradish peroxidase-conjugated (HRP) anti-rabbit (Abcam, catalog number: ab6721, UK) and anti-mouse (Sigma-Aldrich, catalog number: A0168, USA) secondary antibodies in a 1:1000 dilution. Chemiluminescence reagent (Super Signal, Pierce, catalog number: 34080, USA) was added for protein band illumination, and blots were developed on X-ray film (Fuji Medical, Kodak, catalog number: Z358487-50EA, Japan). For quantification, densitometry values were obtained by measuring the pixel density of each band using Image J 1.38 software (NIH, MD, USA). For each protein investigated, the results were confirmed in three sets of experiments. Immunoblotting for β-actin was performed as a control gene for the aforementioned blots. After visualization, the membranes were stripped and reprobed with monoclonal anti-β-actin antibody in 1:1000 dilution (Sigma–Aldrich, catalog number A2228, USA) and protein densitometry was performed. The ratio of each protein/β-actin studied was calculated and compared between the groups.

2.6 Statistical analysis

The densitometric values of the immunoreactive bands (immunoblotting), immunohistochemistry and immunofluorescence were analyzed with the GraphPad Prism software package V6.0 (San Diego, CA, USA). One-way analysis of variance (ANOVA), followed by Dunnett's and/or Tukey's post-hoc tests were used to compare groups. The results were expressed as means ± S.D. All analyses was performed using p-value < 0.05 statistical significance.

3. RESULTS

Glycaemia and body weight

Fast glycaemia was measured on day 22 of diabetes induction, and 4 hours after metformin administration. There was no significant difference in glucose levels between the control group (107.4 mg/dL ± 18.10) and the group that received 200mg/kg of metformin (104.2 mg/dL ± 15.4). However, there were significant differences ($p<0.05$) between the STZ (347.75 mg/dL ± 52.39), STZ+M100 (321.5 ± 39.26), STZ+M200 (333.75 mg/dL ± 56.09) groups and the control groups (control and M200) (figure 1). The same results were observed in all animals after 8h and overnight fasting (data not shown). The average body weight of the STZ (28.85 ± 0.93), STZ+M100 (22.40 ± 2.20) and STZ+M200 (22.50 ± 2.32) groups at the end of the experiment decreased significantly ($p<0.05$) when compared to the control group (28.22 ± 0.93) and the M200 group (27.00 ± 3.30) (figure 2).

Metformin improves memory, especially in diabetic animals

The diabetic animals had a significantly lower score when exploring the T-maze than the animals from the control groups ($p<0.05$). The treatment using 100mg/kg of metformin did not improve the performance of the diabetic animals. However, there was a significant increase in the spatial memory score ($p<0.05$) of diabetic animals that received 200mg/kg of Metformin significant. The non-diabetic animals that also received 200 mg/Kg of metformin had similar results to the control group (figure 3).

Metformin ameliorated neuron survival in the dentate gyrus in mice with diabetes induced by STZ.

Immunohistochemistry analysis for FOX-1 revealed a constitutive stain on mature pyramidal neurons located in the molecular area (arrow) and on granulated neurons in the dentate gyrus (asterisk) in the control group (Figure 4-A). The non-diabetic animals that received 200 mg/Kg of metformin exhibited the same staining pattern as the control group, which indicates a preserved neuron morphology (Figure 4-B). The staining intensity of

mature neurons was lower ($p<0.05$) in the diabetic animal group than in the control group (Figure 4-C). In a similar manner, there was no significant difference between the STZ+M100 group (Figure 4-D) and the STZ group. On the other hand, staining intensity increased ($p<0.05$) following 200 mg/Kg metformin treatment in the diabetic animals (Figure 4-E).

The NeuN marker is a protein produced by mature neurons. After 200mg/Kg metformin, the staining intensity of NGF did not differ from the control group (Figure 5-A-B). In the diabetic group, the staining intensity of neurons adults was significantly lower ($p<0.05$) than in the control and M200 groups. In the STZ+M100 group the result was similar to the STZ group (Figure 5-D). When the diabetic animals were treated with 200 mg/Kg of metformin (Figure 5-E), the staining intensity increased significantly ($p<0.05$) in comparison with the STZ and STZ+M100 groups, and was similar to the control and M200 groups.

Neuronal growth factor (NGF) is an important factor in neuron survival, the differentiation of cells in the CNS and neuron precursor differentiation (SOFRONIEW; HOWE; MOBLEY, 2001). In the diabetic animals, the staining intensity of NGF did not differ from the control group (Figure 6–A, 6-B). However, the STZ+M100 group presented a significant increase in immunostaining ($p<0.05$) (Figure 6-C). Interestingly, the STZ+M200 group showed a reduction in NGF staining when compared to the STZ+100 group. This data was confirmed by Western Blotting analysis (Figure 6-F).

Metformin reduces the astrocyte and microglia activation in the dentate gyrus area of hippocampus.

Glial fibrilar acid protein (GFAP) is a protein expressed in astrocytes and its increase is indicative of astrocyte activation, often termed reactive gliosis. Activated astrocytes present some peculiar morphologic characteristics, differing from non-activated astrocytes. Once activated, astrocytes express shorter cytoplasmic extension, as shown in figure 7-A. The diabetic animals presented a significantly higher expression of GFAP, and also presented a phenotype characteristic of cellular activation (figure 7-C). Only the 200 mg/kg of metformin treatment group resulted in a significant reduction of reactive gliosis ($p<0.05$) in the diabetic group (figure 7-D). Animals from the M200 group did not show any significant differences in terms of astrocyte activation when compared to the control group (figure 7-B).

The protein Iba-1 is a distinct marker of microglia, which are immune specific cells of the CNS. Microglia play an important role in protecting the brain tissue from injuries. In cases of hyperglycaemia, the microglia become activated, which releasing a stimuli for their own

proliferation, followed by morphological changes (figure 8-B). The STZ group showed significant microglia activation ($p<0.05$) when compared to the control and M200. Both of the metformin treatment groups resulted in a significant reduction ($p>0.05$) in microglia activation in diabetic animals. The animals from the STZ+ M100 and STZ+M200 group presented the same level of Iba-1 as the control groups and M200 group (figure 8-A and 8-E, respectively).

Metformin decreases the apoptosis in diabetic mice hippocampus

Chronic hyperglycaemia is directly associated with neuronal apoptosis of the hippocampus, which causes cognitive impairment. The expression of the pro-apoptotic protein Bax (figure 9-A) and the anti-apoptotic protein Blc-2 (figure 9-B) were quantified by immunoblotting. In the control group, both proteins were expressed at physiologic levels (Figure 9). The STZ group exhibited a significant increase of Bax and Blc-2 ($p<0.05$). The expression of the Bcl-2 protein was significantly increased only in the STZ+M100, in comparison with the STZ group.

Metformin increases p-AMPK and eNOS expression and reduces vascular permeability

AMPK is a protein that regulates energy metabolism and is activated by phosphorylation when levels of ATP decrease inside a cell or are activated pharmacologically. Metformin is a drug which acts through the activation of AMPK (AUGUSTO CÉZAR SANTOMAURO JUNIOR, MICHELLE REMIÃO UGOLINI, ANA TEREZA SANTOMAURO, 2008). In the control group, p-AMPK was expressed at physiologic levels. In the STZ and STZ+M100 groups, no significant increase in levels of the p-AMPK protein was observed compared with the control group (figure 10). In contrast, the STZ+M200 group exhibited a significant increase of p-AMPK when compared to the other groups, confirming its pharmacological activation (figure 10).

The p-AMPK pathway has been shown to activate the eNOS enzyme, which subsequently activates the phosphorylation of AMPK, causing a positive feedback loop. The control and diabetic groups presented a basal level of eNOS. Both Metformin treatment groups exhibited a significant increase ($p<0.05$) of eNOS expression compared to the diabetic group (figure 11-A). The data also shows that eNOS activation was not related to the pathogenesis of diabetes, even though the increase in p-AMPK expression induced by metformin helped to increase eNOS expression.

VEGF is a pro-angiogenic protein that acts in the endothelial tissue. During acute inflammation, VEGF increases vascular permeability. Diabetes pathology has many secondary complications, which derive from micro and macro vessel modification, due to increased vascular permeability and endothelial dysfunction. The levels of VEGF were significantly higher among diabetic mice than in the control group ($p<0.05$). Similar results were observed when the STZ+M100 group was analyzed, which may be related to endothelial permeability. The 200ml/kg metformin treatment group displayed decreased expression of VEGF compared to the STZ group ($p<0.05$). No significant statistical difference was found between the STZ+M200 and the control groups (figure 11-B). The findings of the present study suggest that metformin can recover reductions in VEGF levels caused by STZ exposure.

Metformin decreases Iκβα phosphorylation in diabetic mice

The expression of the NFκB inhibitor Iκβα was evaluated using immunoblotting. The phosphorylation of Iκβα stimulates the activation of NFκB, initiating the pro-inflammatory cascade. The phosphorylation of Iκβα was higher in the STZ group than the control group ($p<0.05$). Treatment using 100 and 200mg/kg of metformin resulted in a significant reduction in Iκβα activation in comparison with the STZ group ($p<0.05$) (figure 12-A).

The activation of NFκB was significantly greater in the STZ group compared to the control group ($p<0.05$), indicating the translocation of the transcription. None of the treatments significantly reduced NFκB (figure 12-B). The expression of IL-1β was increased in the STZ group when compared to the control group, which indicates inflammation in the hippocampus. Only the STZ+M200 group had significantly ($p>0.05$) lower IL-1β levels than the other groups (figure 13).

4. DISCUSSION

Metformin hydrochloride, which acts mainly through activation of AMPK, is the most widely prescribed drug for patients with type 2 diabetes (DMT2). Mice that are fed an atherogenic diet follow a model that mimics the characteristics of DMT2. Studies assessing cognitive impairment by HFD induced diabetes have featured variations in the type and consumption time of the diet, as well as in mouse lineage, which results in different effects on the CNS (Arnold et al., 2014). On the other hand, the experimental model of type 1 diabetes *mellitus* (DMT1) induced by STZ is a widely used method to evaluate changes in the CNS caused by hyperglycemia (HERNÁNDEZ-FONSECA et al., 2009; NAGAYACH; PATRO; PATRO, 2014; YE; WANG; YANG, 2011). Although some authors have used STZ to induce

neurotoxicity *in vivo* via intracerebroventricular administration (ISLAM et al., 2013; PATHAN et al., 2006) and *in vitro* (PLASCHKE; KOPITZ, 2014), others have used STZ via i.p injection as a disruptor of insulin signaling to induce impairment memory (ALVAREZ et al., 2009; JOLIVALT et al., 2008, 2010) and reduce hippocampal neurogenesis (BEAUQUIS et al., 2008, 2010). Jolivalt and cols (2010) demonstrated that two intraperitoneal injections of STZ in C57Bl/6 mice induced insulin signaling impairment associated with learning and memory deficits.

The activation of AMPK has been found to have positive effects on CNS neuronal survival (AMATO; MAN, 2011), as well as modulation of long-term potentiation (LTP) (POTTER et al., 2010), neurogenesis and formation of spatial memory (WANG et al., 2012). The effects of AMPK activation by metformin on cognitive impairment caused by hyperglycemia or insulin resistance are controversial. According to studies by Pintana et al. metformin was able to reduce the glycemic level and cognitive impairment in animals fed with an atherogenic diet. (PINTANA et al., 2012). On the other hand, a study by McNeilly et al., showed that dietary supplementation with metformin did not attenuate cognitive impairment in animals fed with HFD, even though it improved glycemic control (MCNEILLY et al., 2012).

In the diabetes induced by STZ model, insulin secretion and signaling are impaired, as there is a destruction of β cells in the pancreas, characteristic of type 1 diabetes mellitus (DMT1). The presence of insulin resistance, and the fact that metformin improves the sensitivity of the insulin receptor, has been shown by using an experimental model of DMT2 induced by HFD (SKOVSØ, 2014). To significantly reduce the glycemic level in animals with DMT1 induced by streptozotocin as well as improve cognitive impairment, Brutada et al. used a dose of 500mg/Kg of metformin per day (Brutada et al., 2010). However when considering dose adjustments based on the metabolism of mice in relation to human doses, 500 mg/kg of metformin for testing in mice is very high (KAMBOJ; KUMAR; KUMAR, 2013). Therefore, in this study metformin hydrochloride did not reduce the glycemic levels in diabetic animals induced by STZ, most likely due to the lack of insulin associated with the destruction of pancreatic β cells.

Recent studies have shown that between the several antidiabetic drugs, the metformin can promote neuronal survival and lead to a significant improvement of memory and cognition (PATRONE; ERIKSSON; LINDHOLM, 2014). Our experiments analyzing the spatial memory of diabetic animals have revealed that there is damage to memory in such cases, and the dose of 200mg/Kg of metformin in diabetic animals improved the damage caused by

hyperglycemia, independent of glycemic control. This data indicates that metformin has a beneficial effect on memory formation and inflammation. Evidence shows that metformin promotes neurogenesis (POTTS; LIM, 2012), while studies of the action of metformin on memory formation in experimental models of Alzheimer's disease have produced inconclusive results (SALMINEN et al., 2011). However, an epidemiological study by Moore et al. indicated that administration of metformin in diabetes patients increased the risk of cognitive impairment (MOORE et al., 2013). However, the evidence from this study was not sufficiently robust to prove that metformin causes cognitive deficits in diabetic patients, as the authors did not produce data regarding the duration and severity of diabetes, duration of treatment with metformin, and use of other hypoglycemic agents (GOODARZI, 2014). The present study shows that the animals from the M200 group did not display any loss of spatial memory, or the activation of astrocyte microglia.

The antibody used in this study was anti-FOX-1, which is able to detect other protein members from the FOX family, namely FOX-1, FOX-2, FOX-3, FOX-4 and FOX-5. Recent studies have shown that the FOX-3 protein is the marker of the nucleus of mature neurons NeuN (DENT et al., 2010; KIM; ADELSTEIN; KAWAMOTO, 2009). Therefore, the anti-FOX-1 antibody was used to analyze neuronal loss in this study, indicating that treatment with 200 mg/kg of metformin was more effective at conserving pyramidal neurons in the dentate gyrus of diabetic mice. Similar results were obtained with immunofluorescence for Neu-N, a marker of mature neurons.

While the animals with diabetes induced by STZ displayed high levels of Bax, no change in Bcl-2 expression was observed. This data confirms the findings of Sima and Li (2005), whose analysis in a spontaneous model of diabetes in BB/Wor mice also demonstrated increased Bax expression, without any alteration in the expression of the mitochondrial apoptosis protein Bcl-xL (SIMA; LI, 2005). Studies looking into endothelial cells have shown that metformin can reduce apoptosis both *in vitro* and *in vivo* (DAVIS et al., 2006). The present study shows that although treatment with metformin tended to reduce Bax levels, there was no significant change when compared with the diabetic group. On the other hand, treatment with 100mg/Kg of metformin induced higher levels of Bcl-1, indicating that metformin appears to have an anti-apoptotic effect on neuronal cells, while treatment with 200mg/Kg of metformin did not result in an increase of Bcl-2 expression, potentially due to the negative feed-back mechanism of the apoptosis signaling pathway. Similar results were obtained from analysis of the expression of NGF, an important marker of neuronal survival,

suggesting that metformin reduces apoptosis and regulates the proliferation of neuronal processes.

Various experimental models report astrogliosis in the brain of animals, which is associated with cognitive impairment (COLANGELO; ALBERGHINA; PAPA, 2014; DUARTE et al., 2012; REVSIN et al., 2005), and the activation of microglia associated with inflammation (DHEEN; KAUR; LING, 2007; NATH et al., 2009). Recent studies have shown that activation of AMPK for resveratrol in a diabetes model induced by streptozotocin reduced astrogliosis (JING et al., 2013). The present study found that pharmacologic activation of AMPK by metformin can produce similar effects, as well as reducing the reactive gliosis of diabetic animals.

The present study is the first to report the effect of metformin on microglia *in vivo.* Studies *in vitro* have shown that metformin inhibits the activation of activated microglia by LPS in the independent mode of AMPK (ŁABUZEK et al., 2010a). On the other hand, the activation of AMPK by resveratrol produced the same effect in cells of microglia activated by morphine *in vitro* (HAN et al., 2014). The *in vivo* results of the present study agree with the *in vitro* findings that show that metformin reduces the inflammation and activation of microglia cells.

The transcription factor NFκB promotes transcription of inflammatory cytokines when activated. The NFκB is a dimer located on the cytoplasm that is linked to its inhibitory molecule Iκβα. Phosphorylation of Iκβα by Kinases IK (IKK) promotes the degradation of Iκβα by the proteasome, with NFκB then activated by phosphorylation. The activated NFκB is translocated to the nucleus where it promotes the transcription of inflammatory proteins, such as IL-1β, IL-6 and TNF-α (HAYDEN; GHOSH, 2008). Although NFκB activates the transcription of the IL-1β gene, post-transcriptional modifications are necessary in order to facilitate the release of IL-1β from the inflammation complex. Studies *in vitro* have shown that metformin treatment resulted in increased IL-1β expression, without the activation of NF-kB in microglia activated by LPS (ŁABUZEK et al., 2010a). However in the present study, metformin decreased phospho-Iκβα expression and negatively regulated the active form of IL-1β (17KDa), resulting in the cleavage of the IL-1β inactive form (31 KDa) by CASPASE 1 (PICCIOLI; RUBARTELLI, 2013). The results show that metformin acts as a modulator for the activation of IL-1β in an NFκB dependent mechanism in hippocampus cells in diabetic animals.

In the hippocampus of diabetic animals treated with metformin, observed the expression of eNOS was observed with both doses, which may have a beneficial effect on endothelial function. The activation of eNOS has been strongly associated with endothelial survival and angiogenesis promoted by increased VEGF expression (DIMMELER; DERNBACH; ZEIHER, 2000). However, in diabetic patients, metformin appears to improve endothelial function and reduces VEGF expression (ERSOY et al., 2008). This study also shows that the hippocampus of diabetic animals treated with 200mg/Kg of metformin resulted in a significant reduction of VEGF. According to Jing et al (2013), the reduction of this growth factor by the activation of AMPK during diabetes may indicate improvement in endothelial function and the blood brain barrier (JING et al., 2013). Furthermore, VEGF can increase the permeability of the blood brain barrier by reducing the expression and arrangement of occludin protein and ZO-1 at the junction of the brain endothelial cells (FISCHER et al., 2002). Therefore, the increase in angiogenesis via eNOS without the up regulation of VEGF expression is an interesting feature observed in the induction of angiogenesis, as it increases the permeability of vessels and inflammation (CHUNG et al., 2008, 2010).

The capacity of metformin to promote neurogenesis is promising for the treatment of patients with cognitive impairment associated with DMT1 and DMT2. The effect of this drug on cognitive impairment induced by hyperglycemia has been little explored. The present study found that metformin is able to significantly reduce neuroinflammation and can decrease the loss of neurons in the hippocampus of diabetic animals, which can subsequently promote improvements in spatial memory. Comparisons between 100 and 200 mg/Kg showed that the last one promoted the better outcome in the STZ experimental analyses. In conclusion, metformin may be a therapeutic alternative for patients with DMT1 that present cognitive impairment. However, further studies are required to evaluate the effect of joint treatment with metformin and insulin therapy in the short and long term.

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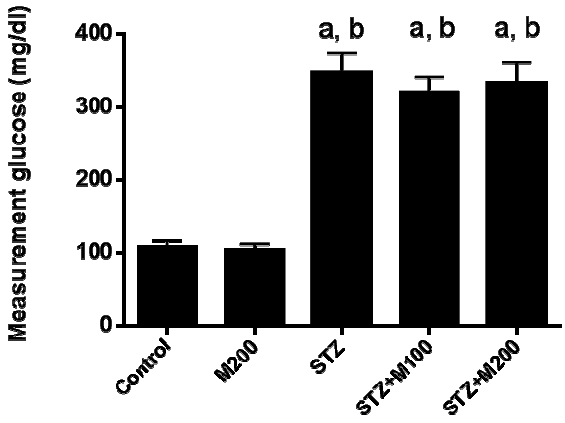

Fig 1 Effects of metformin on glycemic control. Levels of glycaemia (mean ± S.D) 4 hours after metformin was administered using analysis variance (ANOVA), post-hoc Tukey test. $^{a}p < 0.05$ when compared to control group, $^{b}p < 0.05$ when compared with M200 group, $^{c}p < 0.05$ when compared to STZ group, $^{d}p < 0.05$ when compared to STZ+M100 group and $^{e}p < 0.05$ when compared to STZ+M200 group.

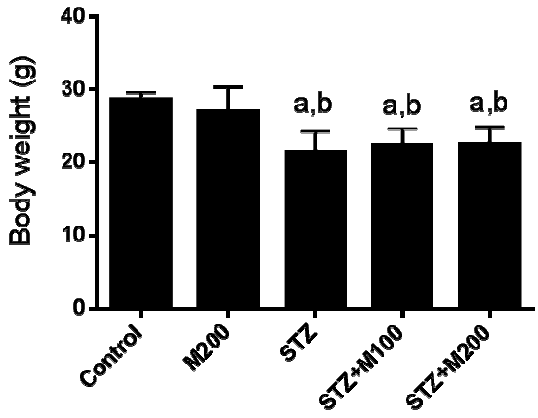

Fig 2 Effects of metformin on body weight (mean ± S.D) at end of study, using analysis variance (ANOVA), post-hoc Tukey test. $^{a}p < 0.05$ when compared to control group, $^{b}p < 0.05$ when compared to M200 group, $^{c}p < 0.05$ when compared to STZ group, $^{d}p < 0.05$ when compared to STZ+M100 group e $^{e}p < 0.05$ when compared to STZ+M200 group.

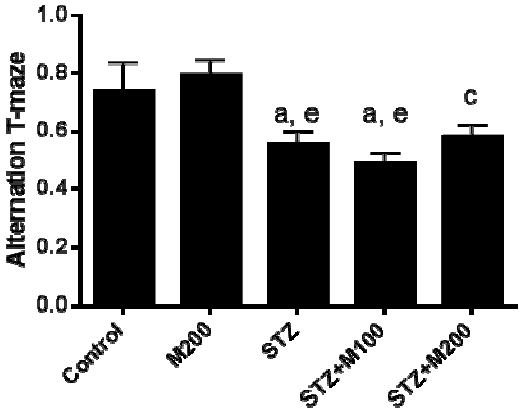

Fig 3 Effects of metformin on memory. Analysis of alternation on T-maze (mean ± S.D), using analysis variance (ANOVA), post-hoc Dunnet test. [a]$p < 0.05$ when compared to control group, [b]$p < 0.05$ when compared to MET200 group, [c]$p < 0.05$ when compared to STZ group, [d]$p < 0.05$ when compared to STZ+M100 group and [e]$p < 0.05$ when compared to STZ+M200 group.

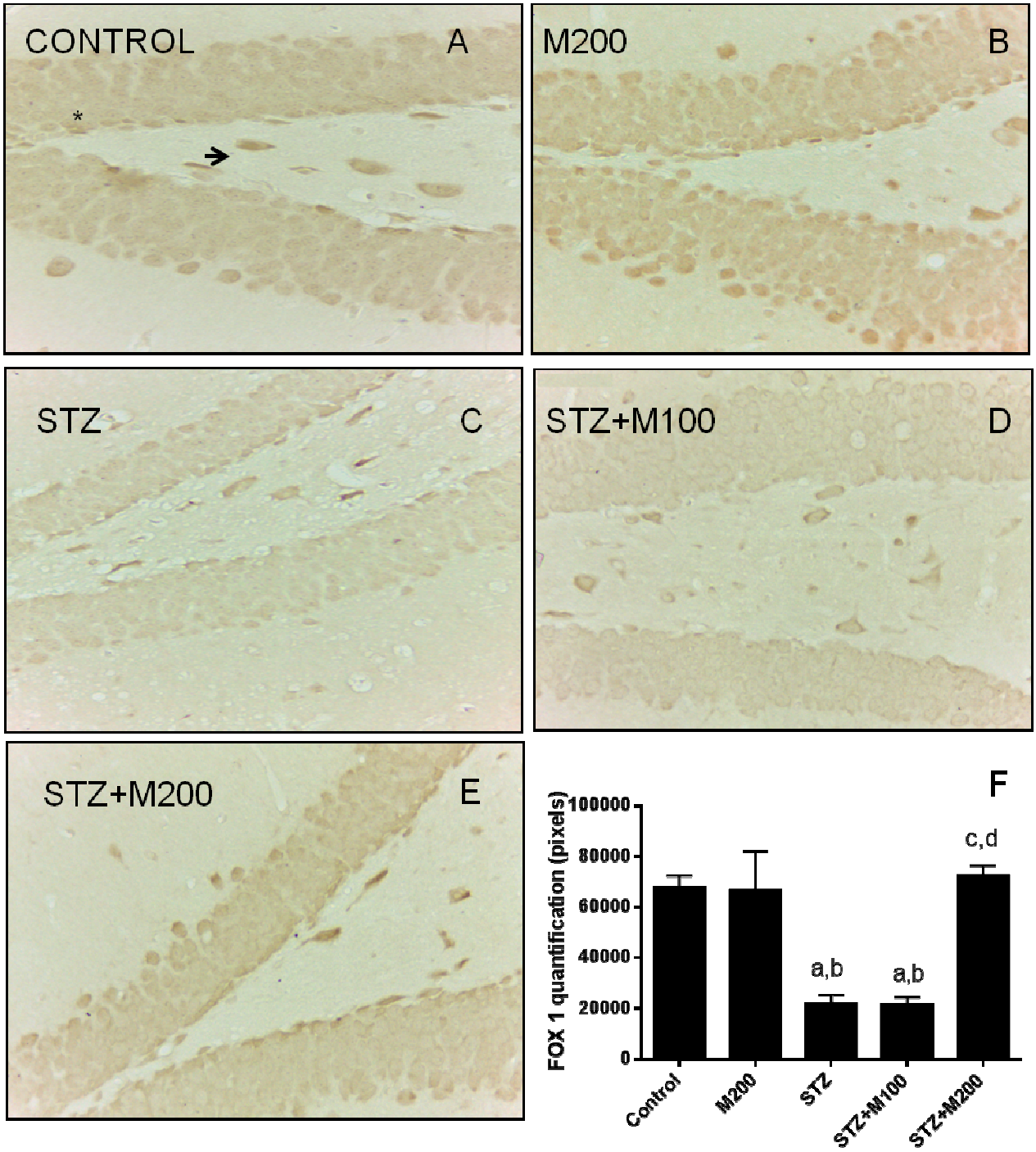

Fig 4 Effects of metformin on neuronal loss. Immunohistochemistry to FOX-1, pixels quantification. Magnification of 400 x. Arrow shows pyramidal neuron and asterisk granular neuron. A) Control, B) MET200, C) STZ, D) STZ+M100, E) STZ+M200 and F) immunohistochemistry quantification (media ± S.D), analysis variance (ANOVA) with Tukey post-hoc test used. $^{a}p < 0.05$ when compared with control group, $^{b}p < 0.05$ when compared with MET200 group, $^{c}p < 0.05$ when compared with STZ group, $^{d}p < 0.05$ when compared with STZ+M100 group and $^{e}p < 0.05$ when compared with STZ+M200 group.

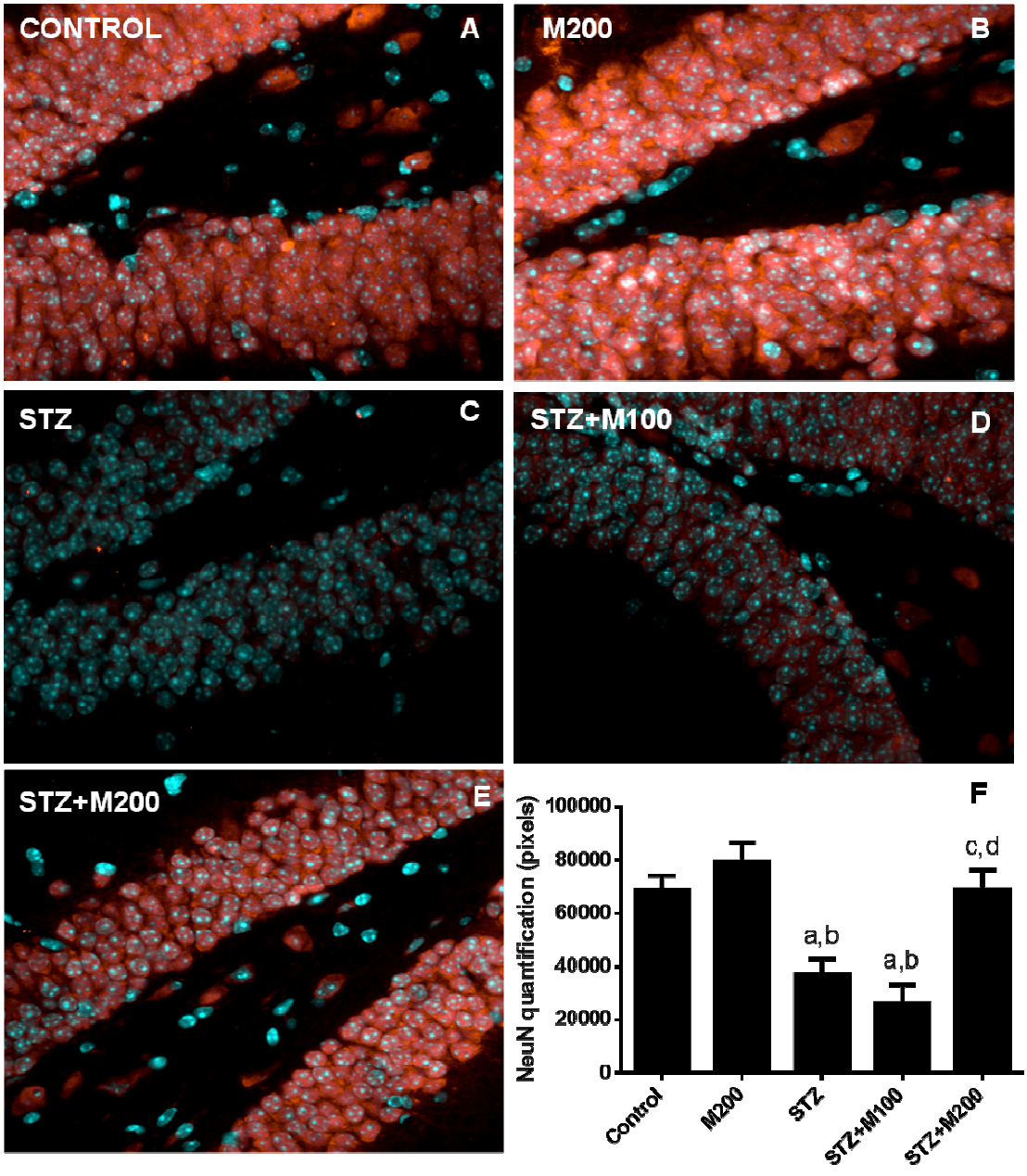

Fig 5 Effects of metformin on neuronal loss. Immunofluorescence to NeuN, pixels quantification. Magnification of 400x. A) Control, B) MET200, C) STZ, D) STZ+M100, E) STZ+M200 and F) Immunofluorescence quantification (media ± S.D), analysis variance (ANOVA) with Tukey post-hoc test used. $^{a}p < 0.05$ when compared with control group, $^{b}p < 0.05$ when compared with MET200 group, $^{c}p < 0.05$ when compared with STZ group, $^{d}p < 0.05$ when compared with STZ+M100 group e $^{e}p < 0.05$ when compared with STZ+M200 group.

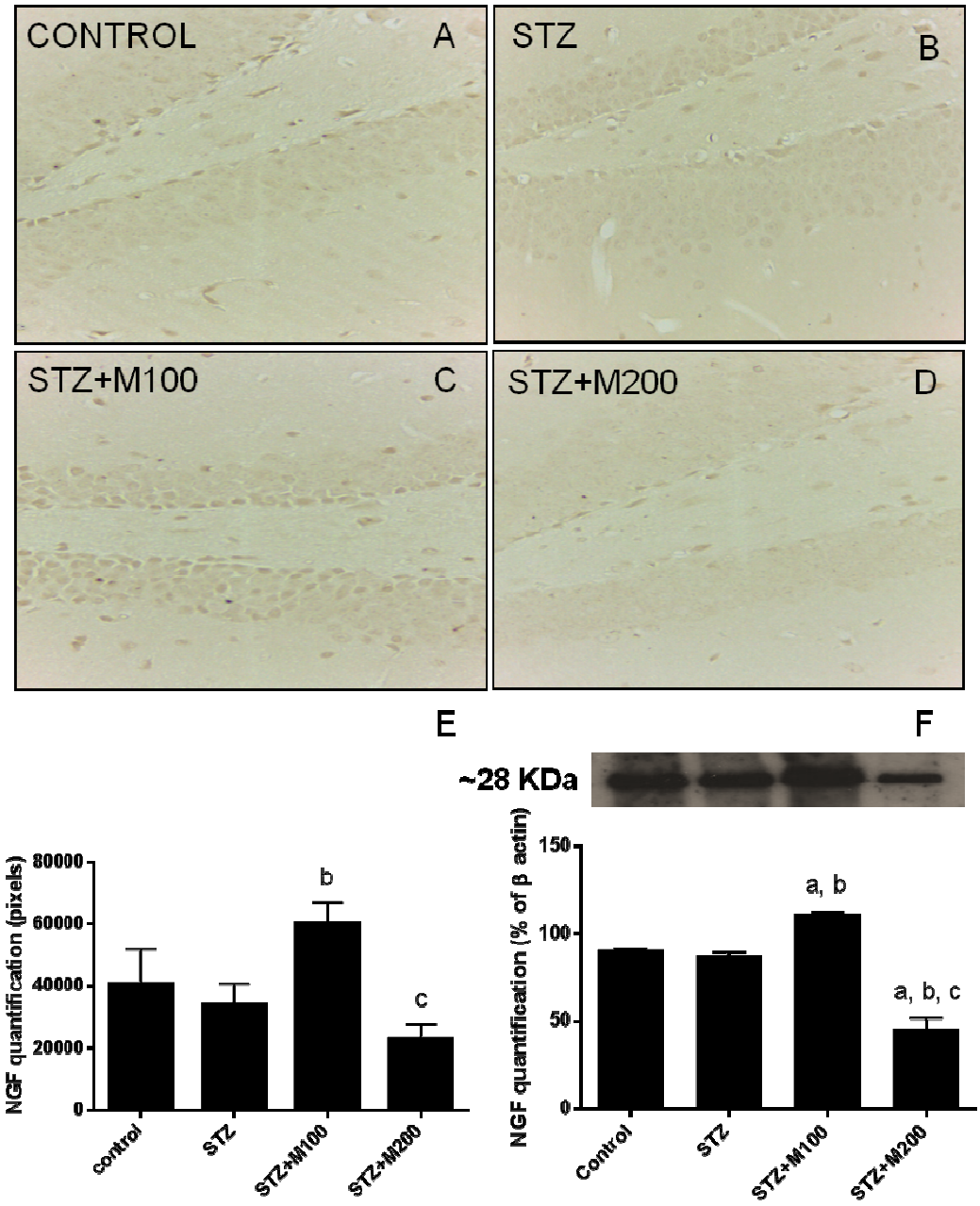

Fig 6 Effects of metformin on tissue hippocampal repair. Analysis of NGF, pixels quantification. Magnification of 400 x. A) Control, B) STZ, C) STZ+M100, D) STZ+M200, E) Quantification of immunohistochemistry results and F) quantification of western blot results (mean ± S.D); 5 animals per group. Analysis variance (ANOVA) with Tukey post-hoc test used. $^{a}p < 0.05$ when compared with control group, $^{b}p < 0.05$ when compared with STZ group, $^{c}p < 0.05$ when compared with STZ+M100 group, $^{d}p < 0.05$ when compared with STZ+M200 group and $^{e}p < 0.05$ when compared with M200 group.

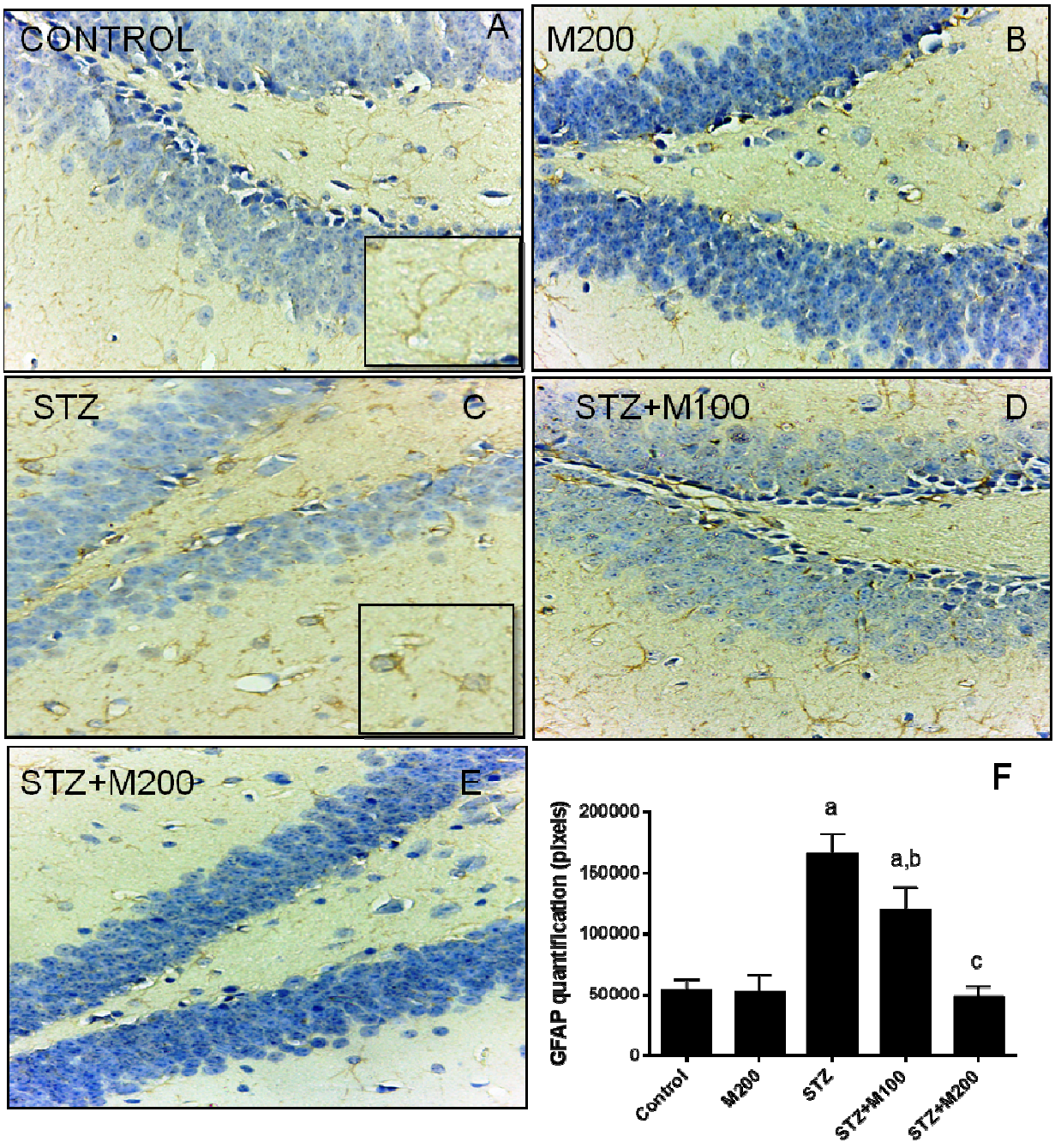

Fig 7 Effects of metformin on astrocyte activation. Immunohistochemistry to GFAP results, pixels quantification. Magnification of 400x. A) Control, B) MET200, C) STZ, D) STZ+M100, E) STZ+M200 and F) Quantification of immunohistochemistry results (mean ± S.D), using Analysis of variance (ANOVA) and Tukey post-hoc test. $^{a}p < 0.05$ when compared with control group, $^{b}p < 0.05$ when compared with STZ group; $^{c}p < 0.05$ when compared with STZ+M100 group, $^{d}p < 0.05$ when compared with STZ+M200 group and $^{e}p < 0.05$ when compared with M200 group.

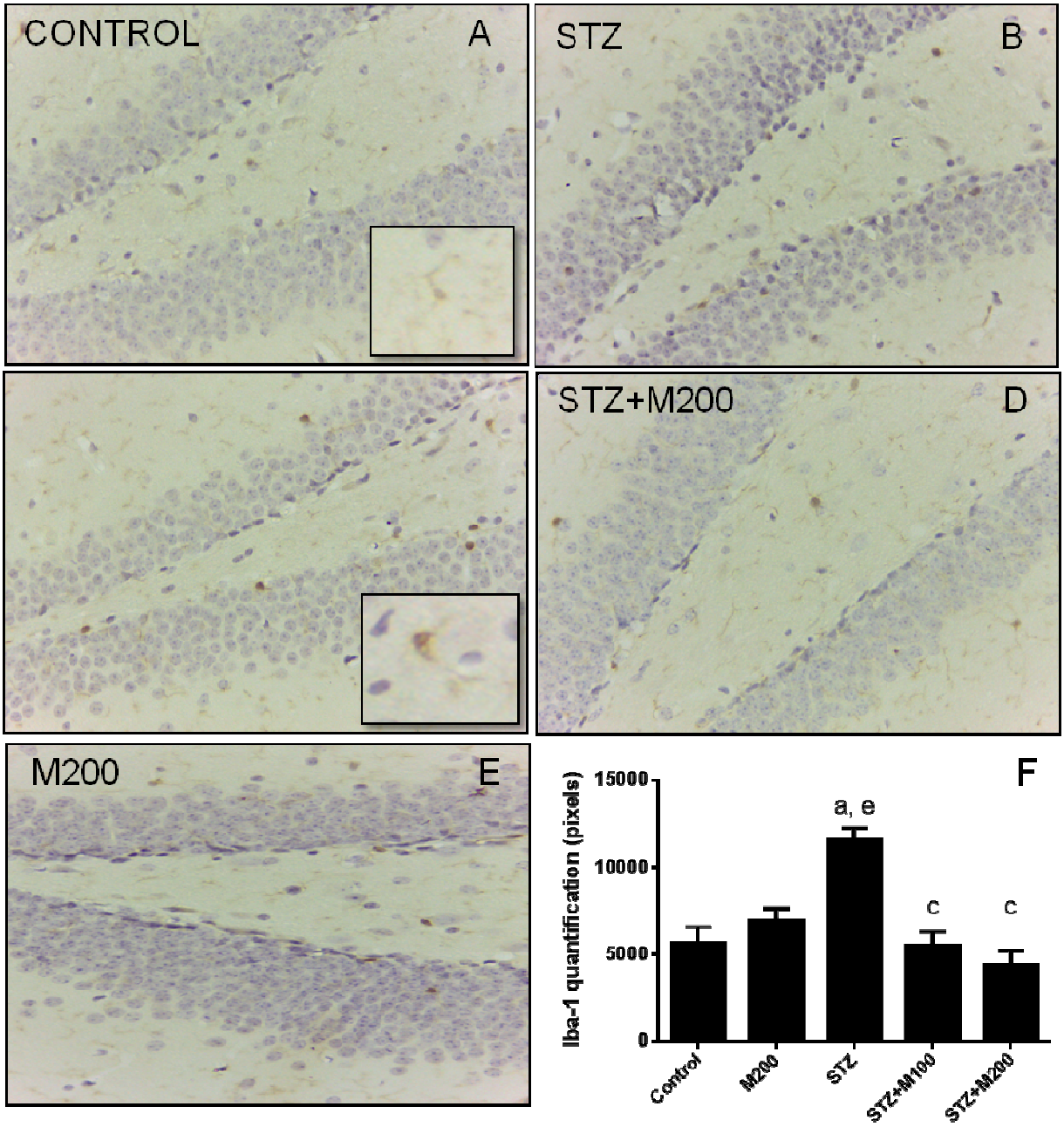

Fig 8 Effects of metformin on microglial activation. Immunohistochemistry to Iba-1, pixels quantification. Magnification of 400x. A) Control, B) M200, C) STZ, D) STZ+M100, E) STZ+M200 and F) Quantification of immunohistochemistry results (mean ± S.D), using Analysis of variance (ANOVA), and Tukey post-hoc test. $^{a}p < 0.05$ when compared with control group, $^{b}p < 0.05$ when compared with M200 group, $^{c}p < 0.05$ when compared with STZ group, $^{d}p < 0.05$ when compared with STZ+M100 group and $^{e}p < 0.05$ when compared with STZ+M200 group.

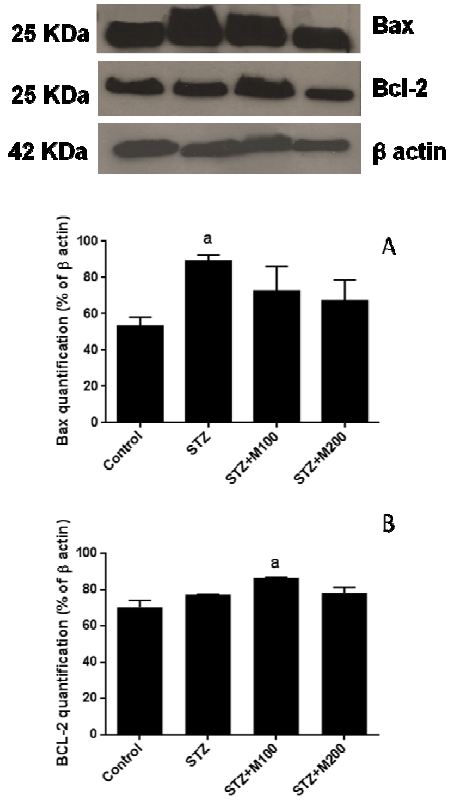

Fig 9 Effects of metformin on apoptosis. Analysis of Bax protein expression (A) for Western blot, using Analysis of variance (ANOVA) and Dunnet post-hoc test. Expression of Bcl-2 protein (B) for Western blot, using Analysis of variance (ANOVA), and Tukey post-hoc test. $^{a}p < 0.05$ when compared to control group, $^{b}p < 0.05$ when compared with STZ group; $^{c}p < 0.05$ when compared with STZ+M100 group and $^{d}p < 0.05$ when compared with STZ+M200 group. Total of 5 animals per each group. Results presented as mean ± S.D.

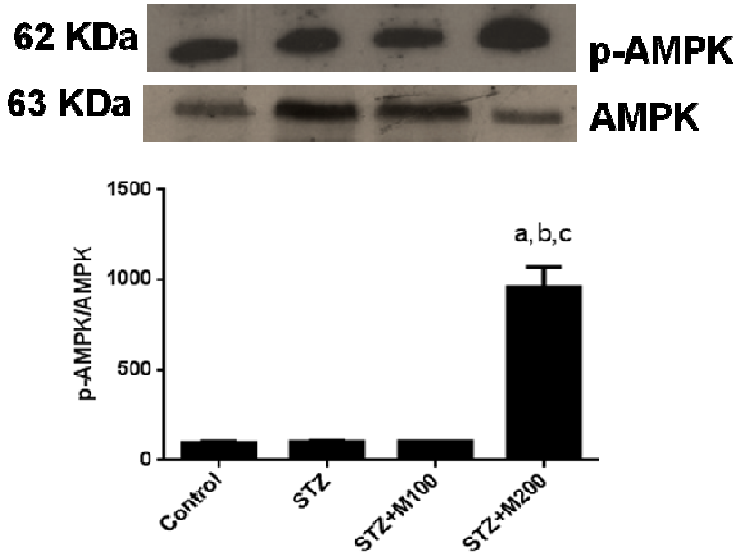

Fig 10 Effects of metformin on AMPK activation. A) Analysis of p-AMPK expression and AMPK for Western blot using analysis of variance (ANOVA) and Tukey post-hoc test Tukey. $^{a}p < 0.05$ when compared with control group, $^{b}p < 0.05$ when compared with STZ group; $^{c}p < 0.05$ when compared with STZ+M100 group and $^{d}p < 0.05$ when compared with STZ+M200 group. Statistical analysis was performed using Analysis of variance (ANOVA) and Tukey post-hoc test Total of 5 animals per each group. Results presented as mean ± S.D.

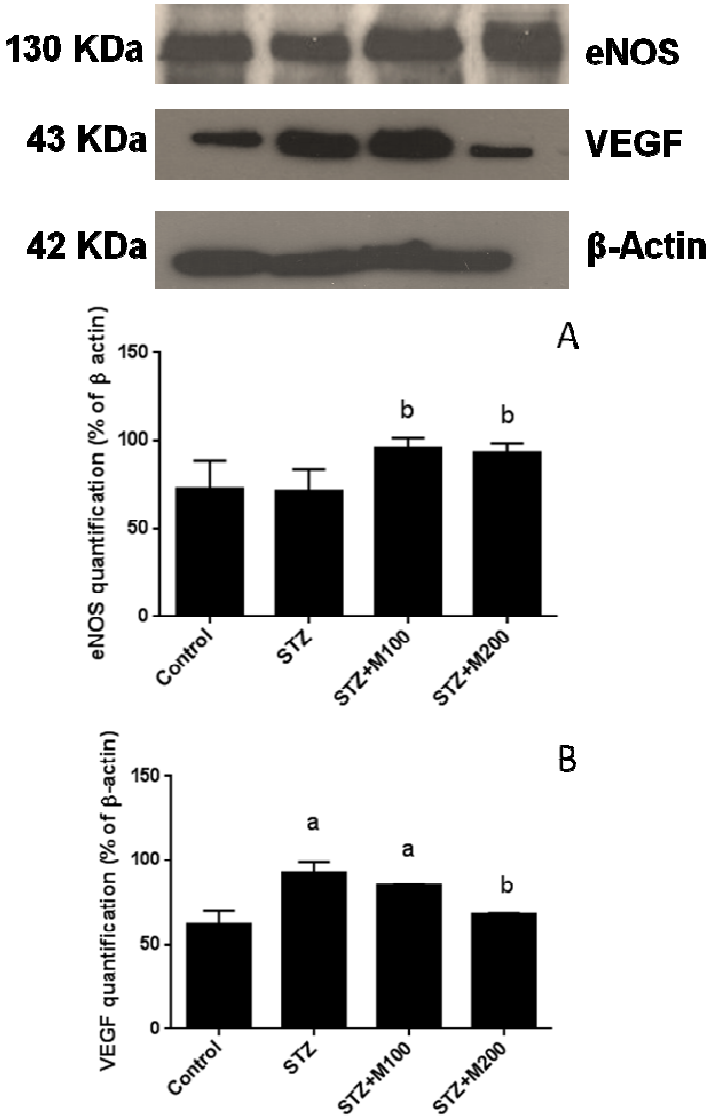

Fig 11 Effects of metformin on eNOS (A) and VEGF (B) expression. Statistical analysis of eNOS protein expression (Western blot) was conducted using Student's t-test, with results presented as mean ± S.D. VEGF protein (Western blot experiment) was statistically analyzed using an Analysis of variance (ANOVA) with Tukey post-hoc test. $^{a}p < 0.05$ when compared with control group, $^{b}p < 0.05$ when compared with STZ group; $^{c}p < 0.05$ when compared with STZ+M100 group and $^{d}p < 0.05$ when compared with STZ+M200 group. Total of 5 animals per group.

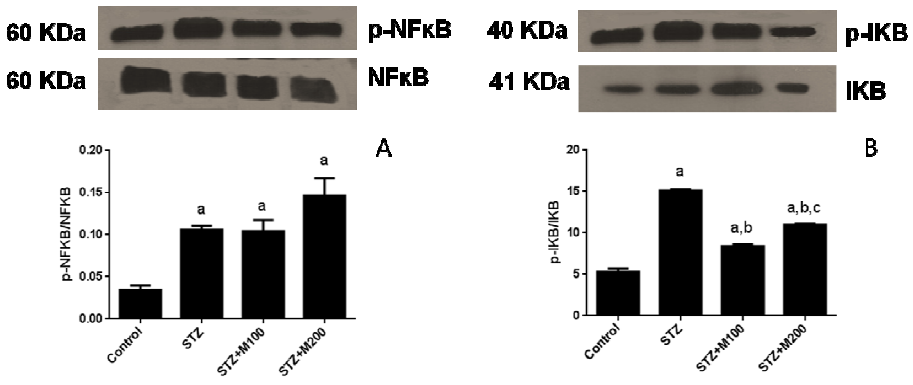

Fig 12 Effects of metformin on activation Iκβα (A) and NFκB (B). Statistical analysis of Iκβα and NFκB expression (Western blot experiment) was conducted using an Analysis of variance (ANOVA) with Tukey post-hoc test. $^{a}p < 0.05$ when compared with control group, $^{b}p < 0.05$ when compared with STZ group; $^{c}p < 0.05$ when compared with STZ+M100 group and $^{d}p < 0.05$ when compared with STZ+M200 group. Results are presented as mean ± S.D, with 5 animals per group.

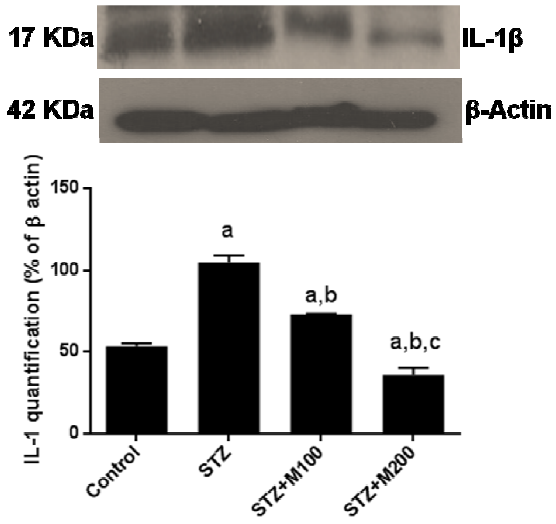

Fig 13 Effects of metformin on IL-1β protein. Statistical analysis of IL-1β protein (Western blot) was conducted using Analysis of variance (ANOVA), with Tukey post-hoc test. Results are presented as mean ± S.D, with 5 animals per group. $^{a}p < 0.05$ when compared with control group, $^{b}p < 0.05$ when compared with STZ group; $^{c}p < 0.05$ when compared with STZ+M100 group and $^{d}p < 0.05$ when compared with STZ+M200 group.

CONCLUSÃO

Nos animais diabéticos, a metformina reduziu a perda neuronal no giro denteado, constatada pelo aumento da marcação de neurônios adultos (FOX-1) e da expressão de NGF, além disso, a metformina reduziu a apoptose, caracterizada pela redução da proteína Bax. Tais resultados indicam uma ação neuroprotetora deste fármaco;

A metformina promoveu a melhora da função endotelial no hipocampo dos animais diabéticos sugere reduzir a permeabilidade vascular, aumentar a ativação de AMPK de maneira dose-dependente, e da expressão da eNOS;

A metformina teve papel anti-inflamatório por reduzir a expressão de IL-1β e Iκβα.

A metformina apresentou efeitos tanto anti-inflamatório como neuroprotetor no modelo de encefalopatia diabética induzida por STZ em camundongos C57Bl/6, além de promover melhora significativa da memória espacial. Portanto, este fármaco demonstra potencial terapêutico para auxiliar o tratamento de pacientes com diabetes mellitus tipo 1 que apresentam perda de memória.

ANEXO

Ministério da Saúde

FIOCRUZ
Fundação Oswaldo Cruz
Centro de Pesquisa Aggeu Magalhães

COMISSÃO DE ÉTICA NO USO DE ANIMAIS

Certificado de Aprovação

Certificamos que o projeto intitulado: **AVALIAÇÃO DOS EFEITOS DA METFORMINA SOBRE A NEURODEGENERAÇÃO NO MODELO DE ENCEFALOPATIA DIABÉTICA EM CAMUNDOBGOS C57BL/6**. Protocolado sob nº 46/2013 pelo (a) pesquisador (a) Dra Christina Alves Peixoto. Está de acordo com a Lei 11.794/2008 e foi aprovado pela COMISSÃO DE ÉTICA NO USO DE ANIMAIS do Centro de Pesquisas Aggeu Magalhães/Fundação Oswaldo Cruz (CEUA/CPqAM) em 20/08/2013. Na presente versão, este projeto está licenciado e tem validade até 28/fevereiro/2015.

Quantitativo de Animais Aprovados	
Espécie	**Nº de Animais**
Camundongo isogênico C57BL/6 macho	**226**

We certify that project entitled **AVALIAÇÃO DOS EFEITOS DA METFORMINA SOBRE A NEURODEGENERAÇÃO NO MODELO DE ENCEFALOPATIA DIABÉTICA EM CAMUNDOBGOS C57BL/6.** Protocol nº 46/2013, coordinated by Dra Christina Alves Peixoto. Is according to the ethical principles in animal research adopted by the Brazilian law 11.794/2008 and so was approved by the Ethical Committee for Animal Research of the Centro de Pesquisas Aggeu Magalhães/Fundação Oswaldo Cruz on August, 20, 2013. In present verson this project is licensed and valid until February, 28,2015.

Recife (PE, Brazil) August, 20, 2013.

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