UNIVERSIDADE FEDERAL DO PARANÁ

SETOR DE CIÊNCIAS BIOLÓGICAS

DEPARTAMENTO DE FARMACOLOGIA

ISABELLA CAROLINE DA SILVA DIAS

INDOLEAMINA-2,3-DIOXIGENASE/QUINURENINA COMO POTENCIAL ALVO FARMACOLÓGICO PARA TRATAR DEPRESSÃO ASSOCIADA AO DIABETES

CURITIBA

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Dissertação apresentada ao Programa de Pós-Graduação em Farmacologia da Universidade Federal do Paraná como requisito parcial para obtenção do título de Mestre em Farmacologia.

Orientadora: Prof[a]. Dr[a]. Janaina Menezes Zanoveli

Co-Orientadora: Prof[a]. Dr[a]. Joice Maria da Cunha

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PARECER DA BANCA EXAMINADORA

Os membros da Banca Examinadora designada pelo Colegiado do Programa de Pós-Graduação em FARMACOLOGIA da Universidade Federal do Paraná foram convocados para realizar a arguição da Dissertação de Mestrado de **ISABELLA CAROLINE DA SILVA DIAS**, intitulada: "**VIA INDOLEAMINA-2,3-DIOXIGENASE/QUINURENINA COMO POTENCIAL ALVO FARMACOLÓGICO PARA TRATAR DEPRESSÃO ASSOCIADA AO DIABETES**", após terem inquirido a aluna e realizado a avaliação do trabalho, são de parecer pela sua APROVAÇÃO, completando-se assim todos os requisitos previstos nas normas desta Instituição para a obtenção do Grau de **Mestre em FARMACOLOGIA**.

Curitiba, 14 de Agosto de 2015.

Prof JANAINA MENEZES ZANOVELI
(Presidente da Banca Examinadora)

Prof ANA MARCIA DELATTRE

Prof ROBERTO ANDREATINI

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ATA DE SESSÃO PÚBLICA DE DEFESA DE DISSERTAÇÃO PARA A OBTENÇÃO DO GRAU DE MESTRE EM FARMACOLOGIA

No dia quatorze de Agosto de dois mil e quinze às 09:00 horas, na sala 107, Centro Politécnico, do Setor de da Universidade Federal do Paraná, foram instalados os trabalhos de arguição da mestranda **ISABELLA CAROLINE DA SILVA DIAS** para a Defesa Pública de sua Dissertação intitulada: "**VIA INDOLEAMINA-2,--DIOXIGENASE/QUINURENINA COMO POTENCIAL ALVO FARMACOLÓGICO PARA TRATAR DEPRESSÃO ASSOCIADA AO DIABETES**". A Banca Examinadora, designada pelo Colegiado do Programa de Pós-Graduação em FARMACOLOGIA da Universidade Federal do Paraná, foi constituída pelos seguintes Professores Doutores: JANAÍNA MENEZES ZANOVELI, ANA MARCIA DELATTRE, ROBERTO ANDREATINI. Dando início à sessão, a presidência passou a palavra a aluna, para que a mesma expusesse seu trabalho aos presentes. Em seguida, a presidência passou a palavra a cada um dos Examinadores, para suas respectivas arguições. A aluna respondeu a cada um dos arguidores. A presidência retomou a palavra para suas considerações finais e, depois, solicitou que os presentes e a mestranda deixassem a sala. A Banca Examinadora, então, reuniu-se sigilosamente e, após a discussão de suas avaliações, decidiu-se pela ...APROVAÇÃO..... da aluna. A mestranda foi convidada a ingressar novamente na sala, bem como os demais assistentes, após o que a presidência fez a leitura do Parecer da Banca Examinadora, outorgando-lhe o Grau de **Mestre em FARMACOLOGIA**. Nada mais havendo a tratar a presidência deu por encerrada a sessão, da qual eu, PATRÍCIA POTT, lavrei a presente ata, que vai assinada por mim e pelos membros da Comissão Examinadora.

Curitiba, 14 de Agosto de 2015.

PATRICIA POTT
Secretaria do Programa de Pós Graduação em FARMACOLOGIA

Prof JANAINA MENEZES ZANOVELI
(Presidente da Banca Examinadora)

Prof ANA MARCIA DELATTRE

Prof ROBERTO ANDREATINI

Dedico este trabalho a todos os meus irmãos de caminhada que me ajudaram a chegar até aqui.

Agradecimentos

Seguindo uma ordem cronológica, primeiramente gostaria de agradecer a minha minha professora de ciências do colégio Euclides da Cunha, por me deixar ansiosa esperando as aulas dela e me despertar o interesse por ciência, muito obrigada profª Sandra.

Ainda na infância outra pessoa que exerceu um papel fundamental foi a minha tia Deise, que conversava sobre ciência e biologia de uma maneira bem didática, como se tudo fosse muito fácil me feazendo adorar esse tipo de conversa, muito obrigada tia Deise não só por incentivar meus estudos mas também por sempre ser o meu colo acessível.

Muito obrigada profª Adriana da UEM, minha primeira orientadora da vida, que me passou muita força no TCC e sempre foi um exemplo de mulher profissional para mim.
Gostaria de agradecer especialmente ao prof Mauro da UEM, o grande responsável pela minha paixão em farmacologia devido as suas aulas maravilhosas e extremamente didáticas, muito obrigada por nos ensinar farmacologia daquele jeito.

Muito obrigada a minha turma de medicina veterinária UEM, que sempre me elogiava nos seminários e foram me passando segurança para traçar o caminho que escolhi.

Obrigada especialmente aos meus amigos Jean, Maurício, Maria, Nathali, Amanda, Ana Cláudia, Camila, Mariah, Taynnara e Tainan, por serem os melhores amigos do mundo e me apoiarem nos momentos difíceis, afinal morar longe da família desde os 17 anos não é uma tarefa fácil, e eu sempre pude (e sei que posso) contar com o colo e torcida de vocês.

Quero agradecer também aos amigos que a UFPR me proporcionou nessa caminhada de 2 anos, mas especialmente as ICs Camila, Jéssica e Dani não só pela amizade mas pela ajuda no trabalho, e a Anne e a Helen por praticamente serem minhas “co-co” orientadoras em meio a tantos conselhos e explicações.

Quero agradecer especialmente as minhas orientadoras profª Janaína e profª Joice, por sempre estarem disponíveis e além de uma bela orientação me proporcionaram também uma verdadeira amizade.

Quero fazer um agradecimento especial a minha avó Odete, que apesar de todas as pedras no caminho sempre me proporcionou o melhor da educação, moral e intelectual, com seu sorriso sereno no rosto; por ser meu exemplo de mulher guerreira que é mãe, aluna, trabalhadora e nunca reclama

de nada, pelo contrário, sempre acha motivo para agradecer. Obrigada por essa oportunidade de ser sua neta nesta encarnação e aprender com os seus exemplos.

Muito obrigada a toda a minha família, minha mãe Iclea, tia Lais, Tia Deise, Tia Rose, Eduardo, vó Odete, Rodrigo e Lais por SEMPRE me apoiarem e me darem asas para voar, sempre percebi que vocês me criaram para o mundo e me fortaleceram para enfrentá-lo.

Alex, meu amor, obrigada pela paciência de aguentar as ocorrências da vida de uma pós graduanda, por me encher de carinho e conselhos quando a caminhada é difícil, e por vir de tão longe dar colo e amizade para sua “melhor amiga”.

Muito obrigada a todos, cada um desenvolveu um papel essencial nessa minha vida de mestranda.

“Se não puder voar, corra. Se não puder correr, ande. Se não puder andar, rasteje, mas continue em frente de qualquer jeito.”

Martin Luther King

NOTA EXPLICATIVA

Esta dissertação é apresentada em formato alternativo, como artigo científico para publicação, de acordo com as normas do Programa de Pós-Graduação em Farmacologia da Universidade Federal do Paraná. A dissertação consta de uma revisão bibliográfica, hipótese do trabalho e o artigo científico formatado conforme normas de revistas científicas internacionais.

RESUMO

Diabetes é uma doença crônica associada à depressão, porém o mecanismo fisiopatológico que associa essas duas condições não foi completamente elucidado. Entretanto, a ativação da Indoleamina-2,3-dioxigenase (IDO), enzima que participa do metabolismo do triptofano levando a diminuição dos níveis de serotonina (5-HT) e que tem sua expressão associada à ativação do sistema imunológico, vem sendo proposta neste trabalho como um dos prováveis mecanismos que pode estar envolvido na associação da depressão ao diabetes. Para testar essa hipótese, animais diabéticos (DBT) e normoglicêmicos (NGL) tiveram os níveis de citocinas (TNF-α, IL-1 β e IL-6), 5-HT e noradrenalina (NA) mensurados em seus hipocampos (HIP). Além disso, o efeito do inibidor da recaptação de serotonina fluoxetina (FLX), do inibidor direto da IDO 1-metil-triptofano (1-MT), do anti-inflamatório e inibidor indireto da IDO minociclina (MINO) ou do anti-inflamatório não esteroidal ibuprofeno (IBU), foi avaliado em ratos DBT no teste da natação forçada modificado (MFST). Após os testes comportamentais, o HIP foi dissecado para posterior análise da expressão de IDO pelo método de *Western blotting*. Ratos DBT exibiram em seus HIP um aumento significativo dos níveis de TNF-α, IL-1 β e IL-6 e diminuição de 5-HT e NA. Eles também apresentaram um comportamento do tipo depressivo mais pronunciado, o qual foi revertido por todos os tratamentos. Interessante notar que o tratamento com drogas que também apresentam uma ação anti-inflamatória (MINO, IBU e FLX) reduziu a expressão elevada de IDO observada no HIP desses animais DBT. Dessa maneira, nossos dados suportam nossa hipótese de que a neuroinflamação no HIP seguida pela ativação da IDO e consequente diminuição de 5-HT pode ser o possível mecanismo patofisiológico que liga a depressão com o diabetes.

Palavras chaves: estreptozotocina, serotonina, depressão, Indoleamina-2,3-dioxigenase, inflamação, hipocampo.

ABSTRACT

Diabetes is a chronic disease associated with depression whose pathophysiological mechanisms that associate these conditions are not fully elucidated. However, the activation of the indoleamine-2,3-dioxygenase (IDO), an enzyme that participate of the tryptophan metabolism leading to a decrease of serotonin (5-HT) levels and whose expression is associated with an immune system activation, has been proposed in this work as a common mechanism that links depression and diabetes. To test this hypothesis, diabetic (DBT) and normoglycemic (NGL) groups had the cytokines (TNFα, IL-1 β and IL-6) and serotonin (5-HT) and noradrenaline (NE) levels in the hippocampus (HIP) evaluated. Moreover, the effect of the selective serotonin reuptake inhibitor fluoxetine, IDO direct inhibitor 1-methyl-tryptophan, anti-inflammatory and IDO indirect inhibitor minocycline or non-selective cyclooxygenase inhibitor ibuprofen was evaluated in DBT rats submitted to the modified forced swimming test (MFST). After the behavioral test, the HIP was obtained for IDO expression by Western blotting analysis. DBT rats exhibited a significant increase in HIP levels of TNFα, IL-1 β and IL-6 and a decrease in 5-HT and NA. They also presented a depressive-like behavior which was reverted by all employed treatments. Interestingly, treatment with drugs that present anti-inflamatory proprieties (MINO, IBU and FLX) reduced the increased IDO expression in the HIP from DBT animals. Taken together, our data support our hypothesis that neuroinflammation in the HIP followed by IDO activation with a consequent decrease in the 5-HT levels can be a possible pathophysiological mechanism that links depression to diabetes.

Keywords: streptozotocin, serotonin, depression, Indoleamine 2,3-dioxygenase, inflammation, hippocampus

LISTA DE ABREVIATURAS

3-HK - 3 hidroxi-quinurenina

5-HT - Serotonina

AGEs - Produtos Finais de Glicação Avançada

BDNF - Brain-derived neurotrophic factor

DE - Disfunção endotelial

DM - *Diabetes Mellitus*

DM1 - *Diabetes Mellitus* tipo 1

DM2 - *Diabetes Mellitus* tipo 2

eNOS - Oxido nítrico sintase endotelial

HPA - Hipotálamo-Pituitária-Adrenal

IDO - Indoleamina 2,3 dioxigenase

IFN - Interferon

IL-1 - Interleucina 1

IL-6 - Interleucina 6

KA - Acido quinurênico

LDL - Lipoproteína de baixa densidade

NO - Óxido nítrico

QA - Ácido quinolínico

ROS - Espécies reativas de oxigênio

SBD - Sociedade Brasileira de Diabetes

STZ - Estreptozotocina

TNF - Fator de necrose tumoral

TRIP - Triptofano

TRYCATs - Catabólitos de triptofano

SUMÁRIO

1 REVISÃO LITERÁRIA

1.0. DIABETES MELLITUS

Diabetes Mellitus (DM) é um distúrbio metabólico caracterizado por hiperglicemia crônica que pode ser resultado de uma falha na ação ou secreção de insulina, que culmina em deficiência relativa ou absoluta desse hormônio (Wayhs et al., 2010). Sabe-se que altas concentrações plasmáticas de glicose levam ao desenvolvimento de distúrbios secundários, como doenças cardiovasculares (Dei Cas et al., 2015), retinopatia (Monaghan et al., 2015), dor neuropática (Downs and Faulkner, 2015) e distúrbios psiquiátricos (Moulton et al., 2015), principalmente depressão (Rotella and Mannucci, 2013).

De acordo com a Federação Internacional do Diabetes (2014), estima-se que existam mais de 387 milhões de pessoas portadoras de DM no mundo. Mais ainda, esse número pode alcançar 592 milhões de pessoas até o ano de 2035. Porém, uma entre duas pessoas que tem DM ainda não foi diagnosticada, portanto esses números estão subestimados. Calcula-se que foram gastos em 2014 cerca de 612 bilhões de dólares devido ao DM e suas complicações, assim como 4,9 milhões de mortes devido a distúrbios secundários ou comorbidades.

Estudos mostram que o aumento da prevalência desta doença tem sido relacionado principalmente com mudanças no estilo de vida das pessoas, como o aumento do sedentarismo, da obesidade e com o aumento da expectativa de vida da população (Schmidt et al., 2009; Shaw et al., 2010).

1.1. CLASSIFICAÇÃO, DIAGNÓSTICO E PATOFISIOLOGIA

De acordo com a Associação Americana de Diabetes (2014), o DM é classificado em tipo 1 (DM1), 2 (DM2), gestacional (DMG), e outros tipos.

No DM1 ocorre uma destruição crônica das células β pancreáticas, por meio de mecanismos autoimunes, mediados por células como linfócitos T e macrófagos. O processo de autodestruição se inicia meses a anos antes do diagnóstico clínico da doença e, dependendo da idade do diagnóstico, cerca de 70 a 90% das células β já foram destruídas após os primeiros sintomas de hiperglicemia (Voltarelli et al., 2009; Associação Americana de Diabetes, 2014). Essa forma de DM1 acomete cerca de 5-10% da população com diabetes e anteriormente era conhecido como insulinodependente ou diabetes juvenil (Associação Americana de Diabetes, 2007). Por sua vez, o DM2 representa cerca de 90% dos casos de diabetes.

Neste tipo de diabetes (DM2), o principal fenômeno fisiopatológico é a resistência à ação da insulina, diminuindo a captação de glicose em tecidos que dependem desse hormônio. Esta resistência é uma condição patológica caracterizada por uma deficiência no mecanismo de sinalização do hormônio para a regulação do açúcar no sangue. Diante disso, os tecidos perdem a sensibilidade à ação da insulina e, consequentemente, a concentração de glicose sanguínea aumenta (Rosak, 2002; Campbell, 2011). Devido a essa resistência, ainda no início da doença, ocorre hiperinsulinemia compensatória, continuando por meses ou anos. Devido a disfunção e redução das células β pancreáticas com o decorrer do DM2, a síntese e a secreção de insulina

poderão ficar comprometidas e, em alguns casos, a terapia com insulina será essencial (Matthaei et al., 2000; Mclellan et al., 2007). Esta forma do DM, que geralmente acomete pacientes com idade igual ou superior a 40 anos de idade, está relacionada com excesso de peso concomitante a uma redução na prática de exercícios físicos. Atualmente, tem sido cada vez mais frequente o surgimento de DM2 em crianças e adolescentes devido ao estilo de vida sedentário (Wilson, 2013; Ramkumar e Tondon, 2013).

O DMG é determinado pela diminuição da tolerância à glicose ou seja, uma deficiência na ação da insulina no período gestacional. O início ou o reconhecimento acontece pela primeira vez durante a gestação, podendo ou não persistir após o parto. No período pós-gestacional há redução da concentração plasmática de hormônios contrainsulínicos, diminuindo as necessidades maternas de insulina e a glicemia volta à normalidade. No entanto, as gestantes que apresentam DMG possuem alto risco de desenvolverem DM2 posteriormente (Schaefer-Graf et al., 2002; Associação Americana de Diabetes, 2007). Além disso, o DMG está associado com o aumento de morbidade e mortalidade perinatal (Diretrizes SBD, 2009).

Na categoria “outros tipos de DM”, destaca-se o *Maturity Onset Diabetes of the Young* (MODY), um subtipo que acomete indivíduos com menos de 25 anos e não-obesos. Caracteriza-se por defeito na secreção de insulina, porém, sem causar dependência da mesma. Há uma herança autossômica dominante, abrangendo, portanto, muitas gerações de uma mesma família (Campagnolo et al., 2005). Além disso alguns relatos de caso reportaram diabetes decorrente de defeitos genéticos das células beta pancreáticas, defeitos genéticos na ação da insulina, endocrinopatias, induzido por químicos ou drogas, complicações de

infecções, doenças do pâncreas e outras síndromes genéticas (Associação Americana de Diabetes, 2014).

Importante mencionar que no nosso estudo o interesse foi voltado ao DM1. Neste sentido, estudos vem mostrando que a prevalência desta forma de DM está aumentando (Chiang et al., 2014; Downs and Faulkner, 2015). O número estimado de pacientes no mundo com DM1 não é conhecido, porém sabe-se que nos Estados Unidos este número está maior que 3 milhões (Prime Group for JDRF, 2011), e que esse número cresceu 21% de 2001 a 2009, o que justifica o interesse crescente em estudar esse tipo de DM e suas comorbidades. Apesar de já ter sido conhecido como “diabetes juvenil”, a maioria dos indivíduos com DM1 são adultos (Chiang et al., 2014). Acredita-se que o número de adultos convivendo com DM1 está crescendo devido principalmente a 2 fatores: 1- o diagnóstico tardio da doença em adultos, mesmo naqueles com diabetes autoimune latente (LADA, em *inglês latent autoimmune diabetes in adults*), e 2- indivíduos que foram diagnosticados com DM1 na infância estão vivendo mais (Miller et al., 2012; Chiang et al., 2014).

Com o objetivo de prevenir complicações ocasionadas pelo DM decorrentes da hiperglicemia, a Associação Americana de Diabetes modificou em 1997 os critérios para diagnóstico desta patologia. Posteriormente, os critérios foram aceitos pela Organização Mundial da Saúde e pela Sociedade Brasileira de Diabetes. Atualmente são três os critérios aceitos para o diagnóstico de DM:

1. Sintomas de poliúria (aumento do volume urinário), polidipsia (aumento de sede) e perda ponderal de peso, acrescidos de glicemia casual (aquela

realizada a qualquer hora do dia independentemente do horário das refeições) acima de 200 mg/dL.

2. Glicemia de jejum > ou = 126 mg/dL. Em caso de pequenas elevações da glicemia, o diagnóstico deve ser confirmado pela repetição do teste em outro dia.

3. Glicemia 2 h após sobrecarga oral de 75 g de glicose acima de 200 mg/dL (Diretrizes SBD, 2009).

Sabe-se que além da doença em si há muitas outras condições patológicas associadas, sendo muitas delas decorrentes do estado hiperglicêmico dos pacientes diabéticos. Dentre estas destacam-se a disfunção endotelial, com perda das propriedades do endotélio vascular, como a alteração no perfil antiaterogênico, causando migração e proliferação de células musculares lisas, agregação de plaquetas, oxidação de LDL, adesão de monócitos, plaquetas e síntese de citocinas pró inflamatórias, contribui para a aterogênese e portanto para as disfunções macrovasculares (Bertoluci et al., 2008). Há também a retinopatia diabética, no qual o indíviduo apresenta alterações na fisiologia ocular derivadas da opacificação do cristalino e de modificações vasculares retinianas (Antcliff e Marshall, 1999; Pereira et al., 2009). Sabe-se que a neuropatia diabética é uma das complicações mais prevalentes acometendo cerca de 90 % dos pacientes diabéticos (Tesfaye et al., 2013), causando uma lesão progressiva das fibras somáticas (sensitivas e motoras) e autonômicas (Ferreira et al., 2011). Portanto, pode-se inferir que a persistência do estado hiperglicêmico é o fator primário desencadeador de complicações macrovasculares, microvasculares, bem como neuronal no indivíduo diabético (Ferreira et al., 2011).

Além das complicações de origem microvascular, macrovascular e neuronal já expostas, outras complicações do diabetes crônico não menos importantes são aquelas relacionadas ao sistema nervoso central, como a demência (Cuklerman et al., 2005), prejuízos cognitivos (Seto et al., 2015) e depressão (Downs and Faulkner, 2015; Moulton et al., 2015; Petrak et al., 2015; Zanoveli et al., 2015), sendo esta última o principal foco de interesse do nosso trabalho.

1.2. DEPRESSÃO

Segundo o Manual diagnóstico e estatístico americano, na sua quinta versão (DSM-V) publicado em maio de 2013, a depressão caracteriza-se pela presença de 5 ou mais determinados sintomas, dentre eles insônia ou hipersônia, retardo ou agitação psicomotor, perda ou ganho de peso, fadiga, sentimentos de inutilidade ou baixa auto estima, ideias ou “tentativa suicida” por no mínimo 2 semanas, onde obrigatoriamente deve haver humor deprimido e/ou anedonia (DSM-V, 2013).

A depressão representa um problema significante e crescente para a saúde pública, pois estima-se que até 2020, seja a segunda maior causa de incapacitação no mundo, gerando um problema socioeconômico (Institute of Medicine, 2001; World Health Organization, 2010)

No Brasil, segundo a Pesquisa Nacional por Amostra de Domicílios divulgada pelo Instituto Brasileiro de Geografia e Estatística, a depressão, identificada por profissional de saúde, atinge 7,8 milhões de brasileiros, o que corresponde a 4,1% da população (IBGE, 2008)

Apesar de ser uma doença antiga, considerando que Hipócatres já a descrevia antes de Cristo como “uma afecção sem febre, no qual o espírito fica

triste e constantemente abatido” (Cuche and Gerard, 1994; Servan-Schreiber, 2004), ainda hoje não se conhece exatamente a neurobiologia da depressão, diante disso, surgiram algumas hipóteses para explicá-la. A hipótese mais conhecida é a monoaminérgica, que foi proposta na década de 60 após a constatação que determinados fármacos exerciam efeito antidepressivo devido a capacidade de aumentar os níveis de monoaminas, como serotonina, noradrenalina e dopamina, na fenda sináptica, portanto, segundo a hipótese monoaminérgica a depressão seria o resultado da diminuição destes neurotransmissores (Schildkraut, 1965; Delgado, 2000). Apesar de estudos posteriores concordarem com essa afirmação, a hipótese monoaminérgica não explica porque ocorre um atraso de 2-3 semanas para pacientes depressivos apresentarem uma melhora clínica, sendo que o fármaco já estabilizou os níveis de monoaminas em 2 dias, assim como também não explica o fato de que nem todas as drogas que melhoram a transmissão serotoninérgica, noradrenérgica ou dopaminérgica são 100% eficazes para combater a depressão (Hirschfeld, 2000). Devido a essas discrepâncias, ao longo do tempo foram surgindo novas hipóteses na tentativa de explicar a neurobiologia da depressão com bases em achados patológicos, entre elas se encontra a hipótese do estresse oxidativo, que afirma que a depressão é o resultado de um desbalanço na produção de espécies reativas de oxigênio e nitrogênio (Behr et al., 2012; Joshi and Pratico, 2014), assim como a hipótese do eixo HPA afirma que devido a uma desregulação desse eixo, os pacientes depressivos apresentam maior quantidade de cortisol, sendo o responsável por ativar certas vias imunológicas que levam aos sintomas depressivos (Martinac et al., 2014; Gold, 2015). Já a hipótese neurotrófica afirma que a depressão

ocorre devido uma diminuição de fatores neurotróficos, como BDNF (Elder et al., 2006; Song and Wong, 2011) em compensação a hipótese inflamatória, de interesse para o nosso estudo, sustenta que a depressão é o resultado de uma estimulação do sistema imunológico pela inflamação (Smith, 1991).

Há tempos estudos relacionam inflamação como parte essencial da patofisiologia da depressão (Smith, 1991; Irwin and Miller, 2007; Miller, 2009; Maes, 2011). Ainda em 1991, Ronald Smith publicou a teoria do macrófago afirmando que a depressão seria o resultado de uma estimulação do sistema imunológico. Para isso ele relacionou a alta incidência de transtornos de humor em doenças inflamatórias, como artrite reumatóide e aterosclerose, assim como explicou que mulheres são mais suscetíveis a sintomas depressivos, devido a capacidade do hormônio estrogenio em ativar o sistema imune (macrófago), e sugeriu também que no Japão a incidência de depressão era menor devido ao alto consumo do óleo de peixe, considerado um anti-inflamatório natural (Smith, 1991), deixando clara uma relação entre sistema imunológico e depressão.

Ainda na década de 90, Maes e colaboradores publicaram que na depressão ocorria uma ativação de monócitos aumentando a produção de interleucina 1 β (IL-1β), IL-6 e fator de necrose tumoral α (TNFα) (Maes et al., 1990 - 1991; 1991; Maes, 1993). Anos após, evidências apontaram um aumento de citocinas pró inflamatórias no sangue e fluído cerebroespinhal de pacientes depressivos (Levine et al., 1999; Gabbay et al., 2009; Lindqvist et al., 2009; Hayley, 2011; Maes, 2011). Mikova e colaboradores publicaram que os níveis plásmaticos de TNFα, IL-6 e IL-8 eram maiores em pacientes depressivos do que em pacientes com esclerose múltipla (Mikova et al., 2001)

Achados clínicos também mostram que pacientes desenvolveram um declínio cognitivo e sintomas depressivos após serem tratados com interferon α (IFN α) (Hoyo-Becerra et al., 2014; Moulton et al., 2015).

Outro aspecto em comum que relaciona depressão e inflamação, é o conhecido “sickness behavior”, que é desencadeado por citocinas pró inflamatórias produzidas por células do sistema imune inato, como forma de defesa do organismo ao entrar em contato com padrões moleculares associados a patógenos (PAMPs). Essas citocinas incluem principalmente IL-1 (α e β), IL-6 e TNFα (Dantzer et al.,2008; Dantzer, 2009). Portanto “sickness behavior” divide características similares entre doenças de cunho inflamatório e depressão, como mal estar, fraqueza, perda de apetite, anedonia e cansaço (Dantzer, 2009; Dobos et al., 2012) entretanto, na doença inflamatória esses sintomas cessam quando o patógeno é retirado, na depressão não (Dobos et al., 2012).

1.3. Diabetes mellitus e DEPRESSÃO

Pesquisas apontam que o risco de pacientes com DM desenvolverem depressão é de 15 a 20% maior do que o risco da população não diabética (Lustman et al., 1992; Gavard et al., 1993; Talbot et al., 2000; Anderson et al., 2001). Especificamente, a prevalência de depressão em pacientes com DM2 varia cerca de 11 a 32% (Ali et al., 2006), enquanto em pacientes com DM1 varia de 8 a 12% (Lustman et al., 2005). Em contrapartida, estudos relataram que alguns pacientes depressivos apresentaram níveis de glicose em jejum elevada (Kahn et al., 2011) tolerância a glicose prejudicada (Hennings et al.,

2010) e intolerância a insulina (Okamura et al., 2000) quando comparados a outros pacientes, indicando que a depressão também é um fator de risco para desencadear algum tipo de diabetes (Eaton et al., 1996; Golden et al., 2008). Portanto, tem sido proposta e discutida uma relação bidirecional entre diabetes e depressão (Golden et al., 2008; Downs and Faulkner, 2015; Prabhakar et al., 2015; Moulton et al., 2015; Petrak et al., 2015; Zanoveli et al., 2015). Interessante que estudos pré-clínicos também demonstraram um comportamento do tipo depressivo mais expressivo em animais com diabetes induzido experimentalmente (Gomez and Barros, 2000; da Silva Haeser et al., 2007; Wayhs et al., 2010, 2013; de Morais et al., 2014). Muitos desses estudos usaram testes comportamentais para detectar o comportamento do tipo depressivo, como o teste da natação forçada (Porsolt et al., 1977, 1978), teste da natação forçada modificado (Detke et al., 1995) e o teste da suspensão pela cauda (Cryan et al., 2005)

Considerando a alta incidência de depressão em pacientes diabéticos (Lustman et al., 1992, 2005; Gavard et al., 1993; Ali et al., 2006) torna-se cada vez mais necessária a busca por tratamentos eficazes, visto que os tratamentos farmacológicos disponíveis muitas vezes não são satisfatórios (para uma revisão ler Zanoveli et al., 2015). Acredita-se que a falta de conhecimento do mecanismo fisiopatológico por trás dessa relação bidirecional, é o maior entrave para se alcançar a cura (Prabhakar et al., 2015). Porém, dentre as hipóteses disponíveis para explicar esse mecanismo, a desregulação do sistema imunológico parece ser a mais plausível (Leonard, 2014), visto que ambas as condições, tanto diabetes quanto depressão, apresentam resposta inflamatória em sua fisiopatologia (Smith, 1991; Downs and Faulkner, 2015).

Nesse contexto, torna-se interessante estudar a Indoleamina-2,3-dioxigenase (IDO), por ser uma enzima ativada por citocinas pró-inflamatórias que desvia a via metabólica do aminoácido triptofano, diminuindo consequentemente a produção de serotonina (5-HT) e produzindo substâncias neurotóxicas.

1.4. INDOLEAMINA-2,3-DIOXIGENASE (IDO)

O triptofano (TRIP) é um aminoácido essencial que em condições fisiológicas é requisitado para a biossíntese de proteínas e outros compostos, como a serotonina (5-HT) (Maes, 2011). Normalmente, a maioria do TRIP ingerido na dieta (95%) é degradado no fígado, entretanto também pode ocorrer metabolismo extra hepático pela via das quinureninas gerando os TRYCATs (catabólitos de TRIP), que podem ser eliminados através da urina ou são gradualmente processados a uma série de outros compostos biologicamente ativos, com importantes implicações fisiológicas e patológicas ao organismo (Maes et al., 2011).

Quando o metabolismo do TRIP é extra-hepático, geralmente ocorre pela via das quinureninas através da enzima IDO, e embora essa oxidação seja quase irrelevante em situações fisiológicas, em situações patológicas exerce grande importância devido a capacidade da IDO de ser ativada por citocinas pró-inflamatórias como interferon γ (IFN-γ), fator de necrose tumoral (TNF-α) e interleucinas (IL-1 e IL-6) (Dantzer , 2009; Maes et al., 2011). A degradação do TRIP pela via das quinureninas (desencadeada pela atividade da IDO) tem grande importância neuropsiquiátrica, pois a degradação desse aminoácido implica na diminuição de produção de 5-HT, consequentemente comprometendo a neurotransmissão serotoninérgica. Considerando que a IDO é expressa no cérebro sua ativação pode afetar diretamente a síntese deste

neurotransmissor (Dantzer et al., 2008). O primeiro metabólito do TRIP que aparece na cascata enzimática da IDO é a quinurenina, que posteriormente é degradada em 3 hidroxi-quinurenina (3-HK), ácido quinolínico (QA) e ácido quinurênico (KA) (Maes, 2011; Maes et al., 2011; Dantzer et al., 2008; Dantzer, 2009), sendo o KA um antagonista NMDA, o QA um agonista NMDA e potente toxina, e o 3-HK um gerador intenso de espécies reativas de oxigênio causando estresse oxidativo.

Vários estudos pré clínicos já mostraram a atividade da IDO diminuindo 5-HT e aumentando TRYCATs em animais que passaram por algum desafio imunológico (Dantzer et al., 2008; Dantzer, 2009; Dantzer et al., 2011; OConnor et al., 2009; Maes, 2011; Maes et al., 2011; Dobos et al., 2012). Outros mostraram que diabetes e inflamação vem sendo associados com alterações nos níveis de dopamina, BDNF e serotonina, ambos implicados também na patofisiologia da depessão (Duman and Monteggia, 2006; Downs and Faulkner, 2015) sugerindo um provável mecanismo em comum.

Com o surgimento da hipótese monoaminérgica para explicar a neurobiologia da depressão, desde a década de 60 estudos relacionam o deficit de níveis de serotonina com sintomas depressivos. Relembrando que o TRIP é o precurssor de 5-HT, vários trabalhos já mostraram que em pacientes depressivos os níveis de TRIP estão diminuídos, seja o TRIP livre no plasma, o TRIP total ou a relação TRIP/outros aminoácidos (Joseph et al., 1984; Moller, 1985; Maes et al., 1990; 1991), mesmo após altas doses de TRIP admnistradas via oral ou intravenosa, pacientes depressivos obtiveram diminuição de TRIP plasmático (Maes and Meltzer, 1995). Consequentemente, a depleção de TRIP periférico implica em menor atividade serotoninérgica no SNC desses

indivíduos (Smith et al., 2000; Yatham et al., 2001). Interessante que drogas que aumentam os níveis de 5-HT ou melhoram sua atividade, diminuiram o comportamento do tipo depressivo em animais em pesquisas pré clínicas (Detke et al., 1995; Cryan and Lucki, 2000; Qiu et al., 2014; Du et al., 2014). Nesta mesma direção, estudos clínicos também mostraram que drogas que inibem a recaptação de serotonina atuaram como potentes antidepressivas (Hochstrasser et al., 2001; Kornstein et al., 2006; para uma revisão ler Fitzgerald and Bronstein, 2013).

Interessante que desregulação de neurotransmissores no SNC também é observado na condição diabética (Gupta et al., 2014a, 2014b; Abraham et al., 2010a, 2010b; Li and France, 2008; Umeda et al., 2007; Bellush et al., 1991). E assim como pacientes depressivos apresentaram níveis plasmáticos de TRIP diminuídos, essa mesma redução de TRIP foi observada em pacientes diabéticos (Maes et al., 1990; Cowen et al., 1989, Herrera et al., 2003; Manjarrez et al., 2006). Mais ainda, animais diabéticos apresentaram diferenças nos níveis de 5-HT de acordo com a região cerebral estudada, quando comparados a ratos normoglicêmicos (Ezzeldin et al., 2014). Por exemplo, os níveis serotoninérgicos de animais diabéticos estavam diminuídos no tálamo/hipotálamo, cerebelo e tronco cerebral, enquanto que no cortéx cerebral e no mesencéfalo esses níveis estavam aumentados (Ezzeldin et al., 2014). Com isso, fica evidente que existe uma desregulação serotoninérgica, tanto na condição depressiva quanto na diabética, que parece ser dependente da área encefálica avaliada. Essa desregulação se extende também sobre alguns tipos de receptores serotoninérgicos, ou seja, estudos mostram alteração na responsividade e/ou atividade de receptores serotoninérgicos

principalmente dos subtipos 5-HT_{1A} e 5-HT_{2A} em diferentes áreas encefálicas (Abraham et al., 2010; Li and France, 2008; Umeda et al., 2007).

Considerando o exposto acima, a via IDO/quinurenina se torna um alvo interessante a ser investigado quando se estuda depressão associada ao diabetes, pois envolve inflamação e desregulação serotoninérgica, ambas condições encontradas nas duas patologias.

1.5. HIPÓTESE

A depressão associada ao diabetes pode ser resultante da ativação do sistema imunológico com consequente ativação da enzima IDO.

2 OBJETIVO

O objetivo do nosso estudo foi investigar o efeito do tratamento subcrônico de inibidores da IDO (direto ou indiretos), sobre o comportamento do tipo depressivo desecadeado por animais diabéticos induzidos por estreptozotocina, no teste de natação forçada modificado.

3 ARTIGO CIENTÍFICO

TITLE PAGE

Indoleamine 2,3-dioxygenase/kynurenine pathway as a potential pharmacological target to treat depression associated with diabetes.

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3.1 Abstract

Diabetes is a chronic disease associated with depression whose pathophysiological mechanisms that associate these conditions are not fully elucidated. However, the activation of the indoleamine-2,3-dioxygenase (IDO), an enzyme that participate of the tryptophan metabolism leading to a decrease of serotonin (5-HT) levels and whose expression is associated with an immune system activation, has been proposed as a common mechanism that links depression and diabetes. To test this hypothesis, diabetic (DBT) and normoglycemic (NGL) groups had the cytokines (TNFα, IL-1 β and IL-6) and serotonin (5-HT) and noradrenaline (NE) levels in the hippocampus (HIP) evaluated. Moreover, the effect of the selective serotonin reuptake inhibitor fluoxetine, IDO direct inhibitor 1-methyl-tryptophan, anti-inflammatory and IDO indirect inhibitor minocycline or non-selective cyclooxygenase inhibitor ibuprofen was evaluated in DBT rats submitted to the modified forced swimming test (MFST). After the behavioral test, the HIP was obtained for IDO expression by Western blotting analysis. DBT rats exhibited a significant increase in HIP levels of TNFα, IL-1 β and IL-6 and a decrease in 5-HT and NA. They also presented a depressive-like behavior which was reverted by all employed treatments. Interestingly, treatment with drugs that present anti-inflamatory proprieties (MINO, IBU and FLX) reduced the increased IDO expression in the HIP from DBT animals. Taken together, our data support our hypothesis that neuroinflammation in the HIP followed by IDO activation with a consequent decrease in the 5-HT levels can be a possible pathophysiological mechanism that links depression to diabetes.

Keywords: streptozotocin, serotonin, depression, indoleamine 2,3-dioxygenase inflammation, hippocampus.

3.2 Introduction

Diabetes, a metabolic condition characterized by chronic hyperglycemia [1], is considered one of the most common metabolic disease worldwide [2-4]. An important feature of diabetes is its ability to develop comorbidities, such as heart disease [5], retinopathy [6], neuropathic pain [7] and psychiatric disorders [8]. Among the psychiatric comorbidities associated with diabetes, depression is the most studied [9] and is characterized by depressed mood, anhedonia, reduced energy, feelings of guilt or low self-worth, sleep or appetite changes and lack of concentration [10]. Notably, it has been reported a higher incidence of depression in diabetic patients when compared to non-diabetics [11-13], for a review see [14], and also there is evidence showing that diabetic patients are more likely to develop depression [13].

Preclinical studies have also shown that diabetic animals exhibit a more pronounced depressive-like behavior when compared to non-diabetic animals [15,16,1,17,18]. In that respect, it has been pointed out that diabetes and depression could have a bidirectional relation [19,20], and possibly share common pathophysiological mechanisms. However, these pathophysiological mechanisms are not fully understood. In an attempt to better understand the relationship between these conditions and its consequences, some hypotheses have been proposed (for a review see [14]). Of interest for this study, it has been shown that inflammation plays an important role in the pathophysiology of depression [21-23] as well as in the diabetes development [24,25,7]. Indeed, it has already been demonstrated that diabetes and/or depression states stimulate the immune system inducing the secretion of pro-inflammatory cytokines [26-28]. Conversely, it has been proposed that the brain overexpression of pro-inflammatory mediators is associated with mechanisms which can lead to depression, *i.e*., an increase of neurotoxic substances and a decrease of serotonin, a monoamine extremely implicated in the pathophisiology of depression [29]. In that sense, an enzyme called indoleamine 2,3-dioxygenase (IDO) seems to be directly involved in this process for being activated by some pro-inflammatory mediators. It is known that IDO is responsible for degrading tryptophan leading a significant decrease of the serotonin synthesis and also raises the production of tryptophan

catabolites with important neurotoxic properties, such as kynurenine, xanthurenic acid and quinolinic acid [30,31,29]. Thus, the activation of IDO can be a causal factor linking depression and diabetes. However, to our knowledge, there are no studies investigating the possible interrelationship between the inflammation and the more pronounced depressive-like response observed in diabetic animals, especially about the characterization of the functional role of the IDO/kinurenine pathway.

Based on the above, we test the hypothesis that the exacerbated depressive like-behaviors in diabetic animals could be associated with a decrease of the hippocampal levels serotonin caused by an increased expression of IDO triggered by brain neuroinflammation and pro-inflammatory cytokines production. For this, diabetic animals were treated with drugs that act inhibiting the activity of the IDO and/or reducing the inflammatory processes and then tested in modified forced swimming test to verify a possible antidepressant-like effect and also a potential effect of hippocampal expression of IDO.

3.3 Material and Methods

3.3.1 Animals

All experiments were conducted in adult male Wistar rats (weighing 180-240 g) provided by the Federal University of Paraná colony. Animals were maintained in a temperature-controlled room (22 ± 2°C) under 12h/12 h light/dark cycle (lights on at 7 a.m.) with food and water available *ad libitum.* All animals (four rats/cage) were housed in plastic cages (41 x 32 x 16.5 cm) changed every day due to polyuria induced by the diabetic condition. Behavioral experiments (modified forced swimming test and open field test) were conducted during the light phase of the cycle (between 9 a.m. and 4 p.m.). All experiments were approved (protocol #748) and conducted in accordance with the rules and laws contained by the Ethics Committee for Research on Animals

UFPR (CEUA/BIO-UFPR). All efforts were made to minimize the number of rats and their suffering.

3.3.2 Drug and treatment

The following substances were used: Streptozotocin (STZ, Santa Cruz Biotechnology Inc., Santa Cruz, California, USA), sodium citrate (Merck S.A. Indústrias Farmacêuticas, Brazil), 1 Methyl-DL-tryptophan (1-MT, Sigma-Aldrich, USA), Ibuprofen (Sigma-Aldrich, USA), Minocycline hydrochloride (Sigma-Aldrich, USA) and Fluoxetine (Daforin – Sigma Pharma, Brazil). STZ (60 mg/Kg, i.p.) was dissolved immediately before use in citrate buffer (10 mM, pH 4.5). 1-MT (1, 3 and 9 mg/kg, i.p.) and tetracycline minocycline (60 mg/kg, i.p.), which directly or indirectly blocks the IDO, respectively, were dissolved in phosphate buffered saline (PBS) with several drops of diluted HCl and NaOH for pH adjustment. The anti-inflammatory ibuprofen (5, 15 and 30 mg/kg, p.o.) and the antidepressant fluoxetine (10 mg/kg, i.p.) were dissolved in sterile saline. The doses were based on previous studies [32-34] or in pilot studies conducted in our laboratory.

3.3.3 Induction of diabetes

The diabetes was induced by a single intraperitoneal (i.p.) injection of streptozotocin (STZ; 60 mg/Kg), freshly dissolved in citrate buffer (10 mM, ph 4.5) in overnight fasten rats. The diabetic condition was confirmed 72 h after the STZ treatment using samples of about 5 µL of blood from the tail vein added to test strips impregnated with glucose oxidase (Accu-Check Active^{TM}, Roche). Only rats with blood glucose levels ≥ 250 mg/dL were considered diabetic and kept in the study.

3.3.4 Open-field test (OFT)

The apparatus consists of a rectangular box (40×50×63 cm) with a floor divided into 6 rectangular units. The OFT was performed as described by de

Morais and collaborators (2014) [18]. In order to assess whether the different treatments or the condition (normoglycemic or diabetic) alter the locomotor activity, all animals were placed in the center of the rectangular apparatus and the number of squares crossed with the four paws was evaluated during 5 min. The open-field was cleaned with a 5% water–ethanol solution before each animal tested to eliminate possible bias due to odors left by previous rats. All tests were video recorded by a camera and analyzed after the experiments.

3.3.5 The modified forced swimming test (MFST)

The forced swimming test was originally described by the Porsolt and collaborators (1977) [35] and modified by Detke et al. (1995) [36]. Briefly, the test was conducted in two sessions. During the pre-test session the rats were placed in opaque plastic cylinder (20 × 20 × 40 cm) containing water at a depth of 30 cm and a temperature of 24 ± 1°C for 15 min. After 24 hs, the animals were submitted to a test session of 5 min. The test session was video recorded by a camera positioned above the cylinder for later analysis. At the end of each 5 s period, the frequency of the active (swimming or climbing) or passive (immobility) behaviors was quantified [36,37]. Immobility is characterized by the display of only a few movements necessary to keep the head above water, without struggling and leading to a float of the rats in the cylinder of water, demonstrating a passive movement. Swimming is described by active swimming motions, more than necessary to maintain his head above water, e.g. moving around the apparatus. Finally, climbing was quantified when animals displayed active movements with forepaws, in and out of cylinder of water, usually directed against the walls vertically [36]. Following the same protocol used in the forced swimming test described by [35], the treatments were conducted 23.5 h, 5 h and 1 h before the MFST. After each session (pre-test or test sessions), the animals were dried with a towel and returned to their home cages.

3.3.6 Determination of noradrenaline and serotonin hippocampal levels by high performance liquid chromatography (HPLC).

The animals were killed by decapitation and the brains were removed from the skull and placed on a cold surface of glass. Next, the hippocampus was dissected out. The HPLC system was equipped with a reverse-phase column (Hypurity Elite C18, 250 mm x 4.6 mm, 5 µm and 100-Å pore diameter particle size; Hypersil, Cheshire, UK), coupled with electrochemical detection. The hippocampus slices were homogenized in 0.2 M perchloric acid containing dihydroxy-benzylamine (DHBA), centrifuged at 15,000 rpm for 20 min at 6 ºC and stored at -70 ºC for 15 days, and 50 µL was injected into the HPLC-EC system. The addition of DHBA to the hippocampus extracts from normoglycemic and diabetic rats served as an internal standard control. The HPLC system consisted of a Shimadzu LC-10 AD chromatograph, with a CBM-10A communication bus module, an online DGU-14A degassing unit, and an L-ECD-6ª electrochemical detector with a glassy-carbon electrode and an LC-10 AD pump. The potential was set at 850 mV (versus an Ag/AgCl reference electrode). The mobile phase containing 150 mM chloroacetic acid, 120 mM NaOH, 0.67 mM EDTA, 0.86 mM sodium octylsulfate, 3.5% acetonitrile, and 2.6% tetrahydrofuran, adjusted to pH 3.0, was filtered and pumped through the system at a rate of 1.2 mL/min. All substances were quantified by comparing the peak areas to standard curves [38].

3.3.7 Quantification of hippocampal levels of pro inflammatory cytokines (TNF α, IL-1 β and IL-6) by ELISA

The animals were sacrificed by decapitation and the brains were removed from the skull for the hippocampus dissection. Then, hippocampal samples were collected in PBS and processed by maceration, and centrifuged at 10,000 rpm (10 min) at 4 °C. The supernatant was used to evaluate the IL-1 β, IL-6 and TNF α levels by immunoenzymatic method (ELISA). Briefly, 96 well plates were coated with 50 µL/well of specific antibody against the protein of interest (Pharmingen, San Diego, CA, USA). This antibody was diluted in binding solution pH 9.0 and incubated for 18-24 hour at 4° C. The plates were then

washed three times with PBS/Tween 20 (0.05% Sigma). The non-specific binding was blocked with 100 µL of PBS/1% BSA for 120 min at room temperature. The samples and standard (standard curve) containing the concentrations for the cytokines were placed on the plates (50 µL) and incubated for 18-24 hour at 4° C. After this period, the plates were washed with PBS/Tween and 50 µL of biotinylated antibodies against specific cytokines were added. After one hour, the plates were washed with PBS/Tween and the avidin-peroxidase conjugate, diluted 1: 5000 was added to each well. After 30 minutes incubation, the plates were washed with PBS/Tween and 100 µL of *orto*-phenylenediamine dihydrochloride (Sigma-Aldrich) were added. The plates were then incubated for 15 to 20 min at room temperature. The reaction was stopped with 50 µl of 1M H_2SO_4 and the optical density measured at 490 nm on a spectrophotometer (Spectra Max 250, Molecular Devices). The results were expressed as picograms/ mg of total protein measured by Braford method [39].

3.3.8 Quantification of expression of hippocampal indoleamine-2,3-dioxygenase (IDO) by Western Blot

The hippocampi was homogenized in ice-cold lysis buffer (25 mM Tris-HCl pH 7,4, 150 mM NaCl, 5 mM MgCl2, 0,3% Triton X-100 and Complete Protease Inhibitor Cocktail (Roche)) and the protein concentration determined using the Bradford assay. Hippocampal lysates (40µg) were boiled in Laemmli sample buffer for 5 minutes at 95ºC for 5 minutes and then subjected to 10% SDS-PAGE under reducing condition followed by transference of proteins to nitrocellulose membranes (GE Healthcare). Membranes were blocked with TBS-Tween 20 (120 mM NaCl, 20mM Tris-HCl pH 7,4 and 0,05% Tween-20) containing 5% nonfat dry milk and analyzed with anti-IDO antibody (Santa Cruz). Anti-βactin antibody (Sigma) was used for protein loading control. Reactions were developed with Westar ECL-Sun (Cyanagen) or Westar SuperNova (Cyanagen) chemiluminescent Substrate for Western Blot and exposed to autoradiogram film (Carestream). The bands were quantified by densitometry analysis using ImageJ software (USA).

3.4 Statistical analysis

The parametric data are reported as mean ± standard error mean (SEM). The data of OFT, MFST, western blotting and cytokines analysis were analyzed by one-way analysis of variance (one-way ANOVA) considering as independent factor the different groups. When appropriated, Newman-Keuls tests were used for *post-hoc* analyses. For the neurochemical analysis, unpaired test *t* de Student was applied. Differences were considered statistically significant when $p \leq 0.05$.

3.5 Experimental design

3.5.1 Experiment 1: Quantification of hippocampal levels of pro inflammatory cytokines (TNFα, IL-1β and IL-6) of diabetic and normoglycemic animals.

To establish the neuroinflammation peak, rats were distributed randomly into 2 groups: normoglycemic (NGL) and diabetic (DBT) animals. Two, three or four weeks after STZ treatment (DBT 2^{nd} , DBT 3^{rd}, DBT 4^{th}), DBT rats were euthanized and had their hippocampus dissected for TNFα, IL-1β and IL-6 quantification by ELISA method. For comparative purposes, the hippocampal levels of these ctytokines were also evaluated in a normoglycemic group.

3.5.2 Experiment 2: Evaluation of levels of noradrenaline (NE) and serotonin (5-HT) in the hippocampus of diabetic and normoglycemic animals.

Four weeks after diabetes induction, STZ-diabetic rats were euthanized for hippocampus dissection. The tissue analysis of serotonin (5-HT) and noradrenaline (NE) levels were evaluated by HPLC method, as described above. Similarly, for comparative purposes, a normoglycemic group had the hippocampal levels of 5-HT and NE evaluated.

3.5.3 Experiments 3, 4, 5 and 6: Behavioral responses of diabetic animals treated with drugs that directly or indirectly inhibit the IDO activity: evaluation in the OFT and MFST.

Four weeks after STZ injection or its vehicle, all animals were submitted to the pre-test session of the MFST and in the next day to the MFST itself. DBT animals were randomly distributed into different groups. For the experiment 3, DBT animals were treated with FLX which was used as a positive control for the antidepressant-like effect and also by acting increasing the 5-HT availability in the synaptic cleft. The following groups were performed: DBT treated with vehicle (VEH) and DBT-treated with antidepressant fluoxetine (FLX; 10 mg/mg; i.p.). For the experiment 4: DBT-VEH and DBT-treated with IDO direct inhibitor 1-methyl-tryptophan (1-MT) at three different doses (1, 3 or 9 mg/kg; i.p.). In the experiment 5, DBT/VEH and DBT-treated with an indirect inhibitor of IDO minocycline (MINO; 60 mg/kg, i.p.). Finally, in the experiment 6: DBT-VEH, DBT-treated with anti-inflammatory ibuprofen at three different doses (IBU; 5, 15 or 30 mg/kg; p.o). In all experiments, as a control of the DBT condition, a normoglycemic (NGL) group treated with VEH (NGL-VEH) was conducted in parallel and submitted to the same protocols. All the treatments (vehicle or drugs) followed the same schedule: 1st injection: after the pre-test session; 2nd injection: 5 hours before the OFT/MFST and finally, the 3rd injection: 1 hour before the OFT/MFST.

3.5.4 Experiment 7: Evaluation of indoleamine-2,3-dioxygenase (IDO) expression in the HIP from treated and untreated diabetic animals.

After the behavioral tests (Experiments 3, 4, 5 and 6), the animals were decapitated and the HIP was dissected to investigate the effects of the different treatments (VEH, FLX, 1-MT, MINO and IBU) on the expression of the IDO by Western blot, as described above. For comparative purposes, a normoglycemic group was also conducted in parallel.

3.6 Results

3.6.1 Levels of pro inflammatory cytokines (TNF α, IL-1 β and IL-6) are increased in the hippocampus of diabetic animals.

Fig. 1 shows that the DBT condition induced a significant increase in the hippocampal levels of TNFα (panel A; [$F(3,23)=4.32$; $p \leq 0.05$], IL-1β (panel B; [$F(3,23)=4.49$; $p \leq 0.05$])and IL-6 (panel C; [$F(3,23)=2.90$; $p \leq 0.05$]). For all cytokines evaluated, this difference was observed only at the fourth week after diabetes induction ($p \leq 0.05$).

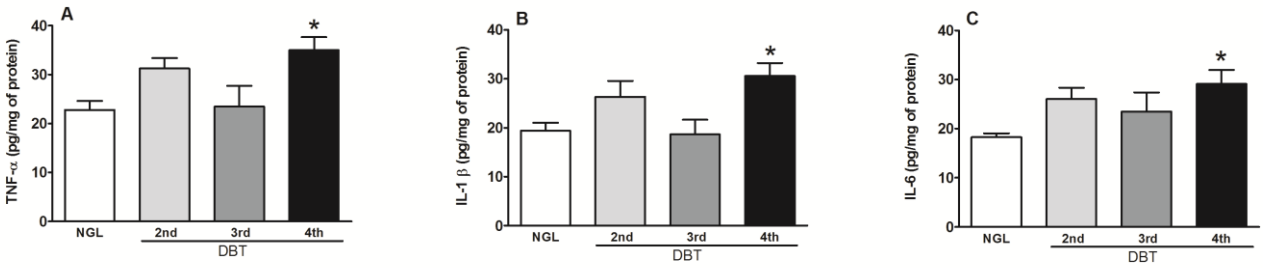

Fig. 1 Hippocampal levels of TNF α (panel A), IL-1 β (panel B) and IL-6 (panel C) in STZ-diabetic (DBT) animals in different weeks (2^{nd}, 3^{rd} and 4^{th}) after diabetes induction. Data are expressed as mean ± SEM (n=6). *p≤0.05 when compared to the normoglycemic (NGL) group.

3.6.2 Levels of noradrenaline and serotonin are decreased in the hippocampus of diabetic animals.

As can be observed in the Fig. 2, DBT animals had a significant reduction in the hippocampal levels of noradrenaline ($t(13)=4.00$; $p \leq 0.05$) and serotonin ($t(13)=2.37$; $p \leq 0.05$).

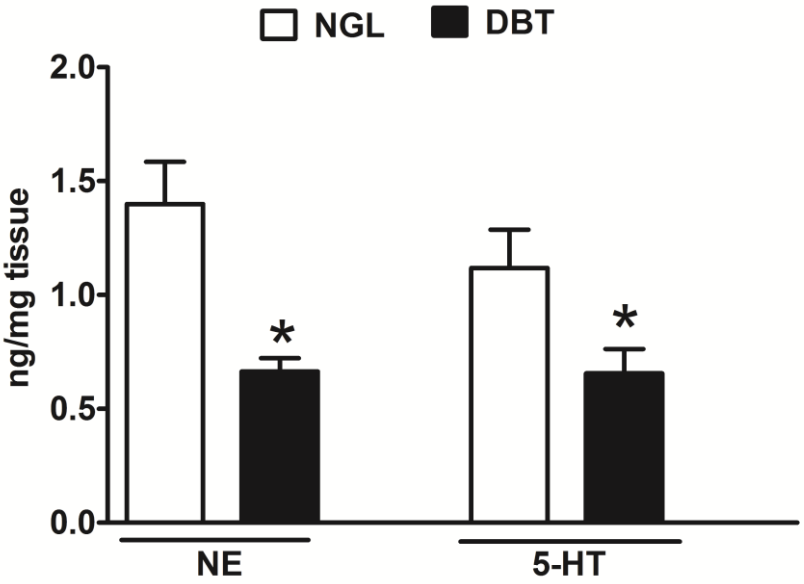

Fig. 2 Levels of noradrenaline (NA) and serotonin (5-HT) in the hippocampus of normoglycemic (NGL) or STZ-diabetic rats (DBT) at the 4th week after diabetes induction. Data are expressed as mean ± SEM (n=7-8). *p≤0.05 when compared to the NGL group.

3.6.3 Effect of treatment with antidepressant fluoxetine (FLX) over behavioral responses of diabetic animals in the MFST.

One-way ANOVA showed a significant difference between the groups concerning the frequency of immobility ([$F(2,17)=6.66$; $p \leq 0.05$]; Fig. 3, panel A), swimming ([$F(2,17)=7.41$; $p \leq 0.05$]; Fig. 3, panel B) and climbing ([$F(2,17)=7.41$; $p \leq 0.05$]; Fig. 3, panel C). When compared to vehicle(VEH)-treated normoglycemic (NGL) rats, VEH-treated DBT animals displayed an increase in the frequency of immobility ($p \leq 0.05$; Fig. 3, panel A) and a decrease in swimming and climbing frequencies ($p \leq 0.05$; Fig. 3, panels B e C). The treatment with FLX (10 mg/Kg) induced a significant decrease of the immobility frequency ($p \leq 0.05$; Fig. 3, panel A) and an increase of the frequency of

swimming in DBT rats ($p \leq 0.05$, Fig. 3, panel B), but no changes in the climbing frequency (Fig. 3, panel C).

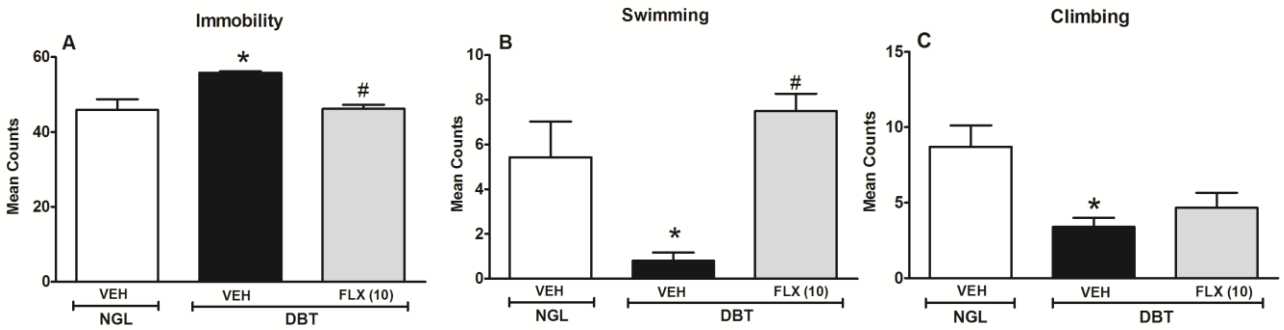

Fig. 3. Effect of treatment with fluoxetine (FLX; 10 mg/Kg, i.p.) on the frequency of immobility (panel A), swimming (panel B) or climbing (panel C) evaluated in the MFST. Data are expressed as mean ± SEM (n =5-7). *p≤0.05 when compared to the normoglycemic (NGL) control group treated with vehicle (VEH). #p≤0.05 when compared to the diabetic (DBT) control group treated with VEH.

3.6.4 Effect of treatment with an IDO direct inhibitor 1 methyltryptophan (1-MT) over behavioral responses of diabetic animals in the MFST.

One-way ANOVA showed difference between the groups when frequency of immobility ([F(4,38)=12.15; $p \leq 0.05$]; Fig. 4 panel A), swimming ([F(4,38)=12.63; $p \leq 0.05$]; Fig. 4, panel B) or climbing ([F(4,38)=4.97; $p \leq 0.05$]; Fig. 4, panel C) was evaluated. Newman Keuls *post hoc* test demonstrated that when compared to NGL group, control DBT animals (treated with corresponding VEH) exhibited a significant increase in the frequency of immobility ($p \leq 0.05$) and a significant reduction of the swimming and climbing frequencies ($p \leq 0.05$). Concerning the effect of the 1-MT, Newman Keuls *post hoc* test showed that the treatment with

doses of 3 or 9 mg/Kg, but not of 1 mg/Kg, significantly decreased the frequency of immobility and significantly increased the frequency of swimming in DBT animals ($p \leq 0.05$). However, the treatment with 1-MT (at all tested doses) was not able to alter the frequency of climbing in the DBT animals ($p > 0.05$).

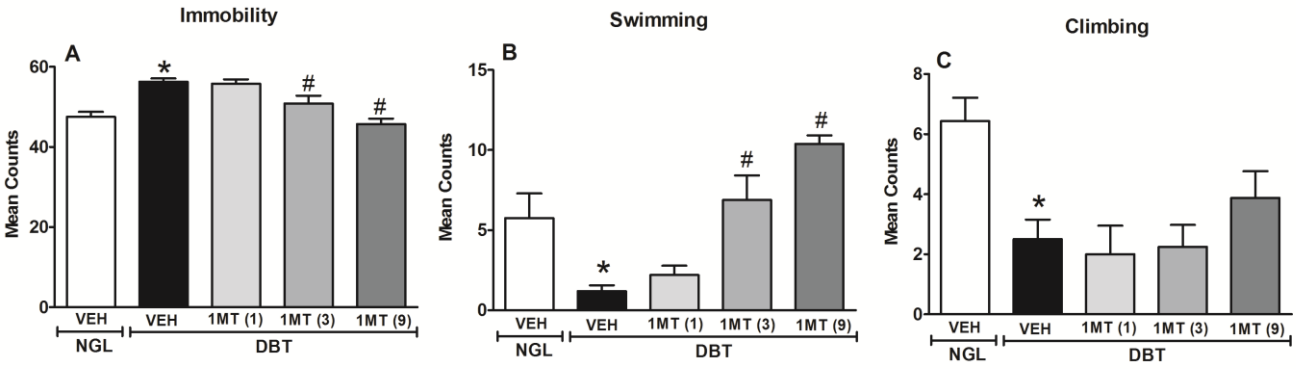

Fig. 4 Effect of treatment with 1 methyltryptophan (1-MT; 1, 3 or 9 mg/Kg, i.p.) on the frequency of immobility (panel A), swimming (panel B) or climbing (panel C) evaluated in the MFST. Data are expressed as mean ± SEM (n=5-10). * p≤0.05 when compared to the normoglycemic (NGL) control group treated with vehicle (VEH). #p≤0.05 when compared to the diabetic (DBT) control group treated with VEH.

3.6.5 Effect of treatment with an IDO indirect inhibitor minocycline (MINO) over behavioral responses of diabetic animals in the MFST.

One-way ANOVA showed a significant difference between the groups when frequency of immobility ([$F(2,24)=6.61$; $p \leq 0.05$]; Fig. 5, panel A), swimming ([$F(2,24)=4.30$; $p \leq 0.05$]; Fig. 5, panel B) and of climbing ([$F(2,24)=6.10$; $p \leq 0.05$]; Fig. 5, panel C) was evaluated. As observed previously, VEH-treated DBT animals exhibited an increase in the passive behavior ($p \leq 0.05$; Fig. 5, panel A) and a decrease in active behaviors ($p \leq 0.05$; Fig. 5, panels B e C). The treatment with MINO (60 mg/Kg) significantly increased the frequency of

swimming in DBT rats ($p \leq 0.05$, Fig. 5, panel B), without altering the others parameters (Fig. 5, panel A and C).

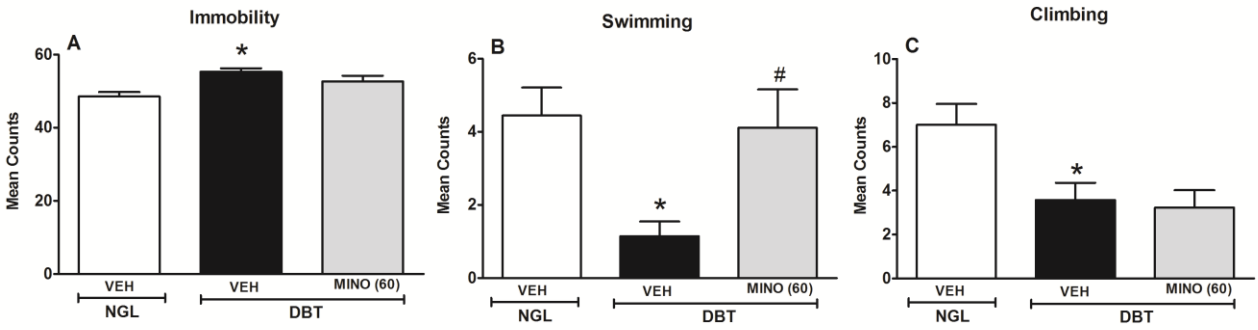

Fig. 5 Effect of treatment with minocycline (MINO; 60 mg/Kg, i.p.) on the frequency of immobility (panel A), swimming (panel B) or climbing (panel C) evaluated in the MFST. Data are expressed as mean ± SEM (n=7-9). * p≤0.05 when compared to the normoglycemic (NGL) control group treated with vehicle (VEH). #p≤0.05 when compared to the diabetic (DBT) control group treated with VEH.

3.6.6 Effect of treatment with a non- steroidal anti-inflammatory ibuprofen (IBU) over behavioral responses of diabetic animals in the MFST.

One-way ANOVA showed a significant difference between the groups regarding the immobility ([F(4,37)=12.17; $p \leq 0.05$]; Fig. 6, panel A), swimming ([F(4,37)=13.63; $p \leq 0.05$]; Fig. 6, panel B) and climbing ([F(4,37)=4.64; $p \leq 0.05$]; Fig. 6, panel C) frequencies. Again, when compared to VEH-treated NGL rats, VEH-treated DBT animals displayed an increase in the passive behavior ($p \leq 0.05$; Fig. 6, panel A) and a decrease in active behaviors ($p \leq 0.05$; Fig. 6,

panels B e C). The treatment with IBU, only at the highest dose (30 mg/Kg) induced a significant decrease of the immobility frequency ($p \leq 0.05$; Fig. 6, panel A) and an increase of the swimming frequency in DBT rats ($p \leq 0.05$, Fig. 6, Panel B), but no changes in the climbing frequency (Fig. 6, panel C).

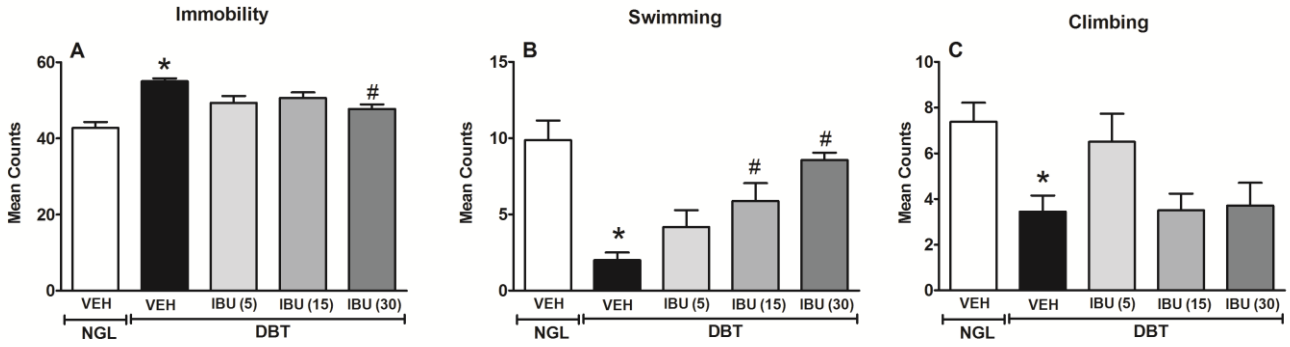

Fig. 6 Effect of treatment with ibuprofen (IBU; 5, 15 or 30 mg/Kg, p.o.) on the frequency of immobility (panel A), swimming (panel B) or climbing (panel C) evaluated in the MFST. Data are expressed as mean ± SEM (n=6-9). * p≤0.05 when compared to the normoglycemic (NGL) control group treated with vehicle (VEH). #p≤0.05 when compared to the diabetic (DBT) control group treated with VEH.

The increase of indoleamine-2,3-dioxygenase (IDO) expression in HIP from diabetic animals was reverted by treatmet with FLX, MINO and IBU.

As can be observed in the Fig. 7, the one-way ANOVA showed difference between the groups ([$F(5,26)=54.88$; $p \leq 0.05$]). Newman Keuls *post hoc* test demonstrated that when compared to NGL group, control DBT animals (treated with corresponding vehicle) exhibited a significant increase in the IDO

expression ($p \leq 0.05$) which was significantly reduced ($p \leq 0.05$) after treatment with FLX (10 mg/kg), MINO (50 mg/kg) or IBU (30 mg/kg).

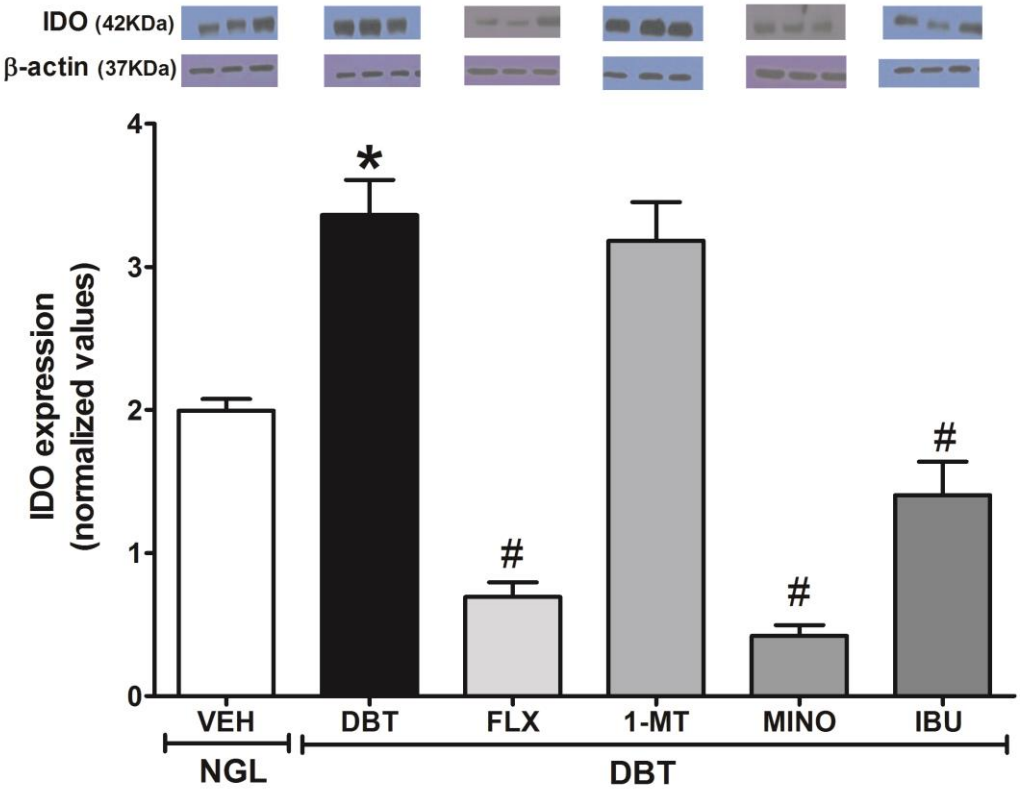

Fig. 7 Effect of treatment with VEH, 1-MT (9 mg/kg; ip), MINO (60 mg/Kg, i.p.), IBU (30 mg/kg; ip) or FLX (10 mg/kg; ip) on expression of IDO into hippocampus. Data are expressed as mean ± SEM (n=4-6). * p≤0.05 when compared to the normoglycemic (NGL) control group treated with vehicle (VEH). #p≤0.05 when compared to the diabetic (DBT) control group treated with VEH.

3.6.7 Treatment with 1-MT, MINO, IBU or FLX did not cause significant changes in the blood glucose, levels, weight gain and in the locomotor activity in diabetic rats.

DBT animals showed a significant decrease in the weight gain ($p \leq 0.05$) and an increased level of blood glucose ($p \leq 0.05$) when compared to NGL group

which were not altered by any treatment employed in this study (see Table 1 - supplementary material). Also, as observed previously by our group [18], DBT rats did not exhibit a significant change in the number of crossings during the OFT. Likewise, none of the treatments in the effective doses were able to induce a significant change of the number of the crossings during the OFT (p>0.05; Table 1 - supplementary material).

Supplementary material – Table 1 - Effect of the condition (diabetic (DBT) or normoglycemic (NGL)) and of the treatments with vehicle (VEH), 1-methyl-tryptophan (1-MT), minocycline (MINO), ibuprofen (IBU) or fluoxetine (FLX) on weight (g), blood glucose (mg/dL) and number of crossings.

Data are expressed as mean ± SEM of 5-10 animals/experimental group. *$p \leq 0.05$ when compared to NGL/VEH.

Experimental Groups	Weight (g; 0 day)	Weight (g; 30th day)	Blood glucose (mg/dL; 3th day)	Blood glucose (mg/dL; 30th day)	Number of crossings (30th day)
DBT /VEH	*202 ± 1.2*	*263± 4.9**	*304.2±15.7**	*306.4±12.7**	*47.1 ± 1.5*
DBT/FLX (10mg/Kg)	*203.5 ± 1.5*	*275.3±7.6**	*391 ± 20.2**	*422.3 ± 24.6**	*49.1 ± 1.7*
DBT/ VEH	*196.1 ± 1.6*	*229.1±4.8**	*422.5± 18.4**	*464.6 ± 15.6**	*49.3 ± 1.8*
DBT/1-MT (1 mg/Kg)	*195.0 ± 2.8*	*224.8±5.4**	*398.2± 31.8**	*438 ± 34.4**	*49.6 ± 3.9*
DBT/1-MT (3 mg/Kg)	*192.6 ± 2.1*	*220.8±4.3**	*395 ± 23.1**	*472.5 ± 15.3**	*44.2 ± 2.1*
DBT/1-MT (9 mg/Kg)	*190.8 ± 2.5*	*229.4±1.8**	*453.1± 25.7**	*480.4 ± 19.6**	*48.6 ± 3.0*
DBT/ VEH	*201.9 ± 1.1*	*276.7±6.5**	*318.6 ±17.7**	*330 ± 22.2**	*48.3 ± 2.8*
DBT/MINO (60 mg/Kg)	*201.3 ± 1.2*	*282.3±3.6**	*380.6 ± 19**	*399.8 ± 21.7**	*47.1 ± 3.2*
DBT/ VEH	*192.6 ± 2.6*	*247.7±6.9**	*481.4± 28.3**	*493.8 ± 21.1**	*50.1 ± 2.9*
DBT/IBU (5 mg/Kg)	*194.2 ± 2*	*261.3±11.4**	*469.3± 16.0**	*508.7 ± 18.2**	40.8 ± *1.1*
DBT/IBU (15 mg/Kg)	*195.8 ± 4.4*	*227.4 ± 6.6**	*401.6± 22.9**	*450.5 ± 32.8**	43.3 ± *1.9*
DBT/IBU (30 mg/Kg)	*205.7 ± 4*	*259.9 ± 9.6**	*514.1± 24.3**	*517.3 ± 28.8**	45.5 ± *2.5*

3.7 Discussion

The major findings of our study are that DBT animals present an increase of depressive-like behaviors associated with a hippocampal increase of pro-inflammatory cytokines and in the IDO expression, besides a significant hippocampal reduction on the 5-HT and NA levels. Furthermore, this is the first study to show that treatment with drugs that block direct or indirectly the IDO enzyme exerted an antidepressant-like effect in DBT animals. Interestingly, treatment with drugs which also have anti-inflamatory properties such as MINO, IBU and FLX was able to reduce the increased IDO expression observed in the HIP from DBT animals. Thus, our data reinforce our hypothesis that inflammation and its consequences may be a common causal factor linking diabetes to depression.

In fact, our data showed that at the fourth week after diabetes induction the DBT animals presented a significant increase in hippocampal levels of pro-inflammatory cytokines (TNFα, IL-1 β and IL-6; Fig. 1). Curiously, the increase of pro-inflammatory cytokines in the HIP from DBT animals was observed only in the fourth week, coinciding with the time required to establish the peak of behavioral alterations associated with depression as observed in previous studies from our lab [18]. Corroborating our data, it has been recently observed that DBT animals showed an increase in the cortex and also in the HIP of neuronal damage, NF-$_K$B, TNFα, IL-1 β, IL-6 and caspase 3 [40]. Also in DBT animals, Dey and colleagues showed an increase of IL-1β and TNFα in HIP induced by chronic treatment with corticosterone [41]. Interestingly, it was observed by Yang and coworkers that the increase of glucose is able to increase the TNFα and IL 6 levels, leading to neuronal apoptosis of hippocampal neurons [42]. The HIP increase of pro-inflammatory cytokines possible is due to the microglia activation as observed by [43]. Previous studies have already demonstrated that high glucose concentrations may lead the apoptosis of the pancreatic β cells by increasing IL-1β production [44,45], compromising the insulin synthesis. Together, our data reinforce the participation of pro-inflammatory cytokines in the pathogenesis of diabetes.

Regarding to the depressive-like response observed in DBT animals, our data support previous findings from our laboratory [18] showing that these

animals have a more pronounced depressive-like behavior when compared to the normoglycemic ones (Figs. 3, 4, 5 and 6). Nevertheless, de Morais and coworkers (2014) [18] used the classical protocol of the forced swimming test proposed by Porsolt et al. (1977) [35] in which only the total immobility time was quantified. Differently, in the present study we evaluated the frequency of the most predominant behavior of the animal every 5 seconds during the modified version of forced swimming test (MFST): the passive behavior of immobility or the active behaviors of swimming or climbing, as proposed by Detke and colleagues (1995) [36]. The main difference of this protocol is that, based on various studies conducted in an attempt to do a predictive validity [46,37,47], it is possible to relate active behaviors with neurotransmitter systems, such as 5-HT and NA. Thus, the increase in swimming behavior is suggestive of an increase in serotonergic neurotransmission, while the increase in the noradrenergic neurotransmission would be indicative of an increase in the climbing behavior [36,46,37,47]. Interestingly, our findings showing that DBT animals have a reduction in 5-HT and NA levels in the HIP (Fig. 2) are consistent with the reduction of swimming and climbing behaviors, respectively, as proposed by many studies validating the MFST [36,46,37,47] and also observed in this study (Figs. 3, 4, 5 and 6). Still, the opposite also seems to be true because in the present study FLX, which mechanism is related to an increase of the 5-HT availability by inhibiting its reuptake, also decreased the immobility and increased the swimming frequencies without interfering in climbing frequency of DBT rats (Fig. 3).

It is well established that a significant dysregulation in 5-HT and NA neurotransmitter systems occurs in depression and diabetes conditions [48-51]. More specifically, studies show that DBT animals presented a dysregulation of the serotonergic system depending on the brain area [48,49], *i.e.* while the 5-HT levels were decreased in the thalamus, hypothalamus, cerebellum and brainstem, it was increased in the cortex and midbrain. Regarding to the NA, it seems that DBT animals have a significant decrease of this neurotransmitter in the cortex, cerebellum and brainstem[49]. Thus, it appears that these changes are brain area-dependent and also may be related to changes in the density or responsiveness of receptors; for example, in relation to serotonergic receptors,

evidence shows these changes occur specially in the 5-HT_{1A} and 5-HT_{2A} receptor subtypes [50,51]. Due to a dysregulation on 5-HT levels, the adaptive changes observed in the serotonergic system and also the well established role of 5-HT in the neurobiology of depression [52,53], it is plausible to hypothesize that the serotonergic system is a multifactorial target involved in the pathophysiology of depression associated with diabetes [54].

Based on the above, it seems that depression associated with diabetes may be due to both inflammation and a dysregulation in the neurotransmitter systems. In our hypothesis, a possible link between these factors may be the activation of IDO enzyme, which plays a key role in tryptophan metabolism and whose expression is increased by pro-inflammatory cytokines. According to our prediction our findings showed an increase in the IDO expression in the HIP from these DBT animals (Fig. 7). This increase has already been described in many experimental situations involving depression and/or inflammation [21,22,55,23]; however, for the first time in the literature this increase was described in the model of diabetes induced experimentally by streptozotocin. We also investigated the functional role of this enzyme on the depressive-like response of these DBT animals by treating these animals with drugs that inhibit direct or indirectly this enzyme. In that way, our data showed that the treatment with the IDO direct inhibitor 1-MT decreased the immobility and increased the swimming frequencies, but not altered the climbing frequency (Fig. 4). Interestingly, this antidepressant-like effect of the 1-MT treatment has already been observed in NGL animals exposed to the FST or to the sucrose preference test [28,56]. Considering the interrelationship of the neurotransmitters and the behavioral responses during the MFST, it is plausible to extrapolate that the increased frequency of swimming in diabetic animals after treatment with 1-MT is associated with increased serotonin consequence of the inhibition of IDO and increased availability of the substrate tryptophan (TRP). Accordingly, it was observed that 1-MT treatment was able to revert the increase of ratio of 3-hydroxykynurenine (3-HK):TRP and the increased in the 5-Hydroxyindoleacetic acid (5-HIAA):5-HT ratios in brain areas of rats submitted to lipopolysaccharide (LPS)- induced depressive-like behavior [55]. Similarly, Dobos and coworkers (2012) [28] observed an increase of IDO in the brainstem

and an increased kynurenine/TRP ratio in the serum of mice treated intracerebroventricularly with LPS. Interestingly, it was reported that inhibition of IDO by 1-MT prevents the development of depressive-like behavior without changing the IDO expression. In that same way, in our study we did not observe any change in the hippocampal IDO expression after treatment with 1-MT (see Fig. 7), suggesting that this compound is acting exclusively by inhibiting the activity, but not the expression of the enzyme. Finally, the lack of the effect of the 1-MT treatment over the climbing frequency may be linked to the absence of effect over the NA neurotransmission.

In the next set of experiment, we investigate the effect of MINO, a drug also described as an indirect inhibitor of IDO activity. There are several reports demonstrating that the tetracycline MINO presents effect as anti-inflammatory, but also as neurotrophic and neuroprotective agent [57-62]. In our study, the treatment with MINO induced a significant increase in the swimming frequency in DBT animals, suggesting an antidepressant-like effect, without significant changes in the climbing and immobility frequencies (Fig. 5). It is important to highlight that the treatment with MINO, which is associated with an indirect inhibition of IDO, improved the swimming frequency, an active behavior associated with the increase in the 5-HT levels. This antidepressant-like effect of MINO had already been shown by Molina-Hernandez and colleagues in normoglycemic animals [32]. Furthermore, it was observed that MINO not only induced antidepressant-like effect, but also attenuated the neuroinflammation by decreasing mRNA levels of IL-1β, IL-6 and also IDO expression in the cortex and HIP of mice submitted to LPS-induced depression [63]. In our study, the anti-inflammatory effect of MINO treatment was observed indirectly by inducing a significant reduction in the IDO expression in the HIP from DBT rats (Fig. 7). It is important to highlight that MINO has been tested as an adjuvant in the treatment for depression in clinical trials [64]. Furthermore, considering the ineffectiveness of the treatments available to treat the depression associated with diabetes (for a review, see [14]), modulating directly or indirectly the IDO activity as well as inflammation in DBT and depressive individuals may be an interesting and promising tool.

Considering the importance of the inflammatory component in the pathogenesis of depression, in the following experiment DBT animals were treated with the non-steroidal anti-inflammatory IBU that acts by unespecifically inhibiting the cyclooxygenase enzymes. The treatment with IBU induced a decrease in the immobility and an increase in the swimming frequencies, without changing the climbing (Fig. 6). These results may indicate that the anti-inflammatory effect *per se* may also alter even indirectly the serotonergic neurotransmission. In that way, our data clearly showed that IBU reduced the elevated IDO expression in the HIP (Fig. 7). This antidepressant-like effect of IBU had also been previously described in animals with experimentally induced Parkinson disease [34] and it has been associated to its neuroprotective effect [65,34]. Curiously, a recent study [33] showed that NGL animals submitted to the stress of swimming and treated with aspirin, the most commonly used non-steroidal anti-inflammatory drug wordwide, showed a decrease in the immobility time in the FST. Moreover, the authors showed that aspirin decreased the elevated levels of cytokine, such as IL-6 and TNFα in the plasma. As these authors, we can also suggest that the anti-inflamatory effect of the non-steroidal anti-inflammatory drugs can exert an important role in the antidepressant-like effect of this class of drugs. Important to highlight that as occurred after MINO or IBU treatments, the same was observed after the treatment with FLX regarding to the decrease in the IDO expression (Fig. 7). In fact, this effect of FLX was not to be surprising, once recent evidence has suggested that antidepressants such as imipramine and FLX evoke neuroprotective and immunomodulatory effects in the brain [66,67]. In one of these studies, using BV2 microglial cell line and primary microglial culture it was observed that FLX significantly inhibited LPS-induced production of TNFα and IL-6. By real time PCR, the mRNA level of these pro-inflammatory cytokines was also attenuated by FLX [66]. Thus, we can suggest that FLX, besides its classical effect over 5-HT reuptake inhibition, may exert anti-inflammatory effects in the central nervous system by modulating glia activation. Once IDO is activated by pro-inflamatory citokynes, this pathway becomes a very interesting target to be better studied.

Finally, in our study all effects observed on behavioral analysis were specific because no significant change on number of crossings was shown when animals were evaluated in the OFT (table 1, see supplementary material). Moreover, neither the treatment with fluoxetine, nor with 1-MT, MINO or IBU was able to change the reduced weight gain and the high levels of blood glucose in DBT rats (table 1, see supplementary material), making it clear that the antidepressant-like effect of these drugs is independent of glycemic control.

Taken together, our findings suggest that inflammatory processes as well as reduction in the 5-HT levels in the HIP, possibly due to the increased IDO expression may be the reason or be the causal factor related to the bidirectionallity between depression and diabetes. Moreover, our findings suggest for the first time in the literature that the IDO/kinurenine pathway may be a remarkable target to an alternative treatment proposition concerning the depression associated with diabetes. However, further studies are clearly necessary to fully evaluate the potential of the IDO/kinurenine pathway inhibition as an intervention for depression associated with diabetes.

Conflict of interest

The authors declare they have no conflicts of interest to disclose.

3.8 Acknowledgements

This study was supported by grants from CNPq (Casadinho/PROCAD Grant No: 479526/2012-5). The authors thank Prof. Dr. Maria ABF Vital for kindly donation of the ibuprofen and minocycline and also the technical assistance of Giuliana Bertozi during the ELISA method.

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4.0. CONSIDERAÇÕES FINAIS

Em conjunto, nossos dados mostram que ratos diabéticos apresentam um aumento de citocinas pró inflamatórias assim como níveis reduzidos de 5-HT e NA no hipocampo, quando comparados com ratos normoglicêmicos. Mais ainda, esses animais DBT apresentaram concomitante a estes achados citados um aumento da expressão da enzima IDO também no hipocampo. Além disso, todos os tratamentos que de alguma forma tem uma ação anti-inflamatória, como MINO, IBU E FLX, foram suficientes para diminuir a expressão da IDO, enquanto que o tratamento com 1-MT não, sugerindo que seu mecanismo de inibição da IDO não se relaciona com diminuição da expressão da enzima. Considerando a inflamação como um importante mecanismo na patofisiologia da depressão e do diabtes, assim como a escassez de tratamentos farmacológicos para tratar a depressão associada ao diabetes, a via IDO/quinurenina parece ser um alvo promissor, necessitando de mais estudos.

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