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EFEITOS DA QUERCETINA NA ATIVIDADE DA ACETILCOLINESTERASE, NA PEROXIDAÇÃO LIPÍDICA E NOS TESTES COMPORTAMENTAIS EM RATOS DIABÉTICOS INDUZIDOS POR ESTREPTOZOTOCINA

TESE DE DOUTORADO

Roberto Marinho Maciel

Santa Maria, RS, Brasil

2013

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Roberto Marinho Maciel

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John Ruskin

RESUMO

Tese de Doutorado
Programa de Pós-graduação em Medicina Veterinária
Universidade Federal de Santa Maria

EFEITOS DA QUERCETINA NA ATIVIDADE DA ACETILCOLINESTERASE, NA PEROXIDAÇÃO LIPÍDICA E NOS TESTES COMPORTAMENTAIS EM RATOS DIABÉTICOS INDUZIDOS POR ESTREPTOZOTOCINA

AUTOR: Roberto Marinho Maciel
ORIENTADORA: Profª. Drª. Sonia Terezinha dos Anjos Lopes
Data e Local da Defesa: Santa Maria, 28 de fevereiro de 2013.

Diabetes melito (DM) refere-se a um grupo de distúrbios metabólicos comuns que compartilham o fenótipo da hiperglicemia, sendo uma condição crônica que surge quando o pâncreas não produz insulina em quantidade suficiente ou quando o organismo não consegue utilizar de modo eficaz a insulina produzida. A condição de hiperglicemia crônica favorece o desequilíbrio entre a produção de radicais livres e a defesa antioxidante endógena, gerando o estresse oxidativo e por fim a peroxidação lipídica de estruturas e membranas celulares, ricas em lipídios. A quercetina (QUE) é um flavonoide que apresenta propriedades antioxidantes e anti-inflamatórias, sendo por isso, investigada como possível adjuvante no tratamento do DM. No presente trabalho foram analisadas as ações do excesso de radicais livres, promovido pelo diabetes melito tipo 1 (DM T1) tanto em nível sistêmico como, principalmente, no sistema colinérgico. Além disso, os possíveis efeitos de proteção antioxidante e anti-inflamatória da QUE. Foram utilizados 130 ratos Wistar, machos, pesando entre 160 a 250 g, distribuidos em 2 grupos: não diabéticos e diabéticos, sendo cada um deles divididos em 5 tratamentos: salina 0,9%, etanol 25% e QUE (5, 25 e 50 mg/Kg). A indução do DM T1 se deu com uma única injeção intraperitoneal de 70 mg/Kg de estreptozotocina (STZ). Quinze dias depois, teve início o tratamento com QUE por 40 dias. Ao término do experimento, foram realizados os testes comportamentais e a coleta de material biológico (sangue e tecidos corporais). O grupo diabético sem tratamento em relação ao controle não diabético apresentou: redução das ilhotas de Langerhans e da população de células beta, perda de peso, hiperglicemia crônica, leucopenia associada à neutropenia, redução na insulina e albumina, elevação na frutosamina, triglicerídeos, ureia, fosfatase alcalina (FA), alanina aminotransferase (ALT), além das frações proteicas de beta e gamaglobulinas. Houve aumento das substâncias reativas ao ácido tiobarbitúrico (TBARS) sérico e redução na atividade de superóxido dismutase hepática. Falha na formação de memória aversiva e comportamento ansioso foram observados nesses animais, bem como, elevação na atividade da acetilcolinesterase (AChE) nas estruturas e sítios cerebrais (córtex, hipocampo, estriado e sinaptossomas) e TBARS (córtex, hipocampo e estriado). Os ratos diabéticos tratados com QUE em relação ao grupo controle diabético: apresentaram elevação na albumina (50 mg/Kg), redução nas betaglobulinas (25 e 50 mg/Kg) e gamaglobulinas (50 mg/Kg), diminuição dos triglicerídeos (5, 25 e 50 mg/Kg). A quercetina nas concentrações utilizadas reverteu a falha de memória aversiva e apresentou propriedades ansiolíticas. A atividade da AChE foi reduzida: no córtex (50 mg/Kg), no hipocampo (5 e 50 mg/Kg) e sinaptossomas (50 mg/Kg). Houve diminuição nos níveis séricos das TBARS no córtex, hipocampo e estriado (5, 25 e 50 mg/Kg). A quercetina elevou a concentração da insulina, nos grupos não diabéticos e diabéticos (5, 25 e 50 mg/Kg). Quando administrada em animais não diabéticos a QUE aumentou a inquietação dos animais (50 mg/Kg); aumentou a atividade da AChE no hipocampo (5, 25 e 50 mg/Kg), estriado (5 mg/Kg) e sinaptossomas (25 mg/Kg), diminuindo no córtex (5 mg/Kg). Além disso, aumentou o nível de TBARS no córtex (25 mg/Kg). A partir desses resultados conclui-se que a QUE possui propriedades antioxidantes e anti-inflamatórias que podem ser utilizadas no tratamento auxiliar do DM. Contudo, também apresentou propriedades não desejáveis, como efeito pró-oxidante, o que sugere a resistência à insulina.

Palavras – chave: Estresse oxidativo. Acetilcolinesterase. Superóxido dismutase. Catalase. Teste das substâncias reativas ao ácido tiobarbitúrico.

ABSTRACT

Doctoral Thesis
Postgraduate Program in Veterinary Medicine
Universidade Federal de Santa Maria

EFFECTS OF ACTIVITY IN QUERCETIN ACETYLCHOLINESTERASE, IN LIPID PEROXIDATION AND BEHAVIOUR IN TESTS IN RATS STREPTOZOTOCIN-INDUCED DIABETIC

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Diabetes mellitus (DM) refers to a group of common metabolic disorders that share the phenotype of hyperglycemia, is a chronic condition that arises when the pancreas does not produce enough insulin or when the body can not effectively use the insulin produced.The condition of chronic hyperglycemia promotes an imbalance between the production of free radicals and endogenous antioxidant defense, causing oxidative stress and finally lipid peroxidation, structures and cell membranes rich in lipids. Quercetin (QUE) is a flavonoid that has antioxidant and anti-inflammatory properties and is therefore investigated as a possible adjuvant in the treatment of diabetes. In this study we analyzed the actions of excess free radicals, promoted by type 1 diabetes mellitus (DM T1) at both systemic and mainly in the cholinergic system. Moreover, the possible effects of antioxidant protection and anti-inflammatory of QUE were analysed. We used 130 male Wistar rats, weighing 160 to 250 g and divided into 2 groups: non-diabetic and diabetic, which are divided into 5 treatments: 0.9% saline, 25% ethanol and QUE (5, 25 and 50 mg/kg).The induction of DM T1 occurred with a single intraperitoneal injection of 70 mg/kg streptozotocin (STZ). Fifteen days later, the treatment with QUE for 40 days started. At the end of the experiment behavioral tests and collection of biological material (blood and tissues) were performed. The untreated diabetic group compared to non-diabetic control showed: reduction of the islets and beta-cell population, weight loss, chronic hyperglycemia, leukopenia associated with neutropenia, decreased insulin and albumin, increase in fructosamine, triglycerides, urea, alkaline phosphatase (ALP), alanine aminotransferase (ALT), in addition to protein fractions of beta and gamma globulin.The increase of thiobarbituric acid reactive substances (TBARS) levels was accompanied by a reduction in the concentration of hepatic superoxide dismutase. Failed aversive memory formation and anxious behavior were observed in these animals, as well as increase in the activity of acetylcholinesterase (AChE) in brain structures and sites (cortex, hippocampus, striatum and synaptosomes) and TBARS (cortex, hippocampus and striatum). Diabetic rats treated with QUE in relation to diabetic control had increased albumin (50 mg/kg), reduction in betaglobulins (25 and 50 mg/kg) and gamma globulins (50 mg/kg), decreased triglycerides (5, 25 and 50 mg/kg). Quercetin reverted the aversive memory failure and showed anxiolytic properties. AChE activity were decreased in the cortex (50 mg/kg), hippocampus (5 and 50 mg/kg) and synaptosomes (50 mg/kg). Levels of TBARS were reduced in the cortex, hippocampus and striatum (5, 25 and 50 mg/kg). Quercetin increased the concentration of insulin in non-diabetic and diabetic groups (5, 25 and 50 mg/kg). When administered in non-diabetic animals, QUE increased the uneasiness of the animals (50 mg/kg) increased AChE activity in the hippocampus (5, 25 and 50 mg/kg), striatum (5 mg/kg) and synaptosomes (25 mg/kg), lowering in the cortex (5 mg/kg), furthermore, increased the level of TBARS in the cortex (25 mg/kg).From these results we conclude that QUE have antioxidant and anti-inflammatory properties which may be used in conjunction with treatment of diabetes. However, it also had undesirable properties, such as pro-oxidant effect and induces insulin resistance.

Keywords: Oxidative stress. Acetylcholinesterase. Superoxide dismutase. Catalase. Thiobarbituric acid reactive substances.

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LISTA DE ABREVIATURAS E SIGLAS

ACh – Acetilcolina
AChE – Acetilcolinesterase
AGL – Ácidos graxos livres
AGPI – Ácidos graxos poliinsaturados
ALT – Alanina aminotransferase
ATP – Adenosina trifosfato
CAT – Catalase
ChAT – Colina-O-acetil-transferase
CS – Estímulo condicionado
C3 – Componente 3 do Complemento
C4 – Componente 4 do Complemento
DM – Diabetes melito
DMID – Diabetes melitoinsulino-dependente
DM T1 – Diabetes melito tipo 1
DM T2 – Diabetes melito tipo 2
ERO – Espécies reativas de oxigênio
ERN – Espécies reativas de nitrogênio
FA – Fosfatase alcalina
GSH – Glutationa reduzida
GLUT 4 – Transportador de glicose sensível à insulina
HDL – Lipoproteínas de alta densidade
HO. – Radical hidroxila
IgA – Imunoglobulina A
IgD – Imunoglobulina D
IgE – Imunoglobulina E
IgG – Imunoglobulina G
IgM – Imunoglobulina M
IRS – Substratos do receptor de insulina
L. – Radical alquila
LO. – Radical alcoxila
LOO. – Radical peroxila
LDL – lipoproteínas de baixo densidade
LPL - Lipoproteína lipase
LPO - Lipoperoxidação
MDA - Malondialdeído
O2.- - ânion radical superóxido
PAS – Coloração ácido periódico-Schiff
PI-3 quinase – Fosfatidilinositol-3’-quinase
RL – Radicais livres
SNC – Sistema nervoso central
SOD – Superóxido dismutase
STZ - Estreptozotocina
TBA – Ácido tiobarbitúrico
TBARS – Teste das substâncias reativas ao ácido tiobarbitúrico
US – Estímulo incondicionado
VAChT – Transportador vesicular de acetilcolina
VLDL – Lipoproteínas de desnsidade muito baixa

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1 INTRODUÇÃO

O diabetes melito (DM) refere-se a um grupo de distúrbios metabólicos comuns que compartilham o fenótipo da hiperglicemia. Existem vários tipos distintos de DM que são causados por uma interação complexa de fatores genéticos e ambientais. Dependendo da etiologia do DM, os fatores que contribuem para a hiperglicemia incluem secreção reduzida de insulina, menor utilização de glicose e maior produção de glicose. A desregulação metabólica associada ao DM acarreta alterações fisiopatológicas secundárias em muitos sistemas orgânicos que impõem uma sobrecarga aos indivíduos com diabetes (POWERS, 2009).

O DM é uma condição crônica que surge quando o pâncreas não produz insulina em quantidade suficiente ou quando o organismo não consegue utilizar de modo eficaz a insulina produzida (ALVARENGA et al., 2005). O DM corresponde a um grupo de distúrbios do metabolismo glicídico, no qual a glicose é subutilizada, produzindo hiperglicemia. Alguns pacientes podem desenvolver episódios hiperglicêmicos agudos, com risco de vida, como cetoacidose ou coma hiperosmolar (SACKS, 1998).

Dentre as endocrinopatias o DM é a mais comum em cães e gatos e pode ser fatal se for incorretamente diagnosticada ou inadequadamente tratada (NELSON, 1998). Ao contrário dos gatos, a maioria dos cães são insulino-dependentes no momento do diagnóstico. A etiologia do diabetes melito insulino-dependente (DMID) não foi caracterizada nos cães, mas provavelmente seja multifatorial (NELSON, 2009). As predisposições genéticas, a destruição imunomediada das células beta, os fatores ambientais como mudanças alimentares, agentes infecciosos e drogas, a obesidade induzindo resistência à insulina e a destruição de células beta, secundárias à pancreatite, são todos fatores potenciais predisponentes (HOENING & DAWE, 1992; GUPTILL et al., 1999).

Em gatos, o DM também é um dos distúrbios endócrinos mais comuns. A carência absoluta ou relativa de insulina, associada com a resistência à insulina, observada em cerca de 80 a 95% dos casos em felinos, é análoga ao DM do tipo 2, em humanos. O restante, cerca de 5 a 20% dos gatos atingidos, apresenta outros tipos específicos de DM (RAND & MARSHALL, 2009).

O DM do tipo 1, resulta da destruição imunomediada das células beta. Essa doença é rara em gatos e apenas casos eventuais de filhotes ou gatos com lesões histológicas ou com anticorpos são consistentes com tal patogênese (WOODS et al., 1994). As lesões patológicas mais comuns em cães com DM são: redução no número e no tamanho das ilhotas pancreáticas, diminuição no número de células beta dentro de ilhotas e degeneração distensível hidrópica das células beta (NELSON, 2009). O DM do tipo 2 é o resultado da diminuição da secreção de insulina combinada com uma ação insuficiente desta. Essa ação é caracterizada como uma sensibilidade diminuída à insulina ou como resistência à insulina. Conforme aumenta a resistência à insulina, mais insulina é necessária para produzir o mesmo efeito de diminuição da glicose do que quando a sensibilidade da insulina é normal. Existem muitas causas de resistência à insulina, algumas das quais podem estar interligadas (RAND & MARSHALL, 2009). A obesidade é um importante fator para a ocorrência da resistência à insulina. Foi observado que gatos com 44% de sobrepeso apresentavam 50% de diminuição da sensibilidade de insulina (APPLETON et al., 2001). A inatividade física também diminui a sensibilidade da insulina, independentemente da obesidade (LEDERER et al., 2003). Atualmente, o sedentarismo é comum, especialmente em zonas urbanas e nos animais confinados em espaços pequenos (RAND & MARSHALL, 2009).

A maioria dos cães tem entre 4 a 14 anos de idade no momento em que é diagnosticado o DM, com um pico de prevalência entre 7 e 10 anos. O DM, de início juvenil, ocorre em cães com menos de 1 ano de idade, mas é incomum. As cadelas são afetadas cerca de duas vezes mais que os machos. Em cães, a predisposição genética influencia no desenvolvimento do DM (Quadro 1) (GUPTILL et al., 1999; HESS et al., 2000). A maioria dos gatos é relativamente idosa no pico da incidência do DM, apresentado entre 10 e 13 anos (RAND, 1999; PRAHL et al., 2003). Os gatos machos castrados apresentam o dobro do risco de desenvolver DM em comparação com as fêmeas castradas (RAND & MARSHALL, 2009).

Raças de alto risco	Raças de baixo risco
Terrier australiano Schnauzer standart Schnauzer miniatura Bichon frisé Spitz Fox terrier Poodle miniatura Samoieda Cairn terrier Keeshond	Pastor Alemão Collie Shetland Retriever Cocker Spaniel Australian Shepherd Labrador Retriever Dobermann Pinsher

Quadro 1 – Predisposição genética ao desenvolvimento do diabetes melito em cães (Estudo em 5.922 cães).

Adaptado de Guptill et al. (1999).

As complicações crônicas do DM afetam muitos sistemas orgânicos e são responsáveis pela maior parte da morbidade e da mortalidade associadas a essa doença (Quadro 2). Essas complicações podem ser dividas em vasculares e não-vasculares. As vasculares, podem ainda serem subdivididas em: microvasculares (retinopatia, nefropatia e neuropatia) e macrovasculares (doença arterial coronariana, doença arterial periférica e doença vascular cerebral). Já as não-vasculares incluem problemas como gastroparesia, infecções e alterações cutâneas (POWERS, 2009).

Ainda segundo POWERS (2009), a hiperglicemia crônica é um fator etiológico importante responsável pelas complicações do DM, porém o mecanismo pelo qual ela induz uma disfunção celular e orgânica tão diversificada é desconhecido. Dentre as várias teorias que buscam explicar como a hiperglicemia promove complicações crônicas do DM, existe a hipótese de que a hiperglicemia acelere o metabolismo da glicose pela via sorbitol.

A glicose intracelular é metabolizada predominantemente por fosforilação e subsequente glicólise, porém, quando aumentada, alguma glicose é transformada em sorbitol pela enzima aldose redutase. A maior concentração de sorbitol altera o potencial de oxidação-redução, eleva a osmolaridade celular, gera espécies reativas de oxigênio e, provavelmente, dá origem a outros tipos de disfunção celular (POWERS, 2009).

Comuns	Incomuns
Hipoglicemia iatrogênica Poliúria, polidpsia, perda de peso persistentes ou recorrentes Cataratas (cão) Uveíte induzida pela lente (cão) Infecções bacterianas, especialmente envolvendo o trato urinário) Pancreatite crônica Cetose recorrente, cetoacidose Lipidose hepática Neuropatia periférica (gato) Hipertensão sistêmica (cão)	Neuropatia periférica (cão) Nefropatia diabética - Proteinúria significativa - Glomeruloesclerose Retinopatia Insuficiência pancreática exócrina Paresia gástrica Hipomotilidade intestinal e diarreia Dermatopatia diabética (dermatite necrolítica superficial)

Quadro 2 – Complicações de diabetes melito em cães e gatos.

Adaptado de Nelson R.W. (2009).

No DM, as lesões neurológicas estão relacionadas a alterações do metabolismo da glicose em nível de sistema nervoso periférico (SNP) (KAPLAN & RICHARDS, 1988), e também à encefalopatia diabética, quando as complicações ocorrem no sistema nervoso central (SNC) (BIESSELS et al., 2002).

Em humanos, a neuropatia diabética ocorre em 50% dos indivíduos com DM tipo 1 e tipo 2 de longa duração. Pode manifesta-se como polineuropatia, mononeuropatia e/ou neuropatia autônoma. Como acontece com outras complicações do DM, o surgimento de neuropatia correlaciona-se com a duração do diabetes e o controle glicêmico (POWERS, 2009).

A encefalopatia diabética pode ser reproduzida em animais experimentais. A relação entre hiperglicemia e dano neuronal no córtex cerebral é demonstrada em ratos diabéticos por estreptozotocina (STZ) e o tratamento com insulina previne o dano neuronal (GUYOT et al., 2001). Diversas drogas como antidepressivos, drogas anti-inflamatórias não esteroidais, anticonvulsivantes e opioides agonistas receptores, estão correntemente sob investigação para o manejo da neuropatia diabética (JAMES et al., 1999). Contudo, tratamentos com essas drogas são limitados por apresentarem efeitos parciais, desenvolvimento de tolerância e potencial toxicidade (COURTEIX et al., 1994; CLARK & LEE, 1995; ARNER & MEYERSON, 1998; GUL et al., 2000).

O DM está associado com alterações cognitivas, estruturais e fisiológicas do cérebro, condição esta denominada encefalopatia diabética. O SNC é protegido de muitos efeitos tóxicos por uma barreira anatômica denominada barreira – hematoencefálica (BHE) (MÉNDEZ-ARMENTA & RIOS, 2007); mesmo assim, alterações nervosas resultantes da degeneração cerebral, são descritas no estado diabético, provavelmente em decorrência de modificações na plasticidade sináptica, o que compromete o mecanismo de regulação da homeostase celular, e tem como consequência a disfunção na atividade dos neurotransmissores na fenda sináptica (BIESSELS et al., 2002).

O SNC é modulado por vários tipos de neurotransmissores que integram o corpo com o seu meio, sendo, uma via informacional para o indivíduo conseguir interpretar os estímulos externos e reagir de acordo com os mesmos (PATEL et al., 2001). Os principais neurotransmissores que desempenham essas funções no SNC são a dopamina, o glutamato, a serotonina, a epinefrina, a norepinefrina e a acetilcolina, entre outros (EVERITT & ROBBINS, 1997; LOPES et al., 1999).

A acetilcolina (ACh) foi a primeira molécula a ser identificada como neurotransmissor e passou a ser amplamente investigada nas sinapses do SNC e SNP (DESCARIES et al., 1997). A ACh desempenha um importante papel na regulação de muitas funções vitais como aprendizagem e memória, processamento da informação sensorial, organização cortical do movimento e controle do fluxo sanguíneo cerebral (MESULAM et al., 2002). A ACh é sintetizada a partir da colina e de acetil-coenzima A, pela colina-O-Acetil-Transferase (ChAT) (VENTURA et al., 2010). Uma vez sintetizada, parte da ACh é transportada e armazenada em vesículas sinápticas. Esse processo é realizado por um transportador vesicular de ACh (VAChT), capaz de elevar em até 100 vezes sua concentração no interior dessas vesículas.

Após ser liberada inteiramente por exocitose, a ACh interage especificamente com os receptores colinérgicos presentes nas membranas pré e pós-sinápticas (FUJII et al., 2008). A ação da ACh cessa quando é hidrolizada em acetato e colina pela enzima acetilcolinesterase (AChE), presente na fenda sináptica. A AChE é uma serina hidrolase que hidrolisa rapidamente a ACh, tanto na sinapse colinérgica, quanto na junção neuromuscular, finalizando a transmissão do impulso nervoso (GRISARU et al., 1999). Por ser uma das mais eficientes e conhecidas catálises

biológicas, a AChE tem sido investigada como um importante alvo terapêutico em várias doenças neurodegenerativas, sendo considerada uma importante enzima regulatória e um bom indicador da atividade colinérgica (APPLEYARD, 1994; SZEGLETES et al., 1999; DAS et al., 2001).

O DM pode modificar funções do SNC (BELLUSH et al.,1991). Disfunções cognitivas observadas em pacientes humanos e modelos experimentais de diabetes têm sido relacionadas com alterações na atividade da AChE, o que pode indicar modificações na neurotransmissão colinérgica (SANCHEZ-CHAVEZ & SALCEDA, 2000). Estudos na atividade da AChE, em ratos diabéticos, demonstraram que a enzima apresentou atividade mais elevada em algumas regiões cerebrais: córtex cerebral, hipocampo, estriado, cerebelo e hipotálamo (SCHMATZ et al., 2009). Por esse motivo, agentes inibidores da atividade da AChE têm sido estudados com o objetivo de diminuir os efeitos hipocolinérgicos, observados quando a atividade da enzima aumenta (DAS et al., 2001), contudo, uma diminuição além do esperado, nessa atividade, pode levar ao acúmulo de ACh, o que resultaria numa hiperatividade colinérgica, convulsão e epilepsia (OLNEY et al., 1986).

A ACh, seus receptores e o aparato enzimático responsável por sua síntese e degradação constituem o sistema de neurotransmissão colinérgica (Figura 1) (BRUNEAU & AKAABOUNE, 2006). O sistema colinérgico realiza uma das mais importantes funções modulatórias no SNC desempenhando um papel fundamental na regulação de muitas funções vitais relacionadas com o comportamento, a aprendizado e a memória, além de atuar na organização cortical do movimento e do controle do fluxo sanguíneo cerebral (PERRY et al., 1999; MESULAM et al., 2002).

Conceitualmente, a memória pode ser definida como o processo de armazenamento e evocação de informações adquiridas através de experiências, quantidade de tipos de memórias e está relacionada com a variedade de experiências vividas (IZQUIERDO, 1989). O principal papel do sistema colinérgico sobre a memória parece ser o de desempenhar um efeito modulatório (SEGAL & AUERBACH, 1997). A memória só pode ser avaliada em animais com a observação de comportamentos modificados durante a evocação (QUILLFELDT, 1994). O encéfalo está constantemente criando e evocando memórias, sendo que a memória não é apenas a capacidade de repetir, mas sim de variar a resposta frente a uma nova aprendizagem. A aprendizagem transforma as experiências em memórias e é o

processo pelo qual os humanos e outros animais, formam o conhecimento (KANDEL et al., 2000).

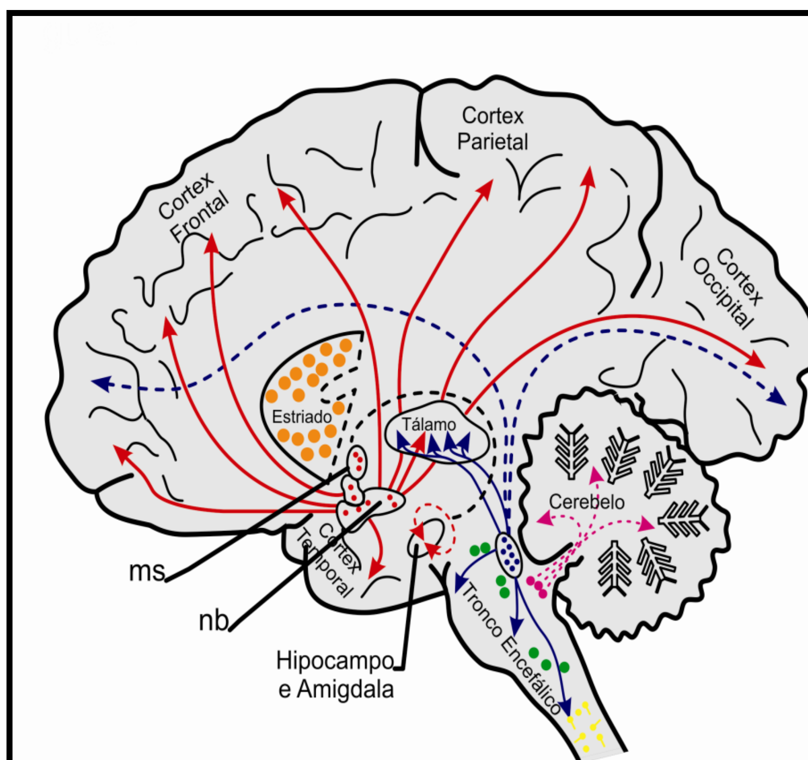

Figura 1 – Sistema colinérgico (encéfalo humano). Fibras colinérgicas em vermelho, núcleo pedúnculo pontilhado em azul, interneurônios estriatais em laranja. (MS = núcleos septo medial) e (nb = núcleo basal). Adaptado de PERRY et al. (1999), modificado por MAZZANTI (2007).

É possível que o nível de atenção, dependente da atividade cortical, dado a uma determinada nova experiência leve à ativação da formação hipocampal, que sofreria alterações plásticas, fazendo com que a nova informação possa ser armazenada de forma mais definitiva nas estruturas corticais mais ascendentes. O hipocampo parece constituir um local transitório onde a informação seria temporariamente armazenada. Outras áreas encefálicas como o telencéfalo basal, principalmente o estriado, o cerebelo e os córtices motores também participam do processamento de memórias com conteúdo procedural ou implícito. Talvez, atuando

de forma paralela e independente de estruturas límbicas ou dos lobos temporais (FUSTER, 1997).

O hipocampo, a amígdala e o córtex entorrinal são interconectados por vias aferentes e eferentes, o córtex entorrinal, adicionalmente, tem conexões com o córtex parietal posterior e córtex pré-frontal. Todas essas estruturas desempenham papeis de forma integrada na memória (QUILLFELDET et al., 1996; IZQUIERDO et al., 1997; LORENZINI et al., 1999).

A contribuição de cada estrutura e região não é idêntica. O hipocampo processa principalmente informações espaciais e contextuais. A amígdala é um importante núcleo modulador da atividade hipocampal e sua função apresenta, como principal substrato, informações com fortes componentes emocionais e aversivos. O estresse é um fator importante no processamento integrado entre hipocampo e amígdala (PITKANEN et al., 1997). O córtex entorrinal está encarregado de integrar as informações provenientes do hipocampo e da amígdala. Assim, o córtex entorrinal participa do processamento e do armazenamento de diferentes conteúdos cognitivos, tanto aversivos contextuais como espaciais (QUILLFELDT et al., 1996; IZQUIERDO et al., 1997).

A observação de alteração no comportamento, durante a evocação da memória, pode ser utilizada na avaliação das condições de reativação das redes sinápticas de cada tipo de memória (QUILLFELDT et al., 1994). Acredita-se que a habituação a um novo ambiente é uma das formas mais elementares de aprendizagem, no qual o decréscimo na exploração como uma função de exposição repetida a um mesmo ambiente é tomada como um índice de memória (THIEL et al., 1998, THIEL et al., 1999).

Uma análise mais comportamental é acompanhada do desenvolvimento de conceitos neurobiológicos de representação. O relatório de um grupo de estudo sobre biologia da aprendizagem (HOLLAND, 1984), aponta três níveis dos sentidos de representação: representação do setor de superfície; representação neural do estímulo presente, que forma a base da percepção e talvez de muitos tipos de memória a curto-prazo; e uma representação armazenada, permanente, de um estímulo que forma a base do que é denominada memória de longo-prazo. Estas representações de eventos são topograficamente organizadas e podem ser encontradas em muitos locais do cérebro (BUENO, 1997).

A análise experimental comparativa da cognição tem levado em conta diferentes níveis de processamento e de diferentes tipos de representação, embora não haja a esse respeito unanimidade entre os pesquisadores. MACPHAIL (1987) defende a possibilidade de uma análise que contemple apenas a formação de associações entre estímulos e respostas, sem referências à noção de representação e a concepções cognitivistas. Estudos experimentais em condicionamento pavloviano com estimulação complexa indicam, portanto, a possibilidade de o organismo animal estar operando não só representações de eventos, mas também representações mais complexas de relações entre os eventos, exigindo um alto nível de processamento no momento do desempenho (HOLLAND, 1990).

O labirinto em cruz elevado é um modelo animal validado do ponto de vista farmacológico, bioquímico e comportamental como teste de ansiedade no rato (PELLOW et al., 1985). Contudo, pesquisadores dedicados ao desenvolvimento de modelos animais de ansiedade se defrontam com vários problemas que começam pela falta de uma definição clara desta patologia. Estudos indicam a possibilidade de que a ansiedade pode não consistir de uma manifestação única, mas de vários sub-tipos de ansiedade que se manifestariam sob exposições a determinados estímulos ou condições (GONZALES et al., 1997). Outras considerações sugerem que a ansiedade estaria associada a outras psicopatologias como a depressão (BUENO, 1997). Vários modelos animais de depressão têm sido elaborados com o objetivo de reproduzir em laboratório mudanças comportamentais próprias desta patologia (HANSEN et al., 1997). Segundo GONZALES et al., (1997), é possível que as duas desordens (ansiedade e depressão) sejam manifestação do mesmo fator etiológico. Além disso, o isolamento social em estádios precoces da vida modifica uma variedade de comportamentos em muitos animais (WONGWITDECHA & MARSDEN, 1996), além de produzir alterações neuroquímicas (PEREZ et al., 1997) e fisiológicas (JIMENEZ & FUENTE, 1993).

Efeitos tóxicos do oxigênio sobre componentes biológicos já eram conhecidos no final do século XIX (LORRAIN-SMITH, 1899). Esses efeitos são resultantes da oxidação de componentes celulares como tióis, cofatores enzimáticos, proteínas, nucleotídios e lípides, principalmente ácidos graxos poli-insaturados (AGPI), mediada por espécies reativas de oxigênio (ERO) e espécies reativas de nitrogênio (ERN), conhecidas genericamente como radicais livres (RL) (GILLER & SIGLER,

1995). As ERO são moléculas contendo oxigênio, formadas a partir de radicais livres durante o metabolismo normal e/ou quando o organismo é exposto a vários estímulos, como radiação ionizante ou biotransformação de xenobióticos. Radicais livres são átomos ou moléculas altamente reativos que possuem elétrons não emparelhados em sua última camada (HALLIWELL & GUTTERIGDE, 2007). As ERO incluem todos os radicais do oxigênio, como o ânion radical superóxido ($O2^{\cdot-}$), radical hidroxila ($HO^{\cdot}$), radical alquila ($L^{\cdot}$), alcoxila ($LO^{\cdot}$) e peroxila ($LOO^{\cdot}$) (BARBER & BERNHEIM, 1967; CHANGE et al., 1979).

Os RL estão relacionados com uma variedade de doenças, incluindo câncer, doenças hepáticas, aterosclerose e envelhecimento (ESTERBAUER et al., 1992; CHIRICO et al., 1993; MORIEL et al., 1999; CHISOLM & STEINBERG, 2000). Na maioria das vezes esta relação se dá pela propriedade que os RL têm de reagir com os ácidos graxos poliinsaturados (AGPI), servindo como iniciadores do processo de peroxidação lipídica ou lipoperoxidação (LPO) (LIMA et al., 2001). Em situações fisiológicas, a formação de RL ocorre normalmente no organismo de seres aeróbicos, especialmente durante a respiração. Na mitocôndria, em torno de 1 a 2% do O_2 participa de reações monoeletrônicas, escapando da redução tetravalente do O_2 pela aceitação de quatro elétrons para sua neutralização, resultando na formação de H_2O. O aumento da formação dessas ERO pode levar à condição de estresse oxidativo (SIES, 1997; SHAW, 1998; HALLIWELL & GUTTERIGDE, 2007).

O estresse oxidativo pode resultar da geração excessiva de ERO, da diminuição da capacidade antioxidante natural do organismo ou ainda da combinação desses fatores (SIES, 1997). Vários fatores podem contribuir para o estabelecimento do estresse oxidativo, como por exemplo, a liberação de metais de transição ou mesmo metais sem ação redox direta, como o Zn^{2+} (MARET, 1998). De um modo geral, o acentuado aumento do metabolismo da glicose, na hiperglicemia diabética, está associado a uma formação aumentada de RL. Nestas condições, pode haver um distúrbio no balanço entre a produção acentuada de RL e as defesas (BAYNES, 1991). A hiperglicemia, marcador através do qual se diagnostica tanto diabetes tipo1 quanto tipo 2, promove o aumento na produção de ERO (BAYNES, 1991; NISHIKAWA et al., 2000).

Ocorrem, também, efeitos bioquímicos secundários, como a produção do ânion superóxido pela auto-oxidação da glicose. O aumento de proteínas glicadas

no plasma dos pacientes está relacionado com o estresse oxidativo, uma vez que essas proteínas interagem com receptores celulares, estimulando a produção de espécies reativas de oxigênio e diminuindo a glutationa intracelular. Esse aumento de produção de ERO contribui para uma série de complicações secundárias ao diabetes, tais como a aterosclerose e a peroxidação das lipoproteínas de baixa densidade (LDL) nas células endoteliais (BAYNES, 1991; NISHIKAWA et al., 2000).

A LPO pode ser definida como uma cascata de eventos bioquímicos resultantes da ação dos RL sobre os lípides insaturados das membranas celulares, gerando principalmente radical alquila ($L^{.}$), radical alcoxila ($LO^{.}$) e radical peroxila ($LOO^{.}$), levando à destruição de sua estrutura, falência dos mecanismos de troca de metabólitos e, numa condição extrema, a morte celular (BENZIE, 1996). A LPO pode provocar uma desrregulação na homeostase iônica, através do aumento da concentração de Ca^{2+} intracelular. Além disso, lesões oxidativas ao DNA tendem a aumentar pelo ataque direto de ERO (TROTTI et al., 1998; MARTINDALE & HOLBROOK, 2002; LEE et al. 2002).

As principais metodologias utilizadas para a avaliação da LPO em sistemas biológicos medem a formação de produtos gerados durante as diferentes fases deste processo (LIMA et al., 2001). A oxidação dos AGPI é acompanhada pela formação de hidroperóxidos dienos conjugados, o que não ocorre em ácidos graxos insaturados não oxidados (HALIWELL &GUTERIDGE, 1999). Uma das técnicas mais utilizadas para se avaliar a oxidação de lípides é o teste do malondialdeído (MDA), um dialdeído formado como um produto secundário durante a oxidação de ácidos graxos poliinsaturados por cisão beta dos AGPI peroxidados, principalmente o ácido araquidônico (LIMA et al., 2001). O MDA é volátil, possui baixo peso molecular, tem uma cadeia curta 1,3-dicarbonil e é um ácido moderadamente fraco (pKa=4,46). Em condições apropriadas de incubação (meio ácido e aquecimento), reage eficientemente com uma variedade de agentes nucleofílicos para produzir cromógenos com alta absortividade molar no espectro visível (JANERO, 1990; BENZIE, 1996). Sua condensação com o ácido tiobarbitúrico (TBA) forma produtos, que podem ser determinados por absorção no visível (532 nm) ou por fluorescência (λ_{exc} = 515 nm e λ_{exc} = 553 nm) (JANERO, 1990). A reação denominada de teste das substâncias reativas ao ácido tiobarbitúrico (TBARS), representa múltiplos métodos que utilizam o TBA, formando o complexo MDA:TBA (1:2), $C_{11}H_8N_4S_2O_4$.H2O, que

tem absorção máxima em 532 nm e apresenta fluorescência (lexc = 515 nm e lem = 553 nm). Neste teste utiliza-se como padrão o MDA obtido pela hidrólise do tetrametoxipropano ou tetraetoxipropano (YU et al., 1986).

Distúrbios do metabolismo energético estão intimamente associados com a elevação do estresse oxidativo, que resulta em oxidação de biomoléculas e inicia danos citotóxicos em células neuronais (OLANOW, 1993; MARTINEZ et al., 1994). Normalmente as células são providas de um mecanismo de defesa denominado sistema antioxidante que pode ser dividido em enzimático, o qual inclui enzimas como a catalase, a superóxido dismutase e a glutationa peroxidase, e em não enzimático, que inclui a glutationa reduzida, a vitamina C e a vitamina E (MATÉS et al., 1999).

O termo flavonoide é derivado do latim *flavus* que significa amarelo, e recebeu este nome por ter sido encontrado originalmente em alimentos de coloração amarelada, mas atualmente já foi encontrado também em outros de colorações diversas (COTELLE, 2001). Os flavonoides são amplamente encontrados em vegetais, frutas, sucos e chás. Eles representam um importante componente da dieta humana (RICE-EVANS et al.1996; HOLLMAN & KATAN, 1999).

Os flavonoides são formados nas plantas pela combinação dos aminoácidos fenilalanina e tirosina com unidades acetato. Existem mais de 8.000 variedades de flavonoides, mas os seis grupos já classificados são flavononas, flavonas, flavonóis, isoflavonoides, antociainas e flavans (COTELLE, 2001).

A presença de grupos hidroxila aromáticos nos flavonoides confere acentuadas propriedades antioxidantes. Eles eliminam ERO e, portanto inibem reações de peroxidação. Além disso, macrófagos também são protegidos do estresse oxidativo pela retenção de glutationa em sua forma reduzida (THIE & CROZIER, 2000).

Está bem estabelecido que os compostos fenólicos exercem efeitos positivos em doenças crônicas, como o câncer e desordens neurodegenerativas. Os principais benefícios fisiológicos dos flavonoides têm sido atribuídos às suas propriedades antioxidantes de eliminação de radicais livres, antibacteriana, anti-inflamatória, anti-alérgica, antiviral, antitumor, anti-isquêmica e vasodilatadora (RICE-EVANS et al., 1996; LEE et al., 2000; MORIMOTO et al., 2003; PILORGET et al., 2003; KIM et al.,2004; NOVAKOVIC et al., 2006).

O efeito neuroprotetor dos flavonoides foi evidenciado em experimentos, onde catequinas originadas de chá verde protegeram as células neuronais de morte em modelos animais de doenças neurodegenerativas como pela toxicidade por MPTP (Doença de Parkinson), pelo peptídeo β-amiloide (Doença de Alzheimer), na isquemia cerebral unilateral ou global e ainda, em modelos de estresse oxidativo por peróxido de hidrogênio (MANDEL et al., 2005). Os flavonoides ainda protegem células neuronais do estresse oxidativo causado por outros tipos de insultos, como do ácido homocisteico, privação de cistina, butionina sulfoximina, hipoglicemia, insultos isquêmicos e peróxido de hidrogênio que podem levar à morte celular induzida por glutamato.

A ação neuroprotetora dos flavonoides pode ocorrer por três mecanismos distintos: alteração do metabolismo da glutationa reduzida (GSH), extinção de ERO, inibição do influxo de Ca^{2+} e manutenção dos níveis de adenosina trifosfato (ATP) (ISHIGE et al., 2001).

A quercetina (QUE) (3,3',4',5,7-pentaidroxiflavona) é um dos mais abundantes flavonoides encontrados em frutas e vegetais, principalmente na cebola, no brócolis, na couve, na maçã, no Ginkgo Biloba, no chá e também no vinho tinto, com uma dose diária de ingestão acima de 25 mg em uma dieta de adulto normal (DAJAS et al., 2003). É formada por anéis catecóis que podem sofrer metabolização oxidativa até ser desmetilada, se conjugando com a glutiona e formando o 2'-glutationil-quercetina, podendo exercer efeitos protetores ou tóxicos nas células (PRIOR, 2003). Além disso, tanto a quercetina quanto a rutina podem também, exibir *in vitro* propriedades antioxidantes em diferentes modelos pró-oxidantes (SPANOS & WROLSTAND, 1992; WAGNER et al., 2006; PEREIRA et al., 2009).

Os efeitos da quercetina são devido às suas propriedades antioxidantes, reduzindo diretamente os radicais livres, inibindo a xantine oxidase e a peroxidação lipídica (PLUMB et al., 1999; FIORANI et al., 2001). Evidências experimentais têm demonstrado que ERO tais como, peróxido de hidrogênio, radical hidroxila e ânion superóxido, tem um papel central no desenvolvimento de complicações crônicas características do estado diabético (RAO et al., 2001; MARITIM et al., 2003).

A QUE é mais potente que outros antioxidantes, como a vitamina C, vitamina E e β-carotenos, que são nutrientes antioxidantes (RICE-EVANS et al., 1995). Além disso, a QUE ajuda na quelação de íons metálicos de transição, incluindo o ferro,

assim prevenindo a ferro-catálise na reação de Fenton (FERRALI et al., 2000). Em modelos animais, a quercetina apresentou o efeito de proteger a memória contra danos causados pela D-galactose, assim como da isquemia cerebral (LU et al., 2006; PU et al., 2007). Dessa forma, a utilização de agentes antioxidantes como a QUE pode representar uma nova abordagem na inibição dos danos provocados pelo estresse oxidativo, servindo como adjuvante na terapia complementar ao DM.

2 OBJETIVOS

2.1 Objetivo geral

Investigar o efeito da quercetina na atividade da acetilcolinesterase, nos testes comportamentais, perfil oxidativo e o efeito anti-inflamatório em ratos diabéticos, induzidos experimentalmente pela estreptozotocina.

2.2 Objetivos específicos

Em ratos diabéticos, induzidos experimentalmente pela estreptozotocina objetiva-se avaliar:

- A resposta hematológica, o perfil eletroforético e a função hepática e renal.
- Os níveis séricos de peroxidação lipídica e atividade das enzimas CAT e SOD hepática e renal.
- As alterações morfológicas hepáticas e pancreáticas.
- O comportamento e a formação da memória aversiva.
- A atividade da acetilcolinesterase e a peroxidação lipídica em diferentes estruturas encefálicas (córtex, hipocampo, estriado e sinaptossomas cerebrais).

3 MANUSCRITOS

Os resultados desta tese estão sob a forma de dois manuscritos científicos. Os itens materiais e métodos, resultados, discussão e referências bibliográficas encontram-se nos manuscritos.

3.1 Manuscrito 1:

Antioxidant and anti-inflamatory effects of quercetin in functional and morphological alterations in streptozotocin-induced diabetic rats

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Manuscrito aceito na Research in Veterinary Science

Antioxidant and anti-inflammatory effects of quercetin in functional and morphological alterations in streptozotocin-induced diabetic rats

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Abstract

The aim of this study was to investigate functional and morphological alterations caused by oxidative stress in streptozotocin (STZ)-induced diabetic rats and to evaluate the antioxidant effect of quercetin (QUE) in this disease. One hundred and thirty male Wistar rats, were randomly distributed in 10 different experimental groups, with ten animals per group: Control Saline (CS), Control Ethanol (CE), Control QUE 5mg/kg (CQ5), Control QUE 25mg/kg (CQ25), Control QUE 50mg/kg (CQ50), Diabetic Saline (DS), Diabetic Ethanol (DE), Diabetic QUE 5mg/kg (DQ5), Diabetic QUE25mg/kg (DQ25), Diabetic QUE 50mg/kg (DQ50). Therefore, hyperglycemia is directly involved in oxidative stress production, as well as in functional and morphological alterations caused by the excess of free radicals. QUE, specially at the dosage of 50mg/kg, can act as an antioxidant and anti-inflammatory agent, becoming a promising adjuvant in the treatment of diabetes mellitus.

Keywords: Diabetes mellitus; hepatic enzymes; proteins electrophoresis; oxidative stress.

Introduction

Diabetes mellitus (DM) is a very complex disease in people and equally so in the dog and cat (Hoenig, 2002). DM is considered to be a common endocrinopathy in dogs, presenting prevalence between 0.3% to 1.3% (Davison et al., 2005). The incidence of diabetes in cats ranges from 1 in 50 to 1 in 400 depending on the population studied (Baral et al., 2003). The most common form of diabetes in companion animals varies with the species. Type 1 DM (T1DM), previously called insulin-dependent diabetes, is most common in dogs, whereas type 2 (T2DM), previously called non-insulin-dependent or adult-onset diabetes, appears to be the more common form of diabetes in cats (Rand et al., 2004). The etiology of canine DM is considered multifactorial and may be broadly divided into insulin resistence and insulin deficiency (Catchpole et al., 2005). Certain breeds have been shown to have either an

increased or a decreased risk of developing the DM, implying the existence of important genetic factors in the aetiology (Davison et al., 2005).

Streptozotocin (STZ) is the drug of choice for the induction of experimental diabetes in rats, once this substance causes a selective destruction of pancreatic beta-cells, resulting in a hyperglycemic model similar to that observed in T1DM (Jin et al., 2010). Even that in this case the mechanism responsible for beta-cells destruction is not an autoimmune process this model remains viable, especially in studies where hyperglycemia is the main cause of the alterations evaluated (Obrosova et al., 2005).

Impaired glucose metabolism observed in diabetes may increase reactive oxygen species (ROS) production (Robertson and Harnon, 2006). An increased oxidative stress is a well-known pathogenic mechanism related to diabetic complications. Induction of diabetes with streptozotocin causes an increase in thiobarbituric acid-reactive substances (TBARS) levels, that can be indirectly used to evaluate free radicals formation (Maritim et al., 2003).

Quercetin (QUE) is a flavonoid with antioxidant properties (De Groot, 1994). This compound is present in fruit, vegetables, and is abundant in red wine (Gaspar et al., 1993). Several researchers have investigated substances with antioxidant effect (Dallaqua and Damasceno, 2011). Currently, over 800 types of plants are used in the treatment of diabetes (Saxena et al., 2004) and most of these have a broad spectrum in the clinic. Thus, it is necessary to explore the phytomedicine area to provide alternative therapies for the treatment of diabetic syndrome (Yeh et al., 2003; Suba et al., 2004).

The objective of the present study was to investigate functional and morphological alterations caused by oxidative stress and to evaluate the antioxidant and anti-inflammatory effects of QUE in STZ-induced diabetic rats. Searching with this, new possibilities for supporting therapy in the treatment of diabetes in veterinary medicine, mainly dogs.

Materials and methods

Animals

One hundred and thirty male Wistar rats (70-90 days old; 160–250g) from the Bioterium of the Federal University of Santa Maria were used in this study. Of this total, 96 rats reached the end of the experiment being used to collect samples. From the remaining 34 rats, 15 rats died between the induction of diabetes and end of the experiment, and the others whose do not reach 250mg/dL blood glucose, were euthanized with halothane (deep anesthesia). The animals were maintained at a constant temperature (23 $\pm$ 1ºC), on a 12h dark/light cycle with free access to food and water. All procedures were approved by the Animal Ethics Committee from the Federal University of Santa Maria (protocol number: 57/2010).

Experimental induction of diabetes

The animals were housed five per cage and submitted to a period of 15 days of adaptation. Diabetes was induced by a single intraperitoneal injection of 70mg/Kg STZ (Sigma Chemical Co, St. Louis, MO, USA). diluted in 0.1 M sodium-citrate buffer (pH 4.5), in the proportion of 0.001mL per 1g of body weight. The age-matched control rats received an equivalent amount of the sodium-citrate buffer. STZ-treated rats received 5% of glucose instead of water for 24h after diabetes induction in order to reduce death due to hypoglycemic shock. Blood samples were taken from the caudal vein 48 h after STZ or vehicle injection to measure glucose levels. Only animals with fasting glycemia higher than 250 mg/dL were considered diabetic and used for the current study. During the experimental period the levels of blood glucose were verified six times (15 days before, on day one, 10, 20, 30, and 40 days after the beginning of treatment).

Treatment with quercetin (QUE)

The rats were randomly divided into ten groups (10 rats per group): Control Saline (CS); Control Ethanol (CE); Control QUE 5mg/Kg (CQ5); Control QUE 25mg/Kg (CQ25); Control QUE 50mg/Kg (CQ50); Diabetic Saline (DS); Diabetic Ethanol (DE); Diabetic QUE 5mg/Kg (DQ5); Diabetic QUE 25mg/Kg (DQ25); Diabetic QUE 50mg/Kg (DQ50). Ten days after diabetes induction, the animals belonging to groups CQ5 and DQ5 received 5mg/Kg of QUE, the animals from CQ25 and DQ25 groups received 25mg/Kg and the rats from groups CQ50 and DQ50 received 50mg/Kg of QUE, while the animals from groups CS and DS received 0,9% saline solution by gavage. The groups CE and DE received 25% ethanol by gavage. QUE, (Sigma Chemical Co, St. Louis, MO, USA), was freshly prepared in 25% ethanol and was administered at between 3 and 4 p.m. once a day during 40 days, by gavage. The dose of QUE was adjusted weekly, according to the body weight of the rats. The volume of saline solution and ethanol were adjusted according to the proportional 0.001mL per 1g of body weiht.

Blood collection

Forty days after DM induction, the rats were anesthetized with halothane and blood was collected by cardiac puncture. Blood samples for biochemical assays were collected in tubes without anticoagulant and serum was obtained by centrifugation of the samples for 10 minutes. Blood samples for hematological analysis were collected in tubes containing EDTA as anticoagulant.

Hematological profile

Hematological analysis was performed using an automatic hematological analyzer (Auto Hematology Analyz, BC-2800 Vet – Mindray). Hematological parameters evaluated were total red blood cell (RBC) and white blood cell (WBC) count, hematocrit (HCT), hemoglobin

(Hb), mean corpuscular volume (MCV) and mean corpuscular hemoglobin concentration (MCHC). For the differential leukocytes count (neutrophils, lymphocytes, eosinophils and monocytes) differential, a blood smear was prepared and the slides were stained with Diff-Quick. The WBC count was performed in groups of 100 leukocytes, and then examined under oil immersion by optic microscope.

Electrophoresis profile of serum proteins determination

The total serum protein was determined through the biuret method, using commercial reagent and the analysis were accomplished in semi automatic spectrophotometer, according to fabricant instructions. The protein fractionation was determined using cellulose acetate strip electrophoresis in a horizontal cube (Labex), with Tris-glycine buffer (pH 8.6), adapted from technique described by Naoum (1999). Samples were applied to the strips and run using a constant voltage of 180 volts for 25 minutes. Strips were then stained with Ponceau for 15 minutes, the excess was removed by washing the strips in 5% acetic acid until background was completely clear. Then strips were fixed in methanol for 30 seconds and washed for 1 minute with a destain solution. Strips were dried at 60ºC for 15 minutes and read by the Descan system. The fractions analyzed were the albumin, alphaglobulin, betaglobulin and gammaglobulin.

Biochemical profile

Biochemical analysis of serum samples was performed using a semi automatic chemistry analyzer (Bioplus, BIO-2000), using commercial kits (Labtest, Minas Gerais, Brazil), according to the recommendations of the manufacturer. Biochemical parameters measured were fructosamine, triglycerides, cholesterol, urea, creatinine, alkaline phosphatase (ALP) and alanine aminotransferase (ALT). Blood glucose was determined by portable glucometer

Accu-Check Nano Performa (Roche, Brazil). Insulin was evaluated by solid phase radioimmunoassay using commercial kit Coat-A-Count, purchased from Diagnostic Products Corporation (Gwynedd, UK).

Determination of lipid peroxidation

Lipid peroxidation in serum was estimated by measuring TBARS according to a modified method of Jentzsch et al. (Levini et al., 1990). Briefly, 0.2mL of serum was added to the reaction mixture containing 1mL of 1% orthophosphoric acid and 0.25mL alkaline solution of thiobarbituric acid (final volume 2mL), followed by 45 min heating at 95ºC. After cooling, samples and standards of malondialdehyde were read at 532 nm against the blank of the standard curve. The results were expressed in nmol MDA/mg protein.

Catalase and superoxide dismutase activities

The determination of CAT activity was carried out in accordance with a modified method of Nelson and Kiesow (1972). For determination of CAT activity in liver and kidney, the tissues were homogenized in 50mM potassium phosphate buffer, pH 7.5, at a proportion of 1:9 (w/v) and 1:5 (w/v), respectively. The homogenate was centrifuged at 2000g for 10 min to yield a supernatant that was used for the enzyme assay. The reaction mixture contained 50mM potassium phosphate buffer (pH 7), 10mM H202 and 20mL of the supernatant. The rate of H_2O_2 reaction was monitored at 240nm for 2 min at room temperature. The enzymatic activity was expressed as nmol CAT/mg protein (one unit of the enzyme is considered as the amount of CAT which decomposes 1µmol of H_2O_2 per min at pH 7 at 25ºC).

Measurement of superoxide dismutase activity is based on the inhibition of the radical superoxide reaction with adrenalin as described by Misra and Fridovich (1972). With the purpose of performing the SOD assay in liver and kidney (Misra and Fridovich, 1972), the

tissues were adequately diluted with Tris-HCl pH 7.4 at a proportion of 1:40 (w/v) and 1:60 (w/v) respectively. Briefly, epinephrine undergoes auto-oxidation at pH 10.2 to produce adrenochrome, a colored product that was detected at 480 nm. The addition of samples (10, 20, 30μL) containing SOD inhibits the auto-oxidation of epinephrine. The rate of inhibition was monitored during 180 seconds. The amount of enzyme required to produce 50% inhibition was defined as one unit of enzyme activity. The enzymatic activity was expressed as UI SOD/mg protein.

Tissue preparation

After blood collection the rats were submitted to euthanasia. Pancreas and liver were quickly removed and placed on buffered formalin. Twenty-four hours later, a fragment of the tissue samples were included in paraffin.

Immunohistochemical analysis of the pancreas

The slides from pancreas were incubated with insulin antibodies (DAKO), Anti Guinea-Pig and Complex ABC (Avidin-biotin-peroxidase) used as secondary markers aiming to identify and measure pancreatic beta-cells. Ten fields per slide were then examined under microscope at 400x magnification.

Hepatic histological and morphometric analysis

The histological and morphometric analysis from hepatic tissue were examined in slides stained by hematoxiline-eosine method (HE), periodic acid-Schiff reactive technique and Harris hematoxiline (PAS). Hepatic morphology and possible alterations were observed by HE technique and the hepatocytes with glycogen accumulation were quantified (PAS technique) in ten standardized microscope fields at 400x magnification.

Statistical analysis

As data presented normal distribution, proven by the Kolmogorov Smirnov test, they were submitted to one-way ANOVA, followed by Tukey's post hoc test, using the statistical program, SPSS 17.0 for Windows. Differences were considered significant when $P<0.05$. All data were expressed as mean ± standard deviation.

Results

Clinical course, body weight and glucose levels

Diabetic rats from this study presented clinical disorders commonly observed in this condition, such as, polydipsia, polyuria, lethargy, polyphagia, and weight loss. The values of body weight and blood glucose levels are presented in Table 1 and 2, respectively. There was not a significant difference in body weight among the groups before the beginning of the study, but fifteen days after administration of STZ, the average body weight of diabetic animals decreased when compared to CS group. Ten days after the start of treatment with QUE, the difference in body weight from DS and CS groups was significant, and remained so until the end of the experiment. Fifteen days after induction of diabetes, blood glucose levels of diabetic animals increased when compared to control animals and remained high during the 40 days of treatment with QUE ($P<0.05$).

Hematological parameters

Evaluating the hematological parameters studied (Table 3), there were changes in the number of total leukocytes and neutrophils ($P<0.05$). Total leukocytes number was significantly lower in diabetic rats when compared to non-diabetic animals, characterizing a significant leukopenia. In DQ5 group, this number reduced, considered statistically similar to CS group

(P<0.05). The diabetic rats also presented neutropenia; the neutrophil count was significantly lower when compared to CS group. In DQ25 group, the number of neutrophils decreased, being statistically similar to those observed in CS group (P<0.05).

Serum proteinogram

The electrophoretic profile is represented by the following parameters: albumin, alphaglobulin, betaglobulin and gammaglobulin (Table 3). DS group showed decreased levels of albumin in relation to CS group. DQ50 group showed a reduction of serum albumin level, similar to those from CS group (P<0.05). In DS group there was an increase in alphaglobulin fraction when compared to CS group. Animals who received 25 and 50mg/Kg of QUE showed a reduction of this fraction when compared to DS group (P<0.05). The concentration of betaglobulin in DS group was higher than CS group. Diabetic rats treated with 25 and 50mg/Kg of QUE, presented increased concentration of betaglobulin, when compared to CS group (P<0.05). The gammaglobulin fraction in DS group increased when compared to CS group (P<0.05). No significant difference was observed in diabetic rats treated with 50mg/Kg of QUE and CS group.

Biochemical and hormonal parameters

The biochemical parameters evaluated in this study are described in Table 4. It was observed a high rate of insulin release in treatments with 5, 25 and 50mg/Kg of QUE, both in CS and DS groups, the last one presenting the highest release rate of this hormone. Diabetic rats showed a decreased in serum concentration of insulin when compared to CS group; DQ50 group showed similar levels to those observed in CS group (P<0.05). The concentration of fructosamine in serum of diabetic rats increased in relation to CS group. In diabetic rats treated with 5, 25 and 50mg/Kg of QUE, the concentration of fructosamine remained higher

compared to CS group (P<0.05). Serum concentration of triglycerides in DC group increased when compared to CS group (P<0.05). In diabetic rats, treated with 5, 25 and 50mg/Kg of QUE, it was found a decrease in concentration of triglycerides to values similar to those observed in CS group.

Concentration of serum urea increased in DS group, comparing with CS group. Diabetic rats treated with 5, 25 and 50mg/Kg of QUE showed an increase, when compared to healthy groups who received this same dosages of this compound (P<0.05). DS group showed ALP activity higher than CS group. In diabetic rats treated with 5, 25 and 50mg/Kg of QUE, the ALP activity remained higher when compared to CS group (P<0.05). When compared to CS group, ALT activity of diabetic rats increased. In diabetic rats treated with 5, 25 and 50mg/Kg of QUE, the enzyme activity remained high in relation to CS group (P<0.05).

Lipid peroxidation, catalase and superoxide dismutase activities

TBARS in serum, SOD and CAT activities in renal and liver tissues were used in this study as biomarkers of oxidative stress (Table 5). The TBARS levels increased in the DS group, when compared to CS group. After treatment with 50mg/Kg of QUE, the levels reverted close to normal values observed in CS group (P<0.05). Regarding the CS group, the SOD activity in both the liver and kidney, decreased in the DS group. However, only in the hepatic tissue was observed significance. In DQ50 group, it was observed a reduction of the enzyme activity, similar to the activity observed in CS group (P<0.05). Regarding the CS group, CAT activity, both as kidney liver, decreased in the DS group, no significant difference in the two organs.

Immunohistochemical, histological and morphometric analysis

Diabetic animals showed a reduction in the number of islet of Langerhans when compared to CS group (P<0.05) (Table 6). Pancreas presented less number of beta-cells in diabetic rats

than CS group animals ($P<0.05$). The relation between the number of beta-cells and the number of islet of Langerhans was lower in diabetic rats when compared to control rats ($P<0.05$).

In comparison to the CS group, histological alterations were detected in the liver tissue of diabetics rats, treated or not with 5, 25 and 50mg/Kg of QUE (Fig. 1-3). The hepatocytes presented tumefaction and it was observed glycogen accumulation (PAS+) in cytoplasm as fine granulation or eventual globules, forming pale areas. Some of the hepatocytes were binucleated. Trabeculae were disaggregated and hepatic sinusoid dilated. Resident macrophages (Kupffer cells), leukocytes and plasmocytes were observed in hepatic sinusoids and portal space. Kupfer cells presented glycogen accumulation (PAS+) as fine granules or big globules. There was no difference among the treatments in relation to the glycogen accumulation (PAS+) in hepatocytes.

Discussion

Fifteen days after STZ administration, glycemia was significantly higher in diabetic rats. STZ is a diabetogenic agent that contains a nitrous substance which liberates nitric oxide during its metabolism, becoming toxic to beta-cells (Szkudelski, 2001). At the end of the experimental period (40 days after DM induction), immunohistochemical evaluation of pancreatic beta-cells (Figure 1) revealed that 91,3% of the cells were destroyed. T1DM characteristics are only evident after destruction of about 80% of beta-cells (Powers, 2009). Thus, when QUE was administered, a huge amount of beta-cells, from diabetic rats, had already been destroyed. Moreover, the absence of beta-cells suggests that QUE did not assist in cellular regeneration.

On the other hand, QUE presented stimulatory activity for insulin secretion, once all rats, diabetic or non-diabetic, who received this compound presented an elevation of insulin concentration when compared to rats who did not receive QUE. Youl et al. (2010) also

observed an increase in insulin release after administration of 20μmol/L of QUE when the effect of this compound was studied in beta-cells INS-1, under oxidative stress conditions caused by H_2O_2. In our study, it was observed an elevated fructosamine concentration in diabetic rats, which means that once hyperglycemia was established, it did not return to physiological values during the experiment. Even in the presence of high levels of insulin, as observed in diabetic rats treated with QUE, it was not observed a reduction of glycemic parameters. Insulin resistance is associated with low regulation of insulin signaling cascade and/or glucose transporter stimulated by insulin (Shulman, 2000). The initiation of insulin resistence in specific tissues becomes physiologically important when the pancreas compensates for whole-body insulin resistance and increases insulin release (Stewart et al., 2009). Previous studies reported beneficial effects: significant reduction in insulin resistance, hyperlipidaemia and inflammation after oral administration of QUE to fatty Zucker rats (Rivera et al., 2008), from a wide range of doses (50 μg $mouse^{-1}$ day^{-1} to 50 mg $mouse^{-1}$ day^{-1}) (Stewart et al., 2009). Thus, the absence of blood glucose reduction in the presence of normal to high concentration of insulin, in the groups treated with QUE, suggests that QUE could influence the sensitivity of insulin receptors, leading to insulin resistance, as well as glucocorticoids and progestins.

In this study, obesity was not observed in diabetic rats, on the contrary, after administration of STZ, the animals presented reduction of body weight as a result of the increase in catabolism. Thus, to compensate difficulties to use blood glucose, diabetic animals present increased tissue metabolism with loss of muscular mass, as it was observed in this study. In our study, triglycerides levels were higher in diabetic rats not treated with QUE. Uncontrolled diabetes is accompanied by an increase in blood concentrations of triglycerides, cholesterol, lipoproteins and free fatty acids. The hypertriglyceridemia is resulted of increased chylomicron and very low density lipoproteins (VLDL) which in turn results from a deficiency of insulin and

restrictions associated with lipoprotein lipase (LpL) (Unger and Foster, 1998). Diabetic rats treated with 5, 25 and 50mg/kg of QUE also exhibited an increase in triglycerides levels, but it was not considered significant. Once increased levels of triglycerides in association with hyperglycemia contribute to development of atherogenesis in diabetic state (Renard et al., 2004), and once QUE was able to maintain triglycerides levels close to those observed in CS group, this substance would have a beneficial effectin lowering triglycerides.

The decline of antioxidant defense mechanisms associated with an excess of free radicals lead to damage of cellular organelles and enzymes, increased lipid peroxidation, and development of insulin resistance (Maritim et al., 2003). Lipid peroxidation results in damage to the cell membrane, caused by ROS (Das et al., 2000). Ramachandran et al. (2004) observed high concentrations of TBARS in liver and kidneys of diabetic rats. In our study we verified that TBARS was increased in diabetic rats treated with 5 and 25mg/kg of QUE. Induction of DM with STZ results in elevation in TBARS concentration, suggesting an elevated free radical production (Montilla et al., 1998). The results obtained with 50mg/Kg of QUE indicate that this flavonoid was able to protect organism from lipid peroxidation. Even after establishment of DM, increased TBARS levels may be reversed by administration of vitamin E combined with vitamin C and β-carotene (Maritim et al., 2003).

In our study, diabetic rats presented a significant reduction in SOD activity, confirming what was observed by other authors (Sekeroglu et al., 2000; Ozkaya et al., 2002; Coskun et al., 2005). However, diabetic rats treated with 50mg/Kg of QUE did not show a significant reduction in SOD activity, suggesting that this flavonoid at this dosage inhibited the effects caused by oxidative stress.

It was not observed a reduction in CAT activity in liver in any of the treatments to which the diabetic rats were subjected. According to Yan and Harding (1997), a reduction in SOD and CAT activities in DM may be due to direct glycation of enzyme protein or loss of weight

observed in diabetic condition (Wohaieb and Godin, 1987). In any way, ROS detoxification depends on a mechanism that acts in a cooperative form (Sies, 1993). CAT requires previous action of enzyme SOD. SOD is responsible to dismutates superoxide anion (O_2^-) into hydrogen peroxide (H_2O_2) and CAT converts H_2O_2 to water (H_2O) and oxygen (O_2) (Fridovich, 1998).

In diabetic animals, a reduction in SOD and CAT activities in liver and kidneys is already well documented (Stanley et al., 2001). However, in our experiment, it was not observed a significant difference in kidneys of diabetic rats treated or not with QUE when compared to CS group ($P<0.05$).

A significant decrease in SOD activity in the liver suggests an impairment of antioxidant defense system in diabetic rats and makes this organ more vulnerable to damage caused by ROS. Increased hepatocyte volume, characterized as cellular tumefaction, has been observed in all treatment regimen from diabetic groups (Figure 2A), as a result of water overload caused by an ionic homeostasis imbalance inside the cell (Schrand et al., 2010).

In diabetic rats from our study, it was not observed an increase of glycogen storage in hepatic samples stained with PAS (Figure 2B). However, as a consequence of the increasing unavailability of ATP, there is higher glycogen consumption through glycolysis, in order to maintain the energetic balance (Myers and McGavin, 2009). The condition of desnutrition observed in diabetic rats suggests an increased depletion of hepatic and muscular glycogen storages. Moreover, glycogen synthesis depends on insulin due to its stimulatory effect. A low insulin availability, as well as the insulin resistance observed in diabetic patients can contribute to decrease of glycogen (Jensen and Lai, 2009). Glycogen depots were found in some macrophage located in sinusoids and portal space. As glycogen synthesis occurs inside hepatocytes, its presence inside Kupffer cells suggests extravasation caused by hepatic injury.

In this study, diabetic rats presented a significant reduction in serum albumin concentration, except rats treated with 50mg/kg of QUE. The low serum levels of albumin may be explained by a chronic inflammation of the liver, when in this case it was observed an increase of immunoglobulins in contrast with a decrease in albumin levels, due to synthesis deficiency (Naoum, 1999).

This study revealed increased ALP and ALT enzymes activities. Elevated liver enzyme activity is usuallys light and due to hepatic lipidosis (Nelson, 2009). Endocrine disorders, such as diabetes, can trigger hepatocellular lipidosis in a variety of species. The lipid accumulation in the liver of a diabetic animal is the result of increased fat mobilization and decreased using of lipids by affected hepatocytes (Cullen, 2009). The high urea concentration observed in diabetic rats from this study indicates an excessive amount of nitrogenated amino acids derived from catabolism and also was an important test to evaluate functional capacity of the liver.

In this study it was also observed that diabetic rats presented a significant leukopenia and neutropenia. Hyperglycemia is associated not only with an increased risk of infection, but also with abnormalities in neutrophil function (Pomposelli et al., 1998). The correlation between infectious diseases in diabetic patients and dysfunction in neutrophils, monocytes and macrophages is well established (Engelich et al., 2001). Neutrophils present reduced capacity to phagocyte and destroy bacteria (Alexiewicz et al., 1995). Patients with severe renal insufficiency present reduced phagocytic activity of neutrophils and macrophages (Vanholder et al., 1991). Creatinine excretion have not been compromised in diabetic rats from this study, but elevated urea concentration observed in this case may have caused a reduction in phagocytic capacity of neutrophils, due to elevated intracellular calcium concentration, iron overload, and uremic toxins (Haag-Weber and Horl, 1996).

In our study, protein electrophoresis showed an increase in gamma fraction in diabetic rats treated with 50mg/Kg of QUE, and in beta fraction in rats who received 25 and 50mg/Kg of this compound. No significant variation was noted in the alpha globulin fraction, probably due to the chronicity of the inflammatory stimuli. Beta globulins, more exactly beta-2 globulins, present C3 and C4 fractions from the complement, and also IgA. This fraction is slightly increased when is observed an elevation of the complement system, caused by inflammation or secondary to intra- or extrahepatic biliary obstruction. Gamma globulin fraction is composed of immunoglobulins, specially IgG, IgA and IgM; and IgD and IgE in less concentration (Szymanowicz et al., 2006).

Many diseases result in increased serum levels of gamma globulins, especially those with chronic evolution in which an accentuated antibody response occurs. Hepatopathies are conditions very often related to an increase in gamma globulin fraction. In hepatitis, a slight increase in beta- and gamma globulins levels are often observed, in association with a decrease in albumin concentration (Ravel, 1995). Hypoalbuminemia and low alpha globulin levels, such as haptoglobin and alpha-1-antitrypsin, are common findings observed in severe hepatic insufficiency and indicate a bad prognosis. High globulin levels associated with an acute phase response indicate a systemic immune stimulus, secondary to an impairment of Kupffer cells, B and T cells function, and immunoglobulin production (Center, 2007). Results observed in diabetic rats treated with 25 and 50mg/Kg of QUE suggest that this flavonoid attenuated the inflammatory process, probably during its acute phase, once this substance presents anti-inflammatory action which has been previously described in other studies (Erlund, 2004).

Conclusion

The results observed in this study suggest that hyperglycemia is directly involved in oxidative stress observed in diabetic rats, as well as in functional and morphological alterations caused by an excess of free radicals. Furthermore, QUE, especially at the dosage of 50mg/Kg presented antioxidant and anti-inflammatory activities, which coud be useful in treatment of DM. Thus, we can conclude that QUE is a promising adjuvant agent in diabetes mellitus therapy.

Conflict of interest statement

None of the authors has any financial or personal relationships that could inappropriately influence or bias the content of the paper.

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Table 1

Body weight of STZ-induced diabetic rats and those treated with quercetin 15 days before, first day, 10, 20, 30, and 40 days after the beginning of treatment

Groups	Body weight (g)					
	15 days before	First day	10 day	20 day	30 day	40 day
Control saline	219,7± 30,7	291,4± 35,1[a]	311,1± 35,6[a]	312,7± 33,2[a]	322,9± 39,0[a]	341,1± 43,6[a]
Control etanol	180,6± 16,9	293,5± 22,8[a]	303,8± 23,1[a]	300,3± 25,2[a]	315,2± 26,9[a]	329,8± 27,7[a]
Control QUE 5mg/Kg	199,3± 14,6	282,1± 31,2[ab]	299,7± 33,6[a]	303,2± 30,1[a]	313,7± 32,5[a]	326,5± 30,5[a]
Control QUE 25mg/Kg	197,9± 36,7	272,2± 25,0[ab]	293,3± 27,2[a]	301,4± 26,7[a]	307,7± 27,3[a]	318,2± 29,3[a]
Control QUE 50mg/Kg	191,2± 24,2	283,5± 21,3[ab]	301,4± 21,0[a]	306,5± 15,3[a]	317,4± 17,2[a]	328,2± 18,1[a]
Diabetic saline	181,1± 15,7	223,4± 23,7[cd]	213,8± 28,1[b]	201,5± 28,3[bc]	198,0± 35,8[b]	200,5± 20,8[bc]
Diabetic etanol	201,0± 25,9	225,1± 26,7[cd]	224,8± 24,6[b]	208,9± 27,3[bc]	214,1± 32,4[b]	198,5± 32,7[bc]
Diabetic QUE 5mg/Kg	193,7± 29,9	214,0± 21,6[d]	213,0± 22,3[b]	197,1± 23,2[c]	196,8± 26,5[b]	183,1± 22,1[c]
Diabetic QUE 25mg/Kg	187,8± 29,2	236,9± 33,3[cd]	227,5± 38,7[b]	208,7± 42,8[bc]	216,3± 47,3[b]	213,4± 53,7[bc]
Diabetic QUE 50mg/Kg	207,8± 34,3	251,8± 22,1[bc]	245,7± 31,6[b]	239,7± 44,5[b]	240,9± 42,4[b]	238,5± 50,9[b]

Groups with different letters, in the same column, are statistically different ($P<0.05$, n=9-10).

Table 2

Glucose levels of STZ-induced diabetic rats and those treated with quercetin 15 days before, first day, 10, 20, 30, and 40 days after the beginning of treatment

Groups	Glucose (mg/dL)					
	15 days before	First day	10 day	20 day	30 day	40 day
Control saline	79,0± 5,2	84,4± 6,6[a]	80,6± 5,2[a]	81,1± 2,4[a]	81,5± 5,7[a]	67,2± 6,3[a]
Control etanol	76,4± 8,1	85,6± 11,0[a]	76,6± 9,7[a]	77,3± 9,9[a]	72,6± 4,8[a]	70,1± 5,2[a]
Control QUE 5mg/Kg	75,2± 8,2	83,0± 15,0[a]	75,4± 8,6[a]	74,1± 6,6[a]	70,1± 4,5[a]	73,6± 6,4[a]
Control QUE 25mg/Kg	76,8± 5,7	85,3± 8,7[a]	79,4± 5,0[a]	74,6± 4,8[a]	69,6± 6,2[a]	75,3± 4,0[a]
Control QUE 50mg/Kg	76,0± 6,0	81,0± 4,4[a]	78,0± 6,8[a]	73,4± 5,3[a]	69,9± 6,1[a]	75,8± 3,7[a]
Diabetic saline	75,1± 6,7	480,7± 105,0[b]	438,7± 133,4[b]	453,4± 124,1[b]	472,3± 102,7[b]	436,8± 69,0[bc]
Diabetic etanol	76,4± 11,0	473,4± 79,4[b]	511,5± 76,4[b]	525,3± 75,2[b]	500,6± 99,1[b]	454,3± 82,3[bc]
Diabetic QUE 5mg/Kg	74,1± 6,6	436,8± 69,6[b]	489,2± 103,7[b]	522,0± 131,6[b]	483,9± 153,9[b]	541,1± 44,7[c]
Diabetic QUE 25mg/Kg	80,6± 8,4	431,0± 112,8[b]	399,7± 178,4[b]	422,5± 166,5[b]	475,1± 153,9[b]	423,9±147,6[b]
Diabetic QUE 50mg/Kg	85,1± 10,1	467,2± 100,4[b]	398,6± 159,9[b]	428,2± 173,9[b]	430,6± 149,2[b]	386,0±161,8[b]

Groups with different letters, in the same column, are statistically different ($P<0.05$, n=9-10).

Table 3
Hematological and serum proteinogran profile of STZ-induced diabetic rats and those treated with quercetin at the end of the experiment

	Groups									
	Control saline	Control etanol	Control QUE 5mg/Kg	Control QUE 25mg/Kg	Control QUE 50mg/Kg	Diabetic saline	Diabetic ethanol	Diabetic QUE 5mg/Kg	Diabetic QUE 25mg/Kg	Diabetic QUE 50mg/Kg
Leukocytes (µL)	5300 ± 1931^{ab}	4370 ± 2073^{abc}	5810 ± 2183^{a}	5290 ± 2072^{ab}	4620 ± 3001^{abc}	2970 ± 1497^{c}	3490 ± 1863^{bc}	4070 ± 2756^{abc}	2930 ± 1514^{c}	2890 ± 2062^{c}
Neutrophils (µL)	1843 ± 751^{a}	1371 ± 383^{ab}	1808 ± 735^{a}	1712 ± 562^{a}	1525 ± 789^{ab}	1030 ± 404^{b}	893 ± 351^{b}	958 ± 687^{b}	1198 ± 752^{b}	858 ± 452^{b}
Total protein (g/dL)	6.45 ± 0.44	6.88 ± 0.79	6.26 ± 0.63	6.11 ± 1.34	6.97 ± 0.67	6.93 ± 0.77	6.56 ± 0.63	6.71 ± 0.64	6.44 ± 0.68	6.60 ± 0.64
Albumin (g/dL)	4.24 ± 0.46^{ab}	4.43 ± 0.39^{a}	3.89 ± 0.24^{abcd}	4.02 ± 0.47^{abc}	4.37 ± 0.45^{a}	3.32 ± 0.37^{cd}	3.45 ± 0.28^{cd}	3.31 ± 0.36^{d}	3.32 ± 0.43^{d}	3.72 ± 0.66^{bcd}
Alpha-1 globulin (g/dL)	0.48 ± 0.18^{ab}	0.47 ± 0.12^{ab}	0.44 ± 0.07^{a}	0.44 ± 0.09^{a}	0.58 ± 0.15^{ab}	0.59 ± 0.07^{ab}	0.61 ± 0.10^{b}	0.62 ± 0.08^{b}	0.58 ± 0.09^{ab}	0.58 ± 0.12^{ab}
Beta globulin (g/dL)	1.05 ± 0.19^{ab}	1.13 ± 0.17^{abc}	0.94 ± 0.21^{a}	0.91 ± 0.24^{a}	1.02 ± 0.21^{a}	1.50 ± 0.32^{d}	1.42 ± 0.17^{cd}	1.55 ± 0.32^{d}	1.36 ± 0.20^{bcd}	1.24 ± 0.15^{abcd}
Gamma globulin (g/dL)	0.92 ± 0.16^{a}	0.89 ± 0.17^{a}	0.87 ± 0.13^{a}	0.91 ± 0.24^{a}	1.01 ± 0.13^{ab}	1.38 ± 0.26^{c}	1.26 ± 0.22^{c}	1.35 ± 0.21^{c}	1.18 ± 0.23^{bc}	0.99 ± 0.13^{a}

Groups with different letters, in the same line, are statistically different ($P<0.05$, n=9-10).

Table 4

Biochemitry of STZ-induced diabetic rats and those treated with quercetin at the end of the experiment

	Groups									
	Control saline	Control ethanol	Control QUE 5mg/Kg	Control QUE 25mg/Kg	Control QUE 50mg/Kg	Diabetic saline	Diabetic ethanol	Diabetic QUE 5mg/Kg	Diabetic QUE 25mg/Kg	Diabetic QUE 50mg/Kg
Insulin (ng/mL)	0.76± 0.03^{d}	0.8± 0.04^{cd}	0.85± 0.05^{bc}	0.89± 0.02^{ab}	0.95± 0.03^{a}	0.14± 0.03^{g}	0.14± 0.02^{g}	0.28± 0.05^{f}	0.53± 0.02^{e}	0.76± 0.09^{d}
Fructosamine (µmol/L)	167± 8.52^{a}	167± 10.34^{a}	159± 10.53^{a}	154± 9.19^{a}	165± 8.91^{a}	238± 17.19^{bc}	258± 38.60^{c}	248± 26.62^{bc}	248± 42.98^{bc}	217± 40.17^{bc}
Triglycerides (mg/dL)	95± 28.33^{a}	79± 30.32^{a}	72± 29.01^{a}	67± 29.99^{a}	73± 22.57^{a}	178± 81.80^{b}	184± 89.45^{b}	111± 50.26^{ab}	127± 18.86^{ab}	127± 18.86^{ab}
Cholesterol (mg/dL)	84± 25.10^{a}	71± 8.19^{ab}	81± 13.89^{a}	71± 27.72^{ab}	81± 14.53^{ab}	63± 13.01^{ab}	71± 26.76^{ab}	51± 20.71^{b}	75± 14.70^{ab}	79± 25.82^{ab}
Urea (mg/dL)	35± 4.94^{a}	41± 8.37^{a}	37± 3.89^{a}	39± 9.31^{a}	41± 6.39^{a}	71± 9.03^{b}	83± 11.99^{b}	83± 18.46^{b}	85± 22.10^{b}	74± 19.10^{b}
Creatinine (mg/dL)	0.50± 0.05	0.51± 0.07	0.52± 0.05	0.53± 0.03	0.53± 0.10	0.55± 0.06	0.49± 0.09	0.51± 0.06	0.53± 0.05	0.57± 0.18
ALP* (UI/L)	258± 89.35^{a}	338± 92.88^{ab}	248± 76.82^{a}	268± 132.35^{a}	324± 170.08^{ab}	1095± 381.11^{c}	1210± 507.73^{c}	1087± 563.74^{c}	1197± 266.61^{c}	770± 487.41^{bc}
ALT ** (UI/L)	71± 16.57^{a}	71± 20.46^{a}	68± 19.03^{a}	79± 30.28^{a}	68± 21.56^{a}	203± 58.90^{bc}	196± 83.00^{bc}	201± 41.45^{bc}	237± 105.87^{c}	154± 85.87^{bc}

Groups with different letters, in the same line, are statistically different ($P<0.05$, n=9-10).

*Alkaline phosphatase, **Alanine aminotransferase

Table 5

Biomarkers of oxidative stress of STZ-induced diabetic rats and those treated with quercetin at the end of the experiment

	Groups									
	Control saline	Control ethanol	Control QUE 5mg/Kg	Control QUE 25mg/Kg	Control QUE 50mg/Kg	Diabetic saline	Diabetic ethanol	Diabetic QUE 5mg/Kg	Diabetic QUE 25mg/Kg	Diabetic QUE 50mg/Kg
TBARS Serum (nmol MDA/mg protein)	9.4 ± 0.9[a]	11.8 ± 4.0[a]	11.0 ± 2.3[a]	10.9 ± 3,5[a]	10.8 ± 2.0[a]	21.0 ± 7.8[b]	23.0 ± 11.1[b]	20.1 ± 7.9[b]	21.1 ± 4.9[b]	16.0 ± 10.7[ab]
Hepatic superoxide dismutase (UI SOD/mg protein)	24.3 ± 6.7[a]	22.0 ± 5.2[ab]	17.2 ± 3.7[ab]	17.7 ± 2,7[ab]	16.3 ± 4.5[ab]	12.3 ± 2.9[b]	12.1 ± 3.5[b]	12.4 ± 3.7[b]	13.8 ± 6.8[b]	15.1 ± 7.5[ab]
Renal superoxide dismutase(UI SOD/mg protein)	22.2 ± 2.2[a]	23.8 ± 5.2[a]	24.3 ± 6.6[a]	23.5 ± 5.5[a]	26.9 ± 7.9[a]	18.3 ± 4.0[a]	21.1 ± 3.0[a]	24.1 ± 5.0[a]	30.0 ± 4.0[a]	25.2 ± 6.5[a]
Hepatic catalase (nmol CAT/mg protein)	111.4 ± 39.7[a]	103.5 ± 29.7[a]	75.3 ± 21.1[a]	93.5 ± 21,7[a]	79.3 ± 42.0[a]	83.1 ± 47.3[a]	87.1 ± 43.7[a]	84.5 ± 39.0[a]	90.1 ± 19.7[a]	116.3 ± 31.1[a]
Renal catalase (nmol CAT/mg protein)	123.5 ± 74.5[abc]	137.6 ± 52.2[ab]	145.4 ± 55.0[a]	138.9 ± 35.6[ab]	146.6 ± 57.3[a]	82.8 ± 9.3[abc]	84.8 ± 10.6[abc]	71.2 ± 19.5[c]	83.5 ± 22.2[abc]	74.5 ± 12.5[ac]

Groups with different letters, in the same line, are statistically different ($P<0.05$, $n=9-10$).

Table 6

Immunohistochemistry pancreatic of STZ-induced diabetic rats and those treated with quercetin at the end of the experiment

	Groups									
	Control saline	Control etanol	Control QUE 5mgKg	Control QUE 25mg/Kg	Control QUE 50mg/Kg	Diabetic saline	Diabetic ethanol	Diabetic QUE 5mg/Kg	Diabetic QUE 25mg/Kg	Diabetic QUE 50mg/Kg
Number of islet per field (100x)	4 ± 1.71[a]	3 ± 1.36[ab]	4 ± 1.88[a]	3 ± 1.55[a]	3 ± 1.10[a]	2 ± 0.69[c]	1 ± 0.74[c]	1 ± 0.67[c]	2 ± 1.00[bc]	2 ± 0.67[c]
Number of β-cell per islet (100x)	74 ± 37.02[a]	100 ± 72.08[a]	122 ± 87.56[a]	89 ± 72.84[a]	86 ± 32.80[a]	6 ± 5.30[b]	6 ± 4.55[b]	6 ± 2.02[b]	6 ± 4.27[b]	7 ± 4.91[b]
Relation β-cell/islet	21 ± 10.59[a]	36 ± 18.72[a]	36 ± 18.51[a]	25 ± 12.96[a]	33 ± 20.91[a]	3 ± 2.19[b]	4 ± 3.60[b]	4 ± 1.89[b]	3 ± 1.86[b]	4 ± 2.74[b]

Groups with different letters, in the same line, are statistically different ($P<0.05$, n=9-10).

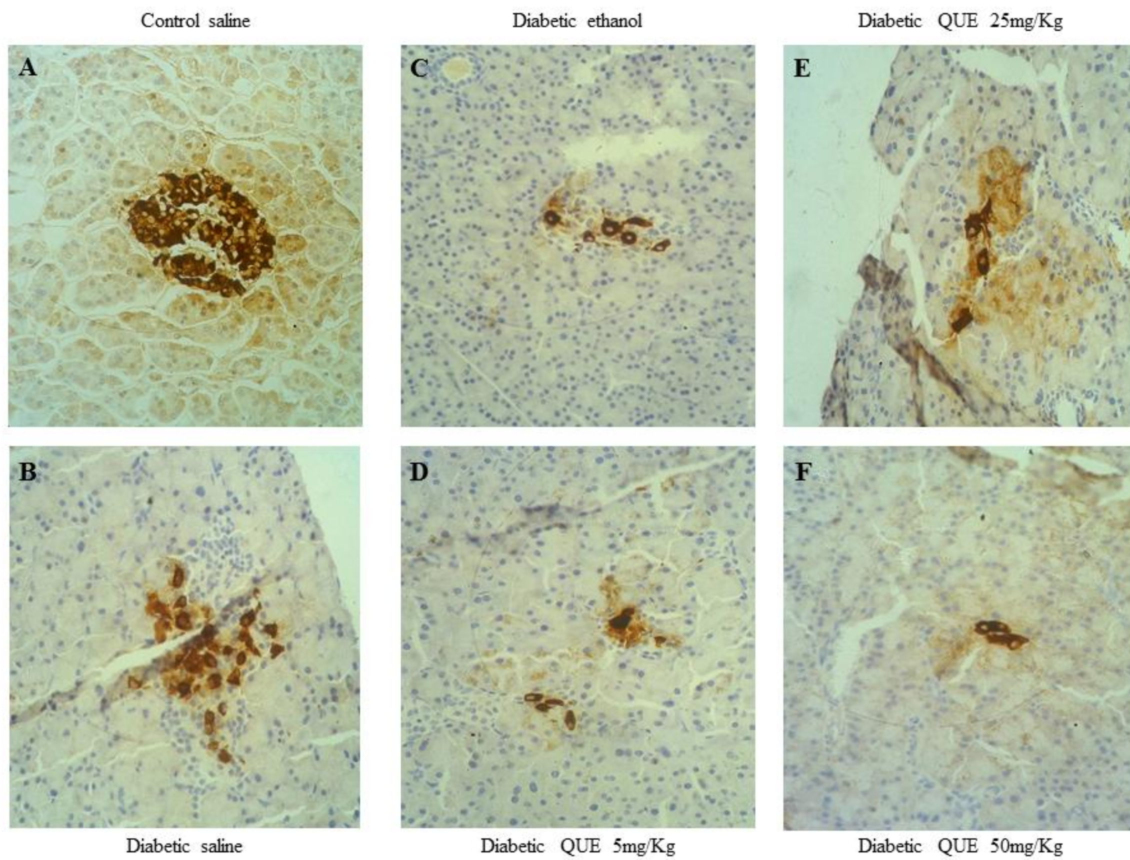

Figure 1 - A: Islet of Langerhans with normal beta cells population; B, C, D, E and F: Unstructured islets of Langerhans and reduced beta cells population. Immunohistochemistry (Avidin-biotin-peroxidase) (400X).

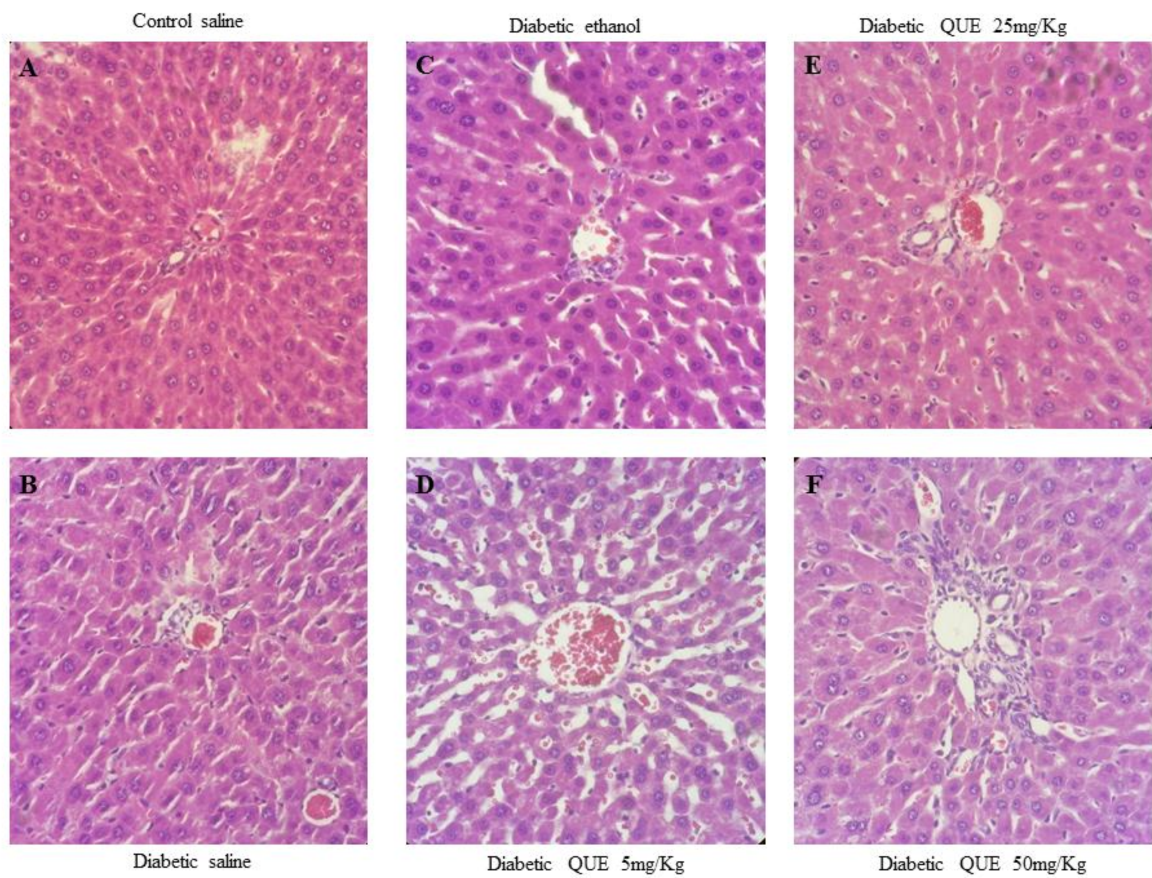

Figure 2 - A: Normal liver structure. B, C, D, E and F: Disaggregated trabeculae and dilated sinusoids, binucleated nuclei. HE staining (400X).

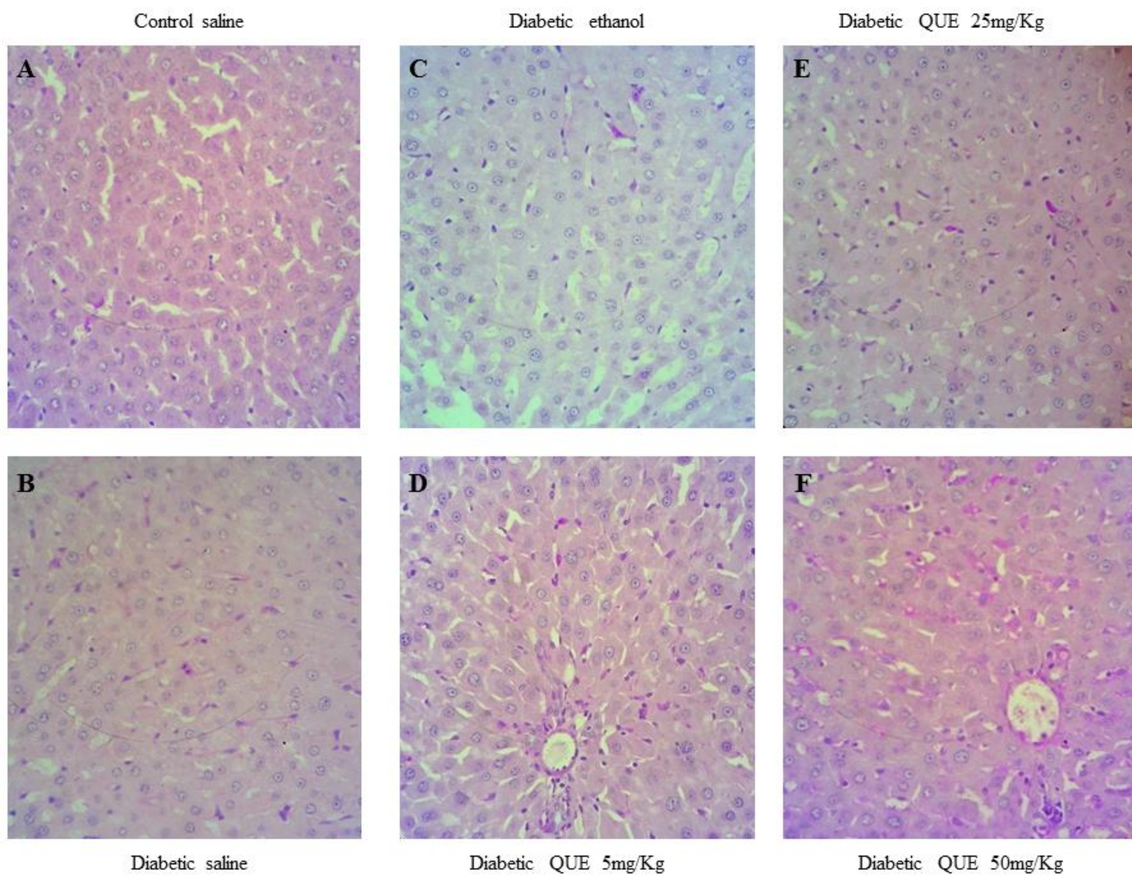

Figure 3 - A: Normal liver structure. B, C, D, E and F: Presence of glycogen accumulation (fine granules or globules in the cytoplasm of hepatocytes) and Kupffer cells. Sinusoids and portal area with Kupffer cells, leukocytes and plasma cells. PAS + staining (400X).

3.2 Manuscrito 2

Neuroprotective effects of quercetin on memory deficits and anxiogenic-like behavior in streptozotocin-induced diabetic rats

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Manuscrito a ser submetido para Brain, Behavior, and Immunity.

Neuroprotective effects of quercetin on memory deficits and anxiogenic-like behavior in streptozotocin-induced diabetic rats

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ABSTRACT

The objective of the present study was to investigate the effect the administration of quercetin (QUE) on memory deficits, anxiety-like behavior and on acetylcholinesterase (AChE) activity in the cerebral cortex, hippocampus and striatum in streptozotocin-induced diabetic rats. One hundred and ten male Wistar rats, aged 70-90 days old weighting 160-250g were used. Type 1 diabetes mellitus was induced by an intraperitoneal injection of 70 mg/kg of streptozotocin (STZ), diluted in 0.1 M sodium-citrate buffer (pH 4.5) in animals from diabetic groups. QUE diluted in 25% ethanol was administered by gavage at the dosages of 5, 25 and 50 mg/kg, to diabetic and non-diabetic rats. The animals were randomly distributed in 8 different experimental groups, with ten animals per group: Control Saline (CS), Control QUE 5 mg/kg (CQ5), Control QUE 25 mg/kg (CQ25), Control QUE 50 mg/kg (CQ50), Diabetic Saline (DS), Diabetic QUE 5 mg/kg (DQ5), Diabetic QUE 25 mg/kg (DQ25), Diabetic QUE 50 mg/kg (DQ50). Diabetic rats not treated with QUE showed: decreased latency, increased anxiety, increased activity of AChE in the cerebral cortex, hippocampus and cerebral synaptosomes, high levels of thiobarbituric acid reactive substance (TBARS) in the cerebral cortex, hippocampus and striatum ($P<0.05$). The results presented in diabetic rats treated with QUE, varied with the concentration of the flavonoid. (5, 25 and 50 mg/kg), onset of anxiety similar to that seen in non-diabetic control, reduction in the level of TBARS in the cerebral cortex, (5 mg/kg): decrease in AChE activity in the cerebral cortex and hippocampus; (5 and 50 mg/kg) reduction in the level of TBARS in the striatum; (25 mg/kg) decrease in the AChE activity in the cerebral cortex and cerebral synaptosomes; (25 and 50 mg/kg) decreased level of TBARS the hippocampus; (50 mg/kg) reduced AChE activity in the cerebral cortex, hippocampus and cerebral synaptosomes. In the cerebral cortex (5, 25 and 50 mg/kg) and cerebral synaptosomes (25 and 50 mg/kg), the decrease in the AChE activity was directly dependent on the concentration of QUE ($P<0.05$). In nondiabetic animals that received QUE, changes were also observed in the results. (5 mg/kg) increase in AChE activity in the striatum; (25 mg/kg): elevation of AChE activity in cerebral synaptosomes and increasing levels of TBARS in the cerebral cortex; (50 mg/kg): increase in time latency in the open arms and decreased latency time in the closed arms, (5, 25 and 50 mg/kg): elevation of AChE activity in the hippocampus ($P<0.05$). In conclusion, the present findings showed that treatment

with QUE prevents the increase in AChE activity and MDA-TBARS levels and consequently memory impairment in diabetic rats, demonstrating that this compound can modulate cholinergic neurotransmission and consequently improve cognition. In addition, we also found that QUE was able to prevent the anxiogenic-like behavior in streptozotocin-induced diabetic rats.

Key words: Diabetes; anxiety; acetylcholinesterase; cholinergic system; oxidative stress.

Introduction

Diabetes mellitus (DM) consists a group of metabolic dysfunction characterized by hyperglycemia resulting from a defect in insulin secretion or insulin action (Maher and Schubert, 2009). Hyperglycemia is indeed the causal link in the evolution of neuropathy and uncontrolled diabetes (Brownlee, 2001). In aging, hyperglycemia is also associated with central nervous system (CNS) damage, a consequence of long-term exposure to glucose (Tomlinson and Gardiner, 2008). Glucose oxidation is believed to be the main source of free radicals (Wolff and Dean, 1987; Jiang et al., 1990). It has been shown that persistent hyperglycemia increases production of reactive oxygen species (ROS) for all tissues across of glucose auto-oxidation and protein glycosylation (Aragno et al., 1999; Bonnefont Rousselot et al., 2000; Robertson, 2004).

The cholinergic systems provide diffuse innervation to practically all of the brain (Kasa, 1986; Woolf, 1991). The broad cholinergic innervation acting via nicotinic acetycholine receptors has been found to influencearousal, attention, sleep, fatigue, anxiety, central processing of pain, and a number of cognitive functions (Rose and Levin, 1991; Levin, 1992; Everitt and Robbins, 1997; Bannon et al., 1998; Adler et al., 1999; Marubio et al., 1999; Dani, 2001). There are two major cholinergic subsystems within the telencephalon. One is the projection subsystem that is composed of various basal forebrain nuclei. Those cholinergic neurons make broad projection mainly throughout the cortex and hippocampus. The second major cholinergic subsystem of the telencephalon is in the striatum, with participation in sensorimotor planning, learnig, and memory (Zhou et al., 2002).

The enzyme acetylcholinesterase (AChE) is found in the cholinergic terminal and is the most efficient enzyme that rapidly hydrolyze the neurotransmitter acetylcholine (ACh) at cholinergic synapses as well as the neuromuscular junction (Gaspersic et al., 1999; Anglister et al., 2008). In addition, the hippocampus and cortical regions of the brain are main sites for cholinergic transmission to monitor learning and memory processing, and seem to be more prone to oxidative damage (McIntosh et al., 1997; Francis et al., 1999). Oxidative damage to the synapse in these regions of rat brain has been reported to contribute in cognitive deficit (Fukui et al., 2002).

Previous studies have shown that the prevalence of depression, anxiety and cognitive deficits in patients with diabetes is considerably higher than in general population samples (Grigsby et al., 2002; Kruse et al., 2003; Frayne et al., 2005; Fisher et al., 2007; Li et al., 2008; Lin et al., 2008; Collins et al., 2009; Egede et al., 2009). Studies from our group have shown that diabetes worsens the memory of rats in the inhibitory avoidance task and increases the AChE activity and induces oxidative stress in different brain structures (Schmatz et al., 2009). In addition to these findings, we also found that resveratrol, an antioxidant compound, prevented the deleterious effects induced by diabetes in rats (Schmatz et al., 2009).

Antioxidants (Hasanein & Shahidi, 2010), antihyperglycemics and insulin sensitizing agents (Ryan et al., 2006) are reported to reduce cognitive dysfunction in diabetic condition. However, at present, no specific treatment is available for the management and/or prevention of cognitive dysfunction in DM (Biessels et al., 2007). Flavonoids have been reported to be potent antioxidants and beneficial in the treatment of antioxidative stress-related diseases (Zhag et al., 2011).

Quercetin (QUE), a flavonoid that possess free radical scavenging properties and may protect from oxidative injury by its ability to modulate intracellular signals and promote cellular survival (Mercer et al., 2005). QUE is a natural antioxidant that is present in a variety of vegetables and fruits which are regularly consumed in our diet (Molina et al., 2003). This compound has been studied due to its wide therapeutic property since neuroprotective activity has been associated with experimental models for neurodegenerative diseases (Chen et al., 1996; Lu et al., 2006; Huebbe et al., 2010; Haleagrahara et al., 2011; Mukai et al., 2012). Furthermore, QUE has been appointed as potent anti-inflammatory (Liu et al., 2012;

Wang et al., 2012) since it is able to regulate pathway of lipoxygenase and arachidonic acid metabolism during inflammation (Mirzoeva and Calder, 1996).

It has been shown that QUE is capable of protecting the memory in diabetic rats (Bhutada et al., 2010) but studies involving oxidative stress and cholinergic system have not been described. In line with these findings, QUE has proved a potent antioxidant with anti-inflammatory and neuroprotective properties in experimental models for cognitive deficits. These data showed a promising beneficial effect of QUE. Thereby, we investigated whether QUE can protect the impairment of memory and worsening of cholinergic neurotransmission in streptozotocin-diabetic rats. In addition, we also investigated the anxiogenic-like behavior in these animals.

Materials and methods

Chemical reagents

The substrates quercetin, streptozotocin, acetylthyocholine, trizma base and percoll were obtained from Sigma Chemical Co (St. Louis, MO, USA). All other chemicals used in this experiment were of the highest purity.

Animals

One hundred and ten male Wistar rats (70-90 days old; 160–250g) from the Central Bioterium the Federal University of Santa Maria were used in this study. Of this total, 76 rats reached the end of the experiment being used to collect samples. Of the remaining 34 rats, 15 rats died between the induction of diabetes and end of the experiment, and the others whose do not reach 250 mg/dL blodd glucose, were euthanized with halothane (deep anesthesia). The animals were maintained at a constant temperature (23 $\pm$ 1°C), on a 12h dark/light cycle with free access to food and water. All procedures were approved by the Animal Ethics Committee from the Federal University of Santa Maria (protocol number: 57/2010).

Experimental induction of diabetes

All animals were maintained an adaptation period of 15 days, at five rats per cage, to acclimatize to the new environment, before the initiation of the experimental protocols. Diabetes was induced by a single intraperitoneal injection of 70 mg/Kg steptozotocin (STZ) diluted in 0.1 M sodium-citrate buffer (pH 4.5). Fifty rats, age-

matched control, received an equivalent amount of the sodium-citrate buffer. STZ-treated rats received 5% of glucose instead of water for 24h after diabetes induction in order to reduce death due to hypoglycemic shock. Blood samples were taken from the tail vein 48 h after STZ or vehicle injection to measure glucose levels. Only animals with fasting glycemia over 250 mg/dL were considered diabetic and used for the current study. During the experiment the levels of blood glucose were verified six times (15 days before, first day, 10, 20, 30, and 40 days after the beginning of treatment). Fifty rats that maintained fasting glycemia higher than 250 mg/dL were considered diabetic and selected for following on experiment. At the end of the experimental period, diabetic rats and the corresponding control animal were euthanized by overdose of halothane anesthesia.

Treatment with quercetin (QUE)

The rats were randomly divided into eight groups (ten rats per group): Control Saline (CS), Control QUE 5 mg/kg (CQ5), Control QUE 25 mg/kg (CQ25), Control QUE 50 mg/kg (CQ50), Diabetic Saline (DS), Diabetic QUE 5 mg/kg (DQ5), Diabetic QUE 25 mg/kg (DQ25), Diabetic QUE 50 mg/kg (DQ50). Ten days after diabetes induction, the animals belonging to groups CQ5 and DQ5 received 5 mg/Kg of quercetin, the animals from CQ25 and DQ25 received 25 mg/Kg and the CQ50 and DQ50 received 50 mg/Kg, while the animals from CS and DS received 0,9% saline solution by gavage. QUE was freshly prepared in 25% ethanol and was administered at between 3 and 4 p.m. once a day during 40 days, by gavage. The dose of QUE was adjusted weekly, to the individual weight.

Behavioral Procedure

Inhibitory avoidance task

At the final day of treatment with QUE (40th day), animals were subjected to training in a step-down inhibitory avoidance apparatus as previously described (Rubin et al., 2000a). Next, twenty four hours after training, the animals were subjected to test in a step-down inhibitory avoidance task. Briefly, the rats were subjected to a single training session in a step-down inhibitory avoidance apparatus, which consisted of a 25×25×35 cm box with a grid floor whose left portion was

covered by a 7×25 cm platform, 2.5 cm high. The rats were placed gently on the platform facing the rear left corner, and when the rat stepped down with all four paws on the grid, a 3-s 0.4 mA shock was applied to the grid. Retention test took place in the same apparatus 24 h later. Test step-down latency was taken as a measure of retention, and a cut-off time of 300 s was established.

Open field

Immediately after the inhibitory avoidance test session, the animals were transferred to an open-field measuring 56×40×30 cm, with the floor divided into 12 squares measuring 12×12 cm each. The open field session lasted 5 min and during this time, an observer, who was not aware of the pharmacological treatments, recorded the number of crossing responses and rearing responses manually. This test was carried out to identify motor disabilities, which might influence inhibitory avoidance performance at testing.

Elevated plus maze task

Anxiolytic-like behavior was evaluated using the task of the elevated plus maze as previously described (Frussa-Filho et al., 1999; Rubin et al., 2000b). The apparatus consists of a wooden structure raised to 50 cm from the floor. This apparatus is composed of 4 arms of the same size, with two closed-arms (walls 40 cm) and two open-arms. Initially, the animals were placed on the central platform of the maze in front an open arm. The animal had 5 minutes to explore the apparatus, and the time spent and the number of entries in open and closed-arms were recorded. The apparatus was thoroughly cleaned with 30% ethanol between each session.

Biochemical Assays

Brain tissue preparation

After behavioral tests, the animals were anesthetized under halothane atmosphere before being killed by decapitation and brain were removed and separated into cerebral cortex (CC), hippocampus (HC) and striatum (ST). The cerebral structures were placed in a solution of Tris–HCl 10mM, pH 7.4, on ice. The brain structures were gently homogenized in a glass potter in Tris–HCl solution. Aliquots of resulting brain structure homogenates were stored at −80 °C until

utilization (Gutierres et al., 2012). Protein was determined previously in a strip that varied for each structure: CC (0.7 mg/ml) HC (0.8 mg/ml) and ST (0.4 mg/ml), as determined by the Coomassie blue method with previously describe (Bradford, 1976), using bovine serum albumin as standard solution.

Cerebral synaptosomes preparation

CC and cerebral synaptosomes (SN) were isolated essentially as previously described (Nagy and Delgado-Escueta., 1984), using a discontinuous Percoll gradient. The CC were gently homogenized in 10 volumes of an ice-cold medium (medium I) containing 320 mM sucrose, 0.1mM EDTA and 5 mM HEPES, pH 7.5, in a motor driven Teflon-glass homogenizer and then centrifuged at 1000xg for 10 min. An aliquot of 0.5 mL of the crude mitochondrial pellet was mixed with 4.0 mL of an 8.5% Percoll solution and layered into an isosmotic discontinuous Percoll/sucrose gradient (10%/16%). The SN that banded at the 10/16% Percoll interface were collected with a wide-tip disposable plastic transfer pipette. The SN fraction was washed twice with an isosmotic solution consisting of 320 mM sucrose, 5.0 mM HEPES, pH 7.5, and 0.1mM EDTA by centrifugation at 15,000 g to remove the contaminating Percoll. The pellet of the second centrifugation was resuspended in an isosmotic solution to a final protein concentration of 0.9 mg/ml. SN were prepared fresh daily and maintained at 0°- 4° throughout the procedure and used for acetylcholinesterase activity assays.

Assay of lactate desydrogenase

The integrity of the SN preparations was confirmed by determining the lactate dehydrogenase (LDH) activity which was obtained after SN lysis with 0.1 % Triton X-100 and comparing it with an intact preparation, using the Labtest kit (Labtest, Lagoa Santa, MG, Brasil).

Assay of acetylcholinesterase activity

The AChE enzymatic assay was determined by a modification of the spectrophotometric method as previously described (Rocha et al., 1993). The reaction mixture (2 mL final volume) contained 100 mM K^{+}-phosphate buffer, pH 7.5 and 1 mM 5,5′-dithiobisnitrobenzoic acid (DTNB). The method is based on the formation of the yellow anion, 5, 5′- dithio-bis-acid-nitrobenzoic, measured by

absorbance at 412 nm during 2 min incubation at 25°C. The enzyme (40–50 μg of protein) was pre-incubated for 2 min. The reaction was initiated by adding 0.8 mM acetylthiocholine iodide (AcSCh). All samples were run in duplicate or triplicate and the enzyme activities were expressed in μmol AcSCh/h/mg of protein. The AChE enzymatic assay was determined as previously described (Ellman et al., 1961).

Brain MDA measurement (TBARS)

Brain MDA levels were determined by the method described previously (Ohkawa et al., 1979), with a few modifications as previously described (Rossato et al., 2002). In short, the reaction mixture contained 200 μL of brain homogenates or standard (MDA-malondialdehyde 0.03 mM), 200 μL of 8.1% sodium dodecylsulfate (SDS), 750 μL of acetic acid solution (2.5 M HCl, pH 3.5) and 750 μL of 0.8% TBA. Since sucrose interferes in the MDA assay, a portion of the brain was weighed, homogenized at a proportion of 1 g of tissue to 10 mL of buffer Tris/HCl 10 mM pH 7.4 plus 10% of sodium dodecylsulfate (SDS) 10%. The mixtures were heated at 95ºC for 90 min. After centrifugation at 1700 g for 5 min, the absorbance was measured at 532 nm. MDA tissue levels were expressed as nmol MDA/ mg of protein.

Protein determination

Protein was measured by the Coomassie Blue method with previously described (Bradford, 1976), using bovine serum albumin as standard.

Statistical analysis

Statistical analysis of latency test was carried by Kruskal–Wallis test. Elevated plus maze task and open field results were analyzed by One-Way ANOVA. Enzymatic activity and TBARS levels were analyzed by one- or two-way ANOVA, followed by Tukey's multiple range tests. $P<0.05$ was considered to represent a significant difference in all experiments. All data were expressed as mean±SEM.

Results

Body weight and blood glucose

Diabetic rats from this study presented clinical disorders commonly observed in this condition, such as, polydipsia, polyuria, lethargy, polyphagia, and weight loss.

The values of body weight and blood glucose levels are available in the first manuscript.

Inhibitory avoidance task

Diabetic rats not treated with quercetin (QUE) showed a significant decrease in latency when compared to non-diabetic control group (Figure 1 - B). When treated with QUE available in three concentrations (5, 25 and 50 mg/kg), diabetics significantly increased the latency time, in relation to diabetic control group, with a lag time similar to that observed in the control group not diabetic.

Open field

Figure 2 (A and B) shows the average results of testing of habituation to open field to crossing and rearing. It can be seen that neither diabetic nor treatment with 5, 25 and 50 mg/kg QUE alter the number of crossing and rearing responses in the open field.

Elevated plus maze task

Diabetic animals not treated with QUE, compared to non-diabetic control group, decreased the number of entries into the open arms of the apparatus (Figure 3 - A), thus increasing the time spent in closed arms (Figure 3 - D) and decreasing spent in the open, between intersections (Figure 3 - E) ($P<0.05$). When treated with 5, 25 and 50 mg/kg of QUE diabetics showed, compared to non-diabetic control group, a raise in the number of entries in the open arms (Figure 3 - A), a decrease in time spent in closed arms (Figure 3 - D) and an increase in the time spent in the open arms between the intersections (Figure 3 - E) ($P<0.05$). The non-diabetic animals treated with 50 mg/kg QUE, compared to non-diabetic control group, showed an increase in time spent in the open arms (Figure 3 - A and B) and a decrease in time spent in the closed arms (Figure 3 - D) ($P<0.05$).

Acetylcholinesterase activity

Regarding the non-diabetic control group, the untreated diabetic animals with QUE showed significant elevation in the activity of acetylcholinesterase (AChE) in the cerebral cortex, hippocampus and cerebral synaptosomes (Figure 4 - A, B and D). In

relation to diabetic control group, mice treated diabetic (5, 25 and 50 mg/kg QUE) which showed decrease in the AChE activity in cortex (Figure 4 - A) to 5 and 50 mg/kg QUE) the decrease of AChE occurred in the hippocampus (Figure 4 - B) with 25 and 50 mg/kg QUE) than the reduction in AChE was observed in brain synaptosomes (Figure 4 - D) ($P<0.05$). When compared to non-diabetic control group, the non-diabetic animals treated with the flavonoid had reduced AChE activity in the cerebral cortex (5 mg/kg QUE) (Figure 4 - A), and elevation of AChE: (5 mg/kg QUE) than in the striatum (25 mg/kg QUE) than (Figure 4 - C) in cerebral synaptosomes (25 mg/kg QUE) (Figure 4 - D) and hippocampus (5, 25 and 50 mg/kg QUE) than (Figure 4 - B) ($P<0.05$).

Tiobarbituric reactivate acid substances activity

Diabetic animals not treated with QUE showed significant increase in TBARS levels in the cerebral cortex, hippocampus and striatum (Figure 5 - A, B and C), compared to non-diabetic control group. In relation to diabetic control group, to which the three concentrations tested (5, 25 and 50 mg/kg) significantly reduced the level of TBARS in the cerebral cortex, hippocampus and striatum (Figure 5 - A, B and C). When comparing the treated diabetic QUE was with the non-diabetic control group, the reduction in the level of TBARS was significantly similar in the hippocampus (25 and 50 mg/kg) and in the striatum (5 and 50 mg/kg) QUE (Figure 5 - B and C). Regarding the non-diabetic control, it was observed that (25 mg/kg) QUE which significantly increased the level of TBARS in the cerebral cortex of non-diabetic animals.

Discussion

Recently, a positive correlation of DM with cognitive impairment or dementia has been demonstrated (Reijmer et al., 2011; Strachan et al., 2011). Specifically in the general population, DM was associated with abnormal performance in the domains of memory, attention and psychomotor speed (van den Berg et al., 2009; Takayanagi et al., 2012). In addition, polyphenolic compounds have recently received considerable attention since they have been shown to protect neurons against a variety of experimental neurodegenerative conditions including cognitive deficit associated with diabetes (Baydas et al., 2003). Quercetin (QUE) a polyphenolic

flavonoid compound present in large amonts in vegetables, fruits, and tea, exhibits its therapeutic potential against many diseases (Amália et al., 2007; González-Gallego, 2007; Tieppo et al., 2009). It contain a number of phenolic hydroxyl groups, which have strong antioxidant activity (Martinez-Florez et al., 2002; Tokyol et al., 2006).

To assess possible changes in behavior or memory, promoted by the diabetic state in rats, we used a model of classical conditioning or Pavlovian fear responses (Kim & Jung, 2006; Maren, 2008). By testing such as inhibitory avoidance, habituation to the open field and elevated plus maze, we got answers that confirmed the influence of chronic hyperglycemia in aversive memory formation as well as the level of protection offered by the management of quercetin (QUE).

The model of Pavlovian conditioning produces rapid responses to aversive stimulus, which may be associated with learning and memory formation (Maren, 2008; Shin & Liberzon, 2010). The results observed in the inhibitory avoidance task (Figure 1 – A and B), showed that diabetic animals not treated with QUE, significantly decreased the latency (time spent on the platform on the metal screen). Working with diabetic rats Schmartz et al. (2009) also found a significant decrease in latency in group of diabetic control compared to non-diabetic control group. The information that suggested that the electrical stimulation received 24 hours prior, not properly processed and stored, causing failure in memory formation and recall of information in these animals. Such classical fear conditioning has primarily been employed using rodents (Kim and Jung, 2006; Maren, 2008). Fear is the product of neural system that envolved to detect danger and produce rapid protective responses automatically, without conscious participation (Rogan and LeDoux, 1996). However, diabetic animals treated with 5, 25 and 50 mg kg QUE, exhibited a latency similar to that observed in the control group. These results suggested that the QUE has promoted protection in aversive memory formation.

The results observed in test elevated plus maze (Figure 3), revealed that the diabetic animals not receiving treatment with QUE, showed an increase in anxiety state. According with Steimer (2002), anxiety is described as a psychological, physiological, behavioral, which can be induced in both animals and humans, from a threat to the well being. The autonomic and neuroendocrine activation induces elevation in the level of expectation and changes in the behavioral pattern of the

animal, which takes from then escape behavior or defense (Steimer, 2011). In the apparatus, the increased anxiety in these animals, reflected in the increase of time spent in the closed arms in disgust and the open spaces. It is important to note that diabetic animals that received treatment with QUE, the 3 concentrations were similar to non-diabetic control. Moreover, nondiabetic mice that received QUE the concentration of 50 mg/kg showed significant increase in the time spent in the open arms and reduction of time when the enclosed arms. This change in behavior suggested that this concentration could inhibit QUE the fear of the animal, reducing the sense of self-preservation, essential to the survival of this species. This test allows the assessment of concomitant innate fear (through exploration of open arms) and learning and memory of a declarative task (discrimination of two closed arms), the other being a safe associated with aversive stimulation (Silva et al., 1997).

Diabetic animals not treated with QUE, showed a significant increase in the activity of acetylcholinesterase (AChE) in the cerebral cortex, hippocampus and cerebral sinaptossomos (Figure 4 – A, B and D). A similar situation was also observed by Schmatz et al. (2009), both in the cerebral cortex, hippocampus and in the comparison between groups of diabetic and non-diabetic control ($P<0.05$). Importantly, diabetic rats that received 5 mg/kg QUE) showed significant reduction in AChE activity in both the cerebral cortex and in the hippocampus. At the concentration of 25 mg/kg QUE a reduction of AChE was observed in the cerebral cortex and cerebral synaptosomes ($P<0.05$). A concentration of 50 mg/kg significantly reduced AChE activity in the cerebral cortex, hippocampus and cerebral synaptosomes.

In the cerebral cortex and in brain synaptosomes, the reduction in AChE demonstrated an inverse relationship to the concentration of administered 5, 25 and 50 mg/kg QUE in the cerebral cortex and 25 and 50 mg/kg in brain synaptosomes. These results suggested that in the cerebral cortex, hippocampus and sinaptossomes neurotransmitter, which inhibit the action of AChE, responsible for the hydrolysis of the neurotransmitter acetylcholine (ACh). The importance of this fact is the possible use of QUE as an adjunct in the treatment of dementia, especially in the elderly who suffer from Alzheimer's disease, since ACh is the main neurotransmitter

of nervous stimuli from one neuron to another, being directly involved in motor processes, cognitive and memory (Patrick, 2001; Rang et al., 2001).

The hypothesis postulated that, if the loss of memory occurring in Alzheimer's Disease (AD) as well as its decline in healthy older adult (Balota et al., 2000) depend on a cholinergic deficit, it should be possible to restore the memory impairment by compensating the cholinergic deficit. For this purpose tacrine, donepezil, rivastigmine and galantamine were developed and marketed. These drugs inhibit AChE and to some extent also butyrylcholinesterase (BuChE), white the exception of donepezil, which is selective for AChE (Weinstock, 1999), increase extracelular ACh levels in the brain of experimental animals (Pepeu, 2000) and restore the cognitive deficits in animal models of dementia (Pepeu, 2000; Pepeu and Giovannini, 2010). On the other hand, was observed in non-diabetic animals, that has significantly increased AChE activity in the hippocampus (5, 25 and 50 mg/kg), striatum (5 mg/kg) and cerebral synaptosomes (25 mg/kg) which suggested the presence of an undesirable property in flavonoid.

Oxidative stress depicts the existence of products called free radical and reactive oxygen species (ROS) wich are formed under normal physiological conditions but become deleterious when not being quenched by the antioxidant systems (Fang et al., 2002). The thiobarbituric acid reactive substance (TBARS) is often said to measure malondialdehyde (MDA) formed in peroxidizing lipid systems, so results are frequently expressed as μmol MDA equivalents (Gutteridge, 1986; Frankel et al., 1989; Frankel, 1991). The results found in the diabetic animals untreated with which showed a significant elevation of TBARS (Figure 5), indicating a significant lipid peroxidation in the cerebral cortex, hippocampus and striatum. Importantly, administration of QUE reduced the level of TBARS in the three brain structures: cerebral cortex (5, 25 and 50 mg/kg), hippocampus (25 and 50 mg/kg) and striatum (5 and 50 mg/kg). These results confirmed the antioxidant properties of QUE, whose degree of protection varied depending on brain structure and concentration employed. However, particularly in the cerebral cortex was observed that among non-diabetic animals, which QUE at a concentration of 25 mg/kg showed significant oxidizing effect. Pro-oxidant effects have been reported in flavonoids (Chan et al. 1999; Constatin & Bracht, 2008; Constantin et al. 2010), the pro-oxidant activity possibly related to the number of hydroxyl groups (Cao & Sofic, 1997).

Conclusion

The results found in this study suggest that hyperglycemia causes changes in learning, memory formation and retrieval of aversive. Moreover, it was shown that quercetin (QUE) can exert antioxidant and anxiolytic and help protect cognitive function. Thus, QUE is a promising adjuvant in the treatment of chronic effects of diabetes mellitus.

Conflict of interest statement

There are no conflicts of interest.

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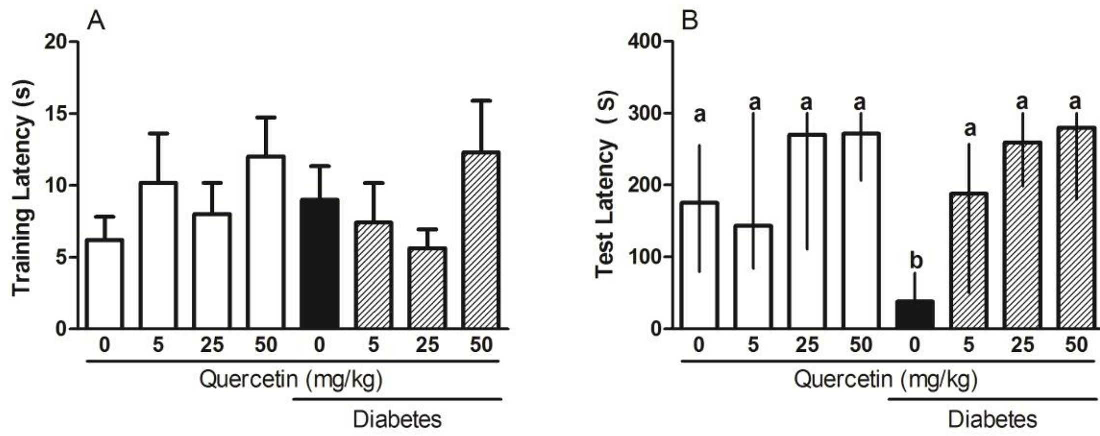

Figure 1: A - training latency (s) and B – test latentency (s) in Iinhibitory avoidance task of STZ-induced diabetic rats and those treated with quercetin at the end of experiment. Groups with different letters are statistically different (P<0.05).

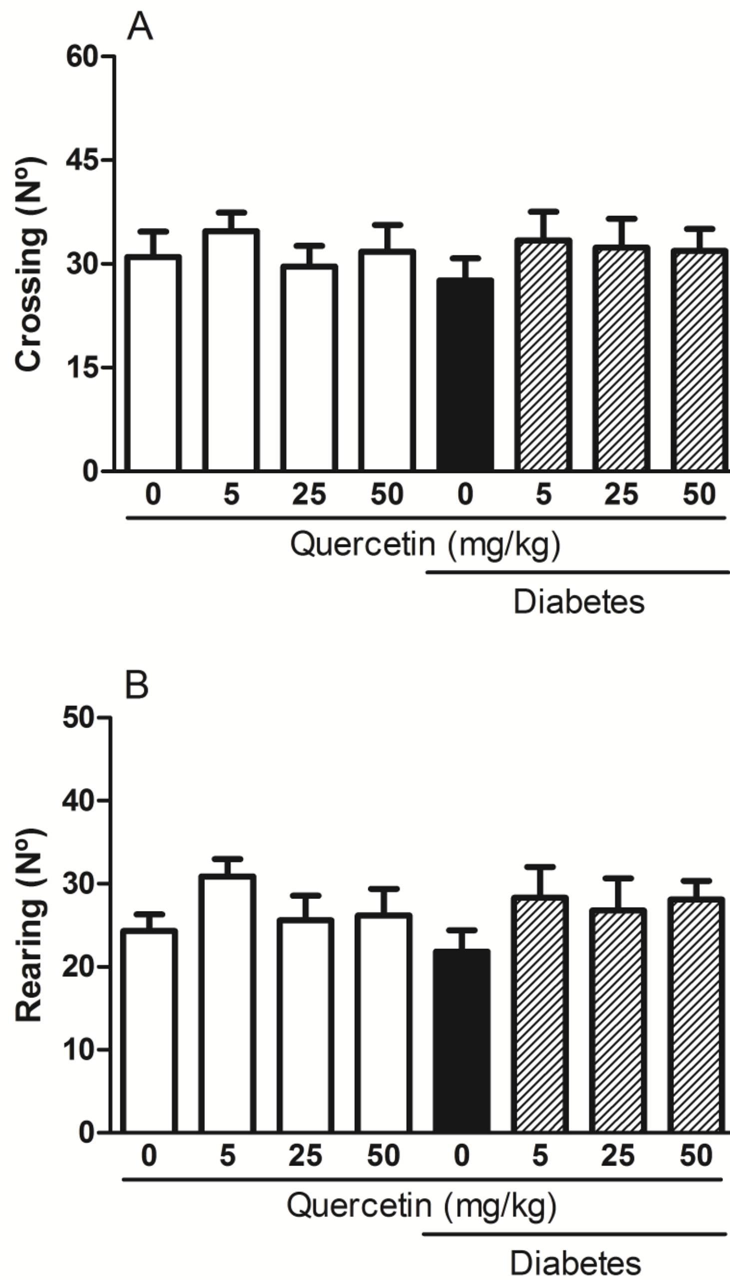

Figure 2: A – Crossing number and B – rearing number in open field of STZ-induced diabetic rats and those treated with quercetin at the end of experiment.

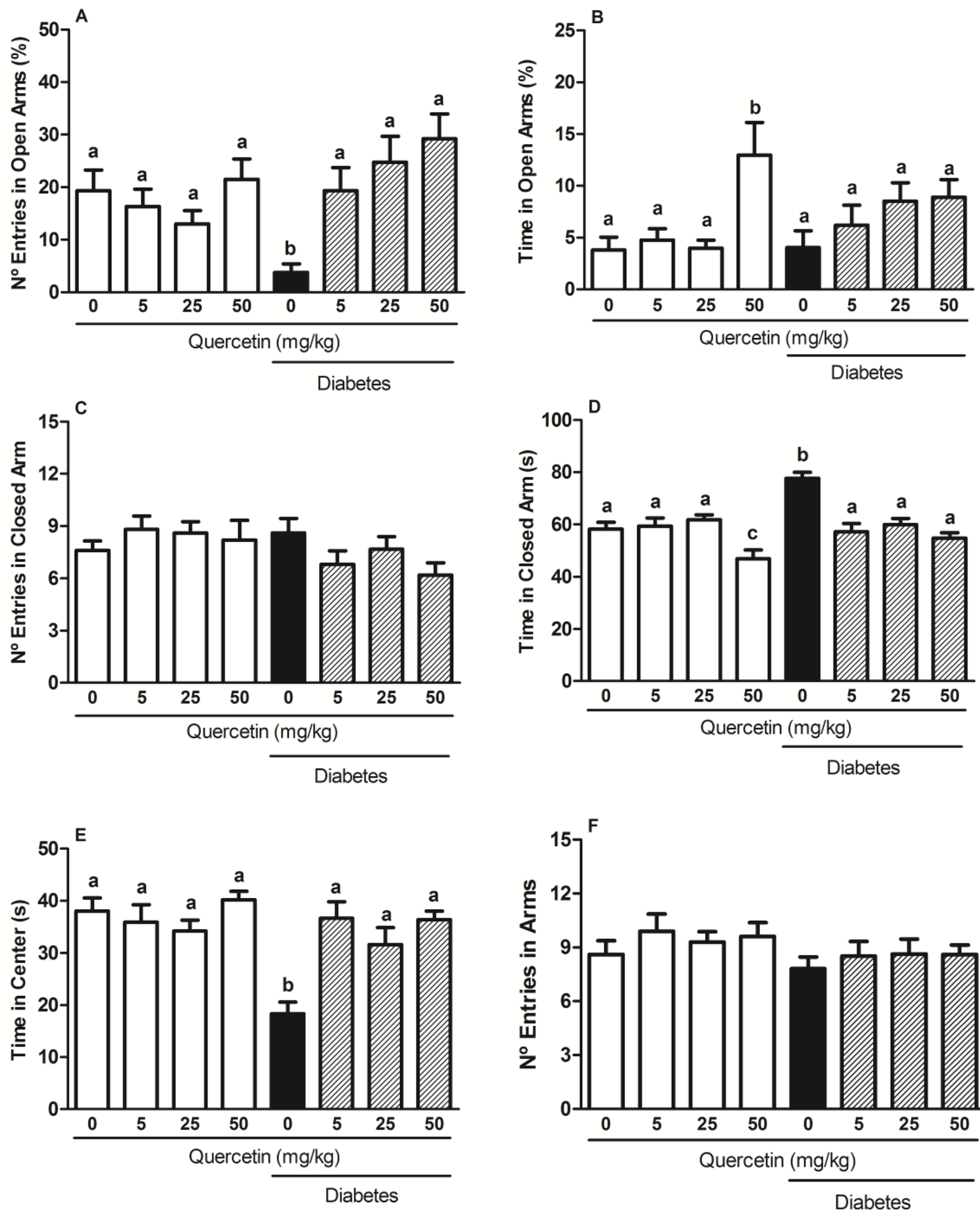

Figure 3: A – number of entries in open arms (%), B – time in open arms (s), C- number of entries in clossd arms, D – time in closed arms (s), E – time in center (s) and F- number of entries in arms in elevated plus maze task of STZ-induced diabetic rats and those treated with quercetin at the end of experiment. Groups with different letters are statistically different ($P<0.05$).

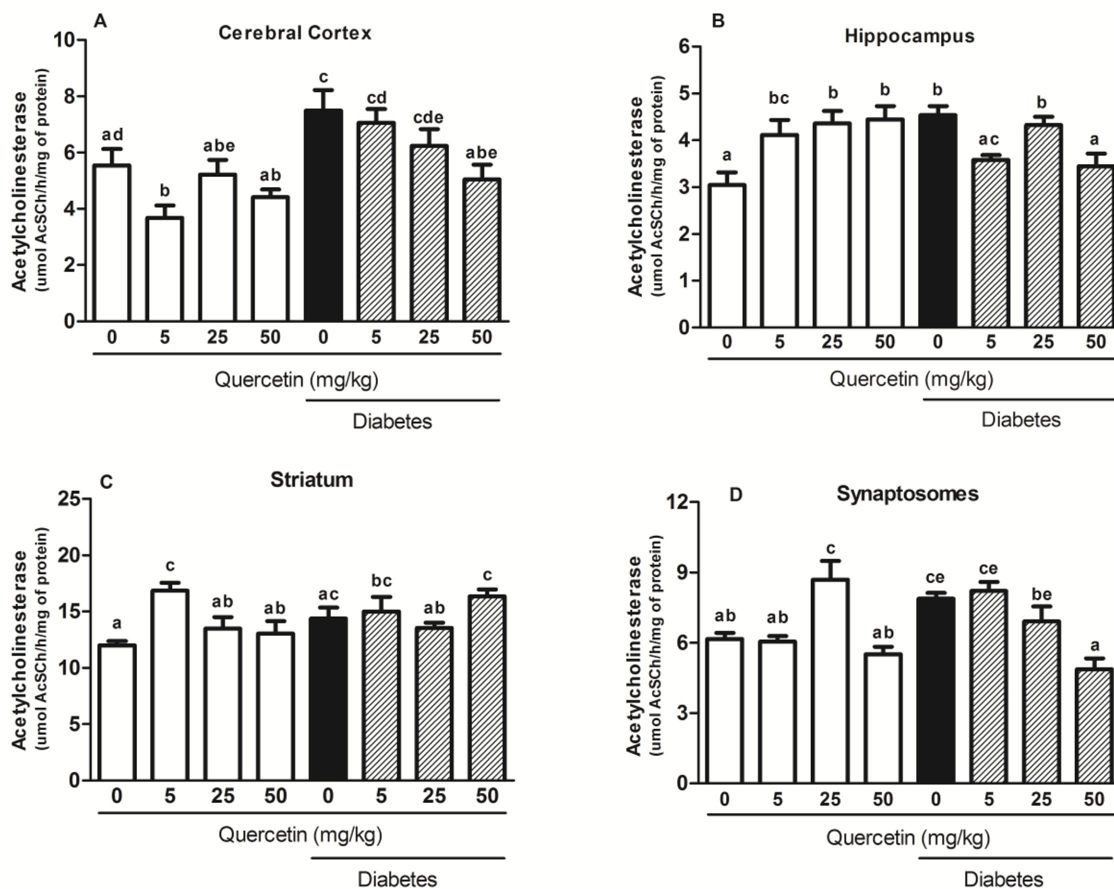

Figure 4: Acetylcholinesterase aticvity (AChE) in: A - cerebral cortex, B – hippocampus, C- striatum and D – cerebral synaptossomes of STZ-induced diabetic rats and those treated with quercetin at the end of experiment. Groups with different letters are statistically different ($P<0.05$).

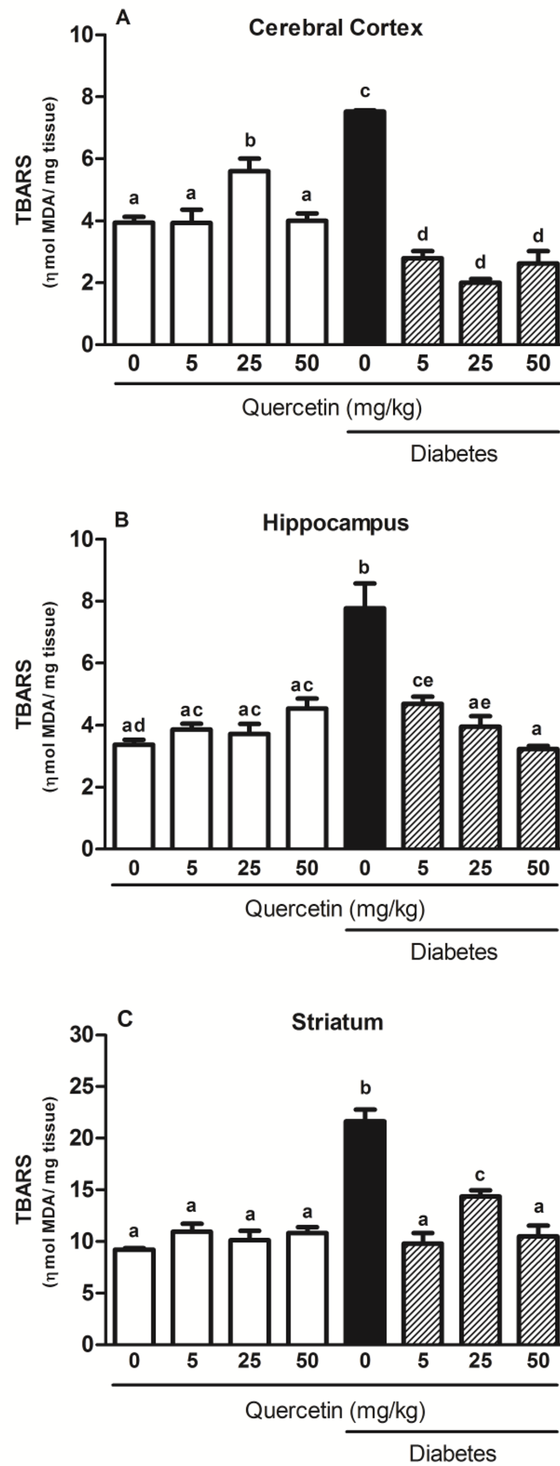

Figure 5: Thiobarbituric acid reactive substance level (TBARS) in: A - cerebral cortex, B – hippocampus and C- striatum of STZ-induced diabetic rats and those treated with quercetin at the end of experiment. Groups with different letters are statistically different ($P<0.05$).

4 DISCUSSÃO

A seguir serão realizadas considerações relativas aos resultados dos dois manuscritos apresentados nessa tese de doutorado. O objetivo do primeiro manuscrito foi avaliar os efeitos antioxidantes e anti-inflamatórios da quercetina (QUE), enquanto no segundo manuscrito objetivou-se avaliar a possível proteção da QUE no sistema colinérgico, principalmente na memória e comportamento de ratos diabéticos.

A avaliação imuno-histoquímica das células beta mostrou que ao término do experimento, que 91,3% dessas células já haviam sido destruídas, o que comprovou a ação diabetogênica da estreptozotocina (STZ). Segundo POWERS (2009), as características do diabetes melito tipo 1 (DM T1), só se tornam evidentes quando cerca de 80% das células beta são definitivamente inativadas. No presente trabalho, 15 dias após a administração da STZ a glicemia dos ratos já estava significativamente mais elevada nos grupos diabéticos. Além disso, a elevada concentração da frutosamina, nesses animais, indicou que a hiperglicemia foi mantida, durante os 55 dias de experimento.

Dessa forma, quando a QUE começou a ser administrada, diariamente e em 3 concentrações (5, 25 e 50 mg/Kg), 15 dias após a aplicação da STZ, uma significativa quantidade de células secretoras de insulina já havia sido destruída. A elevação na concentração da insulina, tanto nos grupos diabéticos, como não diabéticos, tratados com QUE, sugeriu uma possível ação secretagoga, dose-dependente, do flavonoide nas células beta. Esses resultados corroboram com os achados de YOUL et al. (2010), quando trabalharam com linhagem de células beta INS-1 e QUE na concentração de 20μmol/L.

De acordo com a teoria lipocêntrica, da patogênese do diabetes melito tipo 2 (DM T2), a deposição ectópica de lipídios pode desregular o metabolismo dos ácidos graxos, resultando na resistência à insulina (McGARRY, 2002). Segundo ZALIUNAS et al. (2008), o DM T2 está associado à obesidade, e esta é predecessora às evidências de resistência à insulina. Contudo, em nosso estudo, além de utilizarmos um modelo para DM T1, não foi observada obesidade nos animais diabéticos, pelo contrário, foi observada significativa redução no peso corporal (Figura 2 – A e B).

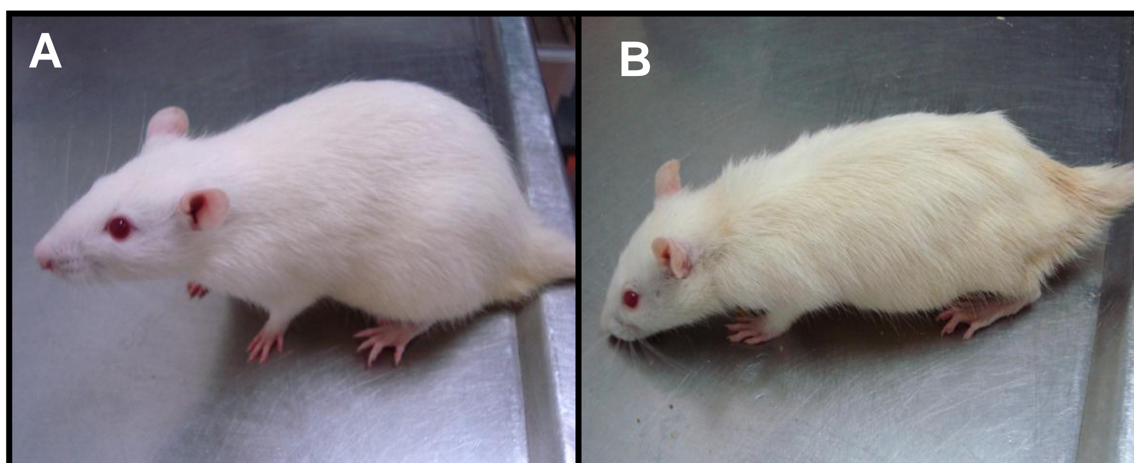

Figura 2 – Aspecto físico de animais de controle: (A) rato não diabético e (B) rato diabético.

Os ratos diabéticos utilizaram o catabolismo protéico, para compensar o não aproveitamento da glicose disponível no sangue (Figura 3 A – B). Por outro lado, a resistência à insulina tem sido indicada, em vários estudos, como uma possível consequência do estresse oxidativo (BLOCH-DAMTI & BASHAN, 2005), o que poderia ter ocorrido entre os animais diabéticos. Contudo, é importante ressaltar, que tanto nos grupos diabéticos como nos não diabéticos, tratados com QUE, não houve redução significativa na concentração de glicose, mesmo diante de concentrações elevadas de insulina. A ligação da insulina ao seu receptor estimula a atividade intrínseca da tirosinoquinase, acarretando a auto-fosforilação do receptor e o recrutamento das moléculas sinalizadoras intracelulares, tais como os substratos do receptor de insulina (IRS). Os IRS e outras proteínas adaptadoras iniciam uma complexa cascata de reações de fosforilação e de desfosforilação, resultando nos efeitos metabólicos e mitogênicos generalizados da insulina. A ativação da via fosfatidilinositol-3'-quinase (PI-3 quinase) estimula a translocação dos transportadores da glicose, o transportador de glicose sensível à insulina (GLUT 4), por exemplo, para a superfície celular, um evento que é de primordial importância para a captação da glicose pelo músculo esquelético e pelo tecido adiposo. Os resultados encontrados sugeriram, principalmente em relação ao grupo não diabético, que em algum momento a QUE influenciou a sinalização da insulina, levando à resistência à insulina, prejudicando a utilização da glicose pelos tecidos-

alvo (músculo, fígado e adiposo) e refletindo em aumento da produção hepática de glicose. Ambos os efeitos contribuem para a hiperglicemia (POWERS, 2009).

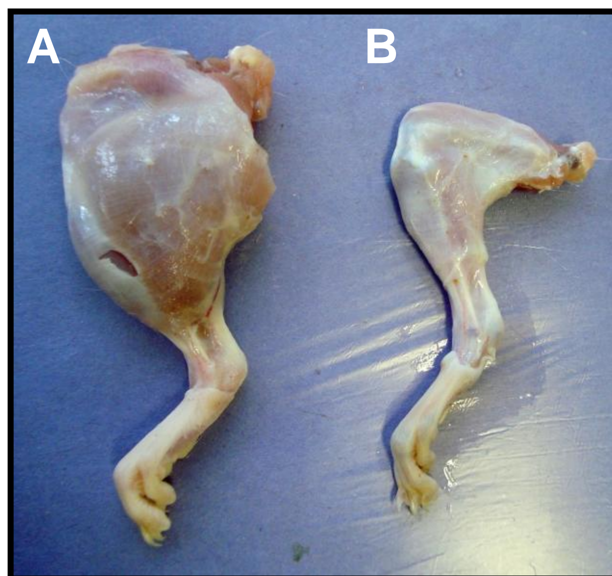

Figura 3 – Comparação entre a massa muscular de animais de controle: (A) rato não diabético e (B) rato diabético.

A condição de resistência à insulina compreende um amplo aspecto de distúrbios, com a hiperglicemia representando um dos aspectos mais prontamente diagnosticados. A síndrome metabólica engloba uma quantidade de desarranjos metabólicos que inclui, entre outros, a dislipidemia (triglicerídeos elevados e lipoproteínas de alta densidade ou HDL colesterol baixo) (POWERS, 2009). No primeiro manuscrito foi verificado, entre os animais diabéticos não tratados com QUE, que além de um discreto declínio na concentração do colesterol, o nível de triglicerídeos subiu significativamente. A hiperlipidemia é comum no paciente diabético não tratado. O DM não controlado é acompanhado pela elevação das concentrações sanguíneas de triglicérides, do colesterol, das lipoproteínas e dos ácidos graxos livres (AGL). A hipertrigliceridemia decorrente do aumento do quilomícron e das lipoproteínas de densidade muito baixa (VLDL) resulta de deficiência da insulina e das restrições associadas à lipoproteína lipase (LpL). Nos cães o colesterol circulante pode estar aumentado em diabéticos, mas o aumento

não é tão evidente quanto dos triglicerídeos. A combinação de lipoproteínas de baixa densidade (LDL) elevadas e HDL diminuídas, pode acelerar o desenvolvimento de doenças ateroscleróticas e coronárias, em humanos (NELSON, 2009). É importante ressaltar que nos animais diabéticos tratados com QUE, a elevação da concentração de triglicerídeos não foi significativa, o que sugeriu que a QUE pode ter uma ação preventiva às doenças ateroscleróticas e coronárias, nos diabéticos.

De acordo com NELSON (2009), as anormalidades identificadas no perfil bioquímico dependem da duração do DM sem tratamento e da presença de doenças concomitantes, mais notavelmente a pancreatite, que pode se manifestar a partir de uma hipertrigliceridemia muito intensa (POWERS, 2009). Foram observadas elevações significativas nas concentrações de ureia, alanina aminotransferase (ALT) e fosfatase alcalina (FA). No diabetes descomplicado, normalmente a ureia e a creatinina séricas estão dentro do intervalo de referência. A capacidade de síntese de ureia fornece informação do estado funcional da massa hepática (HANSEN & POULSEN, 1986). Vale ressaltar que o catabolismo verificado nos animais diabéticos, pela acentuada perda de peso, poderia ser a principal fonte de aminoácidos nitrogenados convertidos em ureia. A elevação da ALT e da FA é prevista no DM, como decorrência da lipidose hepática (NELSON, 2009). No fígado, a FA é formada nos hepatócitos e em células da membrana do trato biliar. Embora a FA de origem hepática possa estar aumentada no soro, devido a uma hepatopatia ativa, a atividade sérica mostra-se especialmente sensível à obstrução do trato biliar (RAVEL, 1997). A ALT é uma enzima glucogênica que, no fígado, tem sido relacionada com acúmulo de gordura visceral (SONG et al., 2008), obesidade (SAKUGAWA et al., 2004), síndrome metabólica (HANLEY et al., 2005; OH et al., 2006; SAITO et al., 2009), resistência à insulina (SCHINDHELM et al., 2005; MARCHESINI et al., 2005) ou como consequência de lesão hepática (SAITO et al., 2009).

No diabetes melito, a hiperglicemia presente favorece ao aumento dos depósitos de glicogênio, principalmente nos hepatócitos, devido à alta permeabilidade dessa célula à glicose (CULLEN, 2009; MYERS & McGAVIN, 2009). No primeiro manuscrito, não foi constatado significativo aumento de depósitos de glicogênio, nas amostras hepáticas, de animais diabéticos, coradas pela reação de PAS. Contudo, como consequência da crescente indisponibilidade de adenosina trifosfato (ATP), a

célula hepática busca o equilíbrio energético, aumentando o consumo de glicogênio, através da glicólise (DE KUMAR et al., 2005; MYERS & McGAVIN, 2009). Assim sendo, o paradoxal estado de desnutrição, verificado nos animais diabéticos, sugeriu a acelerada depleção dos depósitos corporais de glicogênio, tanto hepáticos, como musculares. Além disso, como a síntese de glicogênio depende da estimulação da insulina, a inadequada disponibilidade de insulina, assim como o aumento da resistência à mesma, poderia contribuir de forma importante para a diminuição do glicogênio (JENSEN & LAI, 2009).

Nas frações beta e gama foram observadas elevações significativas nos animais diabéticos tratados com QUE, com exceção dos tratamentos com 25 e 50 mg/Kg na fração beta, e 50 mg/Kg na fração gama, que apresentaram uma diminuição. Não houve variação significativa na concentração das alfaglobulinas, provavelmente, em decorrência do estímulo inflamatório crônico. A fração das betaglobulinas, precisamente na região beta-2, contém, preponderantemente, as frações do complemento C3 e C4, além de IgA que está moderadamente aumentada na elevação do complemento, originária de inflamações ou secundárias a uma obstrução biliar intra ou extra-hepática. A fração protéica das gamaglobulinas é composta, preponderantemente, pelas imunoglobulinas IgG, IgA e IgM, e em menor concentração pelas IgD e IgE (SZYMANOWICZ et al., 2006). As hepatopatias pertencem a um grupo significativo de doenças que podem elevar a concentração das gamaglobulinas. Na hepatite, verifica-se classicamente um aumento relativamente leve dos níveis de betaglobulinas e gamaglobulinas, com redução da albumina (RAVEL, 1995). Na insuficiência hepática grave, a constatação de hipoalbuminemia e a diminuição do teor de alfa-globulina, como haptoglobina ou alfa-1-antitripsina, indicam prognóstico desfavorável. De qualquer forma, juntamente com a resposta de fase aguda, o aumento do teor de globulinas indica um estímulo imune sistêmico, secundário ao prejuízo da função da célula de Kupffer, à disfunção das células B e T e à produção de anticorpos (CENTER, 2007). Os resultados observados nos animais diabéticos tratados com 25 e 50 mg/Kg de QUE, sugeriu que o flavonoide conseguiu interferir no processo inflamatório, provavelmente em sua fase aguda, atenuando seus efeitos, uma vez que esse composto apresenta ação anti-inflamatória, observada em outros estudos (ERLUND, 2004).

As células de Kupffer são conhecidas pelo importante papel na fagocitose de partículas estranhas. Além disso, essas células produzem uma ampla variedade de mediadores químicos, como interferon, interleucina 1, enzimas proteolíticas e fator de necrose tumoral, que podem lesar e destruir outras células (NOLAN, 1981; MICHIE et al., 1988). Foi verificado que as amostras de tecido hepático provenientes de animais diabéticos, em relação às dos não diabéticos, possuíam, além de leucócitos e plasmócitos, uma quantidade maior de células de Kupffer. Alguns desses macrófagos, distribuídos nos sinusoides e espaços porta, exibiam depósitos de glicogênio. Como o glicogênio é sintetizado no interior dos hepatócitos, sua presença nas células de Kupffer sugeriu a ocorrência de extravasamento por lesão das células hepáticas. Além disso, foi observado que os animais diabéticos apresentaram significativa leucopenia por neutropenia. Os neutrófilos são prontamente ativados por citocinas e outros mediadores inflamatórios, e se acumulam principalmente nos sinusoides. Após receberem o sinal para a quimiotaxia, podem migrar e atacar os hepatócitos comprometidos, aumentando a utilização de O_2 e gerando grande concentração de espécies reativas de oxigênio (ERO) (ADAMS et al., 2010). É importante ressaltar que embora, a capacidade de excreção da creatinina não tenha sido comprometida, conforme resultados exibidos no primeiro manuscrito, a elevada concentração sérica da ureia, verificada nos animais diabéticos, pode ter comprometido a capacidade de fagocitose dos neutrófilos, tanto pela elevada concentração intracelular de cálcio, sobrecarga de ferro e toxinas urêmicas, que deprimem essa função no neutrófilo (WATERLOT et al., 1985; HOEPELMAN et al., 1988; BALKE et al., 1995; HORL et al., 1995; HAAG-WEBER & HORL, 1996).

Observou-se que, com exceção do grupo tratado com 50 mg/Kg QUE, os ratos diabéticos apresentaram uma significativa redução na concentração de albumina. Além da síndrome nefrótica, vários fatores podem influenciar na diminuição da concentração sérica de albumina (RAVEL, 1997). Contudo, a hipoalbuminemia moderada pode refletir uma menor síntese de albumina ou até mesmo, um aumento do catabolismo. Ainda assim, o catabolismo protéico promovido por doenças, na maioria das vezes, poupa a albumina e se utiliza da proteína muscular (CENTER, 2007). EREJUWA et al. (2011) atribuíram a hipoalbuminemia, observada nos ratos diabéticos, como consequência de uma nefropatia diabética. Como neste

experimento, a função renal foi preservada, a diminuição do nível sérico de albumina poderia estar mais relacionada à inflamação crônica do fígado, quando o acentuado aumento das imunoglobulinas contrasta com a diminuição de albumina, entre outras proteínas, devido à deficiência de síntese (NAOUM, 1999).

O declínio dos mecanismos de defesa antioxidante frente a uma anormal elevação dos níveis de radicais livres pode levar às lesões de organelas celulares, enzimas, aumento de peroxidação lipídica e ao desenvolvimento da resistência à insulina (MARITIM et al., 2003). A peroxidação lipídica resulta de lesões, nos tecidos e principalmente em membranas celulares, causadas pelas ERO (DAS et al., 2000). RAMACHANDRAN et al. (2004), ao estudarem as substâncias reativas ao ácido tiobarbitúrico (TBARS), no fígado e rim de ratos, observaram significativas elevações da atividade, nos animais diabéticos, quando comparados ao controle não diabético. A indução de diabetes em ratos com STZ resulta em elevação na atividade TBARS, o que evidencia, indiretamente, o aumento da produção de radicais livres (MONTILLA et al., 1998). Observa-se que a TBARS, também foi mais elevada nos ratos diabéticos tratados com 5 e 25 mg/Kg de QUE. Contudo é importante ressaltar, que os resultados obtidos com 50 mg/Kg de QUE, indicaram que esse flavonoide foi capaz de proteger o organismo das ERO.

Atividade da superóxido dismutase hepática (SOD) foi significativamente reduzida nos animais diabéticos, o que está de acordo com os resultados encontrados no DM por SEKEROGLU et al. (2000); OZKAYA et al. (2002); COSKUN et al. (2005). No entanto, nos animais diabéticos tratados com 50 mg/Kg de QUE, a diminuição na concentração de SOD, não foi significativa, sugerindo que nessa dose o flavonoide conseguiu inibir os efeitos do estresse oxidativo. Em todos os tratamentos com ratos diabéticos, foi observada uma redução não-significativa na catalase hepática (CAT). De acordo com YAN (1997), a redução da SOD e da CAT, no diabetes, pode ser devido à glicação direta da enzima proteica ou ao emagrecimento resultante da condição diabética (WOHAIEB & GODIN, 1987).

As possíveis alterações no sistema colinérgico de ratos diabéticos foram avaliadas no manuscrito 2. Para avaliar possíveis mudanças comportamentais ou de memória, promovidas pelo estado diabético nos ratos, utilizamos um modelo de condicionamento clássico ou respostas pavlovianas ao medo (KIM & JUNG, 2006; MAREN, 2008). Através de testes como a esquiva inibitória, a habituação ao campo

aberto e o labirinto em cruz elevado, obtivemos respostas que confirmaram a influência da hiperglicemia crônica na formação da memória aversiva, bem como o nível de proteção oferecida pela administração da QUE.

O modelo de condicionamento pavloviano, produz respostas rápidas ao estímulo aversivo, que podem ser associadas à aprendizagem e formação da memória (SHIN & LIBERZON, 2010). Os resultados observados no teste de esquiva inibitória, mostraram que os animais diabéticos não tratados com QUE, diminuíram significativamente a latência (tempo de permanência na plataforma sobre a tela metálica). O que significou que a recordação do estímulo elétrico recebido 24 horas antes, não foi evocada de forma adequada, nesses animais. Esses resultados coincidem com os encontrados por SCHMATZ et al. (2009), quando trabalharam com ratos diabéticos do grupo de controle positivo (animais diabéticos sem tratamento) em relação ao grupo de controle negativo (animais não diabéticos sem tratamento). Contudo, vale ressaltar, que animais diabéticos tratados com QUE, exibiram uma latência semelhante à observada no grupo de controle. Esses resultados sugerem que a QUE promoveu proteção na formação da memória aversiva.

Os resultados observados no teste de labirinto em cruz elevado, revelaram que os animais diabéticos que não receberam o tratamento com QUE, apresentaram um maior estado de ansiedade. Segundo STEIMER (2002), a ansiedade é descrita como um estado psicológico, fisiológico e comportamental, que pode ser induzido tanto em animais como humanos, a partir de uma ameaça ao bem-estar. A ativação autonômica e neuroendócrina induz elevação no grau de expectativa e alterações no padrão comportamental do animal, que assume a partir de então comportamento de fuga ou de defesa (STEIMER, 2011). No aparelho, o aumento da ansiedade, nesses animais, refletiu na elevação do tempo de permanência nos braços fechados e na aversão pelos espaços abertos. É importante observar, que os animais diabéticos que receberam o tratamento com QUE, nas três concentrações, apresentaram comportamento semelhante ao controle não diabético. Por outro lado, ratos não diabéticos que receberam QUE na concentração de 50 mg/Kg apresentaram significativa elevação no tempo de permanência nos braços abertos e redução de tempo, quando nos braços fechados. Essa alteração de comportamento sugeriu que

nessa concentração a QUE poderia inibir o medo do animal, diminuindo o senso de autopreservação, essencial à sobrevivência dessa espécie.

Os resultados deste experimento mostraram que os animais diabéticos não tratados com QUE apresentaram significativa elevação na atividade da acetilcolinesterase (AChE) no córtex cerebral, hipocampo e nos sinaptossomas cerebrais (Figura 4). Esses resultados concordam com SCHMATZ et al. (2009), que também verificaram elevação significativa da atividade da AChE no córtex cerebral e hipocampo de ratos do grupo de controle positivo. É importante ressaltar que os ratos diabéticos que receberam 5 mg/Kg de QUE apresentaram significativa redução na atividade da AChE, tanto no córtex cerebral, quanto no hipocampo. Na concentração de 25 mg/Kg de QUE a redução da AChE foi observada no córtex e sinaptossomas cerebrais ($P<0,05$). A concentração de 50 mg/Kg de QUE reduziu significativamente a atividade da AChE no córtex, hipocampo e sinaptossomas cerebrais.

No córtex e nos sinaptossomas cerebrais, a diminuição da AChE apresentou uma relação inversa com a concentração de QUE administrada, 5, 25 e 50 mg/Kg no córtex cerebral e 25 e 50 mg/Kg nos sinaptossomas cerebrais. Esses resultados sugeriram que no córtex, hipocampo e sinaptossomas cerebrais, a QUE inibiu a ação da AChE, responsável pela hidrólise do neurotransmissor acetilcolina (ACh). A importância desse fato é a possível utilização da QUE como coadjuvante no tratamento da demência, principalmente em idosos que sofrem com a doença de Alzheimer, uma vez que a ACh é o principal neurotransmissor de estímulos nervosos de um neurônio a outro, estando diretamente envolvida nos processos motores, cognitivos e de memória (PATRICK, 2001; RANG et al., 2001). Por outro lado, foi observado nos animais não diabéticos, que a QUE aumentou significativamente a atividade da AChE no hipocampo (5, 25 e 50 mg/Kg), estriado (5 mg/Kg) e sinaptossomas cerebrais (25 mg/Kg), o que sugeriu a presença de uma propriedade não desejável no flavonoide.

Os resultados encontrados nos animais diabéticos sem tratamento com QUE, revelaram uma significativa elevação da TBARS, indicando importante peroxidação lipídica no córtex cerebral, hipocampo e estriado. É importante ressaltar que a administração de QUE reduziu o nível de TBARS nas três estruturas cerebrais: córtex cerebral (5, 25 e 50 mg/Kg), hipocampo (25 e 50 mg/Kg) e estriado (5 e 50

mg/Kg). Esses resultados confirmaram as propriedades antioxidantes da QUE, cujo grau de proteção variou em função da estrutura cerebral e da concentração empregada. Contudo, particularmente no córtex cerebral, foi observado que entre os animais não diabéticos, a QUE na concentração de 25 mg/Kg, apresentou significante efeito oxidante. Efeitos pró-oxidantes já foram relatados em flavonoides (CHAN et al., 1999; CONSTATIN & BRACHT, 2008; CONSTANTIN et al., 2010), sendo a atividade pró-oxidante, possivelmente, relacionada ao número de grupos hidroxila (CAO & SOFIE, 1997).

5 CONCLUSÕES

5.1 Manuscrito 1

Os resultados observados no presente trabalho sugerem que a hiperglicemia crônica está diretamente relacionada ao estresse oxidativo, observado nos animais diabéticos, bem como em alterações morfológicas e funcionais provocadas pelo excesso de radicais livres. A quercetina apresenta propriedades antioxidantes e anti-inflamatórias, que pode ser utilizadas no tratamento adjuvante do diabetes melito (DM). Contudo, a presença de efeitos não desejáveis como ação pró-oxidante e possível resistência à insulina, indicam a necessidade de novos estudos.

5.2 Manuscrito 2

Os resultados encontrados neste estudo sugerem que a hiperglicemia crônica provoca falhas na formação da memória aversiva, altera o comportamento e promove a peroxidação lipídica em estruturas e sítios cerebrais. A quercetina (QUE), devido às suas propriedades antioxidantes e anti-inflamatórias possivelmente possa ser utilizada como adjuvante no tratamento complementar do diabetes melito, protegendo a formação da memória aversiva, comportamento e sistema colinérgico. Contudo, a presença de efeitos não desejáveis como ação pró-oxidante e elevação na inquietação, indica a realização de novos estudos.

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