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Josiane Bizzi Schlemmer Braun

EFEITO PREVENTIVO DO FLAVONOIDE QUERCETINA NOS SISTEMAS PURINÉRGICO E COLINÉRGICO EM MODELO EXPERIMENTAL DE HIPERLIPIDEMIA

Santa Maria, RS
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Tese apresentada ao Programa de Pós-Graduação em Ciências Biológicas: Bioquímica Toxicológica, da Universidade Federal de Santa Maria (UFSM, RS), como requisito parcial para obtenção do grau de **Doutora em Bioquímica Toxicológica**

Orientadora: Profª. Drª. Daniela Bitencourt Rosa Leal

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Santa Maria, RS
2017

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Vocês também são parte deste trabalho...

RESUMO

EFEITO PREVENTIVO DO FLAVONOIDE QUERCETINA NOS SISTEMAS PURINÉRGICO E COLINÉRGICO DE MODELO EXPERIMENTAL DE HIPERLIPIDEMIA

AUTORA: Josiane Bizzi Schlemmer Braun
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A hiperlipidemia (HL) se caracteriza por alterações no metabolismo lipídico, sendo um dos principais distúrbios metabólicos. A HL é reconhecida como um dos fatores de risco para o desenvolvimento da aterosclerose, sendo esta caracterizada pela presença de disfunção endotelial e por um processo inflamatório. Os nucleotídeos e nucleosídeo extracelulares são importantes moléculas sinalizadoras que modulam as ações dos linfócitos, sendo essenciais para o início e manutenção das reações inflamatórias. Os níveis extracelulares destes nucleotídeos são fisiologicamente controlados pela ação de um complexo enzimático formado pelas enzimas E-NTPDase, E-5'nucleotidase e E-ADA. Estas enzimas estão localizadas na superfície de linfócitos e desempenham um papel importante na manutenção da homeostasia. O processo inflamatório e a resposta imune estão envolvidos na formação da lesão aterosclerótica. As citocinas são responsáveis pela modulação de aspectos da inflamação vascular, alterando a proliferação, diferenciação e função vascular de uma variedade de tipos celulares em que ocorre a comunicação intercelular. Além disto, alterações na homeostase do colesterol também agem negativamente no sistema nervoso central. A acetilcolina é um dos principais neurotransmissores do sistema colinérgico, e é hidrolisada pela acetilcolinesterase, importante enzima regulatória. A AChE é muito importante no controle da transmissão dos impulsos nervosos através das sinapses, sendo por isso considerada um bom indicador da atividade colinérgica. Alterações na atividade da AChE tem sido associadas a déficits de memória e aprendizagem. Diante disto, o interesse pela prevenção de doenças através da alimentação vem aumentando. A quercetina, presente em maçãs, cebolas, brócolis, entre outras, vem sendo estudada por apresentar ações antioxidantes, anti-inflamatórias e neuroprotetoras. Sendo assim, o objetivo deste trabalho foi avaliar se o tratamento preventivo de quercetina é capaz de prevenir as alterações causadas pela HL nos sistemas purinérgico e colinérgico. Foram utilizados ratos Wistar machos, e divididos em 10 grupos (n=7), sendo 5 sem indução da HL e 5 com a indução. Os grupos foram tratados preventivamente com quercetina nas doses de 5, 25 e 50 mg/kg, durante 30 dias. Após os 30 dias, foi administrado o P407 (500 mg/kg) via intraperitoneal, para indução da HL. Sinvastatina (0,04 mg/kg) também foi administrada após a indução como controle positivo. Os resultados monstraram que o tratamento preventivo de quercetina conseguiu amenizar os níveis elevados dos lipídios induzidos pela HL (P<0,05). Também foi observado no teste do reconhecimento de objeto, que houve uma diminuição significativa na memória de ratos hiperlipidêmicos (P<0,001), e esta alteração foi amenizada com o tratamento preventivo de quercetina (P<0,05). A HL também diminuiu a atividade da acetilcolinesterase em 52,6% no hipocampo de ratos hiperlipidêmicos. Também demonstrou-se um aumento na atividade da E-NTPDase, em 72% na hidrólise de ATP e 98,7% na hidrólise de ADP e 78% na atividade da E-ADA em linfócitos dos ratos hiperlipidêmicos e o pré-tratamento com quercetina fez com que este aumento fosse evitado (P<0,01). Níveis elevados de IFN-γ e IL-4 foram observados em ratos com HL (P<0,01), e estes níveis foram diminuídos com o pré-tratamento de quercetina (P<0.05), demonstrando seu efeito anti-inflamatório. Dessa forma, podemos concluir que o tratamento preventivo de quercetina possivelmente inibe as vias inflamatórias, reduzindo os níveis de citocinas pró-inflamatórias, modulando o sistema purinérgico e também revertendo as alterações causadas no sistema colinérgico induzido pela HL.

Palavras-chave: Quercetina. Hiperlipidemia. Sinalização Purinérgica. Sinalização Colinérgica.

ABSTRACT

PREVENTIVE EFFECT OF QUERCETIN FLAVONOID IN PURINERGIC AND COLINERGIC SYSTEMS IN EXPERIMENTAL MODEL OF HYPERLIPIDEMIC

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Hyperlipidemia (HL) is characterized by changes in lipid metabolism, being one of the main metabolic disorders. HL is recognized as a risk factor for the development of atherosclerosis, characterized by the presence of endothelial dysfunction and an inflammatory process. Extracellular nucleotides and nucleosides are important signaling molecules that modulate the actions of lymphocytes and are essential for the initiation and maintenance of inflammatory reactions. Extracellular levels of these nucleotides are physiologically controlled by the action of an enzyme complex formed by enzymes E-NTPDase, E-5'nucleotidase and E-ADA. These enzymes are located on the surface of lymphocytes and play an important role in maintaining homeostasis. The inflammatory process and the immune response are involved in the formation of atherosclerotic lesions. Cytokines are responsible for modulating aspects of vascular inflammation by altering the proliferation, differentiation and vascular function of a variety of cell types in which intercellular communication occurs. Moreover, changes in cholesterol homeostasis also act negatively on the central nervous system. Acetylcholine is one of the main neurotransmitters of the cholinergic system and is hydrolyzed by acetylcholinesterase, an important regulatory enzyme. AChE is very important in controlling the transmission of nerve impulses through synapses and is therefore considered a good indicator of cholinergic activity. Changes in AChE activity have been associated with memory and learning deficits. In view of this, the interest in disease prevention through food is increasing. Quercetin, present in apples, onions, broccoli, among others, has been studied for presenting antioxidant, anti-inflammatory and neuroprotective actions. Thus, the objective of this study was to evaluate whether the preventive treatment of quercetin is able to prevent the changes caused by HL in the purinergic and cholinergic systems. Male Wistar rats were used and divided into ten groups (n=7), five of them without induction of HL and five with induction. Groups were pretreated with quercetin at doses of 5, 25 and 50 mg / kg for 30 days. After 30 days, P407 (500 mg / kg) was administrated intraperitoneally for induction of HL. Simvastatin (0.04 mg / kg) was also administered after induction as a positive control. The results showed that the preventive treatment with quercetin could reduce the elevated lipid levels induced by HL ($P<0.05$). It was also observed in the object recognition test, that there was a significant decrease in the memory of hyperlipidemic rats ($P<0.001$), and this alteration was smoothed with the prevention of the preventive treatment of quercetin ($P<0.05$). The HL also decreased acetylcholinesterase activity in 52.6% in the hippocampus of hyperlipidemic rats. An increase in E-NTPDase activity of 72% in the ATP hydrolysis) and 98.7% in the ADP hydrolysis the 78% in the E-ADA activity in lymphocytes of hyperlipidemic rats was also demonstrated and pretreatment with quercetin avoided this increase ($P<0.01$). Levels of IFN-γ and IL-4 were observed in hiperlipidemic rats ($P<0.01$), and that these levels were decreased with quercetin pretreatment ($P<0.05$), demonstrating its anti-inflammatory effect. Thus, we conclude that the preventive treatment of quercetin possibly inhibits the inflammatory pathways, reducing the levels of proinflammatory cytokines, modulating the purinergic system and also reversing the changes caused in the HL-induced cholinergic system.

Keywords: Quercetin. Hyperlipidemia. Purinergic Signaling. Cholinergic Signaling.

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ARTIGO 1

MANUSCRITO SUBMETIDO

LISTA DE ABREVIATURAS

ACh	Acetilcolina
AChE	Acetilcolinesterase
ADO	Adenosina
ADP	Adenosina difosfato
AMP	Adenosina monofosfato
Apo E	Apolipoproteína E
ATP	Adenosina trifosfato
BChE	Butirilcolinesterase
ChAT	Colina aciltranferase
CT	Colesterol total
DCV	Doença cardiovascular
E-ADA	Ecto-adenosina desaminase
E-NPP	Ecto-nucleosídeo pirofosfato/fosfodiesterase
E-NTPDase	Ecto-nucleosídeo trifosfato difosfoidrolase
HDL	Lipoproteína de alta intensidade
HL	Hiperlipidemia
HMGCoA	Hidroximetil glutaril coenzima A
IDL	Lipoproteína de densidade intermediária
IFN-γ	Interferon gamma
IL-1	Interleucina 1
IL-2	Interleucina 2
IL-4	Interleucina 4
IL-6	Interleucina 6
IL-10	Interleucina 10
INF-α	Fator de necrose tumoral alfa
LCAT	Lecitina colesterol acil transferase
LDL	Lipoproteína de baixa densidade
LDLox	Oxidação do LDL
MP-1	Proteína quimioatrativa de monócitos
P407	Poloxamer - 407
SNC	Sistema nervoso central
SNP	Sistema nervoso periférico
TG	Triglicerídeos
VAChT	Transportador vesicular da acetilcolina
VLDL	Lipoproteína de muito baixa densidade

SUMÁRIO

APRESENTAÇÃO

Esta tese está descrita na seguinte forma: primeiramente são apresentados a Introdução, a Revisão Bibliográfica e os Objetivos.

A seguir, a Metodologia e os Resultados estão apresentados na forma de um artigo e de um manuscrito submetido.

Por fim, os itens Discussão e Conclusão, contêm interpretações e comentários gerais a respeito dos resultados apresentados no artigo e no manuscrito.

As Referências apresentadas no final da tese referem-se às citações que aparecem nas seções Introdução, Revisão Bibliográfica e Discussão.

1 INTRODUÇÃO

De acordo com a Organização Mundial da Saúde (OMS, 2016) cerca de 17,5 milhões de pessoas morrem por doenças cardiovasculares (DCV) todos os anos, representando aproximadamente 31% das mortes do mundo todo, sendo a hiperlipidemia (HL) um dos principais contribuintes para o risco cardiovascular. Deste modo, evidências sugerem que altos níveis de colesterol total (CT), lipoproteínas de baixa densidade (LDL) e triglicerídeos (TG) e baixo nível de lipoproteínas de alta densidade (HDL) são fatores de risco relevantes para as DCV (GONZALO-CALVO *et al.*, 2015).

A HL é caracterizada sorologicamente por níveis plasmáticos elevados de triglicerídeos e colesterol, particularmente a LDL (LUKACH *et al.*, 2014). A HL pode influenciar diretamente na indução de doença e modular indiretamente a inflamação, proliferação celular e também a resposta imune (AUGÉ *et al.*, 1998; COMINACINI *et al.*, 2000; SHAMSHIEV *et al.*, 2007).

A HL é um dos principais fatores de risco para o aparecimento e progressão da aterosclerose, sendo esta uma doença vascular caracterizada pelo acúmulo de lipídios na parede arterial (FISHER *et al*., 2012). O desenvolvimento da aterosclerose é uma cascata complexa, envolvendo alterações predominantes, como por exemplo, alteração no metabolismo lipídico, estresse oxidativo e a ocorrência de um processo inflamatório persistente (LIU *et al.*, 2015). Um evento crítico no início da aterosclerose é a ativação de células endoteliais com expressão de moléculas de adesão dentro da parede do vaso por vários estímulos, incluindo altos níveis de espécies reativas de oxigênio, lipoproteína de baixa densidade oxidada (LDL-ox) e citocinas pró-inflamatórias. Subsequentemente ocorre à aderência de monócitos circulantes ao endotélio vascular e sua transmigração para a camada íntima, que recruta ainda mais células inflamatórias para o local da lesão e, finalmente, forma-se a placa aterosclerótica com seu núcleo rico em lipídios (LIBBY *et al*., 2011; SALVAYRE *et al.*, 2016). A captação de LDL-ox por macrófagos no espaço arterial subendotelial leva à formação de células de espuma, que também é um determinante da ocorrência de lesão aterosclerótica (ZELLER; SRIVASTAVE, 2014).

O processo inflamatório e a resposta imune estão envolvidos na formação da lesão aterosclerótica. As citocinas são responsáveis pela modulação de aspectos da inflamação vascular, alterando a proliferação, diferenciação e função vascular de uma variedade de tipos celulares em que ocorre a comunicação intercelular, gerando doenças cardiovasculares (BIASILLO *et al*., 2010).

Durante o processo inflamatório os nucleotídeos e o nucleosídeo de adenina representam uma importante classe de moléculas extracelulares, que ao interagirem com receptores purinérgicos na superfície celular, sinalizam vias de grande importância que medeiam diversos efeitos biológicos, entre eles o processo inflamatório e a resposta imune (RALEVIC; BURNSTOCK, 1998). A sinalização induzida por estas moléculas correlaciona-se diretamente à atividade de enzimas localizadas na superfície da membrana celular. Estas enzimas pertencem à família das ectoenzimas, as quais regulam as concentrações dos nucleotídeos extracelulares nos tecidos (ZIMMERMANN, 2001).

Dentre as ectoenzimas encontram-se as E-NTPDases que desempenham importante papel na hidrólise de ATP (adenosina trifosfato) e ADP (adenosina difosfato), apresentando funções fisiológicas como participação na regulação de processos como a neurotransmissão, além de funções imunes e inflamatórias (ZIMMERMAN, 2001). A família das E-NPPs hidrolisa o ATP diretamente à AMP (adenosina monofosfato) (YEGUTKIN, 2008; ZIMMERMANN, 2001) e está envolvida na formação óssea e na motilidade celular (GODING *et al.*, 2003; STEFAN *et al.*, 2006). A E-5'-nucleotidase é responsável pela hidrólise de AMP formando adenosina (ADO) (COLGAN *et al.*, 2006). Este nucleosídeo, por sua vez, atua na neuroproteção, supressão da produção de mediadores pró-inflamatórios como o fator de necrose tumoral alfa (TNF-α) e a indução de interleucina-10 (IL-10) (MILLS *et al.*, 2008; RALEVIC; BURNSTOCK, 1998). Além dessas ectonucleotidases, a E-ADA é responsável pela desaminação da adenosina em inosina (YEGUTKIN, 2008). Esta enzima é importante na diferenciação e proliferação de células linfóides, particularmente linfócitos T, e maturação de monócitos possuindo importante função imune em processos inflamatórios (ANTONIOLI *et al.*, 2008; BOTA *et al.*, 2001).

Além de alterações no sistema purinérgico, o colesterol também pode provocar modificações no sistema colinérgico. O colesterol desempenha papel crucial no desenvolvimento e funcionamento normal do sistema nervoso central (SNC). É ele que mantém a plasticidade neuronal, transporta a vesícula sináptica ao longo dos microtúbulos e também participa na liberação de neurotransmissores (KLOPFENSTEIN *et al*., 2002; KOUDINOV *et al*., 2005; MAUCH *et al*., 2001). Porém, sabe-se que desequilíbrios nos níveis de colesterol podem afetar negativamente a função neuronal (DIETSCHY; TURLEY, 2004).

O sistema colinérgico desempenha um dos mais importantes papéis modulatórios no SNC já que possui um papel fundamental na regulação de muitas funções vitais relacionadas ao comportamento, bem como ao aprendizado e memória. A acetilcolina (ACh), um dos

principais neurotransmissores do sistema colinérgico, é reguladapor colinesterases, como a acetilcolinesterase (AChE), que é capaz de hidrolisar este neurotransmissor em muitos tecidos. A AChE é uma enzima regulatória, encontrada principalmente no encéfalo, músculos, eritrócitos e neurônios colinérgicos (MESULAM *et al.*, 2002). A AChE é uma enzima muito importante no controle da transmissão dos impulsos nervosos através das sinapses, sendo por isso considerada um bom indicador da atividade colinérgica (SZEGLETES *et al.*,1999). Além disso, alterações na atividade da AChE tem sido associadas a danos nos processos de memória e aprendizagem (BRAUN *et al*., 2017).

Diante do exposto, sabendo os efeitos causados pela HL, o interesse pela prevenção e também pela cura de doenças através da alimentação vem aumentando muito. Sabe-se que produtos naturais são importantes fontes de agentes terapêuticos. Estudos epidemiológicos sugerem que o aumento do consumo de flavonoides na dieta pode ter efeitos benéficos sobre o sistema cardiovascular (MULVIHILL; HUFF, 2010). Pesquisas indicam que a quercetina, um potente flavonoide, possui efeito cardioprotetor, diminuindo a HL (BHASKAR; HELEN, 2016; CUI *et al.*, 2017; GUO *et al.*, 2016; IMESSAOUDENE *et al*., 2016) sendo que seus efeitos podem ser parcialmente atribuídos às suas propriedades antioxidantes (YI *et al*., 2012) e anti-inflamatórias (KLEEMANN *et al.*, 2011).

A quercetina é um membro da subclasse flavonol e tem recebido considerável atenção pela comunidade científica nos últimos anos devido a suas funções. Como a maioria dos flavonoides, é abundante na dieta humana e um consumo médio diário de aproximadamente 16-23 mg/dia quercetina vem sendo consumido pela população humana (HOLLMAN; KATAN, 1999; NISHIMURO *et al*., 2015).

Contudo, uma vez que a hiperlipidemia é um importante fator de risco no desenvolvimento de DCV, afetando tanto o sistema purinérgico quanto o colinérgico e também o sistema imune e a quercetina possuir efeitos anti-inflamatórios e neuroprotetores, é de interesse investigar se o tratamento preventivo com quercetina é capaz de prevenir as alterações provocadas pela hiperlipidemia.

2 OBJETIVOS

2.1 OBJETIVO GERAL

Avaliar o efeito preventivo da quercetina em alterações dos sistemas purinérgico e colinérgico na hiperlipidemia.

2.2 OBJETIVOS ESPECÍFICOS

Em ratos com e sem hiperlipidemia induzida, tratados previamente com quercetina e tratados com sinvastatina, pretendeu-se:

- Avaliar os efeitos neuroprotetores do pré-tratamento com quercetina.
- Avaliar o efeito preventivo da quercetina em alterações do sistema colinérgico em estruturas cerebrais, como córtex, hipocampo, hipotálamo, estriado e cerebelo.
- Determinar a concentração sérica do colesterol total e suas frações e de glicose.
- Avaliar a atividade das enzimas alanina aminotransferase (ALT), a aspartato aminotransferase (AST) e fosfatase alcalina, assim como concentrações de albumina e ácido úrico em soro.
- Avaliar o efeito preventivo da quercetina em alterações nas atividades das enzimas E-NTPDase e E-ADA na sinalização purinérgica em linfócitos.
- Determinar os níveis séricos de nucleotídeos e nucleosídeo de adenina.
- Avaliar os níveis séricos de citocinas pró e anti-inflamatórias, como o interferon gamma (IFN-γ) e a interleucina 4 (IL-4) respectivamente.

3 REVISÃO BIBLIOGRÁFICA

3.1 HIPERLIPIDEMIA

O consumo de alimentos ricos em gordura saturada pode aumentar o risco do desenvolvimento de diversas doenças como, por exemplo, a obesidade, DCV, doenças metabólicas e distúrbios neurológicos (HOOIJAMANS; KILIAAN, 2008; PASINETTI *et al*., 2007).

Estima-se que, no ano 2020, as DCV sejam responsáveis por mais de 20 milhões mortes/ano, sendo que a maior prevalência dos casos ocorrerá a pacientes do sexo masculino (OMS, 2016). Estes dados epidemiológicos reforçam a necessidade da implantação de medidas voltadas à diminuição e controle dos fatores de risco (BARBOSA, 2011).

Dentre os fatores de risco já bem descritos e estabelecidos para as DCV, têm-se as dislipidemias. A HL se caracteriza como um dos principais distúrbios metabólicos, podendo ser classificada como alterações do metabolismo lipídico que modificam os níveis das lipoproteínas na circulação sanguínea e as concentrações dos seus diferentes componentes (MARTINEZ, 2003). HL é um distúrbio caracterizado pelo aumento nas concentrações de TG, CT e da LDL, determinado por fatores genéticos e/ou ambientais (PEREIRA *et al*., 2010; SOUZA *et al*, 2013).

Dentre os mecanismos propostos sobre o papel dos lipídios na gênese das DCV, destaca-se a sua associação com o estresse oxidativo, um estado em que o excesso de espécies reativas superpõe-se ao efeito dos sistemas antioxidantes, e que pode direta ou indiretamente estar relacionado à fisiopatologia de doenças crônicas como aterosclerose (CATANIA; BARROS; FERREIRA, 2009; SHARGORODSKY *et al.,* 2010).

De acordo com sua etiologia as dislipidemias podem ser classificadas em primárias ou secundárias. As primárias ocorrem em decorrência de fatores genéticos, enquanto que as secundárias podem desenvolver-se a partir de doenças pré-existentes, como a diabetes, assim como devido aos hábitos inadequados, tais como uma alimentação rica em gorduras, tabagismo e alcoolismo (V DIRETRIZES BRASILEIRAS SOBRE DISLIPIDEMIAS E DIRETRIZ DE PREVENÇÃO DA ATEROSCLEROSE, 2013).

Embora o colesterol plasmático em excesso seja visto como um vilão para a saúde, a presença equilibrada dessa molécula é vital para o organismo. O colesterol está presente nos tecidos e nas lipoproteínas plasmáticas na forma livre ou combinado com ácidos graxos de cadeia longa, como éster de colesterol. Além disso, é precursor de todos os esteróides do

organismo, como corticosteroides, hormônios sexuais, ácidos biliares e vitamina D (REINEHR; RUIZ, 2008). Grande parte do colesterol que o indivíduo necessita é sintetizada pelo próprio organismo, em que cerca de 80% é derivada da síntese endógena que ocorre no fígado, sendo necessário obter apenas uma pequena quantidade pela alimentação (LUPATTELLI *et al.*, 2012).

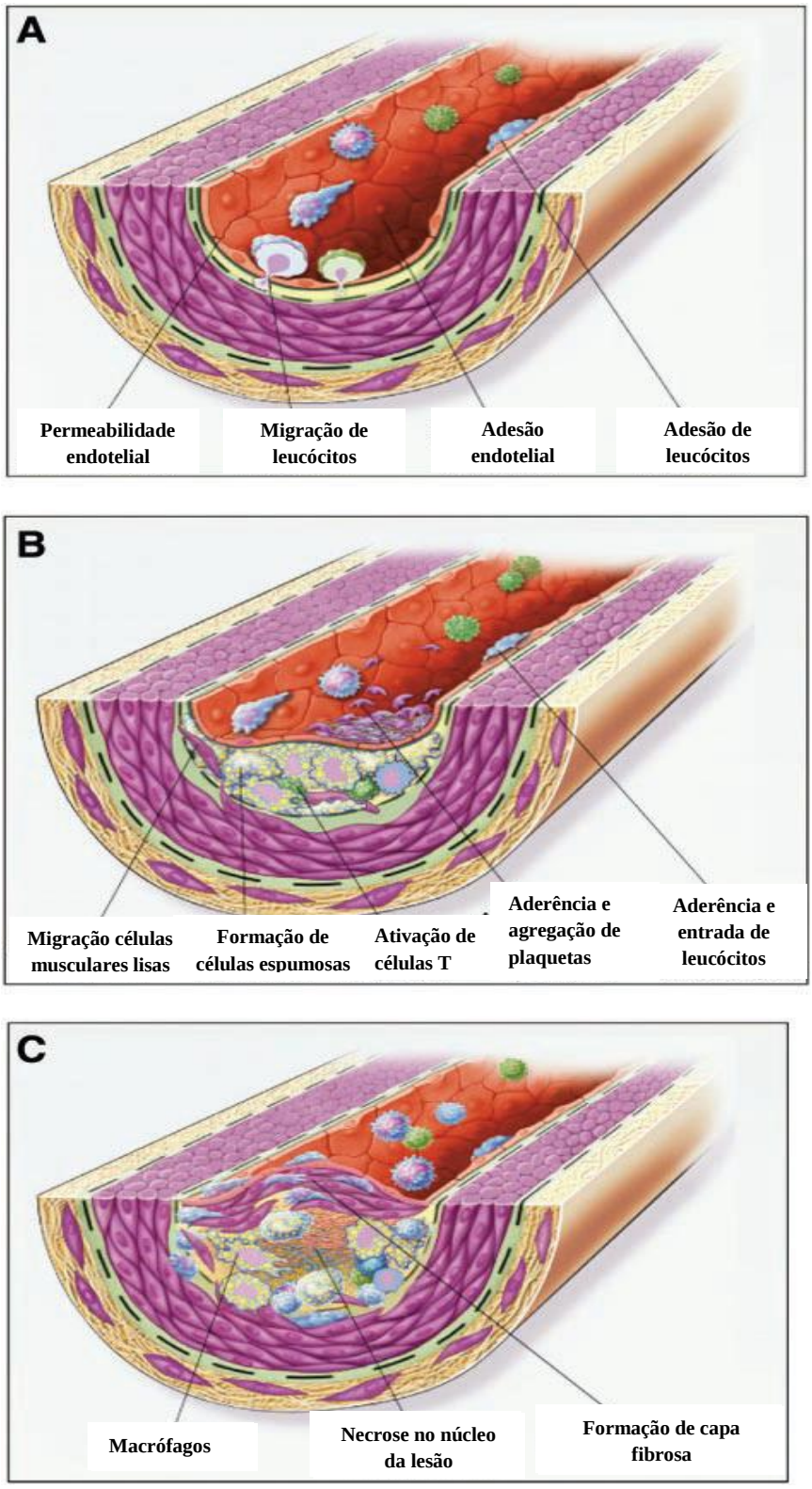
A homeostase do colesterol no compartimento plasmático e nos tecidos é regulada por processos complexos que envolvem a síntese e secreção das lipoproteínas (transportadores) como: quilomícrons, lipoproteínas de muito baixa densidade (VLDL), lipoproteínas de densidade intermediária (IDL), LDL e HDL; além da atividade de receptores celulares específicos para as lipoproteínas; de enzimas lipolíticas e de proteínas de transferência de lipídios (DANIELS *et al.*, 2009; LOTEMBERG, 2009).

Quando ocorre um desequilíbrio no metabolismo das lipoproteínas e/ou enzimas, tem-se o desenvolvimento das dislipidemias. Essas alterações lipídicas têm sido exaustivamente estudadas devido à sua forte relação com as DCV, como por exemplo, a doença aterosclerótica (FORTI; DIAMENT, 2006; FRANÇA; ALVES, 2006).

A HL é um fator chave para o desenvolvimento da aterosclerose em seres humanos, sendo esta uma forma frequente e potencialmente letal (BATISTA *et al.*, 2009). Há evidências de que a rapidez da progressão e a gravidade das lesões da aterosclerose são proporcionais à presença e à agregação dos fatores de risco cardiovasculares, que incluem obesidade (MARANHÃO *et al*., 2011), sedentarismo (CHRISTOFARO *et al.*, 2011), tipo de dieta (NEUTZLING *et al.*, 2010), hipertensão arterial (HOSSEINI-ESFAHANI *et al*., 2011), diabetes mellitus (SHAMIR *et al.*, 2008), síndrome metabólica (FRIEND *et al*., 2013) e dislipidemias (FRANÇA; ALVES, 2006).

A aterosclerose é uma doença multifatorial, lenta e progressiva, resultante de uma série de respostas celulares e moleculares altamente específicas à agressão endotelial, acometendo principalmente a camada íntima de artérias de médio e grande calibre (GOTTLIEB *et al*., 2005). As etapas envolvidas no processo de formação da placa aterosclerótica são: disfunção endotelial; migração de LDL e leucócitos circulantes (linfócitos T e monócitos) para o espaço subendotelial; oxidação do LDL; formação de células espumosas; migração e proliferação das células musculares lisas para o espaço subendotelial e síntese de matriz extracelular e lesão estrutural do endotélio, com deposição de plaquetas e formação de trombos (LUZ; UINT, 2005) (Figura 1).

Figura 1: Variações dos estágios da aterosclerose



Fonte: (Adaptado por Stocker; Keaney, 2004).

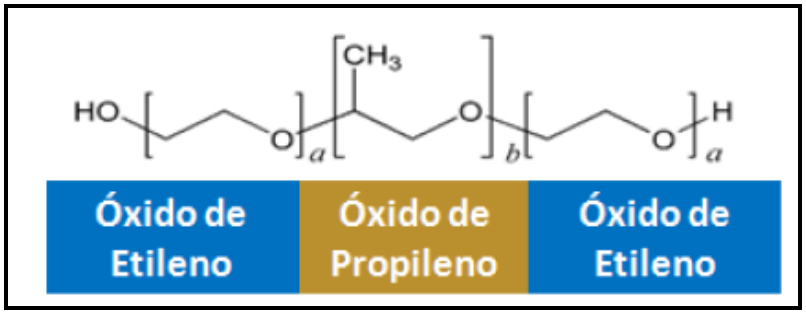
Além disso, outra importante etapa observada neste processo é a deposição de leucócitos sobre o endotélio e sua migração para o subendotélio, mediada por moléculas de adesão (VCAM-1, ICAM-1, E-selectina, ELAM-1). A expressão de moléculas de adesão é regulada por citocinas sintetizadas em pequenas concentrações pelo endotélio arterial, tais como a interleucina-1 (IL-1), interleucina-4 (IL-4), interleucina-6 (IL-6), TNF-α e o interferon gamma (IFN-γ) (GIMBRONE JR, 1994).

Diante do exposto, vários modelos animais têm sido utilizados para estudar os efeitos fisiopatológicos da HL. A HL pode ser induzida por meios dietéticos (alimentação crônica de uma dieta rica em gordura) (BEPPU *et al.*, 2017; BIREM *et al.*, 2017) ou por tratamento com compostos, tais como o Triton (IBRAHIM *et al.*, 2016) e o Poloxamer 407 (BRAUN *et al.*, 2017; KOROLENKO *et al.*, 2016).

3.2 POLOXAMER-407 (P407)

O P407 é um copolímero sintético, da classe mais geral de copolímeros conhecidos como poloxâmeros, surfactante não-iônico, hidrofílico, não tóxico e usado em uma variedade de formas farmacêuticas (oral, parenteral e tópica). É um copolímero em tri-bloco constituído por um bloco hidrofóbico de polioxipropileno e por dois blocos hidrofílicos polioxietileno capaz de formar gel *in situ* (Figura 2) (DUMORTIER *et al.*, 2006; KIBBE, 2012; NIU *et al.*, 2009).

Figura 2: Estrutura química do poloxamer 407 (composto por 95-105 unidades de óxido de etileno *(a)* e por 54-60 unidades de óxido de propileno *(b)*

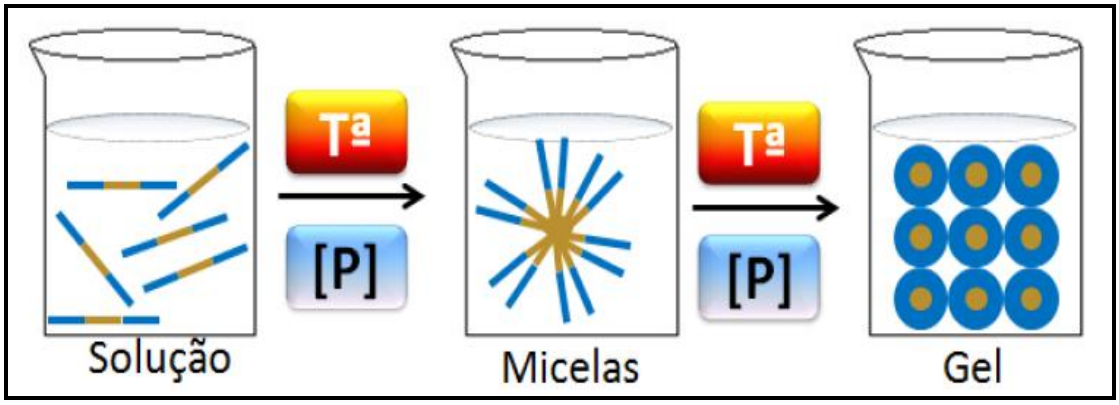


Fonte: (DUMORTIER *et al.*, 2006).

O P407 tem a propriedade de estar em estado líquido a baixas temperaturas, transformando-se em gel semi-sólido a temperaturas elevadas (temperatura de geleificação), o

que depende da condução de calor do ambiente (JOHNSON *et al.*, 2013). Conforme a temperatura aumenta, as moléculas do P407 se agregam formando micelas. A formação de micelas é atribuída à desidratação da região hidrofóbica, composta pelo óxido de propileno, e consiste no primeiro passo para a formação do gel (Figura 3) (DUMORTIER *et al.*, 2006).

Figura 3: Poloxamer 407: formação de gel



Fonte: (DUMORTIER *et al.*, 2006).

O P407 fornece um meio atrativo para a indução da hiperlipidemia devido a sua rápida ação e à aparente falta de toxicidade. O poloxamer é capaz de causar aumento nas lipoproteínas do soro devido ao seu efeito direto no metabolismo lipídico, e por isso é extensamente utilizado em protocolos experimentais de dislipidemia. Dentro de 24 horas após uma injeção intraperitoneal uma hiperlipidemia profunda é detectada (CHAUDHARY; BROCKS, 2013).

O mecanismo de ação para a indução da hiperlipidemia ocorre sobre uma série de enzimas relacionadas ao perfil lipídico. O P407 atua inibindo a lipase endotelial, hepática e a lipase lipoproteica, e, por alteração na atividade dessa última, causa aumento dos níveis de triglicerídeos (WASAN *et al.*, 2003). Tal fato pôde ser visto no estudo de Johnston (2004) onde investigaram o efeito do P407 na atividade da lipase lipoproteica e descobriram que após 3h da injeção intraperitoneal com o composto, a atividade desta enzima diminuiu 95% em relação aos controles. O P407 também atua estimulando indiretamente a enzima 3-hidroxi-3-metilglutaril coenzima A redutase (HMGCoA redutase), resultando em aumento dos níveis circulantes de colesterol total (LEE *et al.*, 2012). Johnston e Zhou (2007) afirmam ainda, uma possível inibição da enzima 7-α hidroxilase, o que prejudica a eliminação do colesterol na

bile, principalmente em doses repetidas de poloxamer, o que contribui para a hipercolesterolemia.

Dessa forma, evidencia-se a ação hiperlipidêmica do P407, já referenciada em outros trabalhos (KOROLENKO *et al.*, 2013; LEE *et al.*, 2012; ZANWAR *et al.*, 2014). O P407 já foi utilizado para induzir hiperlipidemia em vários modelos experimentais de roedores, como camundongos (JOHNSTON; PALMER, 1993), ratos (BRAUN *et al.*, 2017) e coelhos (JOHNSTON *et al.*, 2003). O modelo de hiperlipidemia por P407 tem uma série de vantagens quando comparado a outros modelos vigentes, como por exemplo, não se necessita de animais *knock out* para genes, como a apolipoproteína E (apoE) e proteína quimioatrativa de monócitos (MP-1), o qual se mostra como uma mudança na fisiologia normal de vertebrados. Além disso, mostra-se como um método preciso de hiperlipidemia dose dependente e permite a avaliação da potência de drogas antihiperlipidêmicas de várias classes (estatinas, fibratos, e ácido nicotínico) (JOHNSTON *et al.*, 2002).

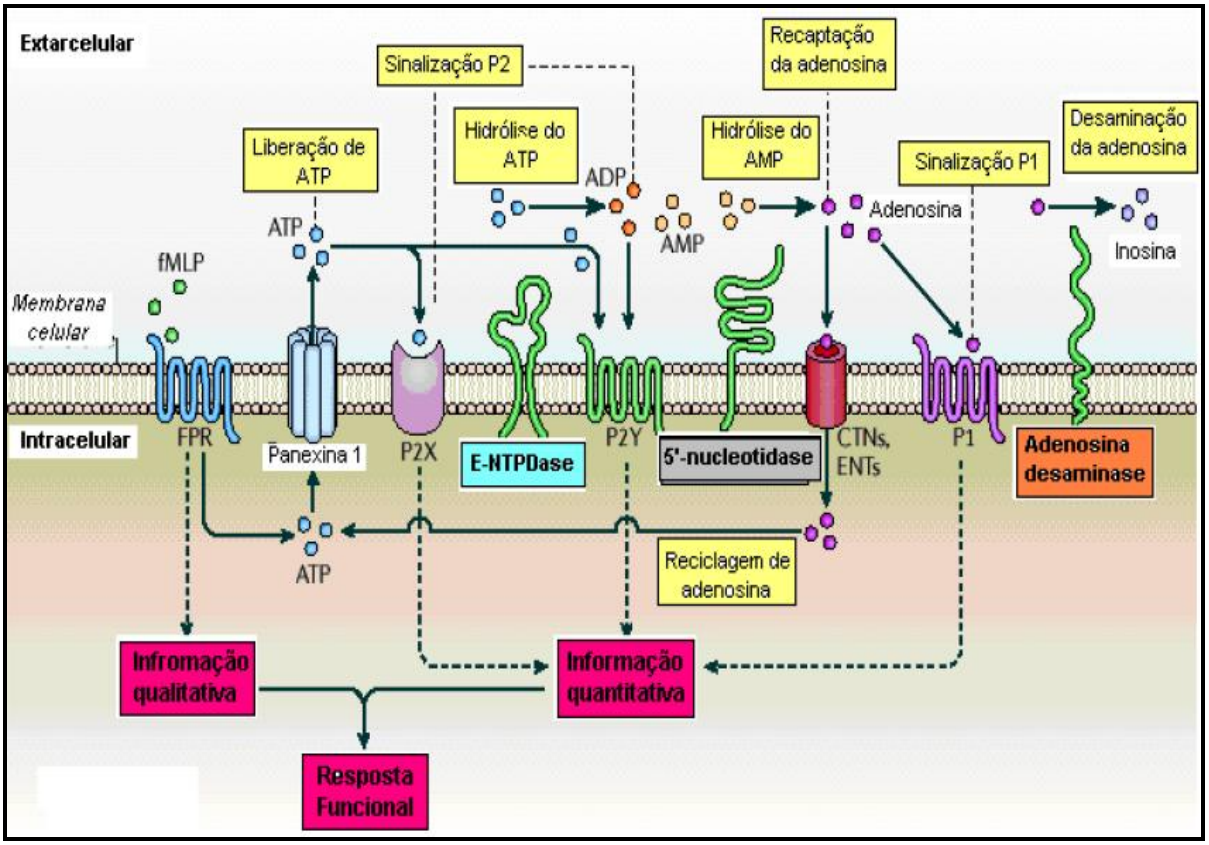
Portanto, o P407 possui a capacidade de induzir a hiperlipidemia, sendo esta capaz de desenvolver a aterosclerose que é caracterizada por um processo inflamatório com acúmulo de linfócitos nas paredes das artérias (HANSSON, 2005; ROCHA; LIBBY, 2009). Diante disto, podemos destacar os nucleotídeos ATP, ADP e AMP, bem como o nucleosídeo ADO que são mediadores capazes de modular as ações dos linfócitos em processos inflamatórios (DI VIRGILIO, 2001). Estes nucleotídeos extracelulares são importantes moléculas sinalizadoras e essenciais para o início e manutenção das reações inflamatórias. Além disso, são também efetivos na regulação da resposta vascular ao dano endotelial (BIRK *et al*., 2002).

3.3 SISTEMA PURINÉRGICO

O sistema purinérgico possui componentes envolvidos em vias de sinalização importantes para a regulação de diversos processos fisiológicos, envolvendo a resposta imune e o processo inflamatório (BURNSTOCK, 2007). Três componentes principais fazem parte da sinalização do sistema purinérgico (Figura 4):

- Nucleotídeos e nucleosídeo extracelulares, que são moléculas mediadoras da sinalização;
- Receptores purinérgicos específicos (P2X, P2Y e P1), através dos quais os nucleotídeos e nucleosídeos exercem seus efeitos;
- As ectoenzimas, responsáveis pela regulação dos níveis dos mediadores no meio extracelular (YEGUTKIN, 2008).

Figura 4 - Representação esquemática dos componentes da sinalização purinérgica



Fonte: (Adaptado de JUNGER, 2011).

Esses componentes da sinalização purinérgica estão presentes em diferentes tipos celulares, como linfócitos, plaquetas, células endoteliais entre outros, permitindo a formação de complexos distintos da sinalização purinérgica (JUNGER, 2011).

3.3.1 Nucleotídeos e nucleosídeo extracelulares

Os nucleotídeos extracelulares, ATP, ADP e AMP, bem como o nucleosídeo ADO, têm sido implicados em um grande número de funções fisiológicas (RALEVIC; BURNSTOCK, 1998). Está bem estabelecido o conceito de que essas moléculas atuam como mensageiros extracelulares, capazes de sinalizar efeitos biológicos no meio extracelular (BURNSTOCK, 2007). Em condições fisiológicas, os nucleotídeos estão presentes no meio extracelular em baixas concentrações, normalmente em quantidades nanomolares, podendo chegar até a quantidade micromolares em determinadas situações (DI VIRGILIO, 2001). Quando em altas concentrações, como no caso do ATP, podem atuar como uma molécula

citotóxica e levar à morte celular, pela formação de grandes poros na membrana plasmática (PODACK *et al*., 1985; YOUNG *et al*., 1986).

O ATP está presente em todas as células vivas e o seu papel intracelular no metabolismo energético já é bastante conhecido, sendo responsável por diversos processos que requerem energia como transporte ativo, motilidade celular e biossíntese de moléculas, dentre outros (BOURS *et al.*, 2006; YEGUTKIN, 2008). Em adição ao seu papel intracelular, o ATP extracelular está envolvido em uma série de processos biológicos como neurotransmissão, contração muscular, vasodilatação, agregação plaquetária, dor e inflamação (BOURS *et al.*, 2006). O ATP é um neurotransmissor armazenado em vesículas sinápticas e é liberado por exocitose juntamente com outros neurotransmissores como, por exemplo, a acetilcolina e o glutamato (ZIMMERMANN, 1999). Além disso, também foi demonstrado que o ATP pode ser liberado através de panexinas (canais protéicos transmembrana, que conectam o espaço intracelular com o extracelular) ou pelas conexinas (originalmente descritas como proteínas de junções ‘‘gap’’, consistindo de dois hemicanais). Os hemicanais isolados podem funcionar como condutores entre o citoplasma e o espaço extracelular, controlando a liberação de ATP das células (SABIROV; OKADA, 2005).

O ATP possui ações pró e anti-inflamatórias, dependendo da sua concentração extracelular, local de ação e tipo de receptor envolvido (BOEYNAEMS; COMMUNI, 2006; DI VIRGILIO, 2005; DI VIRGILIO *et al.*, 2009). Em situações pró-inflamatórias, ocorre a estimulação e proliferação de linfócitos, sendo necessário para a secreção de importantes citocinas dependentes das células T, como INF-γ e interleucina-2 (IL-2), intimamente envolvidas na indução de resposta imune a antígenos estranhos. Também apresentam outros efeitos em muitos processos biológicos, como contração do músculo liso, neurotransmissão, inflamação e dor (RALEVIC; BURNSTOCK, 1998; SNEDDON *et al*., 1999; SITKOVSKY, 1998). Porém, o ATP extracelular também pode desempenhar um papel imunossupressor, por inibir a proliferação de células T, consequentemente, bloqueando a liberação de citocinas pró-inflamatórias (DEAGLIO *et al*., 2007; GESSI *et al*., 2007). Esse mecanismo se dá principalmente quando o ATP está em baixas concentrações extracelulares (submicromolares), no qual é capaz de ativar receptores mais sensíveis, como o P2Y, localizados na superfície dos linfócitos. Estes receptores quando estimulados promovem uma regulação negativa na expressão e liberação de citocinas pró-inflamatórias, estimulam uma resposta Th2, e levam a liberação de citocinas anti-inflamatórias, promovendo um efeito protetor contra danos teciduais excessivos (BOURS *et al.*, 2006). Dessa forma, essas alterações geralmente ocorrem justamente quando o ATP extracelular é encontrado em

concentrações micromolares, podendo assim, atuar como uma potente molécula citotóxica, capaz de levar à morte diferentes classes de células pela formação de grandes poros na membrana plasmática (FILIPPINI *et al.*, 1990).

O ADP não possui um papel definido nos linfócitos (DI VIRGILIO *et al.*, 2001). Porém, nas plaquetas, ele age como um importante mediador da agregação plaquetária e da tromborregulação (ZIMMERMANN, 1999). Em situações de disfunção ou dano vascular, o ADP é liberado do interior de grânulos existentes nas plaquetas, sendo então considerado o agonista mais importante do recrutamento plaquetário e o indutor da formação de trombos no interior de vasos (MARCUS *et al*., 2003). Já o AMP é um metabólito intermediário da hidrólise do ATP (BARSOTTI; IPATA, 2004) que exerce a função de sinalizador em situações de desequilíbrio no metabolismo, servindo também como substrato para a formação da ADO (CUNHA, 2001; LATINI; PEDATA, 2001). A ADO é reconhecida por possuir propriedades anti-inflamatórias (CRONSTEIN, 1994) e vasodilatadoras (JACOBSON *et al*., 2006). No sistema imune, uma das principais funções da ADO é mediar uma resposta imunossupressora para proteger os tecidos dos ataques promovidos pelas células de defesa durante processos inflamatórios (SITKOVSKY, OHTA, 2005).

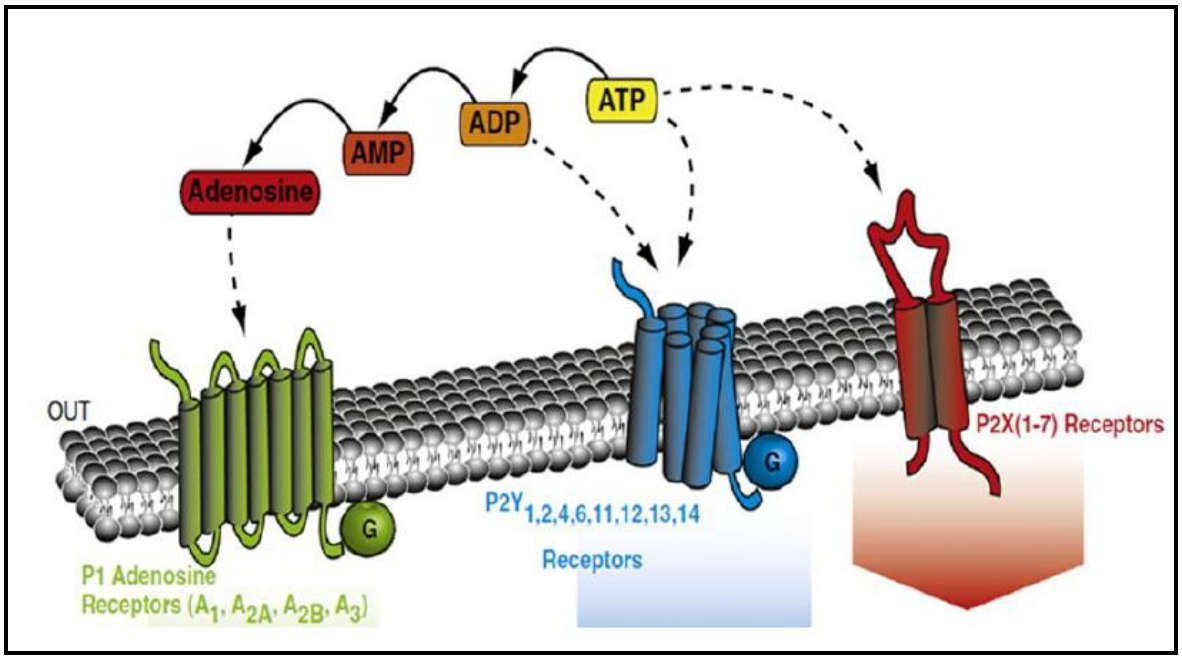
3.3.2 Receptores Purinérgicos

O nucleosídeo e os nucleotídeos extracelulares não atravessam a membrana plasmática, mas podem realizar suas ações biológicas através da sua ligação aos receptores purinérgicos presentes na membrana celular (JUNGER, 2011). O ATP e seus metabólitos extracelulares são reconhecidos por duas famílias de receptores, presentes na superfície de diversas células (BURNSTOCK, 2007). Estes receptores são divididos em dois tipos, o P1 e o P2, que são ativados por adenosina e ATP e ADP, respectivamente (Figura 5) (DI VIRGÍLIO *et al*., 2001).

Os receptores P1 (metabotrópicos) são receptores sensíveis às variações na concentração de adenosina presente no meio extracelular. São descritos quatro subtipos: A_1, A_{2A}, A_{2B} e A_3, com distintas propriedades farmacológicas entre si e todos são acoplados à proteína-G (FREDHOLM *et al.*, 1997; RALEVIC; BURNSTOCK, 1998). Os receptores P1 são amplamente expressos nas células e apresentam diferentes afinidades pela adenosina. Os receptores A_1 e A_{2A} demonstram uma maior afinidade pelo nucleosídeo, enquanto que os receptores A_3 e, particularmente, o receptor A_{2B} apresentam baixa afinidade. Os receptores P2 são subdivididos em duas classes: P2X (ionotrópicos), ligados a canais iônicos e receptores

P2Y (metabotrópicos), acoplados à proteína G. Os receptores P2Y diferem em sua seletividade para nucleotídeos da adenina (ATP e ADP) e da uracila (UTP e UDP), enquanto que os receptores P2X são ativados somente por ATP (BURNSTOCK, 2007).

Figura 5: Receptores envolvidos na resposta fisiológica do nucleosídeo e dos nucleotídeos extracelulares de adenina



Fonte: (http://ars.sciencedirect.com/content/image/1-s2.0-S0005273612000065-gr1.jpg). Data de acesso: 04/05/2017.

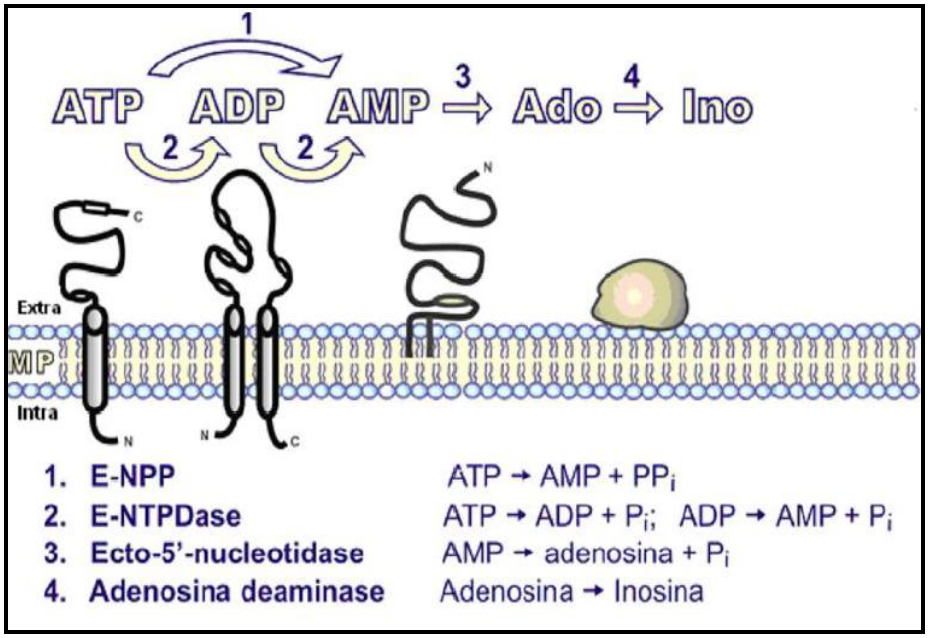
Foram identificados oito subtipos de receptores P2Y em células de mamíferos: P2Y1, P2Y2, P2Y4, P2Y6, P2Y11, P2Y12, P2Y13 e P2Y14 (WHITE; BURNSTOCK, 2006). Esses receptores diferem entre si pela diferença de afinidade aos seus agonistas. Quanto aos receptores P2X, já foram identificados sete subtipos: P2X1, P2X2, P2X3, P2X4, P2X5, P2X6 e P2X7 (DI VIRGILIO *et al*., 2001). Os receptores P2X são exclusivamente ativados por ATP extracelular e desencadeiam seus efeitos via abertura de um canal iônico na membrana celular, permeáveis a Na^+, K^+ e Ca^{2+} (ABBRACCHIO; BURNSTOCK, 1994).

Os níveis extracelulares de nucleotídeos e nucleosídeo de adenina e a consequente sinalização purinérgica por eles induzida através dos receptores são regulados através de uma variedade de enzimas localizadas na superfície celular ou solúveis no meio intersticial, denominadas ecto-nucleotidases (ZIMMERMANN *et al.*, 2007).

3.3.3 Ecto-enzimas

As ecto-enzimas atuam em conjunto, formando uma cadeia enzimática que tem início com a ação da E-NTPDase (Ecto-nucleosídeo trifosfato difosfoidrolase ou apirase; EC 3.6.1.5), e da E-NPP (nucleosídeo pirofosfato/fosfodiesterase; EC 3.1.4.1) as quais hidrolisam o ATP e ADP, formando o AMP, que em seguida é hidrolisado pela enzima E-5'-nucleotidase (EC 3.1.3.5) formando adenosina. Finalmente, a adenosina é desaminada pela E-ADA (ecto-adenosina desaminase) em inosina (Figura 6) (YEGUTKIN, 2008). A cascata purinérgica continua, sendo controlada por enzimas que regulam os níveis de outras purinas como: hipoxantina, xantina e acido úrico, sendo o último uma potente molécula antioxidante (RALEVIC; BURNSTOCK, 2003).

Figura 6: Enzimas envolvidas na degradação extracelular de nucleotídeos e nucleosídeo de adenina



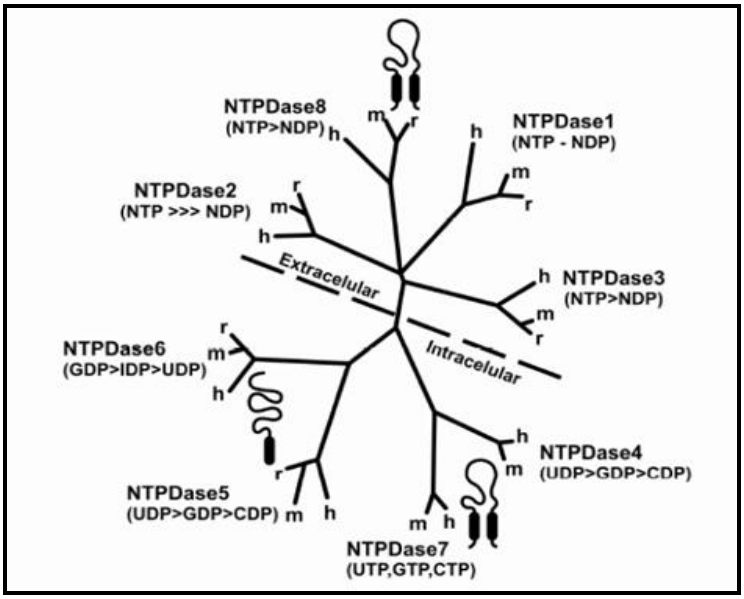
Fonte: (Adaptado de YEGUTKIN, 2008).

As E-NTPDases (CD39; E.C 3.6.1.5) são uma família de enzimas responsáveis pela hidrólise de nucleotídeos di e trifosfatados a seus monofosfonucleotídeos correspondentes (ZIMMERMANN *et al*., 2012). Os membros desta família de enzimas são nomeados de E-NTPDase 1-8 que diferem quanto a localização na célula, distribuição tecidual e especificidade por substratos. Quatro enzimas estão localizadas na membrana celular com seu

sítio catalítico voltado para o meio intracelular (E-NTPDase 4, 5, 6 e 7) e quatro para o meio extracelular (E-NTPDase 1, 2, 3 e 8) (Figura 7) (ROBSON *et al*., 2006).

A primeira a ser identificada e descrita foi a NTPDase-1, que no sistema vascular, desempenha um papel importante no sistema hemostático, uma vez que ela controla os efeitos pró-trombóticos e pró-inflamatórios de nucleotídeos como o ATP e o ADP, prevenindo assim a formação de coágulos e a vaso-oclusão (YEGUTKIN, 2008).

Figura 7: Membros da família da E-NTPDase



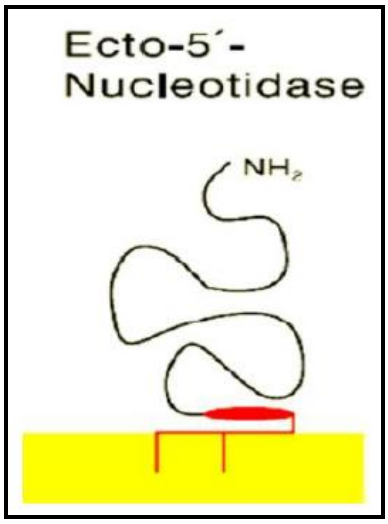
Fonte: (ROBSON *et al*., 2006).

A NTPDase-2, é particularmente associada com as superfícies adventícias dos vasos (ROBSON *et al.*, 2005) e células do sistema nervoso central e periférico (ROBSON *et al*., 2006). Destaca-se por uma atividade hidrolítica preferencialmente a nucleosídeos trifosfatados, podendo induzir consequentemente ativação plaquetária por hidrolisar o ATP a ADP, e desfosforilar o ADP a AMP muito lentamente (KUKULSKI *et al*., 2005; ZIMMERMANN, 2001).

Por sua vez, a enzima E-5'nucleotidase (eN, CD73, E.C. 3.1.3.5) é responsável pela desfosforilação de ribo- e desoxiribonucleossídeos 5' monofosfatados como AMP, CMP, UMP, IMP e GMP, porém com uma maior afinidade pelo AMP, sendo por isto considerada a principal enzima responsável pela formação de adenosina (ZIMMERMANN *et al*., 2012). A eN é uma proteína ancorada à membrana plasmática via um glicosil fosfatidilinositol (GPI)

com seu sítio catalítico voltado para o meio extracelular (Figura 8) (HUNSUCKER *et al.*, 2005; ZIMMERMANN, 2001). Ela é amplamente encontrada em uma variedade de tecidos, como no endotélio vascular, plaquetas e células do sistema imune (COLGAN *et al*., 2006).

Figura 8: Estrutura da E-5’nucleotidase ancorada à membrana



Fonte: (Adaptado de ZIMMERMANN, 2001).

A enzima adenosina desaminase (E-ADA, E.C. 3.5.4.4) também faz parte do conjunto de enzimas responsáveis pela degradação sequencial dos nucleotídeos e nucleosídeo da adenina (YEGUTKIN, 2008).

A E-ADA é responsável pela desaminação irreversível da adenosina e 2'-desoxiadenosina em inosina e 2'-desoxinosina, respectivamente (RESTA *et al.*, 1998; ROBSON *et al.*, 2006). Esta enzima apresenta uma localização citosólica, mas pode também estar localizada na superfície da membrana celular, como uma ectoenzima (YEGUTKIN, 2008). A E-ADA é encontrada em praticamente todos os vertebrados, e em humanos existe na forma de duas isoenzimas, classificadas como ADA1 e ADA2, cada uma com suas particulares propriedades bioquímicas (SHAROYAN *et al*., 2006).

A ADA1 está presente em todos os tecidos humanos, apresentando alta atividade em linfócitos e monócitos, e representa a maior parte da atividade da ADA total. Apesar de sua localização intracelular, a ADA1 pode estar combinada com uma glicoproteína dimérica não específica, designada como proteína combinante (CP), formando o complexo ADA-CP que forma uma E-ADA, encontrada na superfície celular (TSUBOI *et al*., 1995). Sabe-se que a E-ADA é responsável por grande parte do consumo de adenosina circulante nesse meio.

Aparentemente não existem diferenças, tanto catalíticas quanto moleculares, entre a enzima presente no citosol e a ecto-ADA. Esta evidência deve-se ao fato de que apenas 1 gene para a ADA foi encontrado, demonstrando que as sequências protéicas das duas enzimas são idênticas (FRANCO *et al*., 1997).

A ADA2 é a isoenzima predominante no soro e representa a menor parte da atividade da ADA total em tecidos. Diferentemente da ADA1, a ADA2 apresenta diferenças tanto estruturais quanto cinéticas e é encontrada predominantemente no soro de indivíduos normais (UNGERER *et al*., 1992). A ADA2 representa a menor parte da atividade da ADA total em tecidos. A maioria das células humanas contém pequena quantidade de ADA2 e provavelmente sua maior fonte seja o sistema monócito-macrófago. Estudos sugerem que ADA2 no plasma humano pode ser secretada por monócitos ativados em processos inflamatórios, tendo a habilidade de regular a proliferação celular (IWAKI-EGAWA *et al*., 2006).

A E-ADA por ser principalmente encontrada em linfócitos de sangue periférico de humanos, desempenha um papel chave no sistema imune, uma vez que está diretamente relacionada com a proliferação, a maturação e a função destas células (GORELL *et al.*, 2001).

Além de maior porcentagem de células B expressarem ADA na sua superfície, o número de moléculas de ADA na membrana plasmática das células B é muito maior do que das células T, entretanto, a atividade enzimática da ecto-ADA é muito maior em células T do que em células B. Portanto, alterações na atividade da ecto-ADA podem ocorrer como consequência da variação em sua expressão em tecidos e células (FRANCO *et al*., 1997).

A regulação da concentração da adenosina extracelular foi uma das primeiras funções fisiológicas atribuídas a E-ADA, logo após sua descoberta na membrana celular (FRANCO *et al*., 1997). A adenosina é liberada de células, dependendo da sua concentração intracelular ou ser proveniente da degradação do ATP extracelular devido à ação de ecto-nucleotidases. O controle da sinalização adenosinérgica também pode ser exercido através da via de recuperação da adenosina através de transportadores de nucleosídeos, seguida por fosforilação à AMP pela adenosina quinase ou desaminação à inosina pela ADA citosólica (HASKÓ; CRONSTEIN, 2004).

3.4 SISTEMA COLINÉRGICO

No mesmo grau de importância que o sistema purinérgico, podemos citar o sistema colinérgico, sendo este fundamental em várias funções vitais, como o aprendizado, a memória

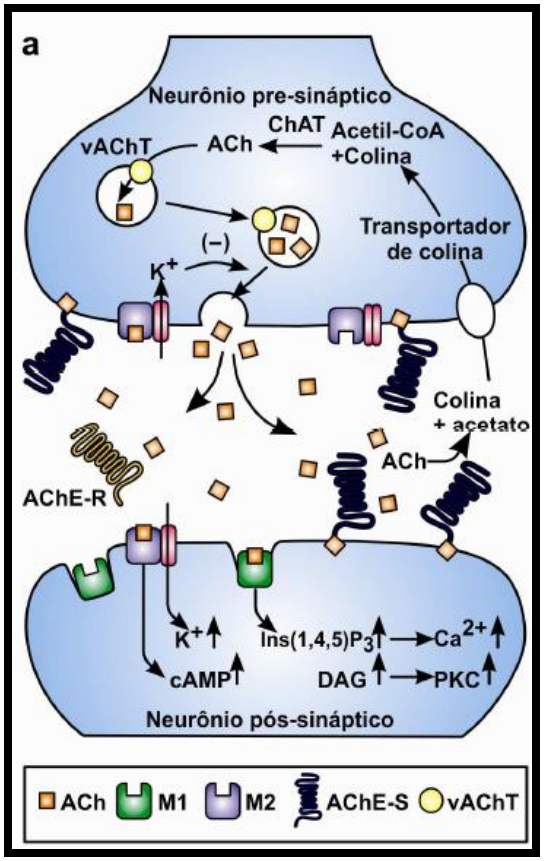
e a organização cortical do movimento (MESULAM *et al*., 2002), assim como também está notavelmente envolvido em ações anti-inflamatórias (DAS, 2007). Alterações na homeostase do colesterol também podem afetar negativamente a função neuronal (DIETSCHY; TURLEY, 2004). Estudos sugerem que uma alimentação rica em gordura e açúcares, e a presença de disfunção metabólica (pré-diabética ou síndrome metabólica), são suficientes para promover o desenvolvimento de distúrbios cognitivos e demência (JEONG *et al.,* 2005; WARD *et al.,* 2005; WHITMER *et al*., 2005).

O sistema colinérgico é uma das mais importantes vias modulatórias dos sistemas nervoso central (SNC) e periférico (SNP) (PRADO *et al*., 2002; SOREQ; SEIDMAN, 2001). No sistema nervoso autônomo, o sistema colinérgico controla a frequência cardíaca (MENDELOWITZ, 1999) e a contração da musculatura lisa gástrica (ROGERS *et al.,* 1999). O conhecimento desse sistema, principalmente das vias de sinalização intracelular e intercelular, que se iniciam pela ativação dos receptores colinérgicos, tem sido utilizado no desenvolvimento de novas abordagens terapêuticas para algumas doenças.

A acetilcolina (ACh) é sintetizada pela enzima colina acetiltransferase (ChAT; EC 2.3.1.6) a partir da colina, um importante produto do metabolismo dos lipídios da dieta, e acetil-CoA, um produto do metabolismo celular (PRADO *et al.,* 2002; SOREQ; SEIDMAN, 2001). Após sua síntese é carregada até as vesículas sinápticas pelo transportador vesicular da ACh (VAChT) onde fica armazenada até a sua liberação (RAND, 2007). Depois de ser liberada, a ACh se difunde na fenda sináptica e ativa os receptores específicos de ACh, posicionados nas células pós-sinápticas. Esses receptores são designados como receptores colinérgicos e, subdivididos em dois grandes grupos: muscarínicos e nicotínicos, que transmitem os sinais por mecanismos diferentes (RANG *et al*., 2004).

Cinco subtipos de receptores muscarínicos foram identificados (M1-M5) e agem via ativação de proteínas G, sendo que os receptores M1 e M2 estão presentes em neurônios do SNC e SNP além de outros tecidos ganglionares (VANDERZEE; LUITEN, 1999). Os receptores nicotínicos são compostos por cinco subunidades conhecidas por α_1, α_2, β, γ e δ e atuam como canais iônicos regulados por ligante e localizam-se, predominantemente, nas sinapses ganglionares (ARIAS, 1998). A ação da ACh é finalizada pela sua hidrólise enzimática na fenda sináptica pela enzima acetilcolinesterase (AChE). A maioria da colina resultante é recaptada pelo terminal pré-sináptico, através de um mecanismo de recaptação de alta afinidade onde poderá ser reutilizada para a síntese de novas moléculas de ACh (MESULAM *et al.,* 2002; SOREQ; SEIDMAN, 2001) (Figura 9).

Figura 9: Sinapse colinérgica. ACh = acetilcolina; M1 = receptor muscarínico tipo 1; M2 = receptor muscarínico tipo 2; VAChT = transportador de ACh vesicular



Fonte: (Adaptado de SOREQ; SEIDMAN, 2001).

3.4.1 Colinesterases

A sinalização colinérgica ocorre através da regulação da concentração de ACh, pelas enzimas AChE e butirilcolinesterase (BChE) (DAS, 2007). As colinesterases desempenham papéis importantes na neurotransmissão colinérgica central e periférica. Estão presentes em tecidos colinérgicos e não colinérgicos assim como no sangue e outros fluídos corporais. A AChE (E.C 3.1.1.7) ou também chamada de colinesterase verdadeira, hidrolisa preferencialmente ésteres com grupamento acetil, e a BChE (E.C. 3.1.1.8) ou pseudocolinesterase, hidrolisa outros tipos de ésteres como a butirilcolina (TAYLOR; BROWN, 1999).

A AChE apresenta ampla semelhança estrutural com a BChE. Porém, a AChE é predominantemente encontrada no cérebro (10 vezes mais abundante que a BChE), junção neuromuscular e eritrócitos enquanto, a BChE é principalmente encontrada no plasma, rins,

fígado, intestino, coração, pulmão e tem uma distribuição neuronal muito mais restrita em relação a AChE (COKUGRAS, 2003; MESULAM *et al*., 2002). Além disso, as duas enzimas são também diferenciadas de acordo com seu comportamento frente a diversos tipos de inibidores (COKUGRAS, 2003).

3.4.1.1 Acetilcolinesterase

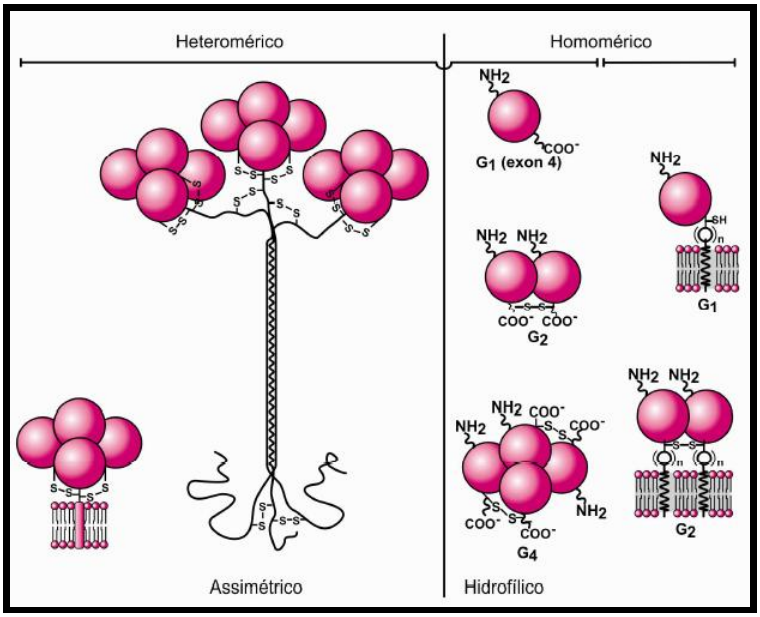
A AChE é uma enzima muito importante no controle da transmissão dos impulsos nervosos através das sinapses, sendo por isso considerada um bom indicador da atividade colinérgica (SZEGLETES *et al.*,1999). Além disso, alterações na atividade da AChE tem sido associadas a danos nos processos de memória e aprendizagem (BRAUN *et al*., 2017). A AChE possui um papel regulatório na neurotransmissão colinérgica. Ela é responsável pela hidrólise rápida do neurotransmissor ACh (MASSOULIÉ *et al*., 1993). A AChE é amplamente distribuída no SNC, além disso, foi localizada e identificada em linfócitos, eritrócitos e plaquetas, onde provavelmente apresenta um importante papel na regulação das funções imunes (KAWASHIMA; FUJII, 2000). Esta enzima também tem potentes efeitos sobre a adesão celular, neurogênese, sinaptogênese e hematopoese, desta maneira um aumento ou inibição desta enzima pode resultar em consequências importantes no cérebro e outros órgãos (SOREQ; SEIDMAN, 2001). Outra importante propriedade atribuída à AChE é atuar como marcador inflamatório. A via colinérgica anti-inflamatória é mediada pela ACh, que é o principal neurotransmissor envolvido e atua através da inibição da produção TNF-α, IL-1 e uma série de outros mediadores inflamatórios e portanto, modula tanto a resposta imune como a neurotransmissão (DAS, 2007).

A enzima AChE ocorre nas formas globular e assimétrica. A forma globular é composta por monômeros (G1), dímeros (G2) e tetrâmeros (G4) da subunidade catalítica. A forma G1 é citosólica e a G4 é ligada à membrana, sendo essa última a mais encontrada no tecido nervoso (ALDUNATE *et al.*, 2004; DAS *et al.*, 2001). Em sangue humano, a AChE é encontrada tanto nos eritrócitos quanto no plasma, onde predominam as formas G2 e G4, respectivamente (RAKONCZAY *et al*., 2005). A forma assimétrica consiste de um (A4), dois (A8) e três (A12), tetrâmeros catalíticos ligados covalentemente a uma subunidade estrutural colagênica chamada Q. Essas formas estão associadas à lâmina basal e são abundantes na junção neuromuscular (ALDUNATE *et al.*, 2004) (Figura 10).

A AChE apresenta algumas características não encontradas em nenhuma outra enzima, tais como, a organização do sítio ativo e o mecanismo catalítico. O sítio ativo da AChE é

encontrado no interior de uma garganta estreita *(gorge)*, e consiste de dois subsítios de ligação, um carregado negativamente ou sítio aniônico, e um sítio esterásico, contendo os resíduos catalíticos, também chamados de tríade catalítica (SHAFFERMAN *et al.*, 1992; TALESA, 1999). A tríade catalítica é composta por três resíduos de aminoácidos, serina, histidina e um resíduo ácido, glutamato (SOREQ; SEIDMAN, 2001). Além do sítio catalítico, a AChE apresenta um sítio aniônico periférico, que está localizado na entrada do sítio ativo *gorge*, este é o sítio de ligação para inibidores e ativadores alostéricos (TAYLOR; LAPPI, 1975). A ACh é hidrolisada a partir de sua ligação ao resíduo de serina no sítio ativo da enzima, formando o intermediário acetil-AChE, liberando colina. Em seqüência, há a hidrólise desse intermediário liberando acetato, e permitindo o "*turnover"* da enzima (SOREQ; SEIDMAN, 2001).

Figura 10: Isoformas da enzima AChE. Estrutura assimétrica (A12) e molecular (G1, G2 e G4) da AChE



Fonte: (http://www.chemistry.emory.edu/ach_inactivation.htm). Data de acesso: 24/06/2017.

3.5 FLAVONOIDES

Contudo, diante de vários estudos relatando que a hiperlipidemia causa alterações tanto no sistema purinérgico como no colinérgico (BRAUN *et al.*, 2017; DUARTE *et al.*, 2007; GUTIERRES *et al.*, 2012; KAISER *et al.*, 2017) novos estudos com o interesse pela prevenção e cura de doenças através da alimentação vem aumentando a cada dia. A fitoterapia

é a modalidade de tratamento que vem crescendo nos últimos anos, principalmente em função do alto custo dos medicamentos industrializados (YUNES *et al.*, 2001).

As plantas representam importante fonte de fármacos considerando a grande quantidade de moléculas com potencial medicinal, podendo contribuir efetivamente na busca de novos produtos bioativos. Há vários relatos de plantas e constituintes químicos com atividade hipoglicemiante, hipotensiva, hipocolesterolêmica, antiaterosclerótica e antitrombótica utilizadas na medicina chinesa (COON; ERNST, 2003; MOLL, 2006). Neste contexto, os flavonoides possuem uma variedade de efeitos benéficos para a saúde, que além de seu efeito antioxidante, já foi descrito que ainda possuem diversas propriedades farmacológicas, tais como: anticarcinogênica, anti-inflamatória, antialérgica, vasoprotetora, neuroprotetora, entre outras (GONÇALVES *et al*., 2010; JUNG *et al.*, 2012; KELLY, 2011; LAKHANPAL; RAI, 2007; SCHMATZ *et al*., 2009). A alta ingestão de flavonoides também está positivamente associada a uma diminuição da incidência de doenças como a dislipidemia e a aterosclerose (BOOTS *et al*., 2008; ISHIZAWA *et al.*, 2011).

O termo flavonoide é derivado do latim *flavus* que significa amarelo, e recebeu este nome por ter sido encontrado originalmente em alimentos de coloração amarela, mas atualmente já foi encontrado também em outras colorações. Os flavonoides são formados nas plantas pela combinação dos aminoácidos fenilalanina e tirosina com unidades acetato (COTELLE, 2001). Os flavonoides são uma classe de compostos fenólicos que diferem entre si pela sua estrutura química e características particulares. Frutas, vegetais, grãos, flores, chá e vinho são exemplos de fontes destes compostos (NIJVELDT *et al.*, 2001). Estes compostos não podem ser sintetizados pelo metabolismo humano e, portanto devem ser adquiridos através da alimentação, deste modo, compõem uma ampla classe de substâncias de origem natural (CHO *et al*., 2003; KANG *et al.*, 2005; PRIOR, 2003).

O teor de flavonoides em alimentos consumidos diariamente é: 44 mg em cereais, 79 mg em batatas, 45 mg em grãos e nozes e 162 mg em vegetais e ervas. A maior parte dos flavonoides consumidos provêm do cacau, cola, café, chá preto, cerveja e vinho, aproximadamente 420 mg/ dia, com um adicional de 290 mg/ dia provenientes de frutas e sucos (PIERPOINT, 1986). Os flavonoides podem ser subdivididos em classes, tais como: antocianidina, flavonol, flavanona, flavona e isoflavona (PEDRIALI, 2005). Muitos estudos relatam que os flavonoides na sua forma livre ou glicosilada são absorvidos no trato gastrintestinal e metabolizados em glucoronidato ou sulfato conjugado. Esses metabólitos circulam no sangue sendo excretados na bile e urina (SESINK *et al*., 2001).

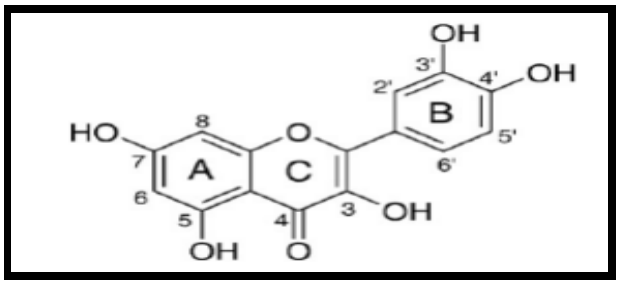
Os flavonoides isolados ou purificados de plantas agem inibindo enzimas da biossíntese e absorção do colesterol como a hidroximetil-glutaril CoA redutase (HMGCoA redutase), bem como enzimas lipogênicas (glicose 6-fosfato desidrogenase e enzima málica) e do metabolismo lipídico como lipoproteína lipase e a lecitina colesterol acil transferase (LCAT). (ANILA; VIJAYALAKSHMI, 2002; JUNG *et al.,* 2006).

3.5.1 Quercetina

Dentre todos os flavonoides podemos destacar a quercetina, a qual é considerada um dos compostos flavonoides mais abundantes sendo encontrados em frutas, legumes e vegetais. Também é um dos componentes mais conhecidos na *Ginkgo biloba* e na erva de São João (*Hypericum perforatum*) (PRIOR, 2003; WILLIANS *et al*., 2004).

A quercetina (3,3',4',5,7 – pentahidroxiflavona) (Figura 11) é o principal flavonoide presente na dieta humana sendo encontrado em diversas frutas e vegetais (CAO *et al*., 1997). Pode conter moléculas de açucares ligado a sua estrutura além de ser uma substância lipossolúvel (KELLY, 2011). Este flavonoide pertence à classe dos flavonóis e é encontrado em plantas na forma de aglicona ou na forma glicosilada (LAKHANPAL; RAI, 2007).

Figura 11: Estrutura química da quercetina



Fonte: (Boots *et al*., 2008).

A quercetina representa cerca de 95% do total dos flavonoides ingeridos. A cebola, maçã e brócolis são suas fontes majoritárias (NIJVELDT *et al.*, 2001; PEDRIALI, 2005). Este flavonoide apresenta baixa toxicidade e a estimativa calculada de ingesta por indivíduo é de aproximadamente 25 mg/dia (CHOI *et al.*, 2003; COOK; SAMMAN, 1996; NATIONAL TOXICOLOGY PROGRAM, 1992). É um composto facilmente absorvido por pequenas células intestinais para posteriormente entrar na corrente sanguínea e ser metabolizado para

desempenhar seus efeitos benéficos. Seus metabólitos quando não utilizados são excretados pela urina. Tem sido observado que a quercetina apresenta diversas atividades biológicas, como ação antiaterogênica, antineoplásica, antioxidante, anti-inflamatória, antiagregante, ansiolítico e antidepressivo, antibacteriano, neuroprotetora, entre outros (BHUTADA *et al.*, 2010; BOESCH-SAADATMANDI *et al.*, 2012; CHOI *et al.*, 2003; DAJAS *et al*., 2015; FILHO *et al*., 2008; JURÍKOVÁ *et al*., 2015; KLEEMANN *et al*., 2011; MOON *et al*., 2006; RIGANO *et al.,* 2007; RUSSO *et al*., 2012; XUE *et al.,* 2017). A quercetina pode melhorar diretamente o metabolismo lipídico, através da modulação dos níveis circulantes das lipoproteínas, e também reduzindo o estresse oxidativo e melhorando a β-oxidação (PFEUFFER *et al.*, 2013; SUN *et al.*, 2015; ZAHEDI *et al.*, 2013).

Em muitas situações, estas ações envolvem sua propriedade antioxidante, devido à sua capacidade de eliminar radicais livres e ligar metais de transição e seus íons (DE SOUZA; DE GIOVANI, 2004; PRIOR, 2003), a qual participa na inibição de enzimas como a ciclooxigenase, lipoxigenase e xantina oxidase que estão envolvidas na citotoxicidade oxidativa (KAHRAMAN, 2003).

A ação anti-inflamatória da quercetina é conhecida por ter impacto no recrutamento de células imunes e na prevenção do desenvolvimento de infecções secundárias após a ruptura da barreira cutânea (SINGH *et al*., 2011). Seu efeito também é devido à inibição de enzimas como a lipoxigenase e pela inibição de mediadores inflamatórios. A quercetina afeta o sistema imune e o processo inflamatório atuando principalmente em leucócitos e direcionando muitas cinases e fosfatases de sinalização intracelular, enzimas e proteínas de membrana, muitas vezes cruciais para uma função celular específica (CHIRUMBOLO, 2010).

O efeito preventivo da quercetina também já foi relatado em outros trabalhos (Cui *et al.,* 2013; Moghbelinejad *et al.,* 2016). Wang *et al.* (2015) demonstrou seu efeito preventivo em camundongos com inflamação induzida por radiação, no qual atribuiu seu efeito em inibir a expressão do NF-κB e a via MAPK. Chen *et al.* (2017) também demonstrou que o pré-tratamento com quercetina protegeu os neurônios do hipocampo de lesão isquêmica e também aumentou significativamente os níveis de expressão das enzimas antioxidantes superóxido dismutase, catalase e glutationa peroxidase nos neurônios do hipocampo de animais com a lesão. Estes estudos confirmam que o pré-tratamento com quercetina apresenta potentes efeitos neuroprotetores, antiflamatórios e antioxidantes.

Diante disto, tendo em vista que a hiperlipidemia é uma condição associada a distúrbios inflamatórios, e também atua negativamente na função neural, torna-se relevante

avaliar se o tratamento preventivo com quercetina é capaz de prevenir os efeitos causados pela hiperlipidemia nos sistemas purinérgico e colinérgico.

4 ARTIGO

Neuroprotective effects of pretreatment with quercetin as assessed by acetylcholinesterase assay and behavioral testing in poloxamer-407 induced hyperlipidemic rats

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Neuroprotective effects of pretreatment with quercetin as assessed by acetylcholinesterase assay and behavioral testing in poloxamer-407 induced hyperlipidemic rats

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ABSTRACT

Hyperlipidemia is a group of disorders characterized by excessive lipids in the bloodstream. It is associated with the incidence of cardiovascular diseases and recognized as the most important factor underlying the occurrence of atherosclerosis. This study was conducted to investigate whether pretreatment with quercetin can protect against possible memory impairment and deterioration of the cholinergic system in hyperlipidemic rats. Animals were divided into ten groups (n = 7): saline/control, saline/quercetin 5 mg/kg, saline/quercetin 25 mg/kg, saline/quercetin 50 mg/kg, saline/simvastatin (0.04 mg/kg), hyperlipidemia, hyperlipidemia/quercetin 5 mg/kg, hyperlipidemia/quercetin 25 mg/kg, hyperlipidemia/quercetin 50 mg/kg and hyperlipidemia/simvastatin. The animals were pretreated with quercetin by oral gavage for a period of 30 days and hyperlipidemia was subsequently induced by intraperitoneal administration of a single dose of 500 mg/kg of poloxamer-407. Simvastatin was administered after the induction of hyperlipidemia. The results demonstrated that hyperlipidemic rats had memory impairment compared with the saline control group ($P < 0.001$). However, pretreatment with quercetin and simvastatin treatment attenuated the damage caused by hyperlipidemia compared with the hyperlipidemic group ($P < 0.05$). Acetylcholinesterase (AChE) activity in the cerebral hippocampus was significantly ($P < 0.001$) reduced in the hyperlipidemic group compared with the control saline group. Pretreatment with quercetin and simvastatin treatment in the hyperlipidemic groups significantly ($P < 0.05$) increased AChE activity compared with the hyperlipidemic group. Our results thus suggest that quercetin may prevent memory impairment, alter lipid metabolism, and modulate AChE activity in an experimental model of hyperlipidemia.

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1. Introduction

Hyperlipidemia is a group of disorders characterized by excessive lipids in the bloodstream. The concentrations of lipids, such as triglycerides (TG), total cholesterol (TC) and low-density lipoprotein (LDL) increase, or the levels of high-density lipoprotein (HDL) decrease in the blood [1]. Cholesterol is the major structural lipid present in the mammalian plasma membrane. It is responsible for mechanical stability and cohesiveness because of its ability to order the membrane [2]. Disorders of cholesterol and lipid metabolism can be associated with the incidence of cardiovascular diseases, obesity, diabetes, hypertension and fatty liver [3]. Hyperlipidemia may be induced by dietary means such as chronic feeding with a high fat diet [4,5], or by treatment with compounds such as Triton [6] and Poloxamer 407 [7–9]. Several animal models have been used to study the pathophysiological effects of hyperlipidemia [4–9].

Poloxamer 407 (P407) is a non-ionic synthetic copolymer surfactant, which provides an attractive means of inducing hyperlipidemia. A rapid onset of a hyperlipidemic state occurs within 24 h of intraperitoneal (i.p.) injection of P407 [10]. P407 increases serum lipoprotein levels by altering lipid metabolism – by inhibiting lipoprotein lipase (hydrolysis of TG) and indirectly stimulating 3-hydroxy-3-methylglutaryl-coenzyme A (HMGCoA) reductase (cholesterol biosynthesis) [11].

The central nervous system (CNS) is protected from many potential toxicants through an anatomically defined barrier called the blood-brain barrier (BBB) [12,13]. Considerable evidence indicates that hypercholesterolemia may also contribute to BBB dysfunction [14–16], affect the BBB integrity and lead to an increase in IgG extravasation in cholesterol-fed rabbits [17,18]. Specifically, a damaged or dysfunctional cerebrovascular system under hypercholesterolemic conditions, with consequent extravasation of serum components into and through the walls of small cerebral vessels, may trigger a persistent activation of perivascular microglia involved in barrier and scavenger functions in the brain [19,20]. Previous studies have shown that hyperlipidemia can cause impairment of learning as well as memory [21–23].

The cholinergic system plays an important role in many functions of both the CNS and the peripheral nervous system. Acetylcholine (ACh) is the main neurotransmitter of the cholinergic system and has vital functions in learning, memory, movement control and modulation of cerebral blood flow [12,13]. ACh levels are regulated by cholinesterases that are capable of hydrolyzing this neurotransmitter in many tissues. Acetylcholinesterase (AChE) is a membrane-bound cholinesterase and can be found mainly in the brain, muscles, erythrocytes, lymphocytes, cholinergic neurons and terminals [24]. AChE is the most efficient cholinesterase and rapidly hydrolyzes ACh at cholinergic synapses as well as at neuromuscular junctions [25,26]. In addition, the hippocampus and cortical regions of the brain are main sites for cholinergic transmission in the monitoring of learning and memory processing, and they seem to be more prone to oxidative damage [27,28]. Oxidative damage to the synapses in these regions of rat brains has been reported to contribute to cognitive deficit [29].

In view of the harmful effects of hyperlipidemia, attention has been focused on health promoting and disease preventing actions of phytochemicals in food [30]. Flavonoids have been studied due to their wide-ranging therapeutic properties, including their protection against several neurodegenerative diseases in experimental models [30–33]. They have been reported to be potent antioxidants and beneficial in the treatment of oxidative stress-related diseases [34]. Quercetin is a flavonoid that possesses free radical scavenging properties and protects against oxidative injury by modulating intracellular signals and promoting cellular survival [35]. It is a natural antioxidant found in a variety of vegetables and fruits that are commonly consumed in the human diet [36]. It is readily absorbed by small intestinal cells and has been reported to exert several biological effects including antiatherogenic, anticancer, antioxidant, anti-inflammatory, antiplatelet, and neuroprotective activities [37,38].

Considering the role of hyperlipidemia in the pathogenesis of neurodegeneration and the potential antioxidant, anti-inflammatory and neuroprotective properties of quercetin in experimental models for cognitive deficits, we hypothesized that quercetin may exert a preventive therapeutic mechanism on memory and anxiogenic-like behavior as well as on AChE activity in a hyperlipidemia rat model. The objective of this study was to investigate whether pretreatment with quercetin can protect against possible memory impairment and deterioration of the cholinergic system in hyperlipidemic rats.

2. Methods

2.1. Chemicals

The chemicals quercetin, simvastatin, acetylthyocholine, poloxamer-407, trizma base and percoll were obtained from Sigma Chemical Co (St. Louis, MO, USA). All other chemicals used in the experiment were of the highest purity.

2.2. Animals

Adult male Wistar heterogenic rats (n = 70; 70–90 days; 250–350 g) obtained from the Central Animal House of the Federal University of Santa Maria (UFSM), Santa Maria, Brazil, were used for this experiment. The animals were maintained at a constant temperature (23 ± 1 °C) on a 12 h light/dark cycle with free access to food and water. All animal procedures were approved by the Ethics Committee on the Use of Animal at the Federal University of Santa Maria (Protocol number: 9894021214/2014).

2.3. Experimental design

Animals were divided into collective boxes polypropylene (41 × 34 × 16 cm). The rats were pretreated with quercetin for one month in three different doses (5, 25 and 50 mg/kg), prior to the hyperlipidemia induction. After induction, simvastatin was administered to some groups. The rats were subdivided into ten groups of 7 animals each; saline control (C), saline + quercetin 5 mg/kg (CQ5), saline + quercetin 25 mg/kg (CQ25), saline + quercetin 50 mg/kg (CQ50), saline + simvastatin 0.04 mg/kg (CS), hyperlipidemia (H), hyperlipidemia + quercetin 5 mg/kg (HQ5), hyperlipidemia + quercetin 25 mg/kg (HQ25), hyperlipidemia + quercetin 50 mg/kg (HQ50) and hyperlipidaemia + simvastatin 0.04 mg/kg (HS). The experiment began with the administration of quercetin for a period of 30 days. After 30 days of pretreatment with quercetin, a single dose of P407 (500 mg/kg) was intraperitoneally administered to hyperlipidemia induction. Simvastatin (0.04 mg/kg) was administered after the induction of hyperlipidemia. The behavioral tests were performed 12 h after induction of hyperlipidemia.

2.4. Pretreatment with quercetin

A month prior to the induction of hyperlipidemia, animals in groups CQ5 and HQ5 received 5 mg/kg of quercetin, CQ25 and HQ25 animals received 25 mg/kg and CQ50 and HQ50 received 50 mg/kg of quercetin, while the C and H animal groups received 0.9% saline solution by gavage. Quercetin (Sigma Chemical Co, St. Louis, MO, USA) was freshly prepared in 25% ethanol and was administered once a day for 30 days by gavage. The choice of the

preventive doses (5, 25 and 50 mg/kg of quercetin) used in this experiment, was based on previous study where the beneficial effects of the compound in the CNS were reported [39]. The group containing only ethanol vehicle was previously shown by Abdalla et al. [39] that no significant differences were observed between the results obtained to the vehicle relative to the control parameter analyzed in this study. The preventive dose of quercetin was adjusted weekly according to the body weight of rats. The volume of saline solution and ethanol were adjusted based on the body weight (0.001 ml/g).

2.5. Induction of hyperlipidemia by Poloxamer 407 (P407)

The animals were randomly divided into hyperlipidemic and control saline rat groups. To induce hyperlipidemia, 500 mg/kg of P407 dissolved in sterile NaCl 0.9% solution was administered via intraperitoneal (i.p.) injection [40]. The control saline rats received the same volume of vehicle alone (cold, sterile 0.9% NaCl solution). After 36 h of induction, the animals with higher total cholesterol, LDL cholesterol and triglycerides levels than the normal values as reported by Chaudhary and Brocks [10]. The animals were subsequently subjected to training and behavioral studies were examined. After, the animals were anesthetized with isoflurane, submitted to euthanasia and blood was collected with cardiac puncture.

2.6. Treatment with simvastatin

After induction of hyperlipidemia, CS and HS groups were given the dose of simvastatin equivalent to the normal dose recommended to human (40 mg) by gavage. This group was administered saline alone prior to the induction of hyperlipidemia. The equivalent dose between mice and humans was calculated according to the method desribed by Reigner and Blesch [41]. The surface area of the animals was determined and represented by Eq. (1) as shown below:

$$BSA^{(m2)} = 1.85\,(W/70)^{2/3} \quad (1)$$

Where BSA is the body surface area, W is the weight in kilograms. In humans, the body surface was considered the average value (1.8 m^2) and 70 kg. Substituting into the equation, a mouse (0.400 kg) consists of a body area 0.0593 m^2. Then, the dose in milligrams per square meter was multiplied by the surface area of the human to find the mouse dose which was 0.04 mg/kg.

2.7. Behavioral procedure

2.7.1. Object recognition test

The object recognition task takes advantage of the rat's spontaneous tendency to explore the environment and does not require punishment or reward [42]. The test was determined as previously described by Frühauf et al. [43] with slight modifications. It consists of three sessions: habituation, training and retention. Animals were left to freely explore for 10 min a round open field (56 cm of internal diameter, which had its floor divided into 10 areas of the same size) in the absence of objects. A light bulb, hanging 60 cm above the behavioral apparatus, provided constant illumination of about 40 lx, and an air-conditioner provided constant background sound isolation. The objects used were glass bottles, each with different shape and colors, but the same size. Throughout the experiments, objects were used in a counterbalanced manner. Animals had not previously displayed a preference for any of the objects. Chambers and objects were cleaned with 30% ethanol immediately before and at the end of each behavioral evaluation. In the first session, rats were individually habituated to the behavioral apparatus and then returned to their home cages. Twenty-four hours later, the animals were subjected to a training session in which the animals were exposed to two of the same objects (object A), and the exploration time was recorded with two stopwatches. Exploration was recorded when the animal touched or reached the object with the nose at a distance of less than 2 cm. Climbing or sitting on the object was not considered exploration. The test session was carried out 4 h after training. Rats were placed back in the behavioral chamber and one of the familiar objects (object A) was replaced by a novel object (object B). The times spent exploring the familiar and the novel object was recorded. The discrimination index was then calculated, taking into account the difference of time spent exploring the new and familiar objects, using the formula: (Tnovel − Tfamiliar)/(Tnovel + Tfamiliar) × 100 (%). The discrimination index was used as a memory parameter.

2.7.2. Elevated plus maze task

The maze test in high cross has been described as a simple method to evaluate anxiety responses in rodents [44]. The protocol used was in accordance with the method of Rodgers and Cole [45]. Briefly, the apparatus consisted of a wooden structure elevated 50 cm from the floor and comprising two opposite open arms, 50 × 10 cm, crossed at right angles by two arms of the same dimensions enclosed by 30 cm high walls, with an open roof. Subjects were initially placed on the central platform of the maze facing an enclosed arm. The behaviors recorded were: the total time spent in the open arms as well as the number of entries into each arm. The apparatus was thoroughly cleaned between the 5-min observation sessions with a 30% ethanol solution.

2.8. Brain tissue preparation

After behavioral tests, animals were anesthetized with isoflurane and submitted to euthanasia. The cranium was opened, the structures were gently excised and separated into the cerebral cortex (CC), hippocampus (HC), striatum (ST), hypothalamus (HT) and cerebellum (CB). All the brain structures were separately homogenized in a glass potter in a solution of 10 mM Tris–HCl, with pH7.4, on ice, at a proportion of 1:10 (w/v). The homogenate was centrifuged at 1800 rpm for 10 min and the resulting supernatant was used for the determination of AChE activity.

2.9. Assay of acetylcholinesterase (AChE) activity

The AChE enzymatic assay was determined according to the method previously described by Ellman et al. [46] with a slight modification of the spectrophotometric method as described previously by Rocha et al. [47]. The reaction mixture (2 ml final volume) contained 100 mM K^+-phosphate buffer, with pH 7.5 and 1 mM 5,5′-dithio-bisnitrobenzoic acid (DTNB). The method is based on the formation of the yellow anion, 5,5′-dithio-bis-nitrobenzoic acid, measured by absorbance at 412 nm during 2 min of incubation at 25 °C. The enzyme was pre-incubated for 2 min. The reaction was initiated by adding 0.8 mM acetylthiocholine iodide. The protein content was adjusted for each structure: CC (0.8 mg/ml), HC (0.8 mg/ml), ST (0.4 mg/ml), HT (0.6 mg/ml) and CB (0.6 mg/ml). All samples were run in triplicate and enzyme activity was expressed in μmol AcSCh/h/mg of protein.

2.10. Biochemical analysis

The serum total cholesterol (TC), low density lipoprotein (LDL), high density lipoprotein (HDL), glucose, triglycerides (TRI), aspartate transaminase (AST), alanine transaminase (ALT), alkaline

phosphatase (ALP), albumin, creatinine and uric acid levels were measured using standard enzymatic methods from Ortho-Clinical Diagnostics® reagents in the fully automated analyzer (Vitros 950® dry chemistry system; Johnson & Johnson, Rochester, NY, USA).

2.11. Protein determination

Protein was measured by Coomassie Blue reagent according to the method previously described by Bradford [48], using bovine serum albumin as standard.

2.12. Statistical analysis

The results ofthe elevated plus maze task and object recognition test were analyzed by One-way ANOVA followed by Bonferroni's post-hoc test. For other tests, Two-way ANOVA tests were used and these were followed by Tukey tests. $P < 0.05$ was considered to represent a significant difference in both analyses used. All data were expressed as mean ±SEM.

3. Results

3.1. Biochemical tests

The results of measurements of TC, LDL, HDL, TG and glucose levels are shown in Table 1. A significant ($P < 0.05$) increase in TC in hyperlipidemic rats (H, HQ5, HQ25, HQ50 and HS groups) compared with control rats (C, CQ5, CQ25, CQ50 and CS) was observed. However, there was a significant ($P < 0.05$) decrease in the total cholesterol levels in the HQ5, HQ50 and HS rat groups compared with the H group. In addition, significant ($P < 0.05$) elevations in LDL and TG of hyperlipidemic rats (H, HQ5, HQ25, HQ50 and HS groups) compared with control rats (C, CQ5, CQ25, CQ50 and CS) were observed. There was also a significant ($P < 0.05$) increase in HDL levels in the CQ25 and CQ50 groups compared with the H group. Glucose measurements were not significantly ($P > 0.05$) diferent among groups.

The results of AST, ALT and ALP activity measurements as well as albumin, creatinine and uric acid levels are shown in Table 2. Significant ($P < 0.05$) elevations in AST, ALT and ALP activities in hyperlipidemic rats (H, HQ5, HQ25, HQ50 and HS groups) compared with control rats (C, CQ5, CQ25, CQ50 and CS) were observed. Non-significant ($P > 0.05$) differences in albumin, creatinine and uric acid levels were observed among all of the rat groups.

3.2. Object recognition test

The results of the object recognition test are shown in Fig. 1. As shown in Fig. 1A, pretreatment with quercetin at the doses of 5, 25 and 50 mg/kg and treatment with simvastatin 0.04 mg/kg caused no statistical ($P > 0.05$) difference in% recognition index compared with the saline control group. The results shown in Fig. 1B revealed a significant ($P < 0.001$) decrease in % recognition indices in the hyperlipidemic (H) rat group compared with the saline control rat group (C). However, pretreatment with quercetin (5, 25 and 50 mg/kg) and treatment with simvastatin significantly ($P < 0.05$) elevated the % recognition index in hyperlipidemic treated rat group (HQ5, HQ25, HQ50 and HS) compared with the H group.

3.3. Elevated plus maze task

The results of the elevated plus maze task are shown in Fig. 2. The results obtained did not show any statistical ($P > 0.05$) differences among the groups.

3.4. Acetylcholinesterase activity

The results of AChE activity measurements in CC, HC, CB, ST and HT homogenates are presented in Fig. 3. In HC (Fig. 3A), AChE activity was significantly ($P < 0.001$) reduced in the hyperlipidemic group compared with the control saline group. However, pretreatment with quercetin (5, 25 and 50 mg/kg) and simvastatin treatment prevented the decrease in AChE activity compared with the hyperlipidemic group ($P < 0.05$). Furthermore, there was no significant ($P > 0.05$) difference in the AChE activity of CC, CB, ST and HT (Fig. 3B–E) among all of the rat groups.

4. Discussion

Hyperlipidemia is generally recognized as the most important factor underlying the occurrence of atherosclerosis (AS) [49,50]. Increased serum TC, TG and LDL levels are considered risk factors for AS whereas elevated serum HDL is well known as an AS-protective factor [51]. Early diagnosis and reversibility of changes observed with AS are important to clinical medicine and to the patients affected by this disease [52].

The present study explored the effects of P407 administration on the serum lipid profile. It has been previously shown that repeated administration of the synthetic copolymer P407 to mice resulted in dyslipidemia, and subsequently atherosclerosis [53,54]. Large increases in lipid levels were seen 36 h after administration

Table 1
Lipid profile and glucose levels after induction of hyperlipidemia.

Groups	TC (mg/dL)	LDL (mg/dL)	HDL (mg/dL)	TG (mg/dL)	Glucose (mg/dL)
C	140.1 ± 9.8^{a}	53.6 ± 8.2^{a}	55.2 ± 9.4^{a}	120.7 ± 21.1^{a}	137.2 ± 12.7^{a}
CQ5	143.0 ± 10.2^{a}	51.5 ± 2.0^{a}	111.8 ± 11.8^{a}	190.0 ± 15.5^{a}	125.1 ± 6.0^{a}
CQ25	138.9 ± 4.1^{a}	50.0 ± 1.5^{a}	119.3 ± 10.3^{b}	214.3 ± 38.5^{a}	122.5 ± 7.8^{a}
CQ50	151.6 ± 10.5^{a}	49.2 ± 1.5^{a}	115.8 ± 6.2^{b}	392.5 ± 150.7^{a}	122.7 ± 4.0^{a}
CS	131.1 ± 7.4^{a}	46.0 ± 1.4^{a}	91.4 ± 23.9^{a}	162.5 ± 18.9^{a}	132.6 ± 13.3^{a}
H	948.2 ± 144.5^{b}	299 ± 14.3^{b}	101.8 ± 9.4^{a}	1830.7 ± 352.9^{b}	130.5 ± 8.2^{a}
HQ5	565.4 ± 133.1^{c}	233.3 ± 34.5^{b}	104.8 ± 9.2^{a}	1690.0 ± 441.5^{b}	119.5 ± 6.5^{a}
HQ25	875.6 ± 94.0^{bc}	293.3 ± 42.2^{b}	90.6 ± 3.1^{a}	2278.8 ± 272.6^{b}	132.8 ± 9.3^{a}
HQ50	545.6 ± 25.0^{c}	212.8 ± 26.6^{b}	120.8 ± 13.0^{b}	1813.1 ± 256.6^{b}	134.4 ± 4.9^{a}
HS	505.6 ± 67.3^{c}	215.2 ± 38.0^{b}	121.8 ± 22.1^{b}	2006.3 ± 387.0^{b}	126.1 ± 4.1^{a}

C: saline control, CQ5: control + quercetin 5 mg/kg, CQ25: control + quercetin 25 mg/kg, CQ50: control + quercetin 50 mg/kg, CS: control + simvastatin 0.04 mg/kg, H: hyperlipidemia, HQ5: hyperlipidemia + quercetin 5 mg/kg, HQ25: hyperlipidemia + quercetin 25 mg/kg, HQ50: hyperlipidemia + quercetin 50 mg/kg, HS: hyperlipidemia + simvastatin 0.04 mg/kg. TC: total cholesterol, LDL: low density lipoprotein, HDL: high density lipoprotein, TG: triglyceride. Values are expressed as mean ± S.E.M. Different letters indicate significant difference between groups when compared to the average of each group to all other groups. Two-way ANOVA followed by post hoc Tukey ($P < 0.05$).

Table 2
Biochemical parameters after induction hyperlipidemia.

Groups	AST (U/L)	ALT (U/L)	ALP (U/L)	Albumin (g/dL)	Creatinine (mg/dL)	Uric acid (mg/dL)
C	193.6 ± 11.6[a]	47.7 ± 2.4[a]	425.3 ± 61.2[a]	4.0 ± 0.2[a]	0.5 ± 0.0[a]	3.1 ± 0.4[a]
Q5	201.8 ± 34.6[a]	56.3 ± 4.6[a]	401.0 ± 47.9[a]	4.4 ± 0.2[a]	0.5 ± 0.0[a]	2.8 ± 0.3[a]
Q25	191.1 ± 25.0[a]	49.8 ± 3.4[a]	520.4 ± 54.7[ac]	4.1 ± 0.2[a]	0.4 ± 0.1[a]	2.0 ± 0.3[a]
Q50	207.1 ± 21.5[a]	57.6 ± 3.1[a]	478.4 ± 48.1[ac]	4.3 ± 0.1[a]	0.5 ± 0.0[a]	2.0 ± 0.2[a]
S	189.0 ± 16.3[a]	46.5 ± 3.2[a]	391.7 ± 64.2[a]	3.9 ± 0.1[a]	0.4 ± 0.0[a]	1.9 ± 0.3[a]
H	416.7 ± 28.3[b]	141.4 ± 10.1[b]	886.6 ± 34.0[b]	4.7 ± 0.2[a]	0.4 ± 0.1[a]	3.1 ± 0.4[a]
HQ5	327.5 ± 37.7[b]	104.8 ± 10.8[b]	681.0 ± 44.6[b]	4.9 ± 0.4[a]	0.4 ± 0.1[a]	3.3 ± 0.4[a]
HQ25	385.0 ± 11.0[b]	130.2 ± 2.8[b]	670.3 ± 35.5[b]	4.7 ± 0.1[a]	0.4 ± 0.0[a]	3.8 ± 0.7[a]
HQ50	336.8 ± 14.4[b]	116.3 ± 12.3[b]	749.6 ± 49.7[b]	4.5 ± 0.1[a]	0.5 ± 0.0[a]	3.3 ± 0.5[a]
HS	370.4 ± 26.0[b]	101.8 ± 20.1[b]	680.4 ± 32.9[b]	4.5 ± 0.2[a]	0.5 ± 0.1[a]	2.6 ± 0.4[a]

C: saline control, CQ5: control + quercetin 5 mg/kg, CQ25: control + quercetin 25 mg/kg, CQ50: control + quercetin 50 mg/kg, CS: control + simvastatin 0.04 mg/kg, H: hyperlipidemia, HQ5: hyperlipidemia + quercetin 5 mg/kg, HQ25: hyperlipidemia + quercetin 25 mg/kg, HQ50: hyperlipidemia + quercetin 50 mg/kg, HS: hyperlipidemia + simvastatin 0.04 mg/kg. AST: aspartate transaminase, ALT: alanine transaminase; ALP: Alkaline phosphatase. Values are expressed as mean ± S.E.M. Different letters indicate significant difference between groups when compared to the average of each group to all other groups. Two-way ANOVA followed by post hoc Tukey ($P < 0.05$).

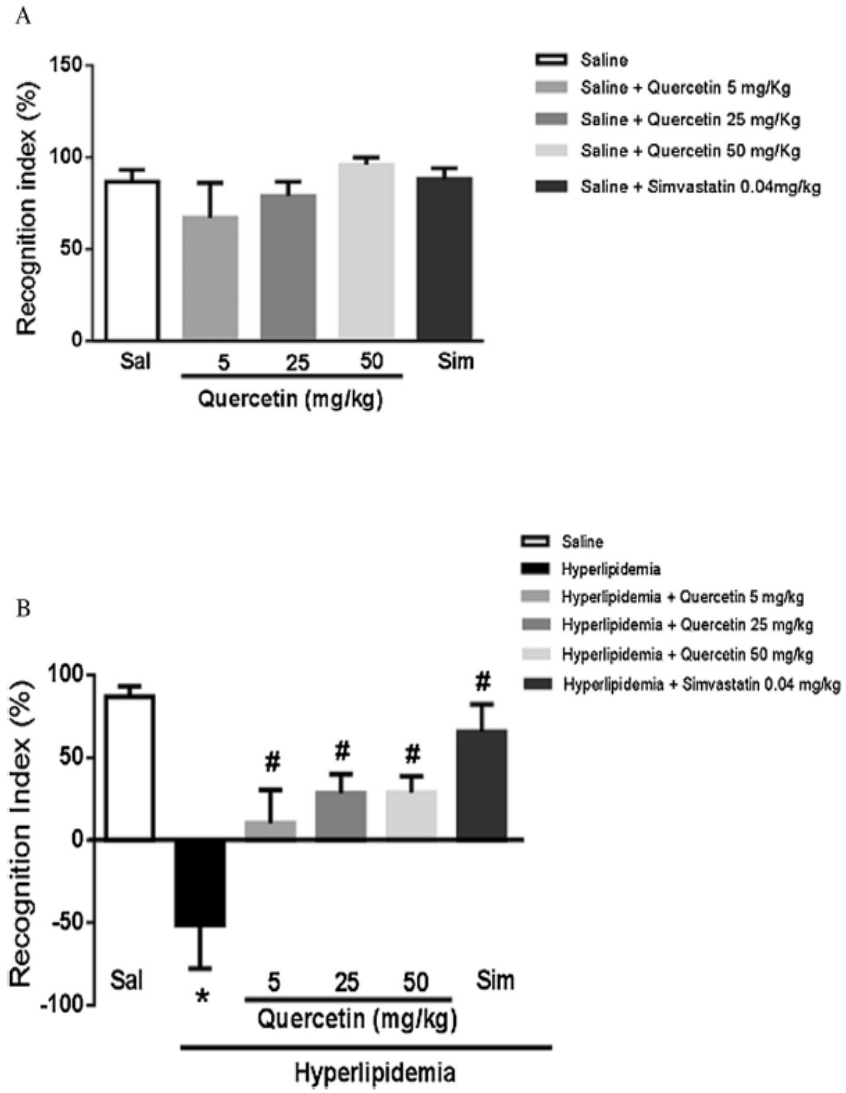

Fig. 1. (A) Effect of quercetin (5, 25 and 50 mg/kg) and simvastatin (0.04 mg/kg) without induction of hyperlipidemia. (B) Preventive effect of quercetin (5, 25 and 50 mg/kg) and simvastatin treatment (0.04 mg/kg) on hyperlipidemia induced by P407 (500 mg/kg) in the test object recognition. The results were analyzed using one-way ANOVA followed by post-hoc Bonferroni and data are expressed as mean ± S.E.M. * Means that the result is significantly different from the saline control group ($P < 0.001$, n = 7). ≠ Means that the result is significantly different in the group with hyperlipidemia ($P < 0.05$, n = 7).

[10]. Ours results follow similar trends with earlier studies where elevations in cholesterol and triglyceride levels were reported in rats administered P407 [10,55]. Johnston et al. [11] also investigated the effect of P407 on lipoprotein lipase activity and found that 3 h after intraperitoneal injection of P407 in rats, the enzyme activity decreased by 95% compared with a normal saline

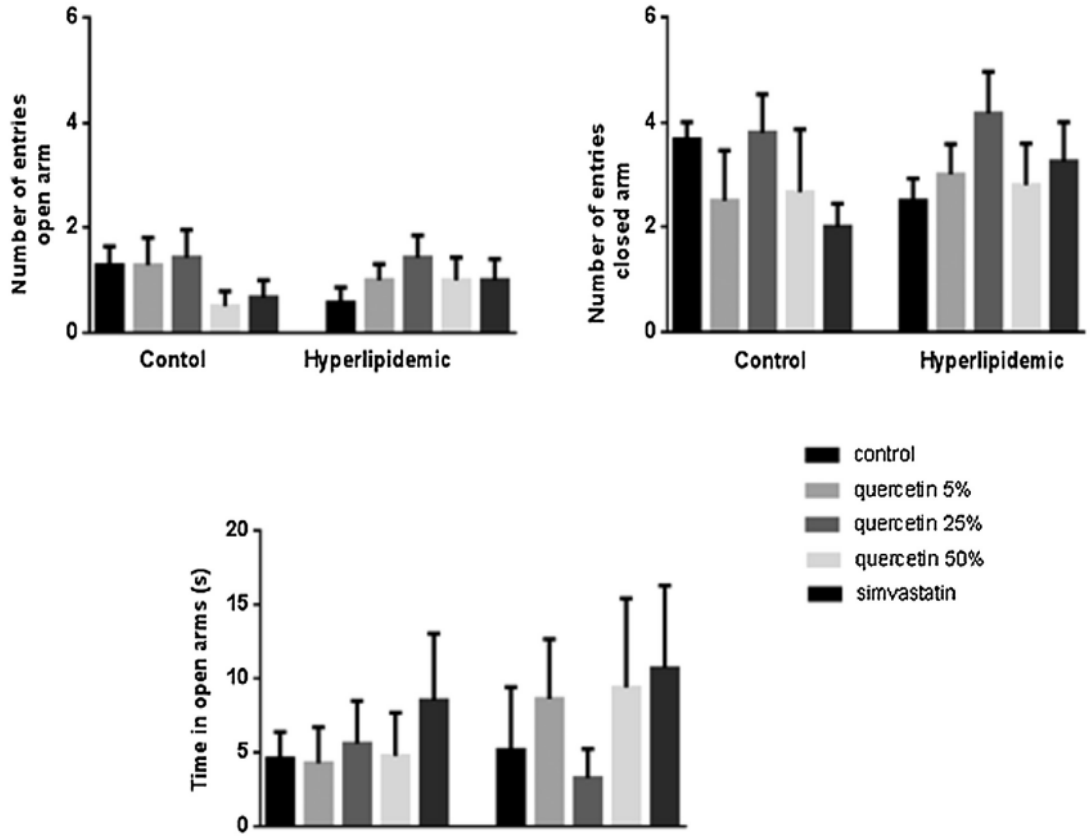

Fig. 2. Effect of hyperlipidemia (500 mg/kg of P407), preventive treatment of quercetin (5, 25 and 50 mg/kg) and simvastatin in high cross maze test. (A) Number of entries into the open arms. (B) Number of entries into the closed arms. (C) Time entries into open arms. The results were analyzed using one-way ANOVA followed by post-hoc Bonferroni and data are expressed as mean ± S.E.M. ($P < 0.05$, n = 7).

treated control. P407 may cause indirect stimulation of HMGCoA reductase, which is involved in the biosynthesis of cholesterol [56,57]. Currently, statins (i.e. simvastatin) are the most commonly used drugs for lowering cholesterol and LDL levels, because they are HMGCoA reductase inhibitors [58]. Furthermore, simvastatin is effective in the treatment of inflammation by directly or indirectly triggering pro-inflammatory signaling pathways [59]. Thus, simvastatin was chosen as the positive control material in our study.

High flavonoid intake has been reported to be positively associated with a decreased incidence of disorders characterized by dyslipidemia, including atherosclerosis, coronary heart disease and diabetes [60,61]. Quercetin is one of the flavonoids found in abundance in apples and onions [62]. Its antioxidant, anti-inflammatory and anti-atherogenic properties have been reported [63–65]. Quercetin derivatives can directly improve lipid and glucose metabolism by reducing oxidative stress and enhancing β-oxidation [66]. In this study, the decreased total cholesterol and increased HDL cholesterol levels in hyperlipidemic rats that were pretreated with quercetin may indicate a partial protective effect of this flavonoid. The results of our study agree with a recent study by Nekohashi et al. [67] where quercetin was reported to reduce high blood cholesterol levels via the inhibition of intestinal cholesterol absorption that may be mediated by Niemann-Pick C1-Like 1 (NPC1L1) cholesterol transporter. Ulasova et al. [68] also suggested that dietary polyphenolic compounds such as quercetin may be effective modulators of plasma cholesterol.

Concentrations of hepatic enzymes such as alanine transaminase (ALT) may be significantly correlated with portal inflammation and fibrosis [69]. Hyperlipidemia, especially high levels of triglycerides, is well known to be a major risk factor for hepatic steatosis [70] and may be responsible for the increased concentration of liver enzymes in the blood [71]. Our data also revealed a significant increase in the activities of the liver enzymes AST, ALT and ALP in the hyperlipidemic rat group compared with the saline control group. Oh et al. [72] reported that obesity may lead to the development of hyperlipidemia and consequently to an increase in ALT activity in serum. Corroborating our study, Warren et al. [73] also demonstrated a slight increase in ALT activity after treatment with P407 and attributed this elevation to the process of Kupffer cell enlargement as well as expansion of the cell lining of hepatocellular plates.

A wide variety of stress agents may affect the function and performance of the brain. A study conducted by de Oliveira et al. [74] showed a positive correlation between plasma cholesterol levels and impairments in cerebral oxidative stress, consequently leading to cognitive dysfunction. In our study, we also evaluated possible changes in AChE activity in hyperlipidemic rats, its

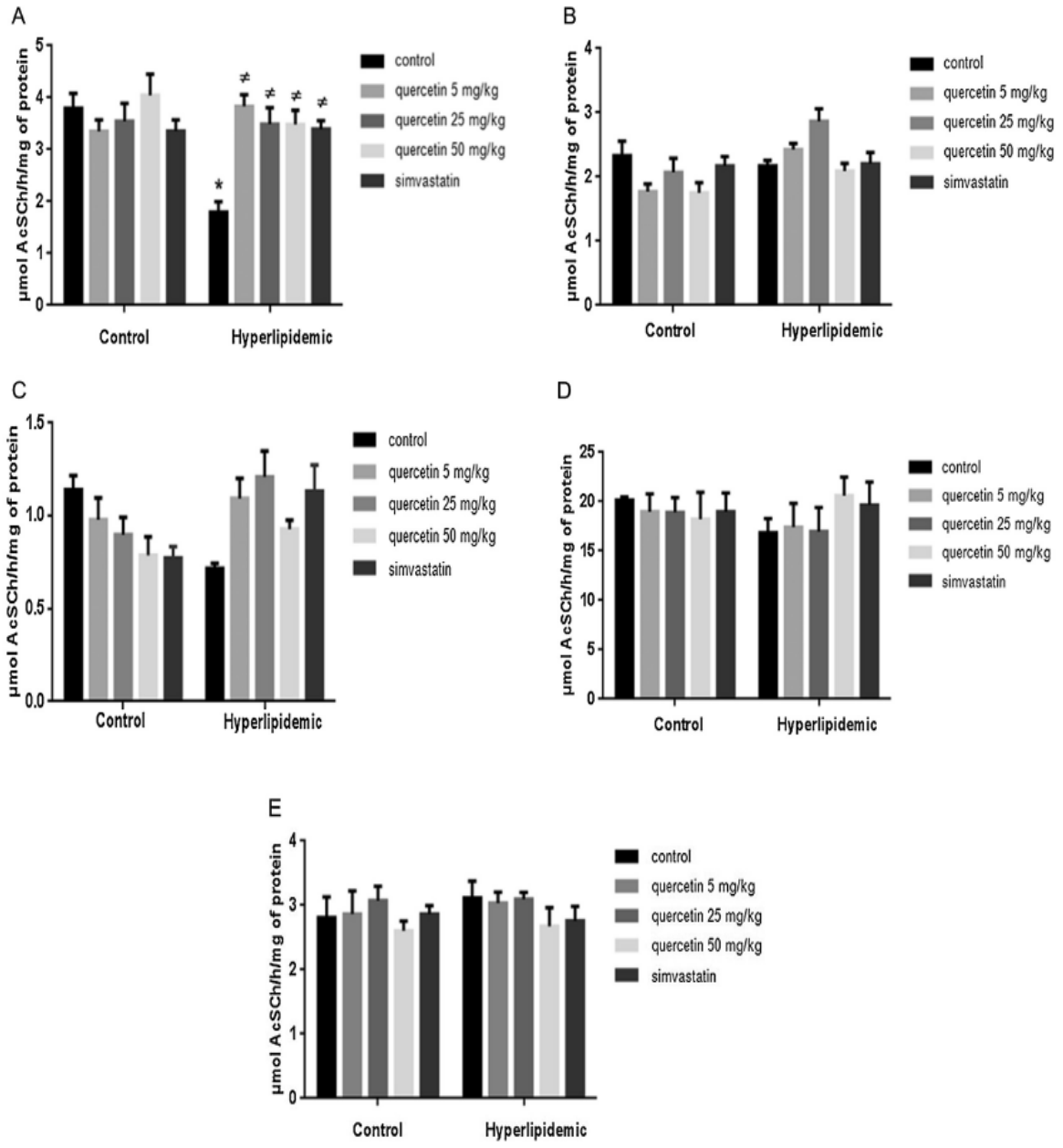

Fig. 3. Activity of the enzyme acetylcholinesterase (AChE) in the hippocampus (A), cortex (B), cerebellum (C), striatum (D) and hypothalamus (E) in hyperlipidemic rats preventively treated with quercetin (5, 25 and 50 mg/kg) and simvastatin treated (0.04 mg/kg). The results were analyzed using two-way ANOVA followed by Tukey test. The results were expressed as the mean ± S.E.M. * Means that the value is significantly different from the saline control group ($P < 0.001$, n = 7). ≠ Means that the value is significantly different from hyperlipidemia group ($P < 0.05$, n = 7).

correlation with behavioral changes, and the effect of pretreatment with quercetin and simvastatin treatment. The object recognition test is a simple behavioral memory test that is based mainly on the innate exploratory behavior of rodents in the absence of rules or externally applied reinforcement [42]. In this study, memory impairment was observed in the hyperlipidemic group compared with the saline control group (Fig. 1). Clarke et al. [75] suggest that increasing neuronal plasma membrane ordering using poloxamer would lead to neuronal membrane dysfunction and a perturbation of neuronal transmission at the cellular level, and that the disruption caused by such cellular effects would underlie decrements in cognitive ability in rats as demonstrated by impaired performance in memory related tasks. Corroborating this, Moreira et al. [76,77] reported that high cholesterol may lead to cognitive impairment in mice as assessed using the object location task. This cognitive impairment was attributed to antioxidant imbalance, oxidative damage and alterations of cholinergic signaling in brain areas associated with learning and memory processes. In a similar study, hyperlipidemia was shown to cause memory impairment, cholinergic dysfunction and inflammation [78].

In the present study, pretreatment with quercetin (5, 25 and 50 mg/kg) and simvastatin treatment attenuated the damage caused by hyperlipidemia compared with the hyperlipidemic group. This suggests a possible protection against the loss of memory and learning-induced hyperlipidemia. Our results follow a similar trend with the report of Abdalla et al. [39] where treatment with quercetin (5, 25 and 50 mg/kg) was able to reverse the memory impairment induced by cadmium intoxication. Quercetin has been studied due to its wide range of therapeutic benefits

including antioxidant effects and neuroprotective capacity [79]. Previous studies have demonstrated the ability of flavonoids to cross the BBB by quantifying them in the CNS [80,81]. Youdim et al. [82] reported the presence of catechins, quercetin, rutin and flavonoid derivatives in the brain of rodents orally administered these compounds.

In another study by Nampoothiri et al. [83], simvastatin (10 mg/kg; a HMG-CoA reductase inhibitor) was shown to antagonize the spatial memory deficit caused by aluminum; thus suggesting a neuroprotective role of simvastatin in correcting cognitive dysfunction. In the same study, simvastatin was also reported to reverse the alteration of the lipid profile by aluminum, indicating an ameliorative role of simvastatin in dementia through correction of dyslipidemia. Lipophilic statins such as simvastatin may cross the BBB more efficiently than hydrophilic ones, suggesting that solubility may be relevant to their effects [84].

The elevated plus maze has been described as a simple method for assessing anxiety responses of rodents [44]. In this study, we observed that P407-induced hyperlipidemia did not alter the response of rats to anxiety. The result of our study was contrary to the report of Korolenko et al. [55], in that a change in animal behavior, such as increased anxiety, was observed when P407 was administered during a one-month period. This may possibly be due to the different dose of P407 used. In our study, we administrated a single dose of P407, suggesting that acute hyperlipidemia may possibly not cause anxiogenic behavior. However, the result of Moreira et al. [77] was similar to that of our study, in that there was no significant difference in the elevated plus maze in high cholesterol fed rats, indicating the absence of anxiogenic/anxiolytic-like responses.

AChE is an important regulatory enzyme, which rapidly hydrolyses ACh. ACh is a neurotransmitter that plays an important role in the regulation of cognitive functions [85]. However, diets rich in choline can increase plasma levels of choline, and consequently affect the brain levels of choline [86]. These changes in choline content may in turn affect the synthesis of ACh in the brain. As observed in our results, decreased AChE activity in the hippocampus of hyperlipidemic rats may possibly be attributed to the large amount of cholesterol in the synaptic membrane of the hippocampus in comparison with other brain structures [87]. The activity of the choline acyltransferase in ChAT-positive neurons in the nucleus of rats with high cholesterol [78] may be reduced and subsequently lead to a reduction in acetylcholine production in the hippocampus. Studies indicate that nicotinic acetylcholine receptors, known to play an important role in learning and memory and general cognitive function, require optimum plasma membrane order to retain normal flux and desensitization capabilities [88]. Poloxamer directly increases plasma membrane order [89] resulting in an increase in rigidity and a decrease in membrane fluidity, altering several processes important to synaptic transmission [90]. Another possible influencing factor could be an inhibition in the synthesis of acetylcholine resulting from interference by dietary factors, thereby leading to inhibition of AChE activity. Furthermore, exposure for a long period to high cholesterol could influence metabolism, thus leading to the repression of enzyme synthesis [91]. Gutierres et al. [92] reported that AChE activity was significantly lower in the hippocampus of a rat group fed with a high fat diet compared with other groups. Kaiser et al. [93] also demonstrated that in animals fed saturated fat for a long period, AChE activity and subsequent acetylcholine synthesis was inhibited in different brain structures. The brain hippocampal region is one of the main sites for the transmission of cholinergic signals, regulation of learning and memory and appears to be more prone to oxidative damage [28].

Decreased activity of AChE can lead to an accumulation of ACh, which may result in cholinergic hyperactivity, epilepsy and seizures [94]. In this study, the observed increase in AChE activity by pretreatment with quercetin (5, 25 and 50 mg/kg) in hyperlipidemic rat may possibly prevent the hyperlipidemic effects on ChAT, thereby normalizing the levels of acetylcholine in the hippocampus to a level similar to that of the saline group. Simvastatin treatment (0.04 mg/kg) also increased AChE activity in hyperlipidemic rats compared with the saline group, and may possibly reverse the hyperlipidemic effects. The alteration of AChE activity to normality may suggest that acetylcholine levels may have returning to baseline levels, and change in memory impairment was modulated in quercetin pretreated groups. Our results follow a similar trend with the report of Abdalla et al. [39], where AChE activity was decreased in the hippocampus (51%) and cortex (53%) of rats exposed to cadmium. However, treatment with quercetin at 5, 25 or 50 mg/kg prevented the reduction in AChE activity. Thus, we suggest that preventive quercetin may modulate cholinergic neurotransmission and memory deficits caused by hyperlipidemia.

5. Conclusions

In summary, our results suggest that pretreatment with quercetin may prevent changes in the metabolism of lipids and ameliorate cardiovascular diseases. Our data also indicate that hyperlipidemia may induce memory impairment and that pretreatment with quercetin could attenuate this effect. In addition, preventive quercetin may modulate the cholinergic system of the nervous system by altering AChE activity. Preventive quercetin may however serve as a novel therapeutic agent for the treatment and management of hyperlipidemia associated with cognitive dysfunction.

Disclosure of interest

The authors declare that they have no conflicts of interest concerning this article.

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5 MANUSCRITO SUBMETIDO

Pre-treatment with quercetin prevents changes in lymphocytes E-NTPDase/E-ADA activities and cytokines secretion in hyperlipidemic rats

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Abstract

Hyperlipidemia (HL) is a condition associated with endothelial dysfunction and inflammatory disorders. Purinergic system ectoenzymes play an important role in modulating the inflammatory and immune response. This study investigated whether the preventive treatment with quercetin is able to prevent changes caused by hyperlipidemia in the purinergic system, through the activities of E-NTPDase and E-ADA in lymphocytes, quantify the nucleotides and nucleoside, and the secretion of anti and proinflammatory cytokines. Animals were divided into: saline/control, saline/quercetin 5mg/kg, saline/quercetin 25mg/kg, saline/quercetin 50mg/kg, saline/simvastatin (0.04mg/kg), hyperlipidemia, hyperlipidemia/quercetin 5mg/kg, hyperlipidemia/quercetin 25mg/kg, hyperlipidemia/quercetin 50mg/kg and hyperlipidemia/simvastatin. Animals were pretreated with quercetin for 30 days and hyperlipidemia was subsequently induced by intraperitoneal administration of 500mg/kg of poloxamer-407. Simvastatin was administered after the induction of hyperlipidemia. Lymphocytes were isolated and E-NTPDase and E-ADA activities were determined. Serum was separated for the cytokines and nucleotides/nucleoside quantification. E-NTPDase and E-ADA activities were increased in lymphocytes from hyperlipidemic rats and pretreatment with quercetin was able to prevent the increase in the activities of these enzymes caused by hyperlipidemia. Hyperlipidemic rats when receiving pretreatment with quercetin and treatment with simvastatin showed decreased levels of ATP and ADP when compared to the untreated hyperlipidemic group. The IFN-γ and IL-4 cytokines were increased in the hyperlipidemic group when compared with control group, and decreased when hyperlipidemic rats received the pretreatment with quercetin. However, pretreatment with quercetin was able to prevent the alterations caused by hyperlipidemia probably by regulating the inflammatory process. We can suggest that the quercetin is a promising compound as adjuvant in the treatment of hyperlipidemia.

Keywords: Flavonoid. Cholesterol. Ectoenzymes. Cytokines.

1. Introduction

Hyperlipidemia (HL), characterized by abnormal levels of serum lipids and the deregulation of lipid metabolism, is the primary risk factor contributing to the formation and progression of atherosclerosis and subsequent cardiovascular disease [1, 2]. Hyperlipidemia may be induced by chronic high fat diet [3], or by acute induction with compounds such as Triton [4] and Poloxamer 407 (P407) [5]. P407 is a non-ionic synthetic copolymer surfactant, which provides an attractive means of inducing hyperlipidemia, since it interferes in the hydrolysis of triglycerides and cholesterol biosynthesis [6, 7]. HL increases the risk of retention and modification of low-density lipoprotein (LDL) in arterial walls, which can undergo oxidation in macrophage-rich tissues [8]. Oxidized low-density lipoprotein (ox-LDL) is immunogenic and can recruit immune cells, including monocytes and T-lymphocytes, to the subendothelial layer of the artery wall. Indeed, T-lymphocytes have been shown to be present at all stages of lesion development in atherosclerosis [9, 10].

In atherosclerosis, large accumulation of lipids within artery walls stimulates a series of inflammatory responses, the stimulated endothelial cells attract T-lymphocytes and monocytes, which transform into macrophages and ingest ox-LDL to become foam cells [8]. Once formed, ox-LDL results in injury or endothelial dysfunction [11]. In addition, some evidence suggests that beta VLDL particles may themselves activate inflammatory functions of vascular endothelial cells [12]. Reverse cholesterol transport effected by high-density lipoprotein (HDL) likely accounts for some of its atheroprotective function. However, HDL particles also can break down oxidized lipids and neutralize their proinflammatory effects [13].

Inflammation and immunity are involved in atherosclerosis lesion formation, and the NF-κB plays an important role in their progression, as in and the activation and development of T and B cells in the adaptive immune response. Also, the activated macrophage abundant in atheroma can produce proteolytic enzymes capable of degrading the collagen that lends strength to the plaque's protective fibrous cap, rendering that cap thin, weak, and susceptible to rupture [14, 15]. Inflammatory mediators regulate tissue factor expression by plaque macrophages, demonstrating an essential link between arterial inflammation and thrombosis [16].

Cytokines are responsible for modulating several aspects of vascular inflammation, altering proliferation, differentiation and vascular function of a variety of cell types where intercellular communication occurs, thus generating cardiovascular diseases. Immune cell products are especially important in the regulation of immune and inflammatory responses

and act on several stages of immunity [17]. It is suggested that cytokines can be classified for their involvement in atherosclerosis in pro-atherogenic cytokines and anti-atherogenic cytokines, and among the first group include interferon-gamma (IFN-γ) [18]. There is evidence that IL-4 plays a role in the development of vascular disease. IL-4 has been shown to be pro-atherogenic in mice models of atherosclerosis [19].

The immune system is essential to host defenses comprising an interactive network of lymphoid organs and immune cells [20]. The inflammatory process is complex and, during the activation of the immune system, interactions between various components occur [21], including molecules of the purinergic system that contribute to the regulation of the inflammatory process [22, 23]. This regulation is performed by extracellular biomolecules such as adenine nucleotides adenosine triphosphate (ATP), adenosine diphosphate (ADP), and adenosine monophosphate (AMP) and their derived nucleoside adenosine [24]. The levels of extracellular ATP, ADP, AMP, and adenosine are dynamically controlled during inflammation by the action of enzymes expressed in immune cells [23].

Extracellular ATP can be hydrolyzed by a variety of ectonucleotidases such as E-NTPDases (nucleoside triphosphate diphosphohydrolases) [25]. The membrane bound enzyme E-NTPDase (CD39; E.C. 3.6.1.5) modulates adenine nucleotides levels, which are fundamental to the modulation of immune responses [26] and this family of enzymes is recognized as a marker of the lymphocyte activation [27]. Furthermore, ecto-adenosine deaminase (E-ADA, E.C 3.5.4.4) is another important enzyme which participates in the degradation of purines by catalyzing the conversion of adenosine into inosine [28, 29].

Previous experimental investigations suggest that quercetin has positive effects on lipid metabolism because of its antioxidant, anti-inflammatory and anti-atherogenic properties [30-33]. Quercetin (3,3', 4', 5,7-pentahydroxyflavone) is one of the most abundant dietary flavonoids and belongs to the flavonols subgroup. It is present in plants in many different glycosidic forms and usually found in conjugated forms with sugars such as glucose, galactose and rhamnose [34]. The properties of quercetin ensure potential benefits for overall health and disease resistance, including anti-carcinogenic and antiviral activities, as well as the ability to inhibit lipid peroxidation, platelet aggregation and capillary permeability, and to stimulate mitochondrial biogenesis [35].

Considering that hyperlipidemia is a condition associated with endothelial dysfunction and inflammatory disorders and the involvement of adenine nucleotides and nucleoside hydrolysis in the modulation of immune system, there is a clinical interest in investigating the preventive therapeutic action of naturals compounds with anti-inflammatory properties, such

as quercetin. The purpose of this study was to evaluate the secretion of proinflammatory and anti-inflammatory cytokines and the activity of E-NTPDase and E-ADA ecto-enzymes in poloxamer-407 induced hyperlipidemic rats pretreated with flavonoid quercetin.

2. Materials and methods

2.1 Reagents

Adenosine 5'-triphosphate disodium salt (ATP), adenosine 5'-diphosphate sodium salt (ADP), bovine serum albumin, Trizma base, Trypan Blue solution, and Coomassie Brilliant Blue G were obtained from Sigma- Aldrich (St. Louis, MO, USA). Ficoll-Hypaque (Lymphoprep) was purchased from Nycomed Pharma (Oslo, Norway). Physiological solution (0.9 g NaCl/100mL distilled water) was obtained from Fresenius KABI (Brazil). K_2HPO_4 was purchased from Reagen (Brazil). Quercetin (Sigma Chemical Co, St. Louis, MO, USA). All chemicals used in the experiments were of analytical grade and of the highest purity.

2.2 Animals

Adult male Wistar rats (n=70; 70-90 days; 250-350 g) obtained from the Central Animal House of the Federal University of Santa Maria (UFSM), Santa Maria, Brazil, were used for this experiment. The animals were maintained at a constant temperature (23±1°C) on a 12 h light/dark cycle with free access to food and water. All animal procedures were approved by the Ethics Committee on the Use of Animal at the Federal University of Santa Maria (Protocol number: 9894021214/2014).

2.3 Experimental Design

Rats were divided into collective boxes polypropylene (41x34x16cm). The animals were pretreated with quercetin for one month in three different doses (5, 25 and 50mg/kg), prior to the induction of hyperlipidemia. After induction, simvastatin was administered to some groups. The rats were subdivided into ten groups of 7 animals each; saline control (C), saline + quercetin 5mg/kg (CQ5), saline + quercetin 25mg/kg (CQ25), saline + quercetin 50mg/kg (CQ50), saline + simvastatin 0.04mg/kg (CS), hyperlipidemia (H), hyperlipidemia + quercetin 5mg/kg (HQ5), hyperlipidemia + quercetin 25mg/kg (HQ25), hyperlipidemia + quercetin 50mg/kg (HQ50) and hyperlipidemia + simvastatin 0.04mg/kg (HS). The experiment began with the administration of quercetin (5, 25 and 50mg/kg) by gavage for a period of 30 days. After 30 days of pretreatment with quercetin, a single dose of P407 (500mg/kg) was intraperitoneally administered to induction of hyperlipidemia. Simvastatin (0.04mg/kg) was

administered after the induction of hyperlipidemia. P407 was diluted in saline and quercetin in ethanol 25% and were administered in a volume of 1mL/kg.

2.4 Pretreatment with quercetin

A month prior to the induction of hyperlipidemia, animals in groups CQ5 and HQ5 received 5mg/kg of quercetin, CQ25 and HQ25 animals received 25mg/kg and CQ50 and HQ50 received 50mg/kg of quercetin for period 30 days, while the C and H animal groups received 0.9% saline solution by gavage. Quercetin was freshly prepared in 25% ethanol and was administered once a day for 30 days by gavage. The choice of the doses (5, 25 and 50 mg/kg of quercetin) used in this experiment, was based on a previous study [5]. The dose of quercetin was adjusted weekly according to the body weight of rats. The volume of saline solution and ethanol were adjusted based on the body weight (0.001mL/g).

2.5 Induction of hyperlipidemia by Poloxamer 407 (P407)

The animals were randomly divided into hyperlipidemic and control saline rat groups. To induce hyperlipidemia, 500 mg/kg of P407 dissolved in sterile NaCl 0.9% solution was administered via intraperitoneal (i.p.) injection [36]. The control saline rats received the same volume of vehicle alone (cold, sterile 0.9% NaCl solution). After 36 hours of induction, the animals were anesthetized with isoflurane, submitted to euthanasia and blood was collected with cardiac puncture.

2.6 Treatment with simvastatin

After induction of hyperlipidemia, CS and HS groups were given the dose of simvastatin equivalent to the normal dose recommended to human (40 mg) by gavage. This group was administered saline alone prior to the induction of hyperlipidemia. The equivalent dose between mice and humans was calculated according to the method desribed by Reigner and Blesch [37]. The surface area of the animals was determined and represented by equation 1 as shown below:

$$BSA^{(m2)} = 1.85\ (W / 70)^{2/3} \quad (1)$$

Where BSA is the body surface area, W is the weight in kilograms. In humans, the body surface was considered the average value ($1.8m^2$) and 70 kg. Substituting into the equation, a mouse (0.400 kg) consists of a body area $0.0593m^2$. Then, the dose in milligrams

per square meter was multiplied by the surface area of the human to find the mouse dose which was 0.04 mg / kg.

2.7 Isolation of lymphocytes from blood

Blood was collected with 7.2 mg dipotassium EDTA as anticoagulant and lymphocyte-rich mononuclear cell were isolated from blood collected with Ethylenediamine tetra acetic acid (EDTA) and separated on Ficoll-Histopaque density [38]. The percentage of lymphocytes was superior to 93%, as previously described [39]. The integrity of lymphocytes preparation was confirmed by determining the lactate dehydrogenase (LDH) activity in intact and disrupted lymphocytes using the kinetic method of the Labquest apparatus (Diagnostics Gold Analyzer). The procedure was repeated before and after the incubation period. The protocol was carried out according to the manufacturer's instructions. Triton X-100 (1%, final concentration) was used to disrupt the lymphocytes preparation. The enzymatic activity is expressed as units per liter, and one unit (1U) corresponds to 1 μmol of NADH formed per minute per liter. The resultant lymphocytes samples were used immediately for enzymatic assays.

2.8 Protein determination

Protein was measured by the Comassie Blue method according to Bradford [40] using serum albumin as standard.

2.9 E-NTPDase activity

E-NTPDase activity in lymphocytes was determined as previously described by Leal *et al*. [41], in which the reaction medium contained 0.5 mM $CaCl_2$, 120 mM NaCl, 5 mM KCl, 60 mM glucose and 50 mM Tris–HCl buffer at pH 8.0, with a final volume of 200 μL. Twenty microliters of the intact mononuclear cells suspended in saline solution was added to the reaction medium (2–4μg of protein), and pre-incubated for 10 min at 37°C; incubation proceeded for 70 min. The reaction was initiated by the addition of substrate (ATP or ADP) at a final concentration of 2.0 mM and stopped with 200 μL of 10% trichloracetic acid (TCA). The released inorganic phosphate (Pi) was assayed by a method previously described by Chan *et al.* [42] using malachite green as colorimetric reagent and KH_2PO_4 as standard. Controls were carried out by adding the enzyme preparation after TCA addition to correct for non-enzymatic nucleotide hydrolysis. All samples were run in triplicate and the specific activity is reported as nmol of Pi released/min/mg of protein.

2.10 E-Adenosine deaminase (E-ADA) activity

E-ADA activity in lymphocytes was measured by the method of Giusti and Galanti [43], which is based on the direct measurement of ammonia produced when ADA acts in excess of adenosine. Briefly, 25 μL of lymphocytes reacted with 21 mM/L of the substrate (adenosine), pH 6.5, and incubation was carried out for 1h at 37°C. The reaction was stopped by adding 106 mM and 167.8 mM sodium nitroprussiate and hypochlorite solution. Ammonium sulfate 75 μM was used as ammonium standard. The protein content for lymphocytes experiment was adjusted between 0.1–0.2 mg/mL. All the experiments were performed in triplicate and the values were expressed in U/L for E-ADA activity. One unit (1U) of E-ADA is defined as the amount of enzyme required to release 1 mmol of ammonia per minute from adenosine at standard assay conditions.

2.11 Separation of blood serum

The blood samples were collected in tubes without anticoagulant and after the clot formation were centrifuged at 1400g for 15min at room temperature. The resultant serum samples were aliquoted in microtubes and kept on ice until the purines quantification and cytokines measurement.

2.12 Purine level measurement

The quantitative determination of adenine nucleotides and adenosine levels were performed in serum by HPLC. At first, proteins were denatured by the addition of 0.6 mol/L of percloric acid. Then, all samples were centrifuged (14,000×g for 10 min). The obtained supernatants were neutralized with 4N KOH and clarified with a second centrifugation (14,000×g for 15min). Aliquots of 40 μL were applied to a reversed-phase HPLC system using a 25cm C18 Shimadzu column (Shimadzu, Japan) at 260 nm with a mobile phase containing 60 mM KH_2PO_4, 5 mM tetrabutylammonium chloride, pH 6.0, in 30% methanol according to a method previously described by Voelter [44]. The peaks of purines (ATP, ADP, AMP and adenosine) were identified by their retention times and quantified by comparison with standards. Results are expressed as nmoles of the different compounds per mL of serum.

2.13 Cytokines measurement

Serum cytokines were simultaneously measured by Cytometric Bead Array (CBA). The Rat IL-4 and IFN-γ Flex Set kit (BD Biosciences, San Jose, CA, USA) was applied

following manufacturer instructions. Quantitative results were generated using Accuri flow cytometer and FCAP Array software. Results are expressed as picogram per milligram of serum.

2.14 Statistical analysis

The statistical analysis was performed using Two-way ANOVA tests were used and these were followed by Tukey tests. $P<0.05$ was considered to represent a significant difference in both analyses used. All data were expressed as mean ± SEM.

3. Results

3.1 Cellular integrity

The lactate dehydrogenase activity measurement showed that approximately 5% of lymphocytes of both groups were disrupted, indicating that the preparation was predominantly intact after the isolation procedure (Data not shown).

3.2 E-NTPDase Activity in Lymphocytes

Figure 1 shows the effect of pretreatment of quercetin on ATP and ADP hydrolysis by E-NTPDase in lymphocytes of poloxamer-407 induced hyperlipidemic rats. Results of lymphocytes E-NTPDase activity with ATP as substrate are shown in Fig. 1A. The activity with ATP as substrate was significantly increased (72%) in the hyperlipidemic group (46.06 ± 2.4; n=7; $P<0.001$) when compared with the control group (26.78 ± 2.7; n=7; $P<0.001$). When quercetin 25 and 50 mg/kg was previously administered in hyperlipidemic group, the increase in E-NTPDase activity caused by HL was prevented by pretreatment with this flavonoid [30.3% (32.12 ± 1.6; n=7; $P<0.05$) and 33% (30.84 ± 2.1; n=7; $P<0.01$) decrease, respectively], and treatment with simvastatin (35.2% decrease, 29.84 ± 1.5; n=7; $P<0.01$). In addition, results obtained for the E-NTPDase activity in lymphocytes with ADP as substrate are shown in Fig. 1B, where the ADP hydrolysis was also increased by 98.7% in the hyperlipidemic group (51.43 ± 4.7; n=7; $P<0.01$) when compared with the control group (25.88 ± 2.1; n=7; $P<0.01$). On the other hand, when quercetin 5, 25 and 50 mg/kg was previously administered in hyperlipidemic group, the increase in E-NTPDase activity caused by HL was prevented by pretreatment with quercetin [27.7% (37.19 ± 2.1; n=7; $P<0.05$), 32.2% (34.88 ± 1.1; n=7; $P<0.01$) and 36.4% (32.73 ± 0.9; n=7; $P<0.01$) decrease respectively] and treatment with simvastatin (37.3% decrease, 32.26 ± 0.8; n=7; $P<0.01$).

3.3 E-ADA Activity in Lymphocytes

Results obtained for adenosine hydrolysis by E-ADA are shown in Fig. 2. The E-ADA activity in the peripheral lymphocytes showed that was significant increase by 78% in the hyperlipidemic group (70.63 ± 3.4; n=7; $P<0.001$) when compared with the control group (39.69 ± 2.8; n=7; $P<0.001$). However, when quercetin 5, 25, and 50 mg/kg was previously administered in hyperlipidemic group, the increase in E-ADA activity caused by HL was prevented by pretreatment with quercetin [39% (43.09 ± 3.8; n=7; $P<0.001$), 43% (40.18 ± 4.4; n=7; $P<0.001$) and 40% (42.35 ± 2.8; n=7; $P<0.001$) decrease respectively] and treatment with simvastatin (41% decrease, 41.56 ± 3.9; n=7; $P<0.001$).

3.4 Purine level measurement

Purine levels in serum were measured by HPLC as shown in Table 1. The levels of ATP, ADP, AMP and adenosine showed no significant alterations in the pretreated groups with quercetin in the doses 5, 25 and 50 mg/kg, as in treated with simvastatin when compared to control group (n=7, $P>0.05$), showing that the quercetin per se did not interfere in the purine level. No significant alterations were observed in the hyperlipidemic group in the level of ATP, ADP, AMP and adenosine when compared to control group (n=7, $P>0.05$). Data also revealed that hyperlipidemic rats receiving pretreatment with quercetin 50 mg/kg and treatment with simvastatin showed decreased levels of ATP (n=7; $P<0.05$), as well as levels of ADP when hyperlipidemic rats receiving pretreatment with quercetin 25 and 50 mg/kg and treatment with simvastatin (n=7; $P<0.001$), when compared to the untreated hyperlipidemic group. The AMP and adenosine levels showed no significant alterations between groups.

3.5 Serum cytokines levels

Serum IL-4 and IFN-γ cytokines levels are shown in Figure 3. Results obtained for serum levels for IL-4 anti-inflammatory cytokine are demonstrated in Fig. 3A. Serum levels for IL-4 cytokine significant increased in the hyperlipidemic group (23.08 ± 2.1; n=7; $P<0.001$) when compared with control group (6.76 ± 0.7; n=7; $P<0.001$). However, there was a significant decrease in the IL-4 cytokine levels in hyperlipidemic rats that received the pretreatment with quercetin 25mg/kg (12.31 ± 1.6; n=7; $P<0.05$), 50 mg/kg (11.64 ± 1.8; n=7; $P<0.05$) and treatment with simvastatin (13.18 ± 2.6; n=7; $P<0.05$) when compared with hyperlipidemic group (23.08 ± 2.1; n=7; $P<0.05$).

As shown in Fig. 3B, the average serum levels for IFN-γ pro-inflammatory cytokine were significantly increased in the hyperlipidemic group (68.85 ± 4.5; n=7; $P<0.01$) when

compared with control group (43.26 ± 7.3; n=7; $P<0.01$). Results also revealed a significant decrease in INF-γ cytokine levels in hyperlipidemic rats pretreated with quercetin 5mg/kg (41.52 ± 2.0; n=7; $P<0.01$), 25 mg/kg (41.62 ± 6.2; n=7; $P<0.01$), 50 mg/kg (42.82 ± 1.5; n=7; $P<0.01$) and treated with simvastatin (41.46 ± 2.3; n=7; $P<0.01$) when compared with hyperlipidemic group (68.85 ± 4.5; n=7; $P<0.01$).

4. Discussion

Quercetin is a flavonoid abundant in many natural plants and has been known to exhibit several biological properties, including anti-inflammatory, antioxidant, vasodilatory and hypolipidemic [33, 45-49]. HL is a risk factor for the development of chronic inflammatory conditions, as atherosclerosis [50, 51].

The inflammatory process can be altered by extracellular adenine nucleotides and nucleosides such as ATP and adenosine, which have been recognized as key components of the purinergic system [23]. The present study was conducted to assess whether pretreatment with quercetin is able to modulate the alterations caused by HL, through the activity of the ectoenzymes of the purinergic system, such as E-NTPDase/E-ADA, the cytokine secretion and purine level measurement of hyperlipidemic rats.

Extensive tissue damage in inflammatory processes may lead to a significant increase in the levels of purine and pyrimidine nucleotides within the involved sites, probably contributing to the amplification of the inflammatory reaction [52]. Our results demonstrate that HL was strongly associated with an increase in E-NTPDase activity both using ATP and ADP as substrate when compared to control group. It has already been demonstrated that the lipids have a role on the regulation of ectonucleotidases activity and localization. Corroborating with our results, Duarte *et al.* [53] found positive a correlation between increased cholesterol levels and platelet ATP and ADP hydrolysis. Robson *et al.* [54] also showed that endothelial cells, when supplemented with saturated or a monounsaturated fatty acid(s), presented an increased ATP and ADP hydrolysis rate, and Papanikolaou *et al.* [55] found that the activity of ENTPDase1/CD39 is directly proportional to the cholesterol levels in cell culture. In addition, Ataman [56] also demonstrated that the elevation of cholesterol concentration was able to increase the ectonucleotidase activities of blood vessels. Studies also suggest that is possible that the high levels of cholesterol measured in diabetic animals could modulate the ectonucleotidase activities [57]. Based on these results, we can suggest that this increased activity of this enzyme leads to the maintenance of nucleotides physiological levels, which can be confirmed by the normal levels of ATP and ADP in the

extracellular medium found in our study. High concentrations of ATP in the extracellular medium activate the proinflammatory purinergic P2X7 receptors and contributes to tissue damage and inflammation [58], such as stimulation and proliferation of lymphocytes and cytokine release [59].

Under physiological conditions, nucleotides are present in the extracellular environment in low concentrations [60], however, in response to different stimulus or conditions, including damage to plasmatic membrane induced by inflammation, increasing concentrations of nucleotides can be released to the extracellular environment increasing its levels [61]. It is possible that the ectonucleotidase activity could be regulated by changes in cholesterol levels at the microdomains of rafts/caveolae or other cholesterol-dependent structures, such as microvilli [55]. It may be assumed that E-NTPDase enzyme has important functions in the modulation of inflammatory response in hyperlipidemia.

Since these enzymes act in a cascade, we can suggest that the E-5′-nucleotidase activity in hyperlipidemic rats could be also increased, however it was not evaluated in our study. This increase would result in a greater amount of adenosine in the extracellular medium to compensate the proinflammatory effects of ATP. However, in our study, hyperlipidemic animals displayed normal levels of ATP, ADP an AMP in the extracellular medium.

In addition to ATP and ADP, adenosine is also among the molecules released as a result of inflammation, which has important functions in the interaction between immune components during activation, course control and resolution of the inflammatory response [23, 62]. Our results demonstrate that E-ADA activity was increased in lymphocytes of hyperlipidemic rats when compared to control group. Corroborating with our results, other studies have also shown that E-ADA activity is increased in infections and autoimmune disorders [63- 65]. A rise in the activity of this enzyme in our study may have led to increased adenosine deamination, thereby causing a reduction in the levels of this nucleoside in circulation. E-ADA activity could be increased by the supposed increase in the activity of E-5'-nucleotidase, since adenosine levels are normal in the extracellular medium in hyperlipidemic animals. Serum E-ADA is increased in various diseases, however, the origin of E-ADA in serum and the mechanism by which serum E-ADA activity is increased have not been fully elucidated yet [66]. E-ADA is another important enzyme in the immune system and it is considered essential for the differentiation, normal growth and proliferation of lymphocytes [67].

The beneficial effects of quercetin, and among them its anti-inflammatory effect, have already been well described in the literature, hence we evaluated the effect of quercetin on the

metabolism of adenine nucleotides. Interestingly, in the control group pretreated with quercetin, the activities of E-NTPDase and E-ADA remained at basal levels, which was also confirmed by the normal serum purine levels found in the extracellular medium. In hyperlipidemic rats pretreated with quercetin and treated with simvastatin, the increases on the E-NTPDase and E-ADA activities were prevented, while the purine levels on serum showed decreased ATP and ADP levels these groups. These results confirm the inhibitory properties of quercetin on the ectonucleotidases activities in pathological conditions and corroborate with some studies that have shown the inhibition of several enzymes by flavonoids. This inhibition has been postulated to be due to the interaction of enzymes with different parts of the flavonoid molecule, e.g. carbohydrate, phenyl ring, phenol and benzopyrone ring [65]. Da Silva *et al.* [68] suggest that chelating property of flavonoids can diminish NTPDase-like, E-5'-nucleotidase and Na+/K+-ATPases activities in cortical membrane preparation. In this way, we propose that the decrease in NTPDase and E-ADA activities in lymphocytes can be due to the chelating action of quercetin. Corroborating our results, a study previously published by our research group [64] also has showed that increased E-NTPDase and E-ADA activities were prevented when rats exposed to cadmium were treated with quercetin. Baldissarelli *et al.* [65] also demonstrated that hypothyroid animals treated with quercetin decreased the activity of adenosine deaminase. As already well reported in the literature, quercetin has anti-inflammatory properties and this compound may have the ability to lower cellular damage [69, 70]. Studies have shown that inhibition in the E-ADA activity may increase the concentration of adenosine in the extracellular environment and potentiate the effects of the nucleoside in cell receptors [71]. These results reinforce evidence that natural compounds could affect purinergic system and may modulate the nucleotide and nucleoside levels present in the circulation [72]. Taking these data together, we suggest that the pretreatment with quercetin may cause a reduction in inflammatory processes caused by hyperlipidemia, since the increased E-ADA activity was prevented resulting in anti-inflammatory effects.

Interactions of various components including molecules of the purinergic system contribute to the regulation of the inflammatory process have been implicated in the activation of the immune system [73]. ATP is involved in proinflammatory functions such as stimulation and proliferation of lymphocytes and cytokine release [74]. Most importantly, ATP appears to be necessary for cytokine secretion in Th1 cells such as IFN-γ, which, once released, activates other subsets of lymphocytes or macrophages [75].

Studies also relate atherosclerosis to systemic inflammatory mechanisms with increase cytokine production and result in endothelial activation, which is considered the first step in the development of atherosclerotic plaques, and is related to monocyte adhesion in the vascular endothelium with its subsequent migration in the wall of the artery [76]. Other parameters evaluated in this study were secretion of pro-inflammatory and anti-inflammatory cytokines in hyperlipidemic rats.

$CD4^+$ cells are the major class of T-lymphocytes present in atherosclerotic lesions. These T-lymphocytes can be activated to differentiate into two major subsets, Th1 and Th2, that release a subset-specific repertoire of cytokines [77]. The predominance of studies has defined the role of Th1 cytokines on the development of atherosclerosis. These have included consistent demonstrations of the atherogenic properties of IFN-γ [78]. Our results demonstrate higher levels cytokine IFN-γ in HL, indicating the occurrence of inflammatory state that is in consonance with possible atherosclerosis development. Inflammation is associated with alterations in lipid metabolism that may be mediated by cytokines. HL is one of the major risk factors leading to early atherosclerotic vascular diseases, involving both the innate and adaptive immune systems, which modulate the initiation and progression of the lesions, and potentially devastating thrombotic complications [79]. Corroborating with the results of our study, Santi *et al.* [80] also found an inflammatory profile in individuals with total high cholesterol level, evidenced by the high levels of IFN-γ. In addition, Mirhafez *et al.* [81] shows that subjects with hypertriglyceridemia had significantly higher serum concentrations of IFN-γ compared to the group with normal triglyceride levels. INF-γ has several functions, as well as immunomodulatory and inflammatory activities it contributes to the progression of atherosclerosis and promotes leukocyte adhesion in vascular injury [82]. The production of INF-γ is well expressed in unstable atheromatous plaques, destabilizing the fibrous cap and promoting the apoptosis of foam cells [83, 84].

Our data also demonstrate decreased levels for INF-γ cytokine in the pretreatment with quercetin 5, 25 and 50 mg/kg and treatment simvastatin. In addition, Zahedi *et al*. [85] also showed that quercetin supplementation reduced the serum concentration of pro-inflammatory cytokines as TNF-α and interleukin-6. Yu *et al.* [86] demonstrated that quercetin represses T-bet-dependent and independent IFN-γ and interleukin-2 production supporting the fact that quercetin could be an effective therapeutic compound for inflammatory and autoimmune diseases. However, we can suggest that pretreatment with quercetin is effective in the reduction of pro-inflammatory cytokines being effective in preventing inflammatory diseases. Previous results, showing that quercetin modulates the production of inflammatory cytokines

in macrophages, strongly suggested quercetin would intensively modulate anti-inflammatory responses [87, 88].

Another cytokine evaluated in our study was IL-4. Results demonstrate IL-4 levels increased in the hyperlipidemic rats when compared with control group. However, when hyperlipidemic rats were pretreated with quercetin 25 and 50 mg/kg, or treated with simvastatin, the levels of IL-4 cytokine were lower in relation to the untreated hyperlipidemic rats. In endothelial dysfunction, the concentrations of IL-4 and IFN-γ are known to increase, stimulating the production of adhesion molecules, thus favoring the recruitment and adhesion of monocytes to the endothelial surface [89]. The effects of IL-4 (Th2 response inducer) are generally considered anti-inflammatory. However, a growing body of evidence indicates that IL-4 may play a role in atherosclerosis through induction of inflammatory responses, such as upregulation of VCAM-1 [90, 91] and monocyte chemoattractant protein-1 (MCP-1) [92]. Moreover, deficiency in IL-4 has been associated with a decrease in atherosclerotic lesion formation [91]. Corroborating with our results, Xu *et al.* [93] also found an increase in the expression of IL-4 and INF-γ in hyperlipidemic rats and, when these rats were treated with quercetin nanoparticles 40 and 80 mg/kg, there was a decrease in the expression of IL-4 and IFN-γ respectively. Tsao *et al*. [94] also shows that IL-4 promotes lipolysis by upregulating the activity of hormone-sensitive lipase, the key enzyme for triacylglyceride degradation. Taken together, these studies suggest that IL-4 may participate in lipid metabolism. In addition, quercetin decreases the eosinophil recruitment, reduces IL-4 level and inhibits NF-KB activation in rats with an inflammatory process, regulating the inflammatory response by decreasing the IL-4 levels [95]. The anti-inflammatory action of quercetin is caused by the inhibition of enzymes such as lipoxygenase, and the inhibition of inflammatory mediators. Quercetin affects immunity and inflammation by acting mainly on leukocytes and targeting many intracellular signaling kinases and phosphatases, enzymes and membrane proteins often crucial for a cellular specific function [96]. Thus, quercetin has the ability to suppress inflammatory cytokine production and decrease blood lipid abnormality by regulating the metabolic system.

In conclusion, ours results demonstrate alterations in enzymes of the purinergic, as well as in the cytokines secretion in hyperlipidemic rats, indicating that these alterations may be due to the inflammatory process caused by hyperlipidemia. It is proposed that the increased ATPasic activity was probably induced as a dynamic response to clean up the elevated ATP levels resulting from injuries promoted by hyperlipidemia. Interestingly, the pretreatment with quercetin was able to prevent the alterations caused by hyperlipidemia probably by

inflammatory process regulation as a result of its anti-inflamatory and hypolipidemic activities. Based on our results, we could propose that quercetin has a therapeutic antiatherogenic effect, not only through the reduction of lipids and plasmatic lipoproteins, but also in the induction of modulator mechanisms of the inflammation, which together contrast the development of atherosclerotic lesions. In this context, we can suggest that the pretreatment with quercetin is a promising as adjuvant in the treatment of hyperlipidemia.

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Conflict of interest

The authors declare that they have no conflict of interest.

Ethical approval

All procedures performed in studies involving animals were in accordance with the ethical standards of the institution or practice at which the studies were conducted.

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Figure Legends

Figure 1: ATP (A) and ADP (B) hydrolysis in lymphocytes of hyperlipidemic rats pretreatment for 30 days with quercetin in the dose of 5, 25 and 50 mg/kg. Enzyme specific activities are reported as nmol of Pi released/min/mg of protein. Groups: saline control (C), saline+quercetin 5 mg/kg (CQ5), saline+quercetin 25 mg/kg (CQ25), saline+quercetin 50 mg/kg (CQ50), saline+simvastatin 0.04 mg/kg (CS), hyperlipidemia (H), hyperlipidemia+quercetin 5 mg/kg (HQ5), hyperlipidemia+quercetin 25 mg/kg (HQ25), hyperlipidemia+quercetin 50 mg/kg (HQ50) and hyperlipidemia+simvastatin 0.04 mg/kg (HS). The results were analyzed using two-way ANOVA followed by Tukey test. The results were expressed as the mean ± S.E.M. $^{(###)}$ Means that the value is significantly different from control group (P<0.001, n= 7). $^{(##)}$ Means that the value is significantly different from control group (P<0.01, n= 7). $^{(**)}$ Means that the value is significantly different from the hyperlipidemic group (P<0.01, n= 7). $^{(*)}$ Means that the value is significantly different from the hyperlipidemic group (P<0.05, n= 7).

Figure 2: Adenosine deamination in lymphocytes of hyperlipidemic rats pretreatment for 30 days with quercetin in the dose of 5, 25 and 50 mg/kg. Enzyme activities are reported as U/L. Groups: saline control (C), saline+quercetin 5 mg/kg (CQ5), saline+quercetin 25 mg/kg (CQ25), saline+quercetin 50 mg/kg (CQ50), saline+simvastatin 0.04 mg/kg (CS), hyperlipidemia (H), hyperlipidemia+quercetin 5 mg/kg (HQ5), hyperlipidemia+quercetin 25 mg/kg (HQ25), hyperlipidemia+quercetin 50 mg/kg (HQ50) and hyperlipidemia+simvastatin 0.04 mg/kg (HS). The results were analyzed using two-way ANOVA followed by Tukey test. The results were expressed as the mean ± S.E.M. $^{(###)}$ Means that the value is significantly different from control group (P<0.001, n= 7). $^{(***)}$ Means that the value is significantly different from the hyperlipidemia group (P<0.001, n= 7).

Figure 3: Serum levels of IL-4 (3A) and INF-γ (3B) cytokines. Groups: saline control (C), saline+quercetin 5 mg/kg (CQ5), saline+quercetin 25 mg/kg (CQ25), saline+quercetin 50 mg/kg (CQ50), saline+simvastatin 0.04 mg/kg (CS), hyperlipidemia (H), hyperlipidemia+quercetin 5 mg/kg (HQ5), hyperlipidemia+quercetin 25 mg/kg (HQ25), hyperlipidemia+quercetin 50 mg/kg (HQ50) and hyperlipidemia+simvastatin 0.04 mg/kg (HS). The results were analyzed using two-way ANOVA followed by Tukey test. The results were expressed as the mean ± S.E.M. $^{(###)}$ Means that the value is significantly different from control group (P<0.001, n= 7). $^{(##)}$ Means that the value is significantly different from control

group (P<0.01, n= 7). $^{(**)}$ Means that the value is significantly different from the hyperlipidemia group (P<0.01, n= 7). $^{(*)}$ Means that the value is significantly different from the hyperlipidemia group (P<0.05, n= 7).

Figure 1

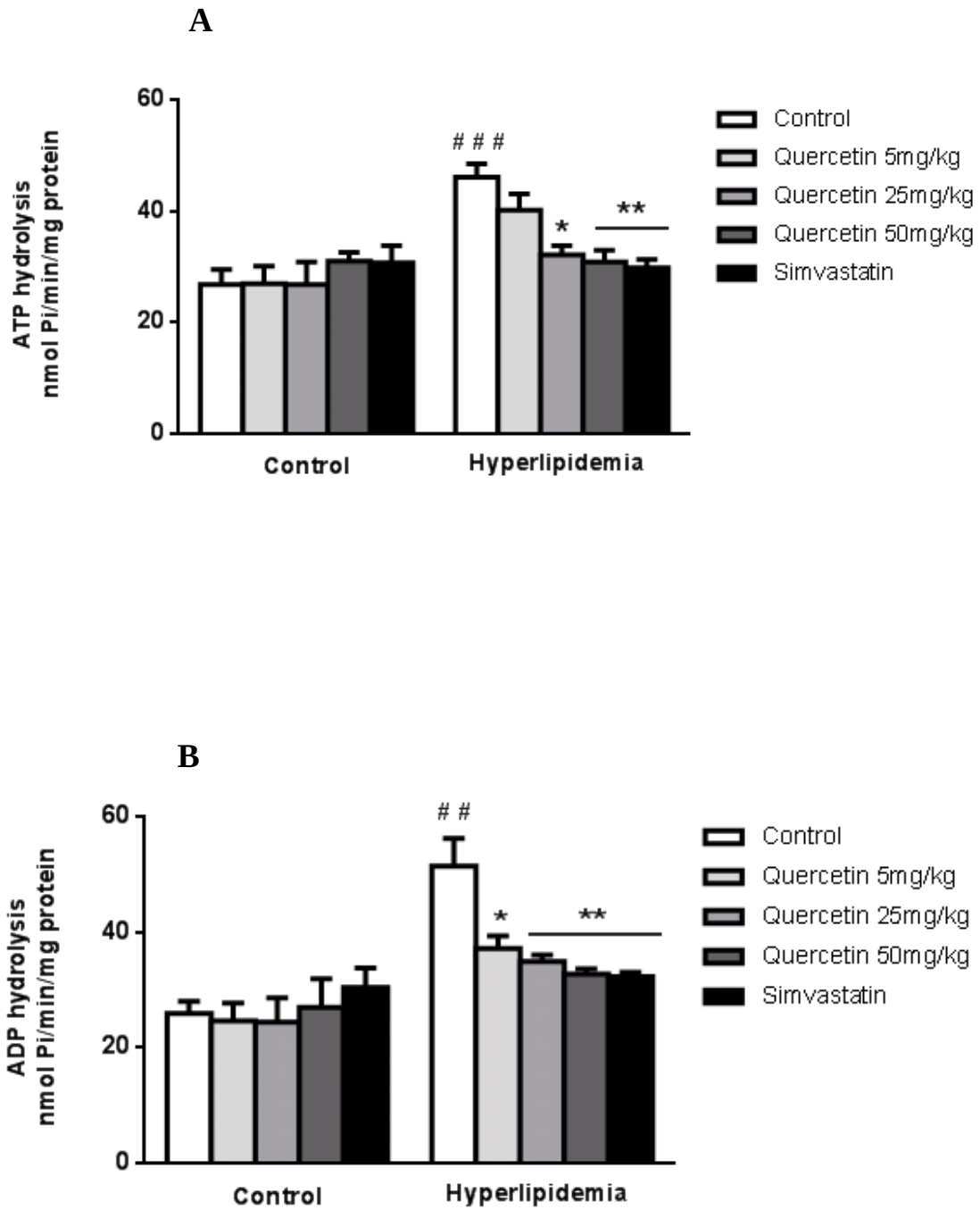
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Figure 2

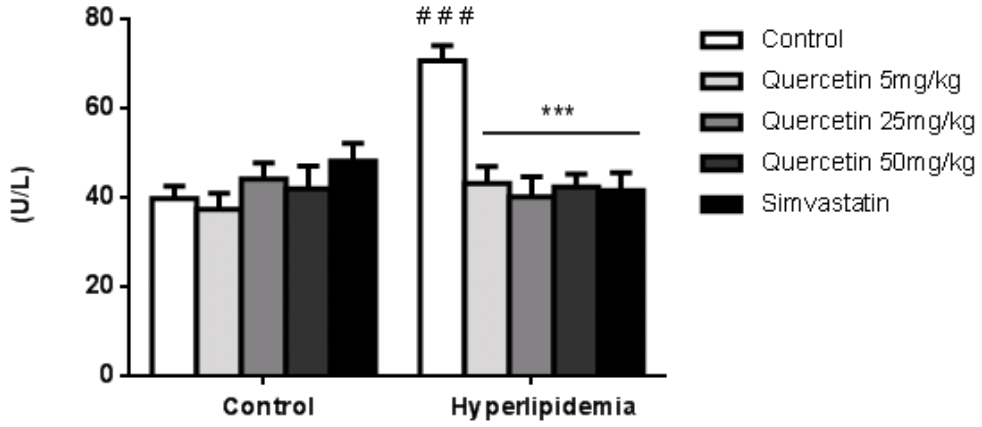
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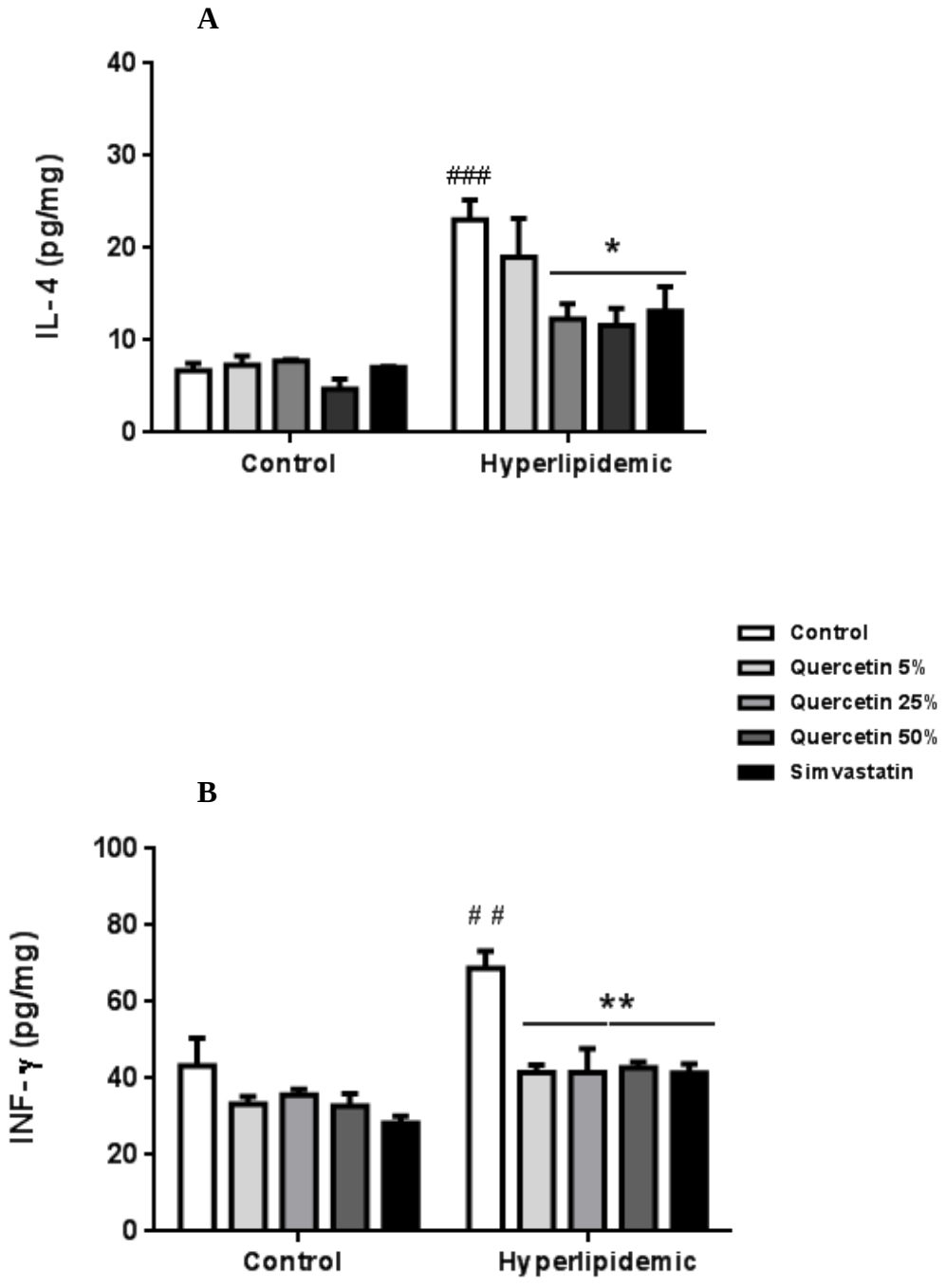
Figure 3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Control
Hyperlipidemic

Table 1: Purine levels in hyperlipidemic rats serum and pretreated with quercetin and treated with simvastatin

	ATP (nmol/mL of serum)	ADP (nmol/mL of serum)	AMP (nmol/mL of serum)	Adenosine (nmol/mL of serum)
C	2.81±0.17[a]	3.16±0.37[a]	0.51±0.02[a]	2.20±0.30[a]
CQ5	3.45±0.50[a]	3.40±0.21[a]	0.54±0.04[a]	2.27±0.34[a]
CQ25	3.09±0.49[a]	3.25±0.30[a]	0.57±0.03[a]	3.05±0.21[a]
CQ50	3.96±0.21[a]	3.08±0.22[a]	0.54±0.06[a]	2.82±0.26[a]
CS	3.51±0.06[a]	3.10±0.19[a]	0.54 ±0.06[a]	2.82±0.41[a]
H	3.01±0.42[a]	3.73±0.38[a]	0.60±0.10[a]	1.99±0.15[a]
H5	2.04±0.42[a]	2.91±0.21[a]	0.40±0.12[a]	2.66±0.29[a]
H25	2.16±0.37[a]	1.90±0.31[b]	0.41±0.08[a]	2.31±0.33[a]
H50	1.08±0.31[b]	2.02±0.26[b]	0.52±0.13[a]	2.72±0.43[a]
HS	1.12±0.26[b]	2.00±0.30[b]	0.29±0.15[a]	2.69±0.43[a]

Adenine nucleotides and adenosine levels measurement in serum of hyperlipidemics rats and pretreated for 30 days with quercetin (5, 25 and 50 mg/kg) and treated with simvastatin. Purine levels measurement were log-transformed and are reported as log of nmol/ml. Groups: saline control (C), saline+quercetin 5 mg/kg (CQ5), saline+quercetin 25 mg/kg (CQ25), saline+quercetin 50 mg/kg (CQ50), saline+simvastatin 0.04 mg/kg (CS), hyperlipidemia (H), hyperlipidemia+quercetin 5 mg/kg (HQ5), hyperlipidemia+quercetin 25 mg/kg (HQ25), hyperlipidemia+quercetin 50 mg/kg (HQ50) and hyperlipidemia+simvastatin 0.04 mg/kg (HS). Bars represent mean ± S.E.M. Groups with different letters are statistically different. (P<0.05; n=7) (two-way ANOVA-Tukey Multiple Comparison Test).

6 DISCUSSÃO

As alterações encontradas em nosso estudo confirmam o sucesso do modelo experimental de hiperlipidemia e o efeito benéfico do tratamento preventivo da quercetina. Um dos objetivos da nossa pesquisa foi avaliar se o pré-tratamento com quercetina possui efeito sobre o perfil lipídico em ratos com hiperlipidemia induzida. Como esperado, foram encontrados aumentos significativos nos níveis de colesterol total e triglicerídeos após 36 horas da indução com P407, confirmando a eficácia da indução. Contudo, os ratos hiperlipidêmicos quando receberam o tratamento preventivo de 30 dias com quercetina e o tratamento com sinvastatina, apresentaram uma diminuição significativa do nível de colesterol total e um aumento do HDL. Corroborando com nosso estudo, Mariee e colaboradores (2012) demonstraram uma redução sérica do colesterol total em 20% e LDL em 31%, e um aumento de 100% do HDL quando ratos com hiperlipidemia receberam a suplementação com quercetina. Juÿwiak e colaboradores (2005) também comprovaram em seu estudo que a quercetina foi eficaz na redução dos níveis séricos de triglicerídeos e colesterol total em animais hiperlipidêmicos. As propriedades do flavonoide foram associadas com a redução da formação de placas ateroscleróticas, sugerindo com isso que a quercetina possui propriedades hipolipidêmicas e antiaterogênicas.

Além da alteração do metabolismo lipídico, estudos indicam que a hiperlipidemia prejudica significativamente a função cognitiva (WINOCUR; GREENWOOD, 2005), a plasticidade sináptica do hipocampo (STRANAHAN *et al*., 2008), e a neurogênese (LINDQVIST *et al.*, 2006). Estudos clínicos também demonstraram que a ingestão elevada de gordura saturada prejudica a cognição (ESKELINEN *et al.*, 2008; KALMIJN, 2006). O colesterol desempenha um papel crucial no desenvolvimento e funcionamento normal do sistema nervoso central. O cérebro é rico em colesterol, e é um importante componente da membrana da bainha de mielina, assim como está presente em células gliais e também constitui as lipoproteínas (DIETSCHY; TURLEY, 2001). O colesterol mantém a plasticidade neuronal (KOUDINOV; BEREZOV, 2005), transporta a vesícula sináptica ao longo dos microtúbulos (KLOPFENSTEIN *et al.*, 2002) e participa na liberação de neurotransmissores (MAUCH *et al.*, 2001).

Em nossa pesquisa, também avaliamos a atividade da acetilcolinesterase (AChE) em diferentes estruturas cerebrais, a qual é uma enzima responsável por regular os níveis de acetilcolina no sistema nervoso, e também verificamos parâmetros comportamentais em ratos hiperlipidêmicos pré-tratados com quercetina e tratados com sinvastatina. Os nossos

resultados mostraram no teste de reconhecimento de objeto que houve um déficit significativo na memória de ratos hiperlipidêmicos, quando comparado com o grupo controle, sugerindo que a hiperlipidemia diminui a aquisição da memória. Francis e colaboradores (2013) revisaram as evidências de que uma dieta ocidental rica em gordura induziu prejuízo no sistema hipocampal e suas funções cognitivas associadas. O efeito da exposição a uma dieta rica em gordura (levando a hiperlipidemia) na aprendizagem e na memória também foi revisto por Cordner e Tamashiro (2015) e Kanoski e Davidson (2011). Estudos em animais sugerem que deficiências na função cognitiva após a exposição dieta rica em gordura estão associadas ao aumento da inflamação do cérebro (PISTELL *et al.,* 2010) e estresse oxidativo (MORRISON *et al.,* 2010) que podem ser consequências indiretas de disfunção metabólica sistêmica. Outros estudos relacionaram o colesterol elevado a um dano na barreira hematoencefálica, com deficiências nas tarefas de memória dependentes do hipocampo (KANOSKI *et al*., 2010).

A neuroinflamação está diretamente relacionada à diminuição das sinapses, o que causa um declínio cognitivo (HONG *et al.,* 2016). Nesta perspectiva, um composto ativo contra um ou mais desses fatores desencadeantes pode ser uma estratégia promissora para o tratamento desta doença. A quercetina, como dito anteriormente, é estudada devido seus amplos benefícios terapêuticos que envolvem seus efeitos antioxidantes e anti-inflamatórios (OLIVEIRA *et al*., 2016) e sua capacidade neuroprotetora (LU *et al.,* 2006). Nossos resultados demonstraram que quando o grupo hiperlipidêmico foi preventivamente tratado com quercetina (5, 25 e 50 mg/kg) e tratados com sinvastatina, o pré-tratamento foi capaz de evitar as alterações na memória causados pela hiperlipidemia, quando comparado com o grupo hiperlipidêmico. Deste modo, podemos sugerir que o pré-tratamento com quercetina é capaz de prevenir a perda de memória e de aprendizagem induzido pela hiperlipidemia. Estudos também relataram que a quercetina conseguiu reverter os déficits cognitivos induzidos pela Doença de Alzheimer (GAYOSO *et al.,* 2017; WANG *et al*., 2016). Esta capacidade dos flavonoides de reverter déficits cognitivos parece estar relacionada com a sua capacidade de interagir com as vias de sinalização neuronal, influenciando assim o sistema vascular periférico, protegendo os neurônios vulneráveis, aumentando a função neuronal existente ou estimulando a regeneração neuronal (SPENCER, 2009). Além disso, é conhecido que a quercetina pode atravessar a barreira hematoencefálica (YOUDIM *et al*., 2004), aumentando a resistência dos neurônios ao estresse oxidativo e à excitotoxicidade, modulando os mecanismos de morte celular (CHOI *et al.,* 2014; LIU *et al*., 2013). Com base nisto,

podemos sugerir que a quercetina pode ser um potencial coadjuvante para a prevenção e terapia de déficit de memória.

O efeito da hiperlipidemia no sistema colinérgico é outro aspecto importante que foi considerado neste estudo. O sistema colinérgico desempenha um papel crucial na regulação da aprendizagem e na memória (MESULAM *et al*., 2002). Nossos resultados demonstram que a hiperlipidemia diminuiu a atividade da AChE no hipocampo de ratos hiperlipidêmicos, e quando estes ratos foram pré tratados com quercetina (5, 25 e 50 mg/kg) e tratados com sinvastatina, houve uma prevenção desta diminuição da atividade desta enzima quando comparado com o grupo hiperlipidêmico. No entanto, não houve diferenças significativas na atividade da AChE nas outras estruturas cerebrais, incluindo córtex, hipotálamo, estriado e cerebelo. Estudos demonstram que o hipocampo, devido a sua maior concentração de colesterol do que outras estruturas cerebrais, responde mais rapidamente à hiperlipidemia do que outras estruturas cerebrais (GAN *et al.*, 2015). Possivelmente a não alteração na atividade da AChE nas demais estruturas cerebrais neste estudo, pode ser atribuída ao curto período de exposição (exposição aguda) ao P407 para o desenvolvimento de hiperlipidemia (36 h). Outras pesquisas também observaram alterações na atividade da enzima, contudo, nestes trabalhos foi observado um aumento significativo em sua atividade. Maciel e colaboradores (2016) mostraram um aumento na atividade da AChE em ratos diabéticos e quando estes ratos foram tratados com quercetina (25 e 50 mg/kg) conseguiu-se prevenir o aumento da atividade da AChE no cérebro (hipocampo e córtex) destes ratos. Pattanashetti e colaboradores (2017) também encontraram um aumento na atividade da AChE em homogeneizado de cérebro de rato com amnésia e quando estes ratos receberam um pré-tratamento com quercetina (25 mg/kg) reduziu significativamente o nível da atividade da AChE. Diante disto, podemos sugerir que a HL é a responsável pela diminuição da atividade da AChE.

A HL além de provocar distúrbios neuronais é capaz de desenvolver a aterosclerose, que é caracterizada por disfunção endotelial e também por um processo inflamatório (DAVIDSON *et al*., 2005; SINGH *et al*., 2002). O processo inflamatório é complexo e durante a ativação do sistema imune, ocorre uma interação entre vários componentes, incluindo moléculas do sistema purinérgico que contribuem para a regulação do processo inflamatório (BOURS *et al*., 2006; MANCINO *et al*., 2001). Em decorrência disto, nosso trabalho também investigou a atividade das enzimas E-NTPDase e E-ADA em linfócitos, as quais são enzimas que se encontram ancoradas na membrana de linfócitos, responsáveis pelo controle dos níveis fisiológicos de nucleotídeos e nucleosídeo no meio extracelular.

Um aumento na atividade da E-NTPDase foi observado em linfócitos de ratos com hiperlipidemia induzida em comparação com o grupo controle. Isto já era esperado uma vez que a E-NTPDase é conhecida como uma enzima chave que desempenha um papel importante na inflamação. Corroborando com nossos resultados, Duarte e colaboradores (2007) também encontraram em seu estudo um aumentado na atividade da E-NTPDase em plaquetas, na hidrólise de ATP e ADP, em pacientes com colesterol elevado, assim como Robson e colaboradores (1997) também demonstraram que as células endoteliais quando suplementadas com ácidos graxos saturados ou monoinsaturados apresentaram aumento na hidrólise de ATP e ADP. Este aumento na atividade desta enzima leva a um aumento na hidrólise dos nucleotídeos ATP e ADP, liberados durante um processo inflamatório, com o intuito de manter seus níveis fisiológicos, já que elevadas concentrações de ATP no meio extracelular acabariam contribuindo para um maior dano tecidual e inflamatório. Com base no exposto, podemos sugerir que a atividade elevada da E-NTPDase poderia agir de forma a normalizar os níveis dos nucleotídeos no meio extracelular. Sabe-se que altas concentrações de ATP no meio extracelular ativam receptores purinérgicos pro-inflamatórios, e que a E-NTPDase tem importante participação na modulação da resposta inflamatória na hiperlipidemia.

As enzimas do sistema purinérgico atuam em cascata, e já que a atividade da E-NTPDase apresentou-se elevada, e a atividade da E-5’nucleotidase não foi avaliada em linfócitos em nosso estudo, podemos sugerir que sua atividade também esteja aumentada a fim de compensar os efeitos pró-inflamatórios da sinalização das moléculas de ATP e ADP. No entanto, nestes animais hiperlipidêmicos, os níveis de ATP, ADP e AMP estão normais no meio extracelular.

A adenosina é um purino nucleosídeo gerado da desfosforilação consequente do ATP, através da ação consecutiva da E-NTPDase e E-5’nucleotidase, como também pode ser encontrada no meio extracelular por ser liberada pelas células endoteliais nos casos de hipóxia (MARSHALL, 2000). Em nossa pesquisa também observamos que a atividade de E-ADA foi aumentada em linfócitos de ratos hiperlipidêmicos quando comparada ao grupo controle. A atividade da E-ADA pode estar aumentada pela suposta atividade aumentada de E-5'-nucleotidase, uma vez que os níveis de adenosina estão em concentrações normais no meio extracelular em animais com hiperlipidemia. Um aumento da E-ADA em linfócitos pode levar a um aumento na produção de inosina extracelular, a qual também apresenta propriedade anti-inflamatória, sendo capaz também de suprimir o TNF-α (HASKÓ *et al*., 2000). Outros estudos também já demonstraram atividade elevada da E-ADA em processos inflamatórios (ABDALLA *et al.*, 2014; RODRIGUES *et al.*, 2014).

Em suma, a regulação dos nucleotídeos através das enzimas E-NTPDase e E-ADA em resposta à inflamação, representa um importante controle na regulação da concentração das purinas no meio extracelular na hiperlipidemia. Com isso, podemos afirmar que as ectoenzimas do sistema purinérgico estão envolvidas e desempenham um papel muito importante nos processos inflamatórios. As alterações na atividade das enzimas nestas células do sistema imune, tais como os linfócitos, só vem a contribuir para a melhora e reversão do processo inflamatório.

As propriedades anti-inflamatórias da quercetina já foram bem descritas na literatura (LIN *et al.* 2017; OLIVEIRA *et al.*, 2016). Nossos resultados demonstram um efeito positivo do pré-tratamento com quercetina. Em ratos hiperlipidêmicos pré-tratados com quercetina e tratados com sinvastatina, o aumento nas atividades da E-NTPDase e E-ADA foram evitadas, enquanto que ATP e ADP no soro mostraram-se reduzidos nestes grupos. Os resultados obtidos em nosso estudo estão de acordo com vários outros estudos que sugeriram que a quercetina inibe as atividades das ectonucleotidases. Schmatz e colaboradores (2013) demonstraram que a quercetina inibe a hidrólise de ATP, ADP e AMP *in vitro* em plaquetas de ratos controle e diabéticos. Baldissarelli e colaboradores (2016) também verificaram que animais com hipotireoidismo quando foram tratados com quercetina apresentaram uma diminuição na atividade da E-NTPDase e E-ADA em sinaptossomas do córtex cerebral. Essa diminuição poderia contribuir com níveis aumentados de adenosina, que atua como uma molécula anti-inflamátória. Estes resultados também confirmam as propriedades inibitórias da quercetina nas atividades de ectonucleotidases em patologia, sendo devido à interação de enzimas com diferentes estruturas das moléculas de flavonoides (BALDISSARELLI *et al.*, 2016). Esses resultados reforçam as evidências de que compostos naturais como a quercetina podem afetar o sistema purinérgico causando uma redução nos processos inflamatórios causados pela hiperlipidemia.

Os nucleotídeos ATP e ADP e o nucleosídeo adenosina representam uma importante classe de moléculas extracelulares envolvidas na modulação de vias de sinalização para o funcionamento normal do sistema imunológico (YEGUTKIN, 2008). Além disso, está bem estabelecido que o ATP atua através de receptores celulares específicos e está envolvido em funções pró-inflamatórias, como estimulação e proliferação de linfócitos e liberação de citocinas, enquanto a adenosina exibe potentes ações anti-inflamatórias e imunossupressoras (DEAGLIO *et al.*, 2007, GESSI *et al.*, 2007).

A aterosclerose é a principal patologia subjacente de muitas DCV, a qual envolve células do sistema imune inato e adaptativo (ROSS, 1999). A patogênese da aterosclerose

geralmente depende da dislipidemia, resultando na retenção de lipoproteínas nas artérias, a modificação local das lipoproteínas retidas e o influxo de monócitos que se diferenciam em macrófagos que absorvem as lipoproteínas modificadas, formando assim as células de espuma. Essas células de espuma, bem como outras células na parede do vaso, produzem citocinas com consequente influxo de monócitos adicionais, bem como uma variedade de células do sistema imune adaptativo, sendo as mais notáveis as células T, que se tornam ativadas localmente (GETZ; REARDON, 2014). As células T predominantemente envolvidas na inflamação aguda e na promoção da aterosclerose são as células Th1, cujo produto primário é o IFN-γ. Por outro lado, os níveis de IL-4, que é produzida pelas células Th2, tem pouco impacto nos lipídios plasmáticos ou lipoproteínas e resultados variáveis na aterosclerose (KLEEMANN *et al.*, 2011; LIBBY *et al.*, 2013; LICHTMAN *et al.*, 2013).

Diante do exposto, outro parâmetro avaliado neste estudo foi os níveis sorológicos de citocinas em ratos hiperlipidêmicos. Nossos resultados demonstram níveis elevados de IFN-γ em ratos com hiperlipidemia induzida quando comparado com o grupo controle. Corroborando com nossos dados, Houssen e colaboradores (2011) também demonstraram níveis plasmáticos elevados de mediadores inflamatórios, entre eles o IFN-γ, em animais ateroscleróticos, quando comparado com o grupo controle. O INF-γ tem funções imunomoduladoras e inflamatórias, pois contribui para a progressão da aterosclerose e promove a adesão de leucócitos nas lesões vasculares (GIRN *et al.,* 2007). Além disso, foi relatado que o IFN-γ induz a expressão de receptores scavenger para a absorção de lipoproteínas modificadas (células de espuma) e estimula a proliferação de macrófagos (RIDKER *et al.,* 2000). Diante do exposto, podemos sugerir que a hiperlipidemia desencadeia um estado inflamatório com aumento dos níveis séricos de IFN-γ, que está em consonância com um possível desenvolvimento da aterosclerose.

Nossos resultados também demonstram que os níveis de INF-γ diminuíram com o pré-tratamento com quercetina 5, 25 e 50 mg/kg e tratamento com sinvastatina. Estudos demonstram que o tratamento com quercetina inibe mediadores pró-inflamatórios (TNF-α, IL-1, IL-6 and IL-8) (CRUZ *et al.*, 2012; PARK *et al.*, 2009), provavelmente devido à inibição de NF-κB (MIN *et al.,* 2007). No entanto, podemos sugerir que o pré-tratamento com quercetina é eficaz na redução de mediadores inflamatórios, sendo eficaz na prevenção da hiperlipidemia.

Outra citocina avaliada em nosso estudo foi a IL-4, cujos resultados demonstram níveis aumentados de IL-4 em ratos hiperlipidêmicos quando comparados com o grupo controle. E quando esses ratos foram pré-tratados com quercetina 25 e 50 mg/kg e tratados

com sinvastatina, houve uma diminuição dos níveis de IL-4. Em consonância com nosso estudo, Gonzalo-Calvo e colaboradores (2015) evidenciaram que quanto mais elevados os níveis de LDL e triglicerídeos em pacientes hipercolesterolêmicos havia uma maior expressão da IL-4. Mito e colaboradores (2000) também demonstraram que a produção de IL-4 e o IFN-γ por linfócitos em ratos obesos é aumentada. Xu e colaboradores (2017) encontraram um aumento nos níveis de IL-4 e IFN-γ em ratos com colesterol elevado, e quando estes ratos foram tratados com quercetina seus níveis foram diminuídos, sugerindo que a quercetina modifica a disfunção do metabolismo lipídico e tem a propriedade de inibir o aumento de citocinas inflamatórias. Além disso, a quercetina diminui o recrutamento de eosinófilos, reduz o nível de IL-4 e inibe a ativação de NF-κB em ratos com processo inflamatório, regulando a resposta inflamatória por diminuição do nível IL-4 (ROGERIO *et al.*, 2010). Em conjunto, podemos sugerir que a IL-4 pode participar do metabolismo lipídico, e a quercetina tem a capacidade de suprimir a produção de citocinas inflamatórias e diminuir a anormalidade lipídica do sangue através da regulação do sistema metabólico.

Diante do exposto, podemos sugerir que a quercetina, um produto bioativo gerado a partir de plantas, possui potentes efeitos antioxidantes, anti-inflamatórios e neuroprotetores. O pré-tratamento com quercetina possivelmente inibe as vias inflamatórias, modulando o sistema purinérgico, assim como reverte os danos causados no sistema colinérgico induzido pela hiperlipidemia.

7 CONCLUSÕES

- O tratamento preventivo com quercetina pode melhorar as alterações do perfil lipídico provocado pela hiperlipidemia, prevenindo o desenvolvimento de doenças cardiovasculares. A prevenção com quercetina parece ter desempenhado um papel hipolipidêmico.

- A hiperlipidemia pode induzir um comprometimento da memória de curto prazo. Já a quercetina pode atenuar este déficit de memória. A quercetina demonstrou ser eficaz em diminuir o dano cognitivo de memória causado pela hiperlipidemia.

- A hiperlipidemia diminuiu a atividade da AChE no hipocampo, e a quercetina foi capaz de reverter os efeitos hipocolinérgicos causados pela diminuição da AChE. O tratamento preventivo com quercetina mostrou-se um potente agente terapêutico coadjuvante para o tratamento da hiperlipidemia associada à disfunção cognitiva.

- A hiperlipidemia aumentou a atividade da E-NTPDase e da E-ADA em linfócitos e o pré-tratamento com as diferentes doses de quercetina preveniu estas alterações. Esta modulação na atividade das enzimas representa um importante controle de nucleotídeos e nucleosídeo extracelulares liberados em resposta ao processo inflamatório desenvolvido na hiperlipidemia.

- O pré-tratamento com quercetina foi capaz de reduzir os níveis de citocinas pró-inflamatórias secretadas durante a hiperlipidemia. A quercetina foi capaz de atenuar as alterações causadas pela hiperlipidemia provavelmente pela regulação do processo inflamatório, devido as suas atividades anti-inflamatórias, antioxidantes e hipolipidêmicas.

- A quercetina possui um efeito antiaterogênico terapêutico, não só através da redução de lipídios e/ou lipoproteínas plasmáticas, mas também na indução de mecanismos moduladores da inflamação. O tratamento preventivo com quercetina levou a uma redução nos processos inflamatórios causados pela hiperlipidemia, sendo um composto promissor como adjuvante no tratamento de doenças.

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ANEXOS

ANEXO 1- CARTA DE APROVAÇÃO

Comissão de Ética no Uso de Animais
da
Universidade Federal de Santa Maria

Santa Maria, 29 de abril de 2015
CEUA N 9894021214

Ilmo(a). Sr(a).
Responsável: Daniela Bitencourt Rosa Leal
Depto/Disc: Microbiologia E Parasitologia

Título do projeto: "EFEITO PREVENTIVO DO FLAVONÓIDE QUERCETINA NO SISTEMA PURINÉRGICO E PERFIL OXIDATIVO EM PLAQUETAS DE MODELO EXPERIMENTAL DE HIPERLIPIDEMIA".

Parecer Concubstanciado da Comissão de Ética no Uso de Animais UFSM

A hiperlipidemia é um fator de risco para o surgimento da aterosclerose, sendo esta uma doença inflamatória crônica caracterizada por anormalidades lipídicas e estresse oxidativo. Dentre os mediadores capazes de modular as ações das plaquetas, durante o processo inflamatório, destacam-se o ATP, ADP, AMP e adenosina, correlacionando-se diretamente à atividade das ectoenzimas E-NTPDase, E-5'-nucleotidase, E-NPP (ecto-nucleosídeo pirofosfatase fosfodiesterase) e Adenosina desaminase (E-ADA). O flavonóide quercetina possui capacidade antioxidante, anti-inflamatória, bem como efeito hipolipemiante. Portanto, este projeto utilizará a quercetina de maneira preventiva, sendo as concentrações de 5, 25 e 50mg/kg/dia, por um período de 30 dias e então será induzida a hiperlipidemia de forma aguda, com 400mg/kg de Poloxamer-407 via intraperitoneal. Tendo como objetivo avaliar a atividade das ectoenzimas E-NTPDase, E-5'-nucleotidase, E-NPP e E-ADA em plaquetas e o perfil oxidativo nos tecido hepático, renal, esplênico e cardíaco de ratos com e sem hiperlipidemia. Para a avaliação do estresse oxidativo serão determinadas as atividades das enzimas catalase e superóxido dismutase, o conteúdo de tióis não-proteicos, o produto da peroxidação lipídica e a carbonilação proteíca. Além disso, serão determinadas as concentrações séricas dos nucleotídeos e nucleosídeo, parâmetros bioquímicos e histologia dos órgãos. Espera-se assim, investigar o potencial efeito preventivo do flavonoide quercetina em ratos hiperlipidêmicos, a fim de contribuir para a busca de novas terapias complementares, que possam beneficiar pacientes e entender melhor a hiperlipidemia.

A Comissão de Ética no Uso de Animais da Universidade Federal de Santa Maria, na reunião de 29/04/2015, **ANALISOU** e **APROVOU** todos os procedimentos apresentados neste protocolo.
1. Comunicar toda e qualquer alteração do protocolo.
2. Comunicar imediatamente ao Comitê qualquer evento adverso ocorrido durante o desenvolvimento do protocolo.
3. Os dados individuais de todas as etapas da pesquisa devem ser mantidos em local seguro por 5 anos para possível auditoria dos órgãos competentes.
4. **Relatórios parciais** de andamento deverão ser enviados **anualmente** à CEUA até a conclusão do protocolo.

Atenciosamente,

Profa. Dra. Vânia Lucia Loro
Coordenadora da Comissão de Ética no Uso de Animais
Universidade Federal de Santa Maria

ANEXO 2- CARTA DE SUBMISSÃO DO MANUSCRITO

---------- Forwarded message ----------
From: **Molecular and Cellular Biochemistry (MCBI)** <em@editorialmanager.com>
Date: 2017-08-09 22:50 GMT-03:00
Subject: MCBI-D-17-00905 - Submission Confirmation
To: Daniela Bitencourt Rosa Leal <danibrl@smail.ufsm.br>

Dear Dr. Leal:

Thank you for submitting your manuscript, "Pre-treatment with quercetin prevents changes in lymphocytes E-NTPDase/E-ADA activities and cytokines secretion in hyperlipidemic rats", to Molecular and Cellular Biochemistry.

The submission id is: MCBI-D-17-00905
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