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Alessandra Guedes Manzoni

AVALIAÇÃO DO EFEITO DE RUTINA E CURCUMINA EM MARCADORES OXIDATIVOS, HEMATOLÓGICOS E PURINERGICOS EM RATOS COM HIPERLIPIDEMIA INDUZIDA

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

Orientadora: Prof.ª Dr.ª Daniela Bitencourt Rosa Leal

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2022

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Dedico este trabalho a minha familia em especial ao meu filho Lorenzo, meu marido Nicolas, meus pais Gilnei e Betânia a e à meu irmão Alexandre.
Vocês são a força e os motivos que me fazem querer ser sempre melhor.

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“Se a educação sozinha não transforma a sociedade, sem ela tampouco a sociedade muda”.

(Paulo Freire)

RESUMO

AVALIAÇÃO DO EFEITO DE RUTINA E CURCUMINA EM MARCADORES OXIDATIVOS, HEMATOLÓGICOS E PURINERGICOS EM RATOS COM HIPERLIPIDEMIA INDUZIDA

AUTORA: Alessandra Guedes Manzoni
ORIENTADORA: Prof.ª Dr.ª Daniela Bitencourt Rosa Leal

O acúmulo de lipídeos, como o colesterol total, triglicerídeos e LDL, na corrente sanguínea é conhecido como hiperlipidemia. Essa condição é caracterizada por gerar danos inflamatórios e oxidativos tanto em vasos sanguíneos quanto em diversos órgãos do corpo, podendo levar ao desenvolvimento da aterosclerose. A E-NTPDase, a 5' nucleotidase e a ADA são enzimas do sitema purirnergico que possuem a capacidade de modular a resposta imune e inflamatória assim como o metabolismo de lipídios. A rutina e a curcumina são polifenóis, conhecidos por apresentarem ações antioxidantes, anti-inflamatórias e também podem ser capazes de modular o sistema purinérgico. O objetivo do presente estudo foi avaliar as alterações hematológicas, bioquímicas, oxidativas e celulares causadas pela hiperlipidemia, bem como avaliar se os compostos rutina e curcumina são capazes de prevenir estas alterações. Ratos machos Wistar adultos foram pré-tratados por gavagem com rutina e/ou curcumina (50mg/kg) durante 30 dias. A hiperlipidemia foi induzida, de forma aguda, mediante administração intraperitoneal de Poloxamer-407 (P-407). Após 36 horas os animais foram eutanasiados e foi coletado o sangue para hemograma e separação de células, e coletado tecidos como coração, baço, rim e fígado para a avaliação da atividade das enzimas oxidantes e antioxidantes, e atividade da NTPDase, 5' nucleotidase e ADA. A indução da hiperlipidemia com P-407 causou alterações hematológicas como a redução no numero de linfócitos e eosinófilos em relação aos leocócitos totais, causou o aumento nos níveis de ácido urico e albumina, além de aumentar os niveis de TBARS e carbonil em fígado rim e coração, e causou a redução das defesas antioxidantes em fígado e rim. Também foram observadas alterações nas atividades das enzimas purinérgicas em membrana de coração e plaquetas. Os pré-tratamentos com rutina e/ou curcumina, foi capaz de reduzir os niveis de TBARS e carbonil e aumentar as defesas antioxidantes no fígado e rim, além de previnir as alterações nos parâmetros hematológicos. Dessa forma, foi demonstrado que curcumina e rutina preveniram a maioria dos danos causados pela hiperlipidemia.

Palavras-chaves: hiperlipidemia, sistema purinérgico, estresse oxidativo.

ABSTRACT

EVALUATION OF THE EFFECT OF RUTIN AND CURCUMIN ON OXIDATIVE, HEMATOLOGICAL AND PURINERGIC MARKERS IN RATS WITH INDUCED HYPERLIPIDEMIA

The accumulation of lipids, such as total cholesterol, triglycerides, and LDL, in the bloodstream is known as hyperlipidemia. This condition is characterized by generating inflammatory and oxidative damage both in blood vessels and in various organs of the body, which can lead to the development of atherosclerosis. E-NTPDase, 5' nucleotidase and ADA are enzymes of the purynergic system that have the ability to modulate the immune and inflammatory response as well as lipid metabolism. Rutin and curcumin are polyphenols, known to have antioxidant and anti-inflammatory actions and may also be able to modulate the purinergic system. The aim of the present study was to evaluate the hematological, biochemical, oxidative and cellular changes caused by hyperlipidemia, as well as to assess whether the compounds rutin and curcumin are able to prevent these changes. Adult male Wistar rats were pre-treated by gavage with rutin and/or curcumin (50mg/kg) for 30 days. Hyperlipidemia was acutely induced by intraperitoneal administration of Poloxamer-407 (P-407). After 36 hours, the animals were euthanized and blood was collected for hemogram and cell separation, and tissues such as heart, spleen, kidney and liver were collected for the evaluation of the activity of oxidant and antioxidant enzymes, and activity of NTPDase, 5' nucleotidase and ADA. The induction of hyperlipidemia with P-407 caused hematological changes such as a reduction in the number of lymphocytes and eosinophils in relation to total leukocytes, increased levels of uric acid and albumin, as well as increased levels of TBARS and carbonyl in liver, kidney and heart, and caused the reduction of antioxidant defenses in liver and kidney. Changes in the activities of purinergic enzymes in heart membrane and platelets were also observed. Pre-treatments with rutin and/or curcumin were able to reduce the levels of TBARS and carbonyl and increase the antioxidant defenses in the liver and kidney, in addition to preventing changes in hematological parameters. Thus, curcumin and rutin were shown to prevent most of the damage caused by hyperlipidemia.
Keywords: hyperlipidemia, purinergic system, oxidative stress.

LISTA DE FIGURAS

LISTA DE ABREVIATURAS E SIGLAS

ADP	Adenosina Difosfato
AMP	Adenosina Monofosfato
AST	Aspartato Aminotransferase
ATP	Adenosina Trifosfato
CAT	Catalase
CD39	NTPDase
CD73	5'-nucleotidase
CEUA	Comitê de Ética no Uso de Animais
CT	Colesterol Total
DMSO	Dimetilsulfóxido
DNA	Ácido Desoxirribonucleico
E-ADA	Ecto-adenosina Desaminase
EDTA	Ácido Etilenodiaminotetratécito
E-NTPDase	Ecto-nucleosídeo Trifosfato Difosfoidrolase
E-5-NT	Ecto-5'-nucleotidase
EROs	Espécies Reativas de Oxigênio
GST	Glutationa S-transferase
HDL	Lipoproteínas de Densidade Alta
LABIBIO	Laboratório de Imunobiologia Experimental e Aplicada
LDL	Lipoproteínas de Densidade Baixa
LDL-ox	Lipoproteínas de Densidade Baixa oxidada
LP	Lipase Lipoproteica
NPSH	Tiois Não-Proteicos
P-407	Poloxamer-407
SOD	Superóxido Dismutase
TBARS	Espécies Reativas ao Ácido Tiobarbitúrico
TG	Triglicerídeo
UFSM	Universidade Federal de Santa Maria
VLDL	Lipoproteínas de Densidade Muito Baixa

INDICE

APRESENTAÇÃO

Esta Tese está subdividida em seções dispostas da seguinte maneira: Introdução, constituída de um embasamento teórico-científico dos principais tópicos abordados no estudo, justificativa, e objetivos, composto das finalidades da pesquisa. As subdivisões Materiais e Métodos e Resultados encontram-se nos Artigos.

O primeiro artigo está publicado na revista *"International Immunopharmacology" IF: 5.714.* O segundo artigo está publicado na revista *" Cardiovascular Drugs and Therapy " IF: 3.947.*

Os itens Discussão e Conclusões contêm interpretações e comentários dos resultados obtidos. Por fim, a seção Referências refere-se às citações mencionadas ao longo das seções Introdução e Discussão.

1- INTRODUÇÃO

1.1 Lipideos na hiperlipidemia

Os lipídeos são moléculas orgânicas geradas de componentes químicos diversos que possuem em comum a característica de serem insolúveis em água. Estas biomoléculas desempenham uma série de funções cruciais no corpo humano, como papel estrutural, participação como cofatores enzimáticos, transportadores de elétrons, agentes emulsificantes do trato intestinal, entre outros (NELSON; COX, 2018).

Um importante exemplo desta classe são os triglicerídeos (TG), ou também chamado de triacilglicerois que são uma classe de moléculas, formados pela união de um glicerol e três ácidos graxos ligados através de ligações éster, que variam de acordo com o tipo do triglicerideo. Estas moléculas fazem parte da classe de lipídeos responsáveis pelo transporte e armazenamento de ácidos graxos dentro de células, como nos adipócitos, e também no plasma (NELSON; COX, 2018).

Outro representante importante da classe dos lipídeos é o colesterol, constituído por um grupo polar e um corpo hidrocarbonado apolar, contendo um núcleo esteroide e a cadeia lateral hidrocarbonada. O colesterol é o principal esterol produzido por mamíferos e desempenha um papel crucial na manutenção da fluidez e permeabilidade da membrana celular, além de atuar como precursor de hormônios esteroidais, ácidos biliares e vitamina D (BHATNAGAR; SORAN; DURRINGTON, 2008).

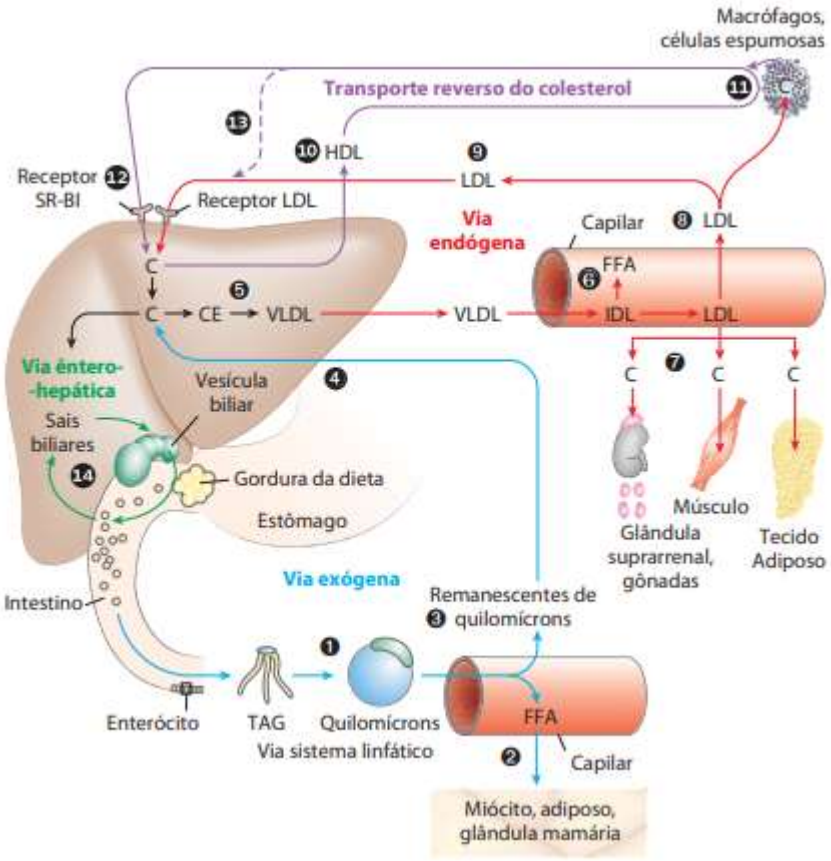
Assim como os demais lipídeos, o colesterol também é insolúvel em água, e assim precisa de uma ligação com determinadas proteínas para que possa ser transportado no sangue. A união de moléculas de colesterol e proteínas forma as lipoproteínas, que são agregados esféricos com lipídeos hidrofóbicos no centro e cadeias laterais hidrofílicas de proteínas com grupos polares de lipídeos na superfície (NELSON; COX, 2018).

As combinações de lipídeos e proteínas produzem partículas de densidades diferentes que são divididas em: lipoproteínas de densidade muito baixa VLDL (*Very low density lipoproteins*) de baixa densidade LDL (*Low density lipoproteins*), e as de alta densidade HDL (*High density lipoproteins*) (NELSON; COX, 2018).

Apesar da importância fisiológica destas moléculas, o seu aumento na corrente sanguínea pode ser extremamente prejudicial. Quantidades superiores a ≥ 160 mg/dl de LDL, é considerado um mau prognóstico para o desenvolvimento de dislipidemias e

progressão para doenças metabólicas e cardiovasculares, e ainda quando acompanhado por um aumento dos níveis de colesterol total (CT) (≥ 200mg/dl) e TG (≥150 mg/dl) na corrente sanguínea, caracteriza uma condição chamada de hiperlipidemia. Por outro lado, temos a molécula de HDL, esta molécula é responsável pela captação do excesso de colesterol circulante, fazendo com que este excesso seja transportado até o fígado para que possa ser eliminado via bile (Figura 1) (OTUNOLA et al., 2010; XAVIER et al., 2013).

Figura 1: Transporte do colesterol



A figura mostra o transporte de colesterol pelas lipoproteínas, o LDL é a lipoproteína responsável por carregar o colesterol presente no fígado ate diversas células do corpo como a glândula suprarrenal, músculos e tecido adiposo, enquanto o HDL fica responsável por carregar o excesso de colesterol presente nas células até o fígado, para que possa ser excretado. Alem disso também temos a participação de outras lipoproteínas como VLDL e quilomícrons. Fonte: Princípios de Bioquímica de Lehninger 6º edição, 2014.

1.2 Hiperlipidemia

Os tipos de dietas e estilo de vida são um fator determinante para o aumento de lipídeos. O excesso de gorduras saturadas, açúcares refinados, e a falta de alimentos saudáveis como frutas e verduras, estão diretamente ligados ao risco elevado de desenvolvimento de algum tipo de dislipidemia. Independentemente dos fatores que causam o excesso de lipídeos, sabe-se que aumento plasmático das concentrações de colesterol, está diretamente associado ao desenvolvimento de doenças cardiovasculares como a aterosclerose (XAVIER et al., 2013).

A hiperlipidemia é um tipo de dislipidemia que é caracterizada por um aumento nos níveis de lipídeos e/ou lipoproteínas. O acúmulo de lipídeos causa diversas alterações dentre as quais se destacam a deposição de lipídeos no endotélio, causando oxidação do LDL e a formação de células espumosas, com ativação e recrutamento de células imunes, além de produção de espécies reativas de oxigênio (EROs) e dano tecidual. Todos estes processos contribuem para a formação do trombo e para a progressão para aterosclerose (VAN DIEPEN et al., 2013).

Estima-se que 16,5% dos brasileiros, 12,9% dos norte-americanos e 36,5% dos chineses apresentem hiperlipidemia, assim como 53% dos norte-americanos teriam LDL elevado (DE OLIVEIRA et al., 2017; KARR, 2017). Para os indivíduos que apresentam hiperlipidemia, o risco de desenvolvimento de doenças cardiovasculares chega a ser duas vezes maior. (FALUDI et al., 2017; KARR, 2017).

A aterosclerose promove um acúmulo de leucócitos e plaquetas nas paredes das artérias e é caracterizada pela presença de inflamação crônica (HANSSON KH, 2005; ROCHA; LIBBY, 2009). Além do mais, estudos epidemiológicos apontam que níveis excessivos de colesterol LDL podem levar ao desenvolvimento de doenças cardiovasculares, mesmo na ausência de outros fatores de risco como fatores genéticos e ambientais (SOZEN; OZER, 2017).

Embora os mecanismos não estejam bem definidos, a associação entre hiperlipidemia e a aterosclerose vem sendo demonstrada por várias décadas, e recentemente têm recebido bastante atenção na prática clínica. Um dos mecanismos pelo qual a hiperlipidemia gera aterosclerose, é através da ação pro-aterogenica do LDL e de outros lipídeos, que se depositam na parede da artéria e sofrem o processo de oxidação dando origem às células espumosas, e também através da imunogenicidade dos macrófagos ativados que geram

uma exacerbação no processo inflamatório (ZÁRATE et al., 2016; FALUDI et al., 2017; FENG et al., 2018). A partir de então, a redução dos níveis de lipídios passou a ser uma das prioridades na manutenção da saúde da população (STEINBERG, 2005). Embora a relação entre hiperlipidemia e doenças cardiovasculares seja conhecida, atualmente sabe-se que ela também pode causar doenças renais, diabetes tipo 2, hipertensão e hipotireoidismo (KARR, 2017).

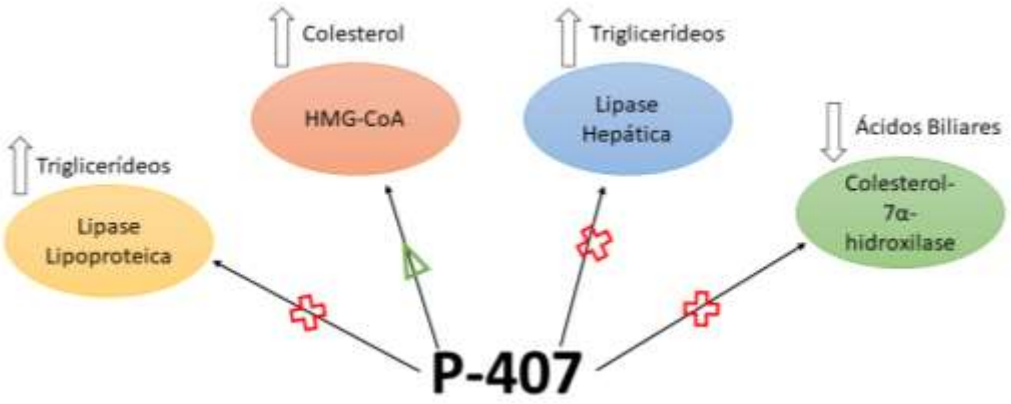
O depósito de lipoproteínas na parede arterial ocorre de maneira proporcional à concentração destas lipoproteínas no plasma (FALUDI et al., 2017). Além disso, a presença contínua de hipercolesterolemia é necessária para a manutenção e progressão da lesão, pelo menos no caso de lesões relativamente precoces. Desta forma, a hiperlipidemia como iniciadora do processo aterosclerótico e a inflamação como resposta à lesão vascular causada pelo acúmulo de lipoproteínas são fatores complementares na patogênese da aterosclerose (STEINBERG, 2002).

1.3 Poloxamer-407 como indutor da hiperlipidemia

Apesar de a hiperlipidemia ser uma condição desencadeada por fatores alimentares, ambientais e comportamentais, alguns compostos naturais ou sintéticos são capazes de mimetizar, em parte, a ação que esta condição causaria. Para investigar a utilização desses compostos, o Poloxamer 407 (P-407), que é um surfactante não iônico hidrofílico da classe mais geral de copolímeros conhecidos como poloxâmeros, vem sendo utilizado como indutor de hiperlipidemia em modelos animais (JOHNSTON; KOROLENKO; SAHEBKAR, 2017).

O principal mecanismo envolvido na indução de hiperlipidemia pelo P-407 é a inibição da enzima lipase lipoproteica (LP), responsável pela hidrólise dos TG, causando um aumento nos níveis destes lipídeos na corrente sanguínea. O P-407 é responsável por estimular a atividade da 3-hidroxi-3-metilglutaril coenzima A (HMG-CoA), uma enzima chave para a síntese de colesterol, causando o aumento na síntese deste lipídeo. Além disso, as enzimas lipase hepática (HL) e colesterol-7α-hidroxilase (C7αH), responsáveis pela hidrólise de TG e síntese de ácidos biliares, respectivamente, podem ser inibidas (Figura 2) (JOHNSTON; PALMERS, 1993; JOHNSTON; KOROLENKO; SAHEBKAR, 2017).

Figura 2: Mecanismo de ação do poloxamer-407

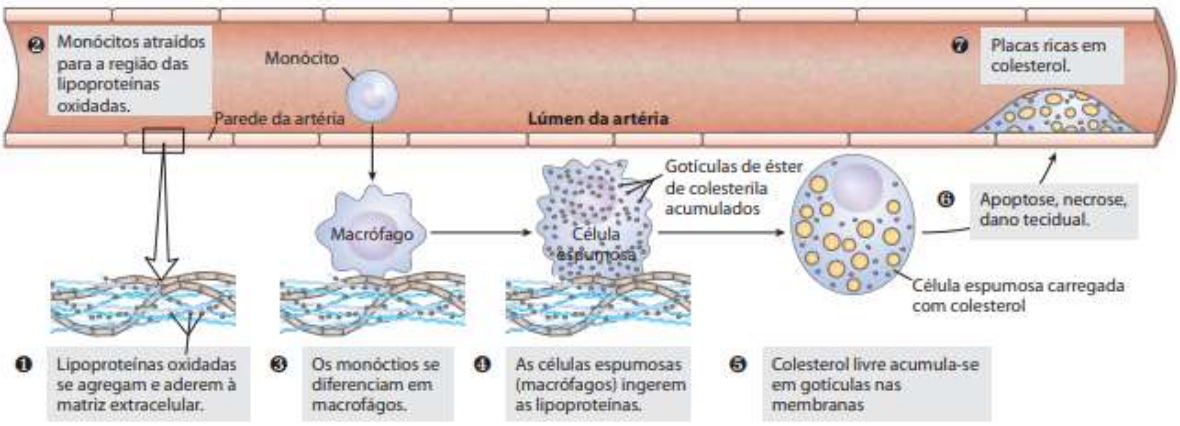


A figura mostra um esquema do mecanismo de ação do poloxamer – 407 (P-407), O X representa o bloqueio da atividade da enzima e o $\triangle$ representa a estimulação da atividade da enzima. Fonte: Elaborada pelo autor

1.4 Alterações celulares causadas pela hiperlipidemia

Sabemos que, na corrente sanguínea, quando os níveis de TG e LDL estão aumentados, e os níveis de HDL insuficientes, o transporte de colesterol é prejudicado, fazendo com que se deposite na parede arterial (LIN et al., 2009). Essas moléculas, quando ficam retidas, sofrem um processo de oxidação que é responsável por torná-las imunogênicas e agravar o processo de agressão ao endotélio vascular. Em resposta a estas alterações os monócitos migram para o espaço subendotelial onde se diferenciam em macrófagos e captam o LDL-oxidado (LDL-ox). Os macrófagos são ativados, assim como outras células inflamatórias, como as células T, que podem estar associadas à evolução da placa aterosclerótica e à amplificação da inflamação através da secreção de citocinas e enzimas proteolíticas (Figura 3) (XAVIER et al., 2013).

Figura 3: Formação da placa aterosclerótica



A figura mostra o esquema de formação de células espumosas e formação da placa aterosclerótica.

Fonte: Princípios de Bioquímica de Lehninger 6º edição, 2014.

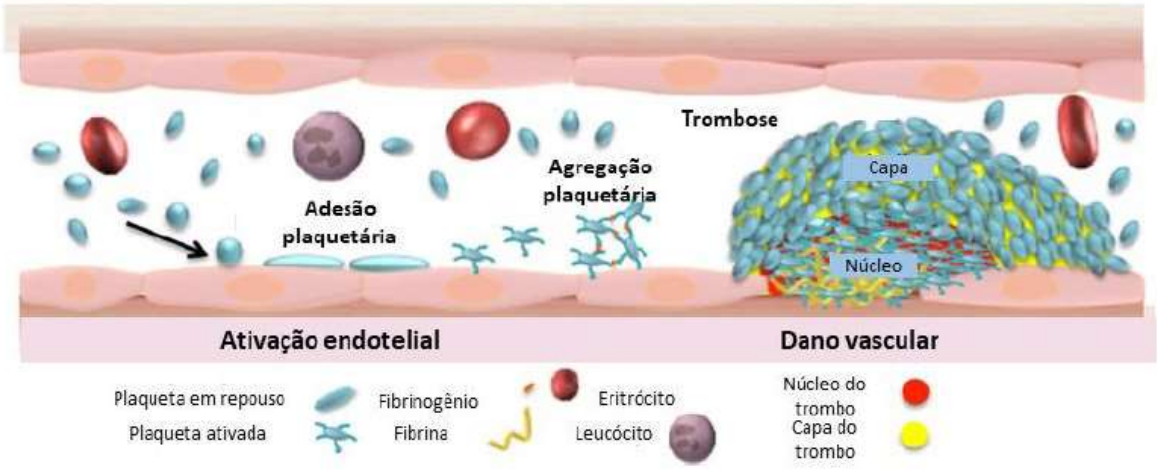
As plaquetas são as principais responsáveis pela coagulação sanguínea e manutenção da hemostasia, intervindo rapidamente quando a integridade do vaso é afetada. Esta intervenção é facilitada pela sua localização próxima a parede do vaso (HOLINSTAT, 2017). Além destas funções, sabe-se que as plaquetas possuem papel importante durante outros processos fisiopatológicos, incluindo imunidade e inflamação (VON HUNDELSHAUSEN; WEBER, 2007).

Em condições patológicas como a aterosclerose, a lesão vascular resulta em hiperatividade plaquetária e consequentemente pode levar à formação de trombo oclusivo, infarto do miocárdio e acidente vascular cerebral (YEUNG; LI; HOLINSTAT, 2018). Assim, o papel das plaquetas na aterogênese vai desde a iniciação da ativação endotelial até à progressão das lesões ateroscleróticas, que resultam muitas vezes em graves complicações tromboembolíticas (Figura 4) (MASSBERG et al., 2002).

Desta forma, a hiperlipidemia pode estar associada a um aumento da reatividade plaquetária, pois quando estas células ficam aderidas ao endotélio comprometido pelo acúmulo de LDL-ox, elas são ativadas e promovem a liberação de citocinas e quimiocinas que contribuem na manutenção do processo inflamatório (MASSBERG et al., 2002; PAWELCZYK et al., 2015). Corroborando com este conhecimento, pesquisas utilizando ratos hipercolesterolêmicos demonstram um significativo aumento de infiltração de

leucócitos e da aterogênese pela ativação da adesão plaquetária (MASSBERG et al., 2002).

Figura 4: Papel das plaquetas na formação do trombo.



A figura mostra o papel das plaquetas durante a formação do trombo aterosclerótico. Fonte: Adaptado de (HOLINSTAT, 2017)

1.5 Alterações teciduais causadas pela hiperlipidemia

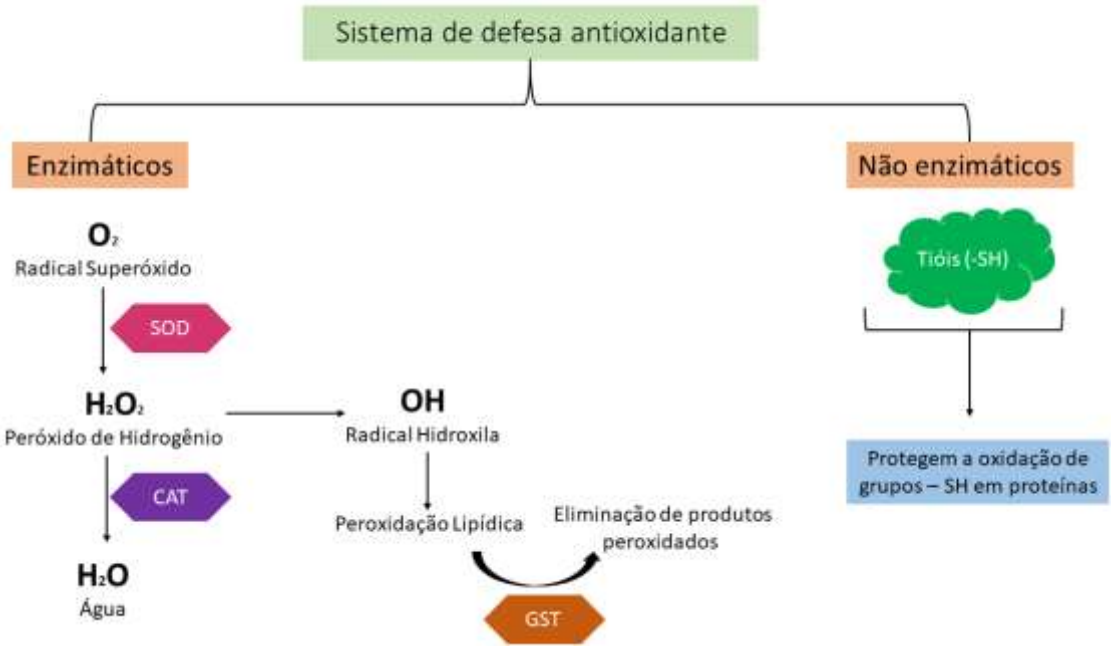
Além das alterações celulares causadas diretamente na corrente sanguínea, o acúmulo de lipídios em tecidos não adiposos como figado, rim, baço e o coração têm um efeito tóxico chamado de lipotoxicidade (BOBULESCU, 2010). Este processo está associado ao desenvolvimento de resistência à insulina, geração de EROs, alterações de vias de sinalização e liberação de mediadores pró-inflamatórios e pró-fibróticos que causam danos às organelas e induzem apoptose (IZQUIERDO-LAHUERTA; MARTÍNEZ-GARCÍA; MEDINA-GÓMEZ, 2016)

A lipotoxicidade já foi descrita por causar inflamação, fibrose e disfunção no fígado, como demonstrado pela esteatose hepática não-alcólica (HIRSOVA et al., 2016) e no rim (IZQUIERDO-LAHUERTA; MARTÍNEZ-GARCÍA; MEDINA-GÓMEZ, 2016). No coração, foi encontrada uma associação entre disfunção e acúmulo de lipídeos em modelos murinos (GOLDBERG; TRENT; SCHULZE, 2013), o que mostra uma outra forma de relação entre a hiperlipidemia e as doenças cardiovasculares, diretamente relacionada ao tecido cardíaco e ainda não totalmente esclarecida em humanos.

Além de induzir o processo inflamatório, a hiperlipidemia também causa estresse oxidativo em diversos órgãos, ocasionando danos teciduais (TRIBBLE, 1999). Um dos principais processos de lesão gerado pelo estresse oxidativo é a lipoperoxidação (LPO). Entretanto, o extresse oxidativo também pode gerar danos às proteínas e ao DNA, provocando diversas alterações na função celular e consequente dano tecidual (SCHNEIDER; REISCHAK DE OLIVEIRA, 2009). O dano à estrutura proteica pode alterar sua atividade biológica, sendo que no caso de enzimas, pode resultar em modificação de sua atividade catalítica (DEAN et al., 1997).

Dentre as defesas antioxidantes do organismo destacam-se os antioxidantes enzimáticos: superóxido dismutase (SOD), catalase (CAT) e a enzima detoxificante glutationa S-transferase (GST). Também temos as defesas não enzimáticas, como por exemplo, o tocoferol, o ácido ascórbico, os carotenoides, os uratos e os tiois totais (TSH) e não proteicos (NPSH), que possuem grande relevância na neutralização das EROs, (Figura 5) (ARAUJO et al., 1995; DEAN et al., 1997; TRIBBLE, 1999).

Figura 5: Sistema de defesa antioxidante enzimático e não enzimático.



A imagem possui uma representação esquemática do sistema de defesas antioxidantes, apresentando as defesas antioxidantes enzimáticas, superóxido desmutase (SOD) e catalase (CAT), e não enzimáticas e ainda mostrando a função detoxificante da enzima glutationa-S- transferase (GST). Fonte: Imagem elaborada pelo autor.

1.6 Papel do sistema purinérgico na hiperlipidemia

Os nucleotídeos e nucleosídeos são secretados por leucócitos, plaquetas e células endoteliais danificadas e desempenham um papel essencial no início e na manutenção das respostas imunológicas. O ATP, ADP e os nucleosídeos de adenina são moléculas capazes de modular as ações das células imunes (DI VIRGILIO et al., 2001). Além disso, ao interagirem com receptores purinérgicos presentes na superfície celular, são responsáveis por desencadear cascatas de reações envolvidas na modulação de diversos efeitos biológicos, como a agregação plaquetária, inflamação e modulação da função cardíaca (ZIMMERMANN, 2000; RALEVIC; BURNSTOCK, 2003).

A sinalização purinérgica desempenha um papel fundamental durante a inflamação, fibrose e reparo tecidual. Já é conhecido que esta via possui uma importante função na aterosclerose (FERRARI et al., 2015). O ATP extracelular e seus derivados são mediadores-chave no sistema imunológico, que podem atuar como pró-inflamatório ou anti-inflamatório, dependendo da concentração e dos receptores expressos em cada tecido em particular (JUNGER, 2011). Os níveis extracelulares de ATP e ADP são modulados principalmente pela enzima ecto-nucleosídeo trifosfato difosfoidrolase (E-NTPDase) conhecida como CD39 (EC 3.6.1.5; CD39), que desfosforila ATP e ADP em AMP. O AMP é posteriormente hidrolisado em adenosina pela ecto-5′-nucleotidase (EC 3.1.3.5; CD73) e a adenosina é então degradada pela ecto-adenosina desaminase (E.C 3.5.4.4, ADA) (ZIMMERMANN; ZEBISCH; STRÄTER, 2012).

As NTPDases constituem uma família de enzimas expressas em todos os tecidos, e possuem um papel fundamental na sinalização purinérgica através do controle dos níveis de nucleotídeos e nucleosídeos. Estas enzimas desempenham suas funções na presença de altas concentrações de Ca^{2+} ou Mg^{2+} (ZIMMERMANN; ZEBISCH; STRÄTER, 2012).

Oito famílias de NTPDases já foram identificadas em mamíferos, sendo quatro delas (NTPDase1, NTPDase2, NTPDase3 e NTPDase8) localizadas na superfície celular e quatro (NTPDase4, NTPDase5, NTPDase6 e NTPDase7) localizadas em organelas intracelulares (KUKULSKI; LÉVESQUE; SÉVIGNY, 2011; ROBSON; SÉVIGNY; ZIMMERMANN, 2006).

A distribuição tecidual da NTPDase1 estávrelacionada às suas funções de modulação da resposta imune celular (DWYERet al., 2007), inflamação, homeostasia do endotélio vascular e trombose (DEAGLIO; ROBSON, 2011). A NTPDase1 é expressa em células imunes, como células NK, monócitos, células dendríticas e subconjuntos de células T ativadas, além de células endoteliais e plaquetas (KOZIAKet al., 1999). Já a NTPDase2 é principalmente expressa na superfície dos vasos sanguíneos, contribuindo para a homeostasia vascular (SÉVIGNYet al., 2002). A propagação da sinalização do tipo P2 em uma variedade de tecidos especializados é ainda realizada pela ação da NTPDase3 e NTPDase8. A NTPDase3 é expressa no cérebro, rim e nos sistemas digestivos, respiratórios e reprodutivos. Por sua vez, a expressão da NTPDase8 é particularmente presente nos tecidos hepático, renal e entérico (KUKULSKI; SÉVIGNY, 2011).

A E-5’-NT controla os niveis de adenosina extracelular, apartir da hidrólise de AMP. Dessa forma, mudanças na expressão e/ou na atividade dessa enzima, assim como no fornecimento de seu substrato AMP, possuem impacto direto na disponibilidade de adenosina no meio extracelular (ZIMMERMANN; ZEBISCH; STRÄTER, 2012). A E-5’-NTpossui uma variada distribuição tecidual, sendo expressa por plaquetas, subpopulações delinfócitos T e B, além demuitas linhagens de células tumorais (JIN et al.,2010). Em relação aos papeis fisiopatológicos da E-5’-NT, estudos demonstram seu envolvimento funcional na imunidade e inflamação (BOURSet al., 2006), regulação da barreira tecidual, adaptação à hipóxia, interações microrganismo-hospedeiro (COLGAN et al.,2006) e no controle imunológico do câncer (ZHANG, 2010).

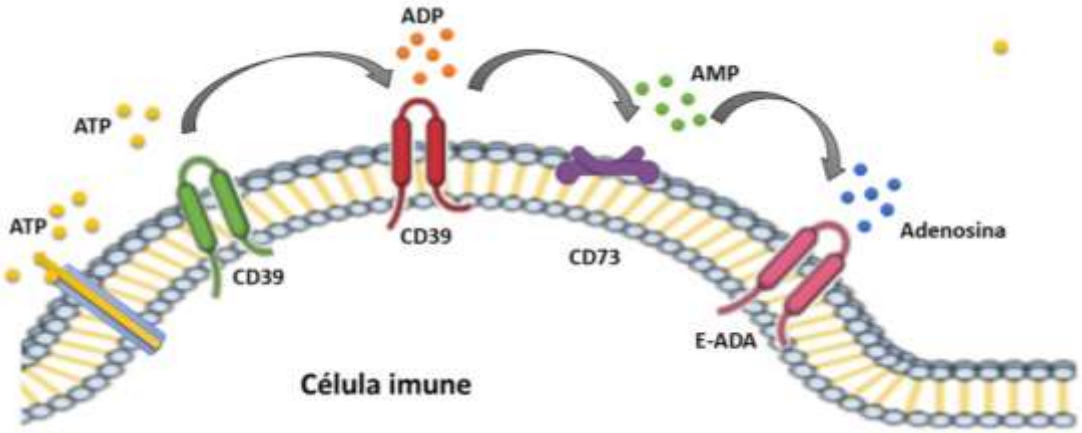
A adenosina desaminase (ADA) catalisa adesaminação irreversível da adenosina em inosina, e está presente em todosos tipos celulares, sendo principalmente encontrada no cérebro, trato gastrointestinal e em tecidos linfoides, especialmente baço e timo (YEGUTKIN, 2008). Esta enzima possuiuma localização tanto citosólica quanto na superfície celular, sendo amplamente expressa na superfície de linfócitos e células dentríticas, onde contribui para a manutenção do estado hiperativo dessas células durante o processo inflamatório através da rápida eliminação de adenosina extracelular (DESROSIERSet al., 2007).

O aumento de lipídeos na circulação causa a liberação de ATP pelas células endoteliais que resulta em liberação de EROs e ativação de linfócitos, macrófagos, neutrófilos e plaquetas (RALEVIC; BURNSTOCK, 2003).

O metabolismo e a sinalização da adenosina estão envolvidos tanto no metabolismo de lipídeos quanto na resposta imune à hiperlipidemia. A interação da adenosina com seus receptores (chamados receptores P1) tem um papel protetor, regulando a lipólise, a formação de células espumosas, o efluxo de colesterol, os níveis de lipídeos circulantes e, consequentemente, a formação da placa aterosclerótica (OHTA; SITKOVSKY, 2001). Além disso, a adenosina desempenha um papel imunossupressor, modulando a resposta imune de forma a reduzir a liberação de mediadores inflamatórios (LEIVA et al., 2017).

Ademais, estudos mostram uma modulação da atividade da ADA no contexto da hiperlipidemia (BRAUN et al., 2017) e da síndrome metabólica (DE BONA et al., 2012). Além disso, a ADA sérica é considerada um marcador de ativação celular e inflamação (Figura 6) (UNGERER; VERMAAK, 1992; SANDS, 2015; VINAPAMULA et al., 2015).

Figura 6: Sistema de degradação do ATP pelas enzimas purinérgicas.

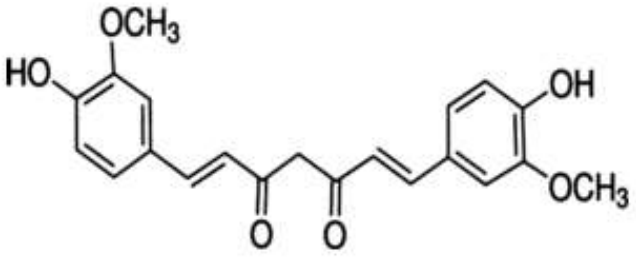


Representação do sistema de degradação do ATP pelas enzimas purinérgicas. Fonte: Imagem elaborada pelo autor.

1.7 Compostos naturais e hiperlipidemia

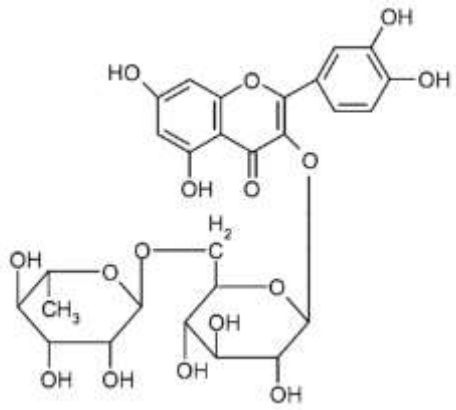
A utilização de compostos naturais, como bioflavonoides têm apresentado diversas aplicações na saúde, devido as suas amplas atividades biológicas, e o seu baixo custo. Um composto natural utilizado na culinária é a curcumina (Figura 7), um pigmento natural presente no açafrão-da-índia, e que tem demonstrado efeitos antioxidantes e anti-inflamatórios. Estudos têm apontado sua utilização frente ao tratamento de doenças como a aterosclerose e a hiperlipidemia (SHIN et al., 2014; SHOME et al., 2016; MANZONI et al., 2019).

Figura 7- Estrutura química da curcumina.



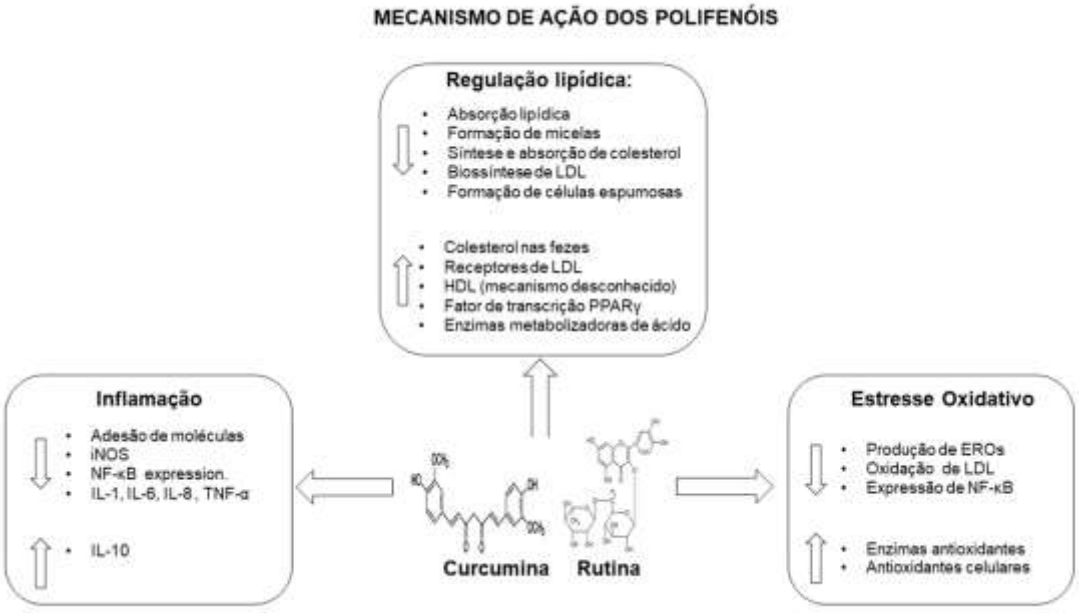
A rutina (Figura 8) é um flavonoide presente em vegetais folhosos e frutas cítricas, o qual tem mostrado uma ampla gama de aplicações farmacológicas, devido as suas propriedades significativas. Em pesquisas atuais foram demonstrados seus benefícios farmacológicos para o tratamento de várias doenças crônicas, como câncer, diabetes, hipertensão e hiperlipidemia (AL-REJAIE et al., 2013; AL-DHABI et al., 2015; MANZONI et al., 2019).

Figura 8 Estrutura química da rutina.



As ações hipolipemiantes, antioxidantes e anti-inflamatórias dos polifenois são devidas a vários mecanismos, sendo que alguns deles já estão bem descritos na literatura. Entre os mecanismos de ação podemos citar a inibição da síntese e absorção lipídica; formação de células espumosas, aumento do receptor de LDL e das lipoproteínas de alta densidade (HDL); modulação nas enzimas metabolizadoras de ácido biliares, respectivamente lipase lipoproteica, enzima 3-hidroxi-3-metilglutaril coenzima A (HMG-CoA), lipase hepática (HL) e enzima colesterol-7α-hidroxilase (C7αH), assim como diminuiçao na produção de espécies reativas, produção de citocinas anti-inflamatórias, entre outros (Figura 9) (JACOB et al., 2007; SIASOS et al., 2013; ZINGG; HASAN; MEYDANI, 2013).

Figura 9: Ação dos polifenois na regulação lipídica, inflamação e estresse oxidativo.



Fonte: Imagem elaborada pelo autor. EROs: Espécies reativas de oxigênio; IL: Interleucina; LDL: Lipoproteina de baixa densidade; NF-kB: Fator nuclear kappa B.

Conhecendo alguns mecanismos de ação da curcumina e da rutina, e sabendo de seus benefícios que já estão descritos na literatura em diversos modelos de doenças, buscamos neste trabalho avaliar se estes compostos isolados ou em conjunto trariam benefícios aos ratos submetidos a indução da hiperlipidemia e também avaliar a segurança da utilização dos compostos em ratos que não foram submetidos a indução. O entendimento da ação desses compostos no modelo de hiperlipidemia pode ser de grande valia na busca de tratamentos preventivos e adjuvantes para a hiperlipidemia.

2- JUSTIFICATIVA

Conhecendo as alterações que podem ser desenvolvidas pelo excesso de lipídeos na corrente sanguínea e os danos que estes podem causar em todo o corpo, torna-se importante entender melhor como esta condição se desenvolve sistemicamente. Além disso, é necessário elucidar qual o papel das enzimas purinérgicas na modulação do sistema imune e na exacerbação do processo inflamatório, na tentativa de reduzir os riscos para o desenvolvimento da aterosclerose.

Uma vez que a hiperlipidemia gera uma exacerbação da resposta imune, causando uma lipotoxicidade no tecido endotelial, e que consequentemente causa uma inflamação, torna-se relevante avaliar quais são as alterações hematológicas, bioquímicas, inflamatórias e purinérgicas em modelo de hiperlipidemia. Verificar o papel do sistema purinérgico na ativação e exacerbação do processo imune, inflamatório e lipotóxico pode ser de grande ajuda para a compreensão da doença e até mesmo para o desenvolvimento de alvos terapêuticos.

Atualmente o tratamento mais indicado para o tratamento da hiperlipidemia são as estatinas que tem o propósito de diminuir os níveis de lipoproteínas plasmáticas ricas em colesterol e reduzir os riscos de doenças cardiovasculares. Porem o uso de medicamentos possui um custo alto e uma baixa aceitação pela parte dos pacientes, sendo a preveção com compostos naturais uma interessante forma de prevenir os efeitos causados pela hiperlipidemia.

Os danos causados pelo excesso de lipídeos no tecido endotelial são amplamente estudados, porém os danos que esta condição pode causar em diversos órgão e tecidos do corpo, também causam preocupação e podem desencadear uma série de doenças. Sendo assim, torna-se extremamente importante conhecer estes danos, ampliando as informações sobre a hiperlipidemia e os riscos associados a ela. Sendo que, compostos naturais têm ações benéficas na saúde em geral, e essencial esclarecer como sua utilização pode beneficiar os indivíduos afetados pela hiperlipidemia. Sendo assim, a investigação dos efeitos da rutina e/ou curcumina em modelo de hiperlipidemia pode trazer informações importantes que permitam avaliar o uso destes compostos na prevenção de complicações causadas pela hiperlipidemia.

3 OBJETIVOS

3.1 Objetivo geral

Avaliar os efeitos causados pela hiperlipidemia em células sanguíneas, órgãos e tecidos de ratos adultos, assim como avaliar o possível efeito preventivo da rutina e da curcumina em seu desenvolvimento.

3.2 Objetivos específicos

Em ratos submetidos ou não à indução de hiperlipidemia pelo poloxamer-407 pretende-se:

- Determinar a concentração sérica da albumina, ácido úrico, fosfatase alcalina e creatinina;
- Verificar a atividade sérica das enzimas: a alanina aminotransferase (ALT) e aspartato aminotransferase (AST);
- Avaliar parâmetros hematológicos;
- Determinar a atividade das enzimas NTPDase, 5'-nucleotidase e adenosina desaminase em plaquetas e membranas de células do coração;
- Verificar parâmetros de estresse oxidativo do fígado, rim, baço e coração;
- Avaliar se os pré-tratamentos com rutina e/ou curcumina são capazes de prevenir as alterações causadas pela hiperlipidemia;
- Avaliar se os pré-tratamentos com rutina e/ou curcumina são capazes de gerar imunotoxicidade quando utilizados *per se*.

4- MÉTODOS E RESULTADOS

4.1- ARTIGO 1: Hyperlipidemia-induced lipotoxicity and immune activation in rats are prevented by curcumin and rutin, International Immunopharmacology, 2020.

Hyperlipidemia-induced lipotoxicity and immune activation in rats are prevented by curcumin and rutin

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Abstract

We assessed the effects of curcumin, rutin, and the association of rutin and curcumin in organs of hyperlipidemic rats. Rutin and curcumin have notable antioxidant and anti-inflammatory actions, so we hypothesized that their association potentialized their beneficial effects. Hyperlipidemia results in lipotoxicity and affects several organs. Lipotoxicity is not only an outcome of lipid accumulation in non-adipose tissues but also a result of the hyperlipidemia-associated inflammation and oxidative stress. Wistar rats were treated with rutin and curcumin for 30 days before the induction of acute hyperlipidemia by Poloxamer-407. After 36 hours, the animals were euthanized, for the collection of blood and organs. Untreated hyperlipidemic rats showed higher uric acid and albumin levels in the serum and increased spleen size and ADA activity. Rutin curcumin and the association reduced the spleen size by 20% and ADA activity by 23, 28, and 27%, respectively. Rats pretreated with rutin showed reduced lipid damage in the liver (40%) and the kidney (44%), protein damage was also reduced in the liver (75%). The lipid damage was decreased by 40% in the liver, and 56% in the kidney of rats pretreated with curcumin. The association reduced lipid damage by 50% and 36% and protein damage by 77% and 64% in the liver and kidney, respectively. Rutin better prevented the decrease in the antioxidant defenses, increasing SOD by 34%, CAT by 246 % and GST by 84% in the liver, as well as SOD by 119% and GST by 190% in the kidney. Also, analyses of blood and spleen parameters of untreated and pretreated non-hyperlipidemic rats showed no signs of immunotoxicity. Despite showing protective effects, the association did not perform better than the isolated compounds. Here, we showed that rutin and/or curcumin reestablished the immune homeostasis and redox balance disrupted by hyperlipidemia in peripheral organs of rats.

Keywords: hyperlipidemia, immune activation, adenosine deaminase, antioxidants, lipotoxicity, oxidative stress.

1 INTRODUCTION

Lipotoxicity is caused by the accumulation of lipids in non-adipose tissues, or as a consequence of inflammation and oxidative stress. Several organs, such as the liver, spleen, heart, and kidneys are affected by lipotoxicity [1,2]. Hyperlipidemia is a condition caused by elevated serum levels of total cholesterol, low-density lipoprotein cholesterol and triglycerides which accumulate in the body and may induce lipotoxicity affecting different organs and systems [3]. Hyperlipidemic individuals are not only more prone to develop cardiovascular disorders [4] but are also at risk of developing liver [5] and kidney diseases [1].

Lipid deposition in the arterial walls elicits immune activation and inflammation by LDL oxidation and formation of foam cells [6], changing the profile of circulating immune cells [7], and is likely to affect secondary lymphoid organs such as the spleen. The spleen maintains peripheral tolerance, modulates of both innate and adaptive immune responses and reacts to different pathological conditions [8,9].

Polyunsaturated lipids include cytotoxic lipids that impact on cellular homeostasis, even small changes in quantity, composition, or location of such lipids can have profound effects on cellular viability and function. Lipid antigens are recognized by splenic natural killer T (NKT) cells, initiating the adaptive response by releasing cytokines [10,11].

Due to the anatomic proximity, inflammatory mediators originated in the spleen may reach the liver and kidney, increasing toxicity [11,12]. The liver is a central organ in the metabolism of fatty acids, proteins, and glucose, and is sensitive to lipid toxicity and inflammation, which may result in the accumulation of toxic products [5]. Lipotoxicity in the kidney affects the lipid metabolism, favors insulin resistance, and increases generation of ROS generation, that are important causative factors in chronic kidney dysfunction and acute renal failure [1,13].

Lipotoxicity is caused not only by the accumulation of lipids in organs but also by the proinflammatory mediators and reactive species produced in response to the endothelial damage caused by hyperlipidemia [14]. The production of reactive species is tightly regulated by an endogenous antioxidant system that counteracts their excess.

However, several diseases may induce tissue damage by creating an imbalance between the production and the neutralization of these reactive species [15].

Currently, there is a growing interest in plant-based functional foods, enriched in polyphenols. The latter are bioactive molecules with diverse properties represent the most common antioxidants in the human diet. They are plentifully present in vegetables, coffee, fruits, and cereals [16–18]. Rutin, found in fruits and dark-leaved vegetables, and curcumin, present in turmeric, are polyphenols with a wide range of pharmacological applications. Antioxidant [19–21], anti-inflammatory, and hypolipidemic [7] are some of the properties of these polyphenols.

The inflammation and oxidative stress associated with hyperlipidemia are important physiological and pathological events in the development of lipotoxicity as well as in liver and kidney diseases. Thus, we sought to evaluate the potential of rutin and curcumin in preventing splenic immune activation, and hepatic and renal redox imbalance in rats with induced hyperlipidemia. We also investigated the effects of the association of both compounds that, to the best of our knowledge, have not yet been explored in this context. In order to discard the immunotoxic effects of the compounds per se, we assessed immunotoxicity parameters in non-hyperlipidemic rats.

2. Materials and methods

2.1 Reagents

Coomassie Brilliant Blue G-250, Malondialdehyde bisdimethylacetal (MDA, 99%, Aldrich 108383), (-) epinephrine (+) bitartrate salt, and 2-thiobarbituric acid (sodium derivative), rutin, curcumin and Poloxamer-407 (P407) were obtained from Sigma-Aldrich (St.Louis, MO, USA). Physiological solution (0.9g NaCl/100 mL distilled water) was purchased from Fresenius KABI (Brazil). We ensured analytical grade and maximum purity for all chemicals used in the experiments.

2.2 Animals

Experimental animals consisted of adult male, conventional and heterogenic, Wistar rats (250-300g) from UFSM Central Vivarium. The animals were kept at a constant temperature (23±21°C) on a 12-hour light/dark cycle with freely available food and water. All procedures involving the animals were approved by the Ethics Committee on the Use

of Animal at the Federal University of Santa Maria (Protocol number: 1006200117, dated 01/2017).

2.3 Induction of hyperlipidemia and treatment with natural polyphenols (or with curcumin and rutin)

The animals randomly assigned to the hyperlipidemic groups received 500 mg/kg of poloxamer 407 (P407) suspended in sterile NaCl (0.9%) solution via intraperitoneal injection [22]. The control groups received equal volume of vehicle alone (cold, sterile 0.9% NaCl solution). The animals were anesthetized with isoflurane and euthanized 36 hours post-induction, followed by blood drawing by cardiac puncture. The study was designed with eight groups as seen in table 1.

The animals belonging to the groups designated for pretreatment were given rutin and/or curcumin or saline by gavage for 30 days prior to the induction. Rutin and curcumin were diluted in corn oil. Initially, there was a group who received only corn oil. However, this group was excluded from the study because it did not present a significant difference in the enzymatic activities compared to the group that were given saline (control).

Table 1: Design of study groups

Group	Pretreatment	Induction	Number of animals
Saline	Saline	Saline	*n=5*
Rutin	Rutin 50 mg/kg	Saline	*n=6*
Curcumin	Curcumin 50 mg/kg	Saline	*n=6*
Rutin and Curcumin	Curcumin 50 mg/kg + Rutin 50 mg/kg	Saline	*n=6*
Hyperlipidemic saline	Saline	Poloxamer 407	*n=5*
Hyperlipidemic Rutin	Rutin 50 mg/kg	Poloxamer 407	*n=6*
Hyperlipidemic Curcumin	Curcumin 50 mg/kg	Poloxamer 407	*n=6*

Hyperlipidemic Rutin and Curcumin	Curcumin 50 mg/kg + Rutin 50 mg/kg	Poloxamer 407	*n=6*

Separation of blood serum

Serum was separated from blood samples drawn in serum separation tubes and spun at 1400×g at room temperature for 15 min and frozen for later analyses.

2.5 Biochemical analyzes

Blood was drawn with Ethylenediamine tetracetic acid (EDTA) and complete blood count was analysed using SYSMEX XT-1800i, Roche Diagnostic, USA.

Serum levels of aspartate aminotransferase (AST), alanine aminotransferase (ALT), albumin, alkaline phosphatase, creatinine, and uric acid were evaluated in a Thermo Plate Analyzer Plus®, Thermo plate using Bioclin/Quibasa commercial kits.

2.6 Preparation of liver, kidney and spleen homogenates

Liver and kidney were collected and homogenized on ice in 1 mL Tris HCl buffer (50 mM pH 7.4). Samples were further centrifuged at 3000×g for 10 min (4°C). Spleen samples were collected and homogenized on ice in 1 mL of phosphate buffered saline followed by centrifugation at 1800×g for 10 min at 4°C. All the supernatants were kept in microtubes at −80°C until tested.

2.7 Protein and lipid damage assays in the liver and kidney

Protein carbonyls levels were quantified using 2,4-dinitrophenylhydrazine (DNPH) according to Levine et al. [23]. The results were presented as nmol of protein carbonyl/mg protein.

Lipid peroxidation was estimated by measuring TBARS generated during an acid-heating reaction according to a method described previously by Draper [24]. The absorbance was measured at 532 nm in a microplate reader. Malondialdehyde (MDA) was used as standard and results were given as nmol MDA/mg protein.

2.8 Antioxidant analyzes in the liver and kidney

The radical superoxide reaction to adrenalin was inhibited to quantify SOD activity, and the adrenochrome release was read at 480 nm. The product was given as U SOD/mg of protein, where one unit of SOD is the amount of enzyme necessary to inhibit 50% of autoxidation of adrenalin [25]. CAT activity was measured by monitoring the decrease in H2O2 absorbance at 240 nm [26]. The enzymatic activity was expressed as μmol/min/mg protein.

Glutathione S-transferase (GST) activity was analyzed as described previously [27]. Absorbance was monitored at 340 nm and the enzyme activity was further calculated by using the molar extinction coefficient (9.6 mM/cm). A unit of GST was defined as the amount of enzyme required to catalyze the conjugate 1 mol of 1 chlorine 2-4 dinitro benzene (CDNB) with GSH/min at 25°C. Data were reported as μmol GS-DNB/min/mg protein.

Total thiol or total hydrosulfide groups (T-SH), which consists in the reduction of 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB) in pH 7.0. The results were expressed in nmol T-SH/ mg of protein. Non-protein hydrosulfide group (NPSH) were assessed by Ellman method [28] and reported as nmol SH/ mg of protein.

2.9 Immune activation parameters in the spleen

Before euthanasia, all the animals were weighted. After dissection, the spleen was weighed in an analytical balance. The individual variation was compensated by calculating the ratio between the spleen weight (g) and the body weight (g) (SW/BW).
Adenosine deaminase (ADA) activity was measured in homogenized spleen samples according to Giusti and Galanti [29]. This method is based on the direct quantity of ammonia produced when ADA hydrolyses adenosine. All tests were run in triplicate and the values of ADA activity were reported as μMNH3/min/mg protein.

2.10 Protein content

Coomassie blue method [30] was used as a reference to determine the proteins content in the liver, kidney and spleen homogenates.

2.11 Immunotoxicity assessment

To assess whether rutin and/or curcumin *per se* had an immunotoxic effect, we compared the blood parameters and SW/BW between the control group (pretreated with saline), and each of the pretreated controls groups (rutin and/or curcumin), along with ADA activity in the spleen. Regarding the blood parameters, we considered cells counts and albumin levels.

2.12 Statistical analysis

We used T-test or one-way analysis of variance (ANOVA) plus Tukey's Multiple Comparison Test as required. All data were expressed as mean ± standard error of the mean (SEM). Correlations were tested using Pearson correlation coefficients. Values of $P<0.05$ were regarded as statistically significant.

RESULTS

3.1 Confirmation of hyperlipidemia

After 36 hours of induction of hyperlipidemia, there was a peak of serum levels of lipids. An increase in the levels of total and high-density lipoprotein cholesterol and triglycerides was observed, confirming the induction of hyperlipidemia by P407. These results were published in a previous paper [6].

3.2 Biochemical Parameters

AST and ALT activity and albumin, uric acid, creatinine, and alkaline phosphatase levels are shown in Table 2. The activity of AST and ALT was not significantly different among all groups. In comparison to the control group, the untreated hyperlipidemic group presented a significant raise in the albumin levels. However, the levels of albumins were not significantly different among the groups. With respect to uric acid levels, the hyperlipidemic groups show an increase compared to the control group.

3.3 Protein and lipid damage

Carbonyl levels in liver and kidney are shown in figure 1. The carbonyl levels are expressed in (nmol of protein carbonyl/mg protein). In the liver (Fig.1A), carbonyl levels

were significantly higher in untreated hyperlipidemic rats (20.4±1.3, P<0.001) when compared to control rats (5.4±1.1, P<0.001). All pretreatments, rutin (5.2±1.9, P<0.001), curcumin (4.1±1.0 P<0.001) and the association (4.7±0.4, P<0.001), were able to prevent the changes in levels of carbonyl protein in the liver compared to the hyperlipidemic group.

In the kidney (Fig.1B), the levels of carbonyl in untreated hyperlipidemic rats (27.3±5.2, P<0.01) were increased when compared to control (3.9±0.4, P<0.01). The pretreatments with curcumin (11.3±1.7 P<0.05) and rutin-curcumin association (9.8±3.3, P<0.05) avoided the increased in carbonyl levels when compared to the hyperlipidemic group (27.3±5.2, P<0.05).

Figure 2 shows levels of TBARS in liver (Fig.2A) and kidney (Fig.2B). The TBARS levels are expressed in nmol MDA/mg protein. In the liver (0.1±0.009, P<0.001) and kidney (0.25±0.03, P<0.001), rats with untreated hyperlipidemia showed a significant increase in TBARS levels in comparison to the control group (liver: 0.06±0.003, P<0.001, kidney: 0.08±0.004, P<0.001). Regarding the pretreatments, all of them managed to significantly reduce TBARS levels when compared to the control group. All the pretreatments prevented the changes in TBARS levels, rutin in the liver (0.06±0.004, P<0.01) and kidney (0.14±0.01, P<0.01), curcumin in the liver (0.06±0.007, P<0.01) and kidney (0.11±0.01, P<0.001), and the association in the liver (0.05±0.002, P<0.001) and kidney (0.16±0.01, P<0.05).

3.4 Antioxidant defense

The results of SOD and CAT in liver and kidney tissues are shown in Figure 3. The activity of SOD was expressed as U SOD/mg of protein, and CAT activity in μmol/min/mg protein. Comparing the control group (54.8±0.5, P<0.01) to the untreated hyperlipidemic group (37.4±0.9, P<0.01), SOD levels in the liver. (Fig.3A) were significantly reduced in the second group. Pretreatment with isolated rutin (50.4±4.1, P<0.05) prevented the decline in SOD levels when compared to the untreated hyperlipidemic group.

In the kidney (Fig.3B), SOD levels were also significantly lower in the hyperlipidemic group (27.4±1.1, P<0.01) compared to control (48.2±6.0 P<0.01), and

rutin pretreatments (60.2±4.9, P<0.001) and curcumin (47.1±2.9, P<0.05) were able to prevent these changes compared to the hyperlipidemic group.

CAT activity in liver (1.4±0.1, P<0.01) (Fig.3C) and kidney (1.3±0.1, P<0.05) (Fig.3D) showed a significant reduction in the untreated hyperlipidemic groups, when compared to the respective controls (liver 3.8±0.5, P<0.01; kidney 2.9±0.1, P<0.05). Rutin pretreatment (4.5±0.4 P<0.01) was able to prevent the changes in hepatic CAT levels, when compared to the untreated hyperlipidemic group.

GST activity in the liver (Fig.4A) and kidney (Fig.4B). The activity of GST is expressed in μmol GS-DNB/min/mg protein. A significant reduction in GST activity in liver (0.12±0.007, P<0.01) and kidney (0.10±0.01, P<0.01) tissues in untreated hyperlipidemic rats, when compared to control rats (liver: 0.23±0.01, P<0.01; kidney; 0.25±0.02, P<0.01). Concerning the pretreatments, rutin was able to avoid the changes in GST activity in both the liver (0.22±0.02 P<0.01) and kidney (0.29±0.02, P<0.001) in contrast to the hyperlipidemic group. The association in the kidney, (0.21±0.03, P<0.001) avoided the reduction in GST activity compared to the untreated hyperlipidemic group.

The levels of total hydrosulfide group (T-SH) and non-protein hydrosulfide group (NPSH) are expressed in nmol T-SH/ mg of protein and nmol SH/ mg of protein, respectively. No significant difference in T-SH levels between the pretreatments groups were seen in the kidney (Fig.5B) while decreased levels of the T-SH were found in the liver of untreated hyperlipidemic group (21.2±0.3, P<0.05) in comparison to the control group (23.4±0.1, P<0.05) (Fig.5A). Regarding NPSH levels, a significant reduction was observed to the untreated hyperlipidemic groups (19.1±0.1, P<0.05) (8.4±0.2, P<0.05) when compared to the control group (22.3±0.7, P<0.05) (12.1±0.9, P<0.05), in the liver (Fig.5C) and kidney (Fig.5D), respectively. Regarding the pretreatments, no statistical difference was observed between the groups.

3.5 Spleen weight/body weight ratio

Untreated hyperlipidemia caused an increase in the spleen weight/body weight ratio (SW/BW) (0.0021±0.0001 P<0.001) in comparison to the control group (0.0015±0.00008 P<0.001). Rutin (0.0017±0.00007, P<0.05), curcumin (0.0017±0.00005, P<0.05) and the association (0.0017±0.00003, P<0.05) were effective in preventing the increase in the SW/BW) in hyperlipidemic rats (Figure 6).

3.6 ADA activity in spleen homogenates

Figure 7 illustrates the results for splenic ADA activity. The ADA activity is expressed in μM NH_3/min/mg protein. Untreated hyperlipidemic rats (66.02±1.3, P<0.001) showed an ADA activity twice as high as the control group (33.9±2.5, P<0.001). All treatments were effective in preventing the increase in ADA activity (Rutin (50.7±5.2, P<0.05), Curcumin (47.11±2.5, P<0.01) and Rutin and curcumin (47.9±1.1, P<0.01) in relation to the untreated hyperlipidemic group.

3.7 Comparison between the pretreatments

Table 3 shows the differences of each distinct pretreated hyperlipidemic group in percentage using the untreated hyperlipidemic group as absolute value.

Rutin pretreated rats showed reduced levels of damage to proteins by 75% and lipids by 40% in the liver when compared to the untreated hyperlipidemic group. However, in the kidney, rutin decreased the levels of lipid damage (44%). Regarding the antioxidant enzymatic activities in the liver, rutin prevented the reduction of SOD (34%), CAT (234%), and GST (84%). In the kidney, only SOD (119%) and GST (190%) were increased when rutin pretreated rats were compared to the hyperlipidemic group. Pretreatment with rutin reduced both the SW/BW ratio (20%) and the activity of ADA in the spleen (23%) in comparison to the hyperlipidemic group.

Curcumin prevented damage to the lipids by 40% and 58% and protein by 80% and 56% damage in the liver and kidney, respectively. Nevertheless, rats pretreated with curcumin only showed increased activity of SOD (72%) in the kidney in comparison to hyperlipidemic rats. Regarding the spleen analyses, the pretreatment with curcumin reduced both the SW/BW ratio (20%) and the activity of ADA (28%), when compared to the hyperlipidemic group.

The association of rutin and curcumin prevented lipid and (50%,36%) protein (77%,74%) damage both in liver and kidney, respectively. Rats pretreated with the associating showed increased GST activity in kidney (110%). In the spleen, pretreatment with the association was able to reduce both the SW/BW ratio (20%) and the activity of ADA (27%), when compared to the hyperlipidemic group.

3.8 Immunotoxicity parameters

The blood cell counts, serum albumin, SW/BW and ADA activity in the spleen showed no significant differences among the saline-pretreated and the rutin and/or curcumin pretreated non-hyperlipidemic rats (Table 4).

3.9 Correlations between lipotoxic markers

We found a positive correlation between uric acid levels and ADA activity in the serum of hyperlipidemic rats ($R=0.8121$, $P<0.05$). Also, the activities of ADA in the serum and the spleen of these rats were strongly correlated ($R= 0.9363$, $P<0.05$). The results of ADA in the serum of these hyperlipidemic animals have been previously published [7].

DISCUSSION

4.1 Hyperlipidemia induced inflammation and immune activation

Hyperlipidemia elicits inflammatory and immune responses due to the accumulation of lipids in the circulation [7]. Here, we found that hyperlipidemia increased the SW/BW and ADA activity in the spleen, indicating spleen enlargement and immune activation, respectively. Spleen reacts to several conditions such as infections, malignancies, hematological, hepatic, metabolic, and cardiovascular diseases, all of which cause this organ to enlarge [31]. Moreover, hyperlipidemia in rats [32] and individuals with familial lipoprotein lipase deficiency [33] were shown to cause splenomegaly.

The immune response to the systemic proinflammatory environment associated with hyperlipidemia triggers the spleen enlargement seen in untreated hyperlipidemic rats. ADA hydrolyzes adenosine which downregulates immune activation [34] and plays an important role in the proliferation of lymphocytes [35], which contributes to spleen enlargement. Thus, the increase in activity of ADA and spleen size in the hyperlipidemic rats reflect an activation of the immune cells in the spleen.

Activated immune cells release proinflammatory cytokines and reactive species, prompting oxidative damage and toxicity [36]. The activity of ADA in the serum is an unspecific marker of immune activation an inflammation [37] and activated cells release ROS that mediates the oxidation of lipids. Unsurprisingly, lipid oxidation, ROS levels,

and ADA activity have been reported to be increased in metabolic disorders [7,38,39]. On a previous study, we have shown that ADA activity in the serum correlates directly with the production of oxygen reactive species (ROS), suggesting that both indicate the level of immune cells activation [7].

In this study, ADA activity in spleen and serum correlate directly, showing a relationship between systemic and splenic immune activation. In a previous study, we have shown that curcumin, as well as its association with rutin, had a hypotriglyceridemic effect. Moreover, these pretreatments lessened the immune activation and inflammation associated with hyperlipidemia [7]. In this study, rutin, curcumin, and their association prevented splenomegaly and the increase in the activity of ADA in the spleen of hyperlipidemic rats.

4.2 Curcumin and/or rutin are not immunotoxic

Chemicals substances, drugs, and even natural compounds may have toxic effects on the immune cells and tissues. Thus, we analyzed blood and spleen parameters in untreated and pretreated non-hyperlipidemic rats. We found no signs of immunotoxic effects of the compounds and their association. SW/BW ratios, hematologic parameters such as total white blood cell counts, and albumin levels are basic parameters of immunotoxicology. Changes in the blood cell counts are indicative of immune activation, inflammation or immunosuppression. Also, increased or decreased spleen weights may be indicators of higher or lower rates of lymphocyte proliferation, respectively [40].

We have also evaluated the activity of ADA in the spleen to discard immune activation caused by the compounds *per se*. Although ADA activity is not a classical marker of immunotoxicity, it is a marker of cellular immune activation which may result in immunologic tissue impairment [41]. These findings corroborate with our previous report showing that ROS levels and ADA activity in the serum were not affected by the compounds in non-hyperlipidemic rats [7].

4.3 Biochemical parameters changed in hyperlipidemia

In this study, we found that hyperlipidemia increased UA levels which we showed to be directly correlated with serum ADA activity. This result was expected since UA is a product of the downstream metabolism of adenosine. Uric acid levels have been shown to correlate directly with several other inflammatory markers [42]. Increased serum

concentrations of UA represent an important risk factor for several diseases such as gout, cardiovascular and renal diseases [43,44], and metabolic syndrome [45,46], contributing to organ damage [42]. Only curcumin and the association prevented the increase in UA levels.

Hyperlipidemic rats have also shown increased albumin levels. Albumin is responsible for the transport of lipids into the bloodstream, whose increased levels are linked to the appearance of hyperlipidemia [46]. All pretreatments prevented the increase in the albumin levels. The levels of ALT, AST, creatinine and alkaline phosphatase have remained unchanged, which may be due to the acute nature of hyperlipidemia, which don't have an enough time for changed these parameters.

4.4 Oxidative biomarkers are dangers in hyperlipidemia context

Several biomarkers have emerged as useful indicators of oxidative damage in kidney and liver disease such as lipid peroxidation markers, carbonyl protein content, and the activity of antioxidant defense enzymes [47,48]. Peroxidation of membrane lipids induced by oxidative stress leads to inactivation of membrane-bound receptors or enzymes and may consequently increase tissue permeability and abnormal cellular function [15,49]. We observed a significant increase in lipid and protein damage in liver and kidney tissues of hyperlipidemic rats, as given by the levels of TBARS and carbonyl protein, respectively. Our results are in line with a previous study that found increased lipid levels raise oxidative stress in the liver [50]. All pretreatments were effective in preventing the increase in lipid and protein damage.

4.5 Antioxidant defenses an important mechanism for control de hyperlipidemia damages

Antioxidant enzymes are endogenous cellular defense mechanisms to control the potentially harmful effects caused by free radicals. Although SOD plays a prooxidant role through its peroxidase activity, its primordial role is to neutralize O2 and H2O2 levels, acting in synergism with CAT [49,51,52]. SOD and CAT activities in rats with hyperlipidemia were reduced, in agreement with other studies using animal models of diabetes, hyperlipidemia, and hyperglycemia [52,53].

Curcumin had previously exhibited a hepatoprotective against oxidative damage in models of hyperlipidemia [54] and diabetes [55], as well as in the kidney in a model of diabetes [56]. Rutin has shown to be effective in protecting against hypercholesterolemia-induced hepatoxicity [57] and, when combined with ascorbic acid, renal injury in rats [58]. We found that both CAT and SOD activities in liver were increased in hyperlipidemic rats pretreated with rutin, whilst in the kidney only SOD activity was enhanced. Curcumin failed to increase the antioxidant defenses, except for SOD activity in the kidney. The association did not prevent any changes in the activity of SOD and CAT activities.

GST is one of the main detoxifying enzymes and has redox potential due to sulfhydryl (SH), besides eliminating ROS and lipid peroxides [49]. We observed a reduction in the activity of GST in untreated hyperlipidemic rats. In the liver, only rutin pretreatment increased GST activity, suggesting an increase in organ detoxification potential. In the kidney, rutin alone and the rutin-curcumin association increased GST activity, which returned to basal levels.

T-SH and NPSH detoxifiers that maintain redox homeostasis, extinguish free radicals to safeguard cellular constitution and function [52,53]. Hepatic T-SH and NPSH levels were reduced in the untreated hyperlipidemic rats, whereas only renal NPSH levels were reduced in these animals. However, in both organs, the pretreatments failed to restore T-SH and NPSH levels.

4.6 Differences between the polyphenol treatments

We found differences between the effects of the different polyphenols tested. Although in different levels of effectiveness, all pretreatments prevented the increase in oxidative damage in liver and kidneys, as well as preventing the immune activation in the spleen.

The action of rutin was the most effective concerning enzymatic antioxidant defenses and oxidative parameters. We believe this is due to the high antioxidant power of rutin [59]. Metabolites of rutin have been found in the liver [59], which may explain why its effects were more pronounced in this organ.

In a previous study, we found that curcumin presented a better hypolipemiant action than rutin and the association [60]. Here, we have shown that among all the

antioxidant defense parameters tested, curcumin only improved the activity of SOD activity in the liver. However, curcumin prevented oxidative damage, which we believe is an indirect result of reducing lipid levels and consequent reduction in lipid toxicity. Regarding the levels of immune activation, curcumin presented very similar results to rutin, being even slightly better than rutin in reducing ADA activity in spleen.

The association has shown the least efficient results when compared to the isolated compounds. Rather than the expected synergistic effect between curcumin and rutin, both known for their antioxidant effects, their association has not shown a better protective effect against protein and lipid damage and immune activation. Besides, the association failed to raise the antioxidant defense with results comparable to curcumin, whereas rutin was the most effective in preventing the decrease in the antioxidant defenses in the liver and kidney. Even so, it was able to significantly prevent immune activation in the spleen and reduce the levels of damage in lipids and protein, in the liver and kidney.

The differences found in the intensity of effects, in different tissues and tested pretreatments, may reflect different mechanisms of action employed by these compounds, as well as antagonistic and synergistic effects that the association can exert on the body. The antioxidant mechanisms of polyphenols include the inhibition ROS production, NF-κB pathway, and metal chelation as well as stimulation of antioxidants enzymes and antioxidant molecules such as vitamin E and glutathione [59]. In addition, when interacting with metal ions, different flavonoids requires different ideal pH values to potentiate the reaction [60].

5 CONCLUSIONS

The results of this study highlight the occurrence of immune activation in the spleen and oxidative damage in the liver and kidney caused by hyperlipidemia-associated lipotoxicity. We found that the association of rutin and curcumin did not perform better than the separate polyphenols. More importantly, we showed that rutin and/or curcumin reestablished the immune homeostasis and redox balance disrupted by hyperlipidemia.

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The authors declare that they have no conflicts of interest

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TABLES

Table 2 Biochemical parameters after induction of hyperlipidemia in rats pretreated with rutin, curcumin and association of rutin and curcumin

Groups	Albumin	ALT	AST	Uric acid	FA	Creatinine
S	3.12±0.049	62.4±4.27	133.2±26.5	4.42±0.21	748±0.07	0.8±0.032
R	3.07±0.14	49.5±2.22	138.2±17.8	4.28±0.39	718±102	0.82±0.025
C	3.733±0.25	55.3±3.68	126.1±9.4	7.35±1.11	324±26.7	0.85±0.022
R+C	3.46±0.13	53.8±4.36	105.8±9.0	7.72±1.35	738±122	0.92±0.04
H+S	5.18±0.28**	50.4±4.95	116.8±13.9	30.56±3.17***	739±110	0.8±0.07
H+R	4.62±0.68	57.6±6.98	97.2±10.9	28.12±6.72***	702±22.6	0.78±0.05
H+C	4.00±0.20	51.5±4.35	132.1±8.0	16.95±2.95	714±60.6	0.66±0.04
H+RC	3.90±0.45	50.8±6.86	135.0±15.0	17.88±3.59*	320±32.0	0.74±0.02

AST: aspartate transaminase (U/L). ALT: alanine transaminase (U/L); Albumin (g/dL); Uric acid (mg/dL); Alkaline phosphatase (mg/dL); Creatinine (mg/dL). Groups: saline control, saline + rutin 50 mg/kg, saline + curcumin 50 mg/kg, saline + rutin and curcumin 50 mg/kg, hyperlipidemia, hyperlipidemia + rutin 50 mg/kg, hyperlipidemia + curcumin 50 mg/kg, hyperlipidemia + rutin and curcumin 50 mg/kg. Results were analyzed using two-way ANOVA-Tukey's multiple comparison tests and expressed as mean ± SEM. *The value is significantly different from the control group (P<0.05, n= 6). **The value is significantly different from the control group (P<0.01, n= 6). ***The value is significantly different from the control group (P<0.001, n= 6.

Table 3 Comparison between the hyperlipemia treatments and untreated hyperlipidemia.

TESTS	Hyperlipidemic Rutin	Hyperlipidemic Curcumin	Hyperlipidemic rutin and curcumin
TBARS in liver	↓40%	↓40%	↓50%
TBARS in Kidney	↓44%	↓56%	↓36%
CARBONIL in liver	↓75%	↓80%	↓77%
CARBONIL in kidney	-	↓58%	↓64%
SOD in liver	↑34%	-	-
SOD in kidney	↑119%	↑72%	-
CAT in liver	↑246%	-	
CAT in kidney	-	-	-
GST in liver	↑84%	-	-
GST in kidney	↑190%	-	110%
SW/BW ratio	↓20%	↓20%	↓20%
ADA in spleen	↓23%	↓28%	↓27%

Table 4 Comparison of blood and spleen parameters among control groups

	Lymphocytes	**Neutrophils**	**Platelets**	**Eosinophil**	**Monocytes**	**Leucocytes**	**SW/BW**	**Albumin**	**Splenic ADA**
Saline	5460±422.8	2015±29.1	1181400±762	181.4±65.0	400.8±104.2	8100±502	30.9±2.5	3.12 ± 0.04	30.9±2.5
Rutin	4935±766.0	2211±429.6	1276000±888	251.0±96,2	182.8±72.6	7580±677	40.8±4.0	3.04 ± 0.06	40.8±4.0
Curcumin	4348±544.3	2379±171.8	1182000±114	115.3±54.85	176.3±45.1	6280±640	40.7±3.2	3.06 ± 0.13	40.7±3.2
Rutin and curcumin	3956±529.1	1970±244.7	1214833±114	64.83±25.2	285.8±67.4	6250±802	33.5±4.3	3.73 ± 0.24	33.5±4.3

FIGURES

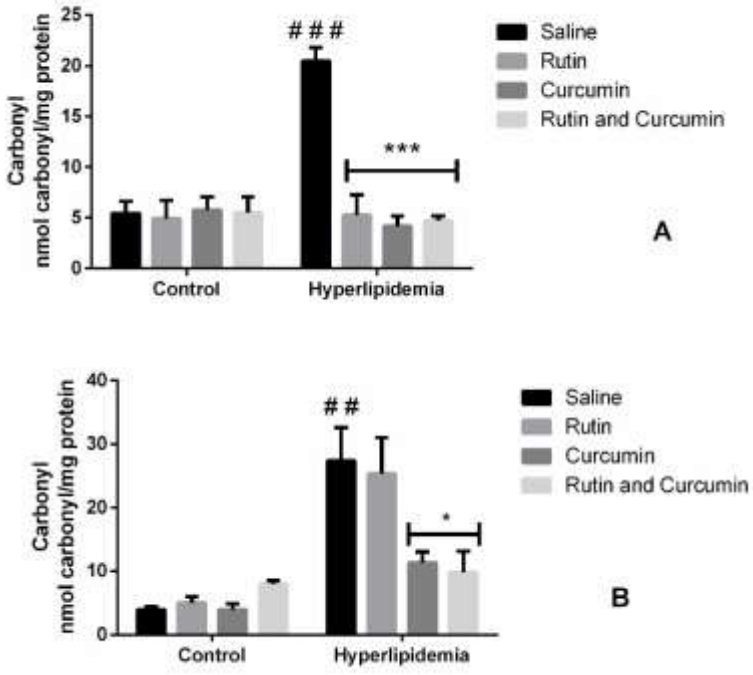

Fig. 1. Protein carbonyl levels in tissues of hyperlipidemic rats pretreated for 30 days with rutin and/or curcumin at the dose 50 mg/kg. (A) Protein carbonyl levels in liver. (B) Protein carbonyl levels in kidney. Groups: saline control, saline + rutin 50 mg/kg, saline + curcumin 50 mg/kg, saline + rutin and curcumin 50 mg/kg, hyperlipidemia, hyperlipidemia + rutin 50 mg/kg, hyperlipidemia + curcumin 50 mg/kg, hyperlipidemia + rutin, and curcumin 50 mg/kg. The results were analyzed using two-way ANOVA followed by Tukey test. The results were expressed as the mean ± SEM. # compare to the control group * compare to the hyperlipidemic group, *P<0.05; ***P*<0.01 and ****P*<0.001.

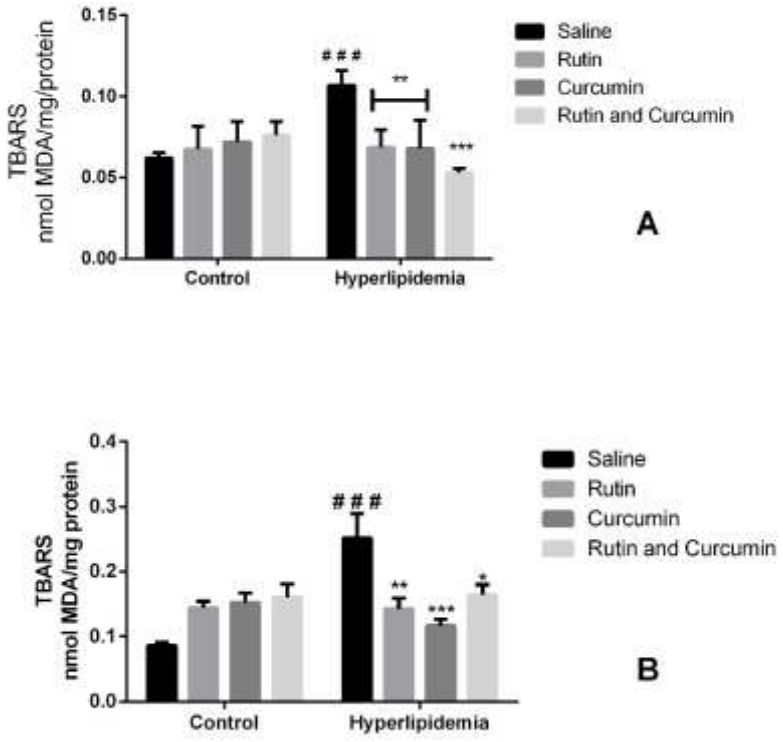

Fig. 2. Lipid peroxidation in tissues of hyperlipidemic rats pretreated for 30 days with rutin and/or curcumin at the dose 50 mg/kg (A) TBARS levels in liver. (B) TBARS levels in kidney. Groups: saline control, saline + rutin 50 mg/kg, saline + curcumin 50 mg/kg, saline + rutin-curcumin association 50 mg/kg, hyperlipidemia, hyperlipidemia + rutin 50 mg/kg, hyperlipidemia + curcumin 50 mg/kg, hyperlipidemia + rutin, and curcumin 50 mg/kg. The results were analyzed using two-way ANOVA followed by Tukey test. The results were expressed as the mean ± SEM. # compare to the control group * compare to the hyperlipidemic group, *P<0.05; **P<0.01 and ***P<0.001.

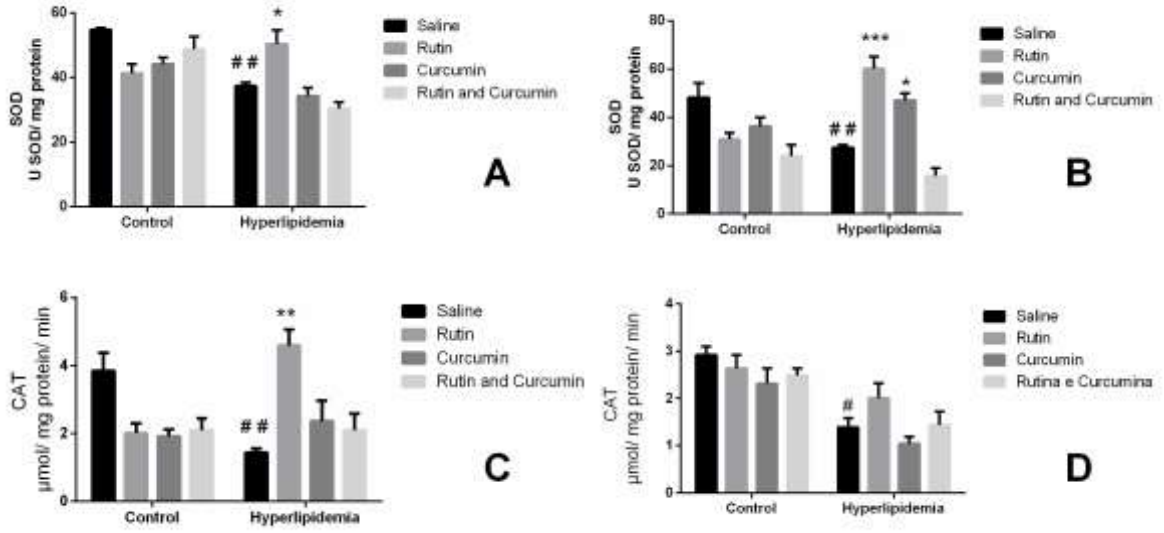

Fig. 3. Antioxidants enzymes activities in tissues of hyperlipidemic rats pretreated for 30 days with rutin and/or curcumin at the dose 50 mg/kg. (A) SOD activity in liver. (B) SOD activity in kidney. (C) CAT activity in the liver. (D) CAT activity in kidney. Groups: saline control, saline + rutin 50 mg/kg, saline + curcumin 50 mg/kg, saline + rutin and curcumin 50 mg/kg, hyperlipidemia, hyperlipidemia + rutin 50 mg/kg, hyperlipidemia + curcumin 50 mg/kg, hyperlipidemia + rutin, and curcumin 50 mg/kg. The results were analyzed using two-way ANOVA followed by Tukey test. The results were expressed as the mean ± SEM. # compare to the control group * compare to the hyperlipidemic group, **P*<0.05; ***P*<0.01 and ****P*<0.001.

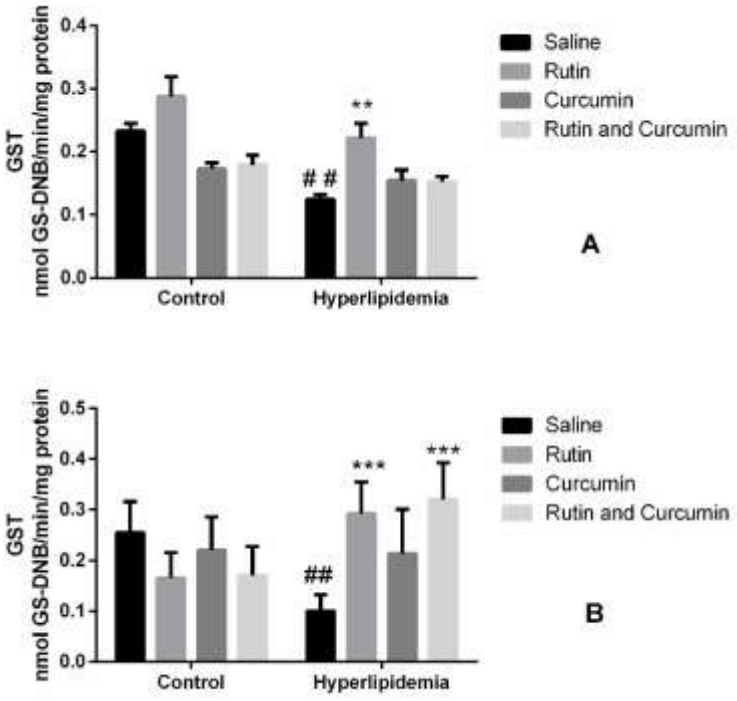

Fig. 4. Antioxidants enzymes activities in tissues of hyperlipidemic rats pretreated for 30 days with rutin and/or curcumin at the dose 50 mg/kg. (A) GST activity in liver. (B) GST activity in kidney. Groups: saline control, saline + rutin 50 mg/kg, saline + curcumin 50 mg/kg, saline + rutin and curcumin 50 mg/kg, hyperlipidemia, hyperlipidemia + rutin 50 mg/kg, hyperlipidemia + curcumin 50 mg/kg, hyperlipidemia + rutin, and curcumin 50 mg/kg. The results were analyzed using two-way ANOVA followed by Tukey test. The results were expressed as the mean ± SEM. # compare to the control group * compare to the hyperlipidemic group, *P<0.05; **P<0.01 and ***P<0.001.

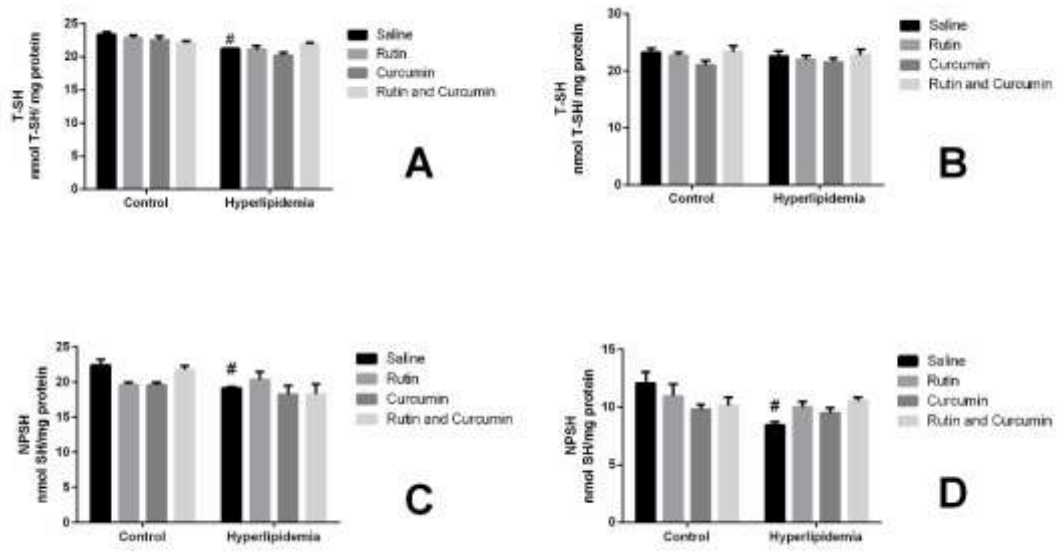

Fig. 5. Non-enzymatic antioxidants levels in tissues of hyperlipidemic rats pretreated for 30 days with rutin and/or curcumin at the dose 50 mg/kg. (A) T-SH levels in liver. (B) T-SH levels in the kidney (C) NPSH levels in the liver. (D) NPSH levels in kidney. Groups: saline control, saline + rutin 50 mg/kg, saline + curcumin 50 mg/kg, saline + rutin and curcumin 50 mg/kg, hyperlipidemia, hyperlipidemia + rutin 50 mg/kg, hyperlipidemia + curcumin 50 mg/kg, hyperlipidemia + rutin, and curcumin 50 mg/kg. The results were analyzed using two-way ANOVA followed by Tukey test. The results were expressed as the mean ± SEM. # compare to the control group * compare to the hyperlipidemic group, *P<0.05; **P<0.01 and ***P<0.001.

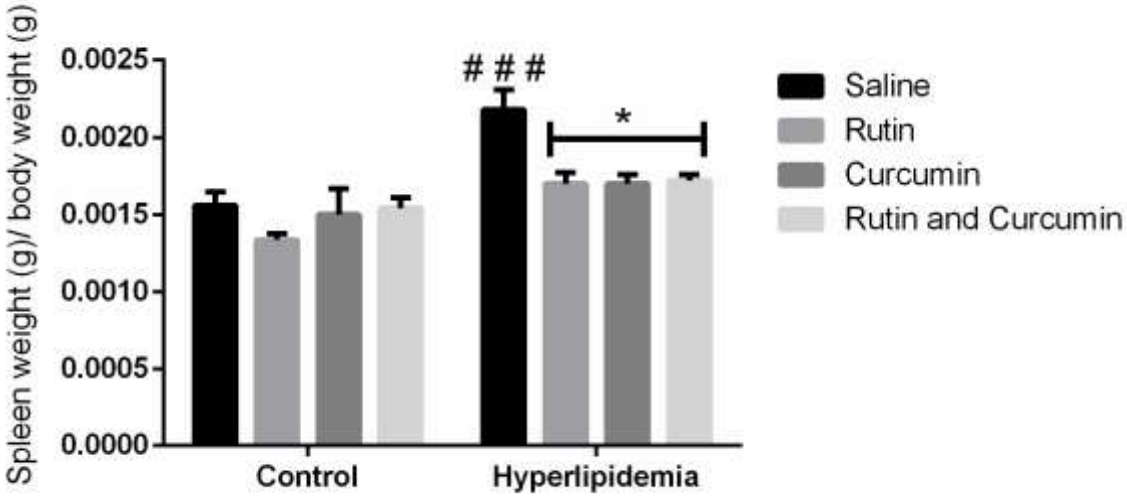

Fig. 6. Spleen weight/body weight ratios in hyperlipidemic rats pretreated for 30 days with rutin and/or curcumin at the dose 50 mg/kg. Groups: saline control, saline + rutin 50 mg/kg, saline + curcumin 50 mg/kg, saline + rutin and curcumin 50 mg/kg, hyperlipidemia, hyperlipidemia + rutin 50 mg/kg, hyperlipidemia + curcumin 50 mg/kg, hyperlipidemia + rutin, and curcumin 50 mg/kg. The results were analyzed using two-way ANOVA followed by Tukey test. The results were expressed as the mean ± SEM. # compare to the control group * compare to the hyperlipidemic group, **P*<0.05; ***P*<0.01 and ****P*<0.001.

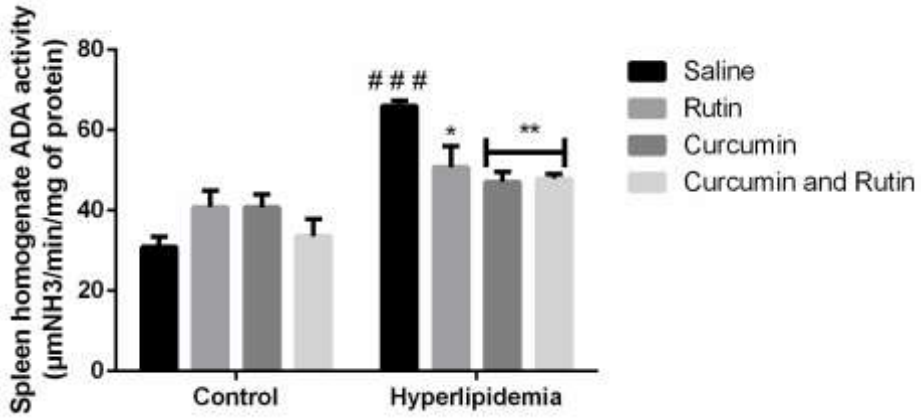

Fig. 7. Adenosine deaminase activity in homogenates of spleen of hyperlipidemic rats pretreated for 30 days with rutin and/or curcumin at the dose 50 mg/kg. Groups: saline control, saline + rutin 50 mg/kg, saline + curcumin 50 mg/kg, saline + rutin and curcumin 50 mg/kg, hyperlipidemia, hyperlipidemia + rutin 50 mg/kg, hyperlipidemia + curcumin 50 mg/kg, hyperlipidemia + rutin, and curcumin 50 mg/kg. The results were analyzed using two-way ANOVA followed by Tukey test. The results were expressed as the mean ± SEM. # compare to the control group * compare to the hyperlipidemic group, **P*<0.05; ***P*<0.01 and ****P*<0.001.

4.2- ARTIGO 2: Purine Metabolism in Platelets and Heart Cells of Hyperlipidemic Rats. Cardiovascular Drugs Therapy, 2020

Purine metabolism in platelets and heart cells of hyperlipidemic rats

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ABSTRACT

Purpose: Hyperlipidemia, characterized by an increase in circulating lipid levels, doubles the chance of developing cardiovascular diseases. It prompts inflammation, immune activation, and oxidative stress in the bloodstream and organs of rats. Thus, we theorized that the metabolism of purines, an immunomodulatory mechanism, is altered in cells involved in the development of cardiovascular diseases.

Methods: Hence, we induced acute hyperlipidemia in Wistar rats with Poloxamer-407 and euthanized the animals 36 hours later. The leucocyte differential, the rate of purine metabolism in the surface of platelets and heart cells, and markers of oxidative stress in the heart tissue were evaluated. Also, these parameters were assessed in animals pretreated for 30 days with curcumin and/or rutin. Hyperlipidemic rats exhibited a reduced percentage of eosinophils and lymphocytes.

Results: Hyperlipidemia increased the hydrolysis of adenosine triphosphate (ATP), adenosine diphosphate (ADP), and adenosine monophosphate (AMP) in platelets. In heart cells, the metabolism of ATP and adenosine (ADO) were increased, while ADP hydrolysis was reduced. Also, lipid damage and antioxidant defenses were increased in heart homogenates.

Conclusion: Together, these findings are indicative of an increased risk of developing cardiovascular diseases in hyperlipidemic rats. The pretreatments with antioxidants reverted some of the changes prompted by hyperlipidemia preventing detrimental changes in the cells and tissues.

Keywords: hyperlipidemia, purine metabolism, platelets, heart, oxidative stress.

INTRODUCTION

Hyperlipidemia, a condition defined as the elevation of circulating lipids, such as triglycerides (≥150 mg/dl), total cholesterol (≥ 200mg/dl), and low-density lipoproteins (≥ 160 mg/dl), doubles the risk of cardiovascular diseases [1]. Lipid and inflammatory cell build-up in the arterial walls and oxidative stress are the most common effects of hyperlipidemia, leading to systemic immune activation and inflammation, increasing the risk of atherosclerosis, heart failure, and coronary disease [2]. Poloxamer-407 is a well-established method used to induce hyperlipidemia in murine models [3], causing an increase in TC, TG, and LDL levels [4]. P407 induces hyperlipidemia by inhibiting the enzymes involved in triglyceride hydrolysis, cholesterol synthesis, and bile acid synthesis [5].

Excessive levels of circulating lipids impair the transport of cholesterol in the bloodstream, causing it to be deposited in the arterial wall that leads to endothelial damage and increasing the permeability of the endothelial cell membranes. Retained LDL particles oxidize and become immunogenic, aggravating the process of aggression to the vascular endothelium. In response, monocytes migrate to the subendothelial space where they differentiate into macrophages and capture the oxidized LDL. Macrophages are activated, along with other inflammatory cells, such as T cells, and lead to the progression of the atherosclerotic plaque and amplification of inflammation through the secretion of cytokines and proteolytic enzymes. In addition, the endothelial dysfunction prompts the pro-fibrotic agents and platelet aggregation as a response to the damage [6].

In addition to the vasculature, several organs are affected by hyperlipidemia-induced lipotoxicity. Lipid deposition and toxic lipids, along with reactive oxygen species and inflammatory mediators, cause organ damage and dysfunction by lipotoxicity [7,8]. Hyperlipidemia causes cardiovascular impairment through various pathologic mechanisms. Blockade of blood flow and oxygen to the heart and accumulation of toxic lipids in this organ are direct mechanisms of cardiovascular impairment. Moreover, the heart is affected by the systemic inflammation and oxidative stress caused by hyperlipidemia. These mechanisms alter crucial signaling pathways that maintain homeostasis and preserve the function of this organ [9].

Inflammation and oxidative stress are strongly associated with cell and tissue damage (REF). Extracellular purine nucleosides are signaling molecules that activate purinergic receptors present on the surface of cells, and these interactions trigger several physio-pathological processes such as platelet aggregation, immune response, inflammation, and

modulation of cardiac function [10]. The degradation of these molecules by purinergic enzymes has a critical immunomodulatory role that allows the maintenance of immune homeostasis. Adenosine triphosphate (ATP) is a purine nucleoside that mediates inflammation. When released from injured or stressed cells in high concentrations, it interacts with the P2X7 receptor that activates the NLRP3 inflammasome in immune cells [11].

The downstream metabolism of ATP results in adenosine diphosphate (ADP), adenosine monophosphate (AMP), and adenosine (ADO). These purines are hydrolyzed by Ecto-nucleoside triphosphate diphosphohydrolase (EC3.6.1.5, E-NTPDase), ecto-5'-nucleotidase (EC 3.1.3.5; E-5´-NT), and ecto-adenosine deaminase (E.C 3.5.4.4, ADA), respectively. ADP has an important role in platelet function and thrombus formation, when interacting with the P2Y12 receptor in platelets it prompts platelet activation [12].

ADO is a cardioprotective nucleoside with proven antioxidant and anti-inflammatory properties. ADO plays a multiplicity of immunomodulatory effects, including the promotion of angiogenesis, tissue regeneration, and inhibition of platelet aggregation [13]. In the heart, ADO regulates mechanisms that are vital to the maintenance of heart function, such as contractility, coronary flow, and heart rate [14]. ADO is degraded by ADA, an important biomarker that increases activity in activated immune cells, and serum in proinflammatory environments [15,16].

Curcumin is a natural pigment present in turmeric with antioxidant and anti-inflammatory properties. Recent studies have explored its effects on inflammatory diseases such as atherosclerosis to which hyperlipidemia is a risk factor [17,18]. Present in leafy vegetables and citrus fruits, rutin is a bioflavonoid with a wide range of pharmacological applications. Thus, current research has highlighted its pharmacological benefits for the treatment of various chronic diseases such as cancer, diabetes, hypertension, and hyperlipidemia [19].

Hyperlipidemia prompts inflammation, immune activation, and oxidative stress in the bloodstream and organs of rats [15,16]. Thus, we theorized that the metabolism of purines, an immunomodulatory mechanism, would be altered in cells directly involved in the development of cardiovascular diseases. Being hyperlipidemia closely linked to cardiovascular impairment, we explored the purine metabolism in platelets and heart cells of hyperlipidemic rats, as well as oxidative stress in the heart.

Based on evidence that rutin and curcumin are antioxidant and anti-inflammatory [15,16] and capable of preventing atherosclerosis [20,21], we also assessed the protective role of rutin and curcumin in platelets and heart cells of hyperlipidemic rats.

METHODS

Animals

The animals used in this study were conventional adult male Wistar rats, obtained by outbreeding in the UFSM animal house. Before any experiment was conducted, all procedures in the study were cleared with the local Animal Use Ethics Committee (Protocol number: 1006200117), which follows international and Brazilian legislation in animal experimentation. The animals used in this study are the same used in previous studies [15,16].

Groups

The experiment was conducted with eight groups of animals containing five or six animals each. The treatments applied to each group are described below:

Control groups: S: Saline (n=5); R: 50 mg/kg of rutin (n=6); C: 50 mg/kg of curcumin (n=6); R + C: 50 mg/kg of curcumin plus 50 mg/kg of rutin (n=6);

Hyperlipidemic groups: H+S: Saline (n=5) (untreated hyperlipidemic group); H+R: 50 mg/kg of Rutin (n=6); H+C: 50 mg/kg of curcumin (n=6); H+RC: 50 mg/kg of curcumin plus 50 mg/kg of rutin (n=6).

Induction of hyperlipidemia

The induction of acute hyperlipidemia was given by a single intraperitoneal injection of Poloxamer-407 (P407) (Sigma-Aldrich, St. Louis, MO, USA) in the dose of 500 mg/kg dissolved in sterile 0.9% NaCl solution (Fresenius KABI, Brazil) [3]. NaCl sterile solution only was administrated to the animals assigned to the control group (non-hyperlipidemic).

Rutin and/or curcumin pretreatments

Prior to the induction of hyperlipidemia, some animals were randomly selected for a 30-day pretreatment by gavage with rutin (Sigma-Aldrich, St. Louis, MO, USA), curcumin (Sigma Chemical Co., St. Louis, MO, USA), or a combination of curcumin and rutin (50 mg/kg).

Rutin and curcumin were prepared in corn oil. According to previous studies published in our research group, corn oil does not interfere with E-ADA activity [22]. Thus,

this group was excluded from the study because it did not present a significant difference in the enzymatic activities compared to the control group.

Euthanasia and collection of samples

The animals were anesthetized with 3% isoflurane, and the euthanasia was done by exsanguination 36 hours post-induction [3]. The blood samples were drawn by cardiac puncture and the organs were collected.

Isolation of blood platelets

Sodium citrate (3.5%) collection tubes (BD Vacutainer) were used for the blood samples. Platelet-rich plasma was separated from whole blood using the method developed by Pilla et al. [23].

Activity of E-NTPDase and E-5´-NT in platelets

The enzymatic activity of E-NTPDase (hydrolysis of ATP and ADP) in platelets was performed by a colorimetric assay that quantifies the inorganic phosphate released in the nucleotide hydrolysis reaction, according to Pilla et al. [23] and E-5´-NT (hydrolysis of AMP) by the same method with some modifications. The hydrolysis of the substrates was analyzed by measuring the release of the inorganic phosphate as described by Chan e col. (1986) [24].

Preparation of tissue homogenates

The hearts were homogenized on ice in 1 mL Tris HCl buffer (50mM pH 7.4) (Sigma Chemical Co., St. Louis, MO, USA). Samples were further centrifuged at 3000×g for 10 min (4°C) and kept at −80°C.

Preparation of heart cell membranes

The hearts were homogenized on ice in 1 mL of phosphate-buffered saline 1x Ph 7.2 (Sigma Chemical Co., St. Louis, MO, USA). The homogenate was spun at 10000×g for 2 hours (4°C). The supernatants were stored at −80°C, using the method developed by Capiotti et al. [25].

Activity of E-NTPDase and E-5´-NT in membranes of heart cells

The hydrolysis of ATP and ADP by E-NTPDase and the hydrolysis of AMP by E-5´-NT was measured using a colorimetric assay that quantifies the inorganic phosphate released

in the nucleotide hydrolysis reaction. The substrate (ATP or ADP) (Sigma-Aldrich, St. Louis, MO, USA) hydrolysis was analyzed by the release of the inorganic phosphate [25]. Results were given in nmol/Pi/min/mg protein.

Adenosine deaminase activity in membranes of heart cells

The degradation of ADO by ADA was measured in membranes of heart cells according to Capiotti et al. [25]. The activity was measured in μMNH_3/min/mg protein.

Lipid damage assays in heart tissue

Lipid peroxidation was estimated by measuring in nmol/MDA/mg protein of malondialdehyde (MDA) using the thiobarbituric acid reactive substances (TBARS) [26]. The absorbance was measured at 532 nm and malondialdehyde (MDA).

Antioxidant defense analysis in heart tissue

The superoxide dismutase (SOD) activity was measured according to previous methods [27]. The product was given as U SOD/mg of protein. Catalase (CAT) activity was measured according to a method described in the literature [28]. The enzymatic activity was expressed as μmol/min/mg protein.

Protein content

The coomassie blue method [29] was used to determine the proteins content in platelets, membranes of heart cells, and heart homogenates.

Statistical analysis

We used the Student T-test or two-way analysis of variance (ANOVA) plus Tukey's Multiple Comparison Test as required. All data were expressed as mean ± standard error of the mean (SEM). Correlations were tested using Pearson correlation coefficients. Values of *P* smaller than 0.05 were regarded as statistically significant.

RESULTS

Lipid profile

The induction of hyperlipidemia was confirmed by the significant increases found in total cholesterol and triglycerides levels in the untreated hyperlipidemic group compared to the control group. These results were published elsewhere [15].

Hematological parameters

In the leukocyte differential (Table 1), the untreated hyperlipidemic group presented a reduced percentage of eosinophils (1.3±0.33) and lymphocytes (24.2±2.8) when compared to the control group (3.6±0.33 for eosinophils $P<0.01$ and 67.2±1.2 for lymphocytes, $P<0.001$).

Purine metabolism

In platelets

The hydrolysis of ATP (Figure 1.A) was significantly increased in the untreated hyperlipidemic group (6.30±0.81, $P<0.001$) when compared to the control group (2.32±0.40). ADP hydrolysis (Figure 1.B) was also significantly higher in the untreated hyperlipidemic group (18.36±2.35, $P<0.001$) when compared to the control group (4.50±1.08). AMP hydrolysis (Figure 1.C) was elevated in the untreated hyperlipidemic group (14.2±0.79, $P<0.001$) when compared to the control group (4.72±0.55).

All pretreatments prevented the increase in the hydrolysis of ATP (Figure 1.A) when compared to the untreated hyperlipidemic group, where rutin (2.74±0.52) was the pretreatment with the highest statistical difference ($P<0.01$). All pretreatments prevented the increase in ADP hydrolysis: rutin (10.3±2.50 $P<0.05$), curcumin (10.1±1.22, $P<0.05$), and the combination of rutin and curcumin (9.9±2.69, $P<0.05$) when compared with the untreated hyperlipidemic group (18.36±2.35) (Figure 1.B). All pretreatments were able avoided the increase in the hydrolysis of AMP (rutin: 4.1±2.0 $P<0.001$; curcumin: 3.1±1.2, $P<0.001$; rutin+curcumin: 4.3±1.2, $P<0.001$) in comparison to the untreated hyperlipidemic group (14.2±0.7) (Figure 1.C).

In heart cell membranes

ATP hydrolysis (Figure 2A) was increased in the untreated hyperlipidemic group (34.0±2.4, $P<0.05$) when compared to control group (24.8±1.4), as well the hydrolysis of adenosine (Figure 2D) (2.6±0.07, $P<0.001$) when compared to the control group (1.1±0.09). A significant reduction in the hydrolysis of AMP (Figure 2B) was observed in the untreated hyperlipidemic group (0.77±0.3, $P<0.001$) when compared to the control group (5.2±1.5).

The antioxidant defense

SOD (Figures 3.A) and CAT activity (Figure 3.B). SOD (19.1±1.8, $P<0.01$) and CAT (1.3±0.1, $P<0.05$) were increased in the untreated hyperlipidemic group when compared to the control group (8.7±1.0 and 0.4±0.02, respectively).

All pretreatments failed to avoid the increase in SOD. However, curcumin (0.6±0.2, $P<0.05$) and curcumin+rutin pretreatments (0.04±0.07, $P<0.05$) prevented the increase in CAT activity when compared to the untreated hyperlipidemic group (1.3±0.1).

The lipid peroxidation

The levels of TBARS in heart homogenates (Figure 4) were also increased in the untreated hyperlipidemic group (0.1±0.04, $P<0.001$) when compared to the control group (0.05±0.005). The pretreatments with rutin (0.09±0.009, $P<0.001$), curcumin (0.08±0.003, $P<0.001$), and curcumin+rutin (0.8±0.002, $P<0.001$) avoided the increase in TBARS levels in comparison with the hyperlipidemic group.**Correlations**

We found a strong direct correlation between ADA activity in the membranes of heart cells and cholesterol levels of hyperlipidemic rats ($R=0.992$, $P<0.001$). Also, the hydrolysis of ATP in the membranes of the heart cells of these rats was strongly and directly correlated with TBARS levels ($R= 0.895$, $P<0.05$). The results of cholesterol in the serum of these hyperlipidemic animals have been previously published [15]. The graphics of these tests are in the supplementary files.

DISCUSSION

Untreated hyperlipidemic rats showed altered purine metabolism, leukocyte differential and oxidative damage parameters when compared to non-hyperlipidemic rats. They exhibited a reduced percentage of eosinophils and lymphocytes in the differential blood counts. The hydrolysis of ATP, ADP, and AMP were increased on the surface of the platelets of these animals. In the surface of their heart cells, the metabolism of ATP and ADO were increased, while AMP hydrolysis was reduced. Also, lipid damage and antioxidant defenses in the heart tissue were increased.

Leucocytes are important in the context of inflammation and atherosclerosis, however, the specific role of each leukocyte subpopulation in cardiovascular diseases is not yet well-defined. The leukocyte differential is emerging as a useful biomarker to predict the occurrence and prognosis of cardiovascular diseases. Moreover, results from a large clinical trial have shown that reduced eosinophil and lymphocyte counts are associated with the onset of cardiovascular diseases [30]. In our study, the reduction in the percentage of eosinophils and lymphocytes of hyperlipidemic rats suggests that they have an increased risk of developing cardiovascular complications.

In a previous study, we reported changes in the blood counts of hyperlipidemic rats; however, we have not found an increase in the platelet counts [15]. Here, we found an increase

in the metabolism of ATP, AMP, and ADP in the surface of platelets which may indicate that these cells, despite not increased in number, are hyperactive. The levels of extracellular ATP increase in contexts of inflammation and immune activation. Besides, extracellular ATP is known to stimulate lipogenesis in rat adipocytes [31]. The increased activity of E-NTPDase in this work suggests that the levels of ATP are increased in response to the lipid levels in the serum.

ADP is secreted by platelet granules and particularly important in this setting because of its role as a platelet agonist. By binding to specific receptors, ADP favors platelet aggregation and activation and subsequent formation of thrombus and atherosclerosis [12]. In contrast, AMP has been recently revealed as a biomolecule with antiplatelet effects by inhibiting thrombus formation and reducing the release of inflammatory mediators associated with atherosclerosis [32]. Therefore, the increased hydrolysis of ADP and AMP found in this study suggest a regulatory mechanism to decrease the levels of these nucleotides and maintain homeostasis. Corroborating with this idea, another study with hyperlipidemia found an increase in ATP, ADP and AMP levels and a reduction in ADO levels in the serum of hyperlipidemic rats [33].

Purinergic enzymes, such as those that hydrolyze ATP and ADO are considered markers of immune activation, and their function is altered in immune cells and serum of rats and humans with inflammatory diseases [15]. Considering the evidence linking lipid accumulation and toxicity, inflammation, and oxidative stress, we sought to investigate the immune response in the heart of hyperlipidemic rats through purine metabolism. We had a particular interest in heart cells because these parameters are not yet well explored in this tissue. Although there are reports on the expression of E-NTPDase [34], the activity of this enzyme in heart cells was not yet reported. However, the protective role of E-NTPDase against lipid accumulation was demonstrated in an *in vitro* study with adipocytes where E-NTPDase decreased the levels of ATP and, consequently, the accumulation of triacylglycerol caused by this nucleoside [35]

In the membranes of heart cells, ADO plays a crucial role in physiological and pathological processes. The primary sources of ADO in the heart are the myocytes and infiltrating immune cells. The production of ADO occurs both intra- and extracellularly through the E-NTPDase / E-5´-NT pathway [14]. These enzymes respond to increased levels of purines by hydrolyzing them to reduce their levels. Here, we found an increase in ATP hydrolysis by E-NTPDase and the deamination of ADO by ADA. In contrast, the hydrolysis of AMP by E-5´-NT was reduced. Collectively, these findings imply that ADO levels may be decreased in

the heart in hyperlipidemic rats. ADO seems to be not only insufficiently produced but degraded at a greater rate.

Hyperlipidemic rats have also had an increase in TBARS, an oxidative stress and lipid peroxidation marker in the heart. Although the link between hyperlipidemia, cardiovascular disease, and systemic oxidative damage is well-known, we understood that assessing the lipid peroxidation in the heart tissue would better expose the changes caused by hyperlipidemia in this specific organ. Besides, lipid damage has been associated with lipotoxicity in the kidneys [16,36], liver [16,37], and heart [38,39].

Lipid damage was also associated with cholesterol levels and the hydrolysis of ATP (REF). We observed a positive correlation between cholesterol levels and ATP hydrolysis in the heart, which may indicate that ATP levels are increased in response to elevated lipid levels. Also, we found a positive correlation between ATP hydrolysis and TBARS levels, corroborating with the idea that oxidative stress, ATP, and lipid levels are critical factors in cardiovascular impairment. Besides, a study with diabetic patients found increased TBARS levels in those with cardiovascular diseases when compared to those without cardiovascular diseases, indicating that TBARS is a relevant marker of cardiovascular diseases [40].

SOD and CAT are enzymes that take part in the antioxidant defense against oxidative stress. The activities of SOD and CAT were increased in the hearts of hyperlipidemic rats when compared to the control group. A study with diabetes has found similar results [40]. These findings indicate that the heart increased its antioxidant defenses to protect itself from oxidative damage.

Polyphenols are an important class of compounds readily available in fruits and vegetables. These compounds have shown well-known antioxidant, anti-inflammatory, and antilipidemic effects [17,41,42]. In this study, we assessed the effects of two polyphenols, named rutin and curcumin. We aimed to investigate their ability to prevent the changes in the purine metabolism in platelets and heart tissue. However, we observed that, in platelets, the pretreatments prevented the increase in the hydrolysis of ATP, ADP, and AMP, as well as in TBARS levels. Also, curcumin and its association with rutin prevented the increase in CAT activity when compared to the untreated hyperlipidemic group. These results indicate that the use of these polyphenols is beneficial in the prevention of cardiovascular impairment in hyperlipidemic rats.

Purine signaling and metabolism are important modulators of immune responses, promoting a shift from a pro-inflammatory to an anti-inflammatory state. Besides, the importance of immune mechanisms in the onset, prognosis, and treatment of heart failure has

recently started to be recognized [43]. Our results suggest that these changes in the purine metabolism, along with oxidative damage, in the hyperlipidemic rats, indicate an increased risk of cardiovascular impairment.

CONCLUSIONS

The differences observed in the purine metabolism in hyperlipidemic rats are a response to the hyperlipidemia-related immune activation and oxidative stress. In platelets, these changes reflect the hyperactivation of these cells and a pro-thrombotic environment. In the heart, the increased metabolism and decreased production of adenosine indicate that this organ´s response to damage is impaired. Together, these findings are indicative of an increased risk of developing cardiovascular diseases in hyperlipidemic rats.

The effectiveness of the compounds in preventing some of the changes in purine metabolism and oxidative damage markers indicates they may be useful against cardiovascular impairment.

Declarations

Ethics approval and consent to participate

This work was approved by the Ethics Committee on the Use of Animal at the Federal University of Santa Maria (Protocol number: 1006200117).

Consent for publication Not applicable.

Availability of data and material

The authors confirm that the data supporting the findings of this study are available with in this article.

Conflict of interest

The authors declare that they have no conflicts of interest.

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TABLE

Table 1. Differential leukocyte counts in non-hyperlipidemic and hyperlipidemic rats

	Non-hyperlipidemic	Hyperlipidemic
Total Leucocytes counts (TL)	8100±502.0 n=5	6620±513.2 n=5
Eosinophil (% of TL)	3.6±0.33 n=5	1.3±0.33 n=5 **
Neutrophil (% of TL)	25.2±1.3 n=5	24.2±2.8 n=5
Lymphocytes (% of TL)	67.2±1.2 n=5	24.2±2.8 n=5 ***
Monocytes (% of TL)	5.4±1.077 n=5	4.200±1.1 n=5

The percentage of leucocytes were measured in the non-hyperlipidemic group (control) (n=5) and hyperlipidemic group (n=5). The results are expressed in % of total leucocytes. The results were analyzed using the Student t-test expressed as mean ± standard deviation. ** The value is significantly different from the control group ($P<0.01$). *** The value is significantly different from the control group ($P<0.001$).

FIGURES

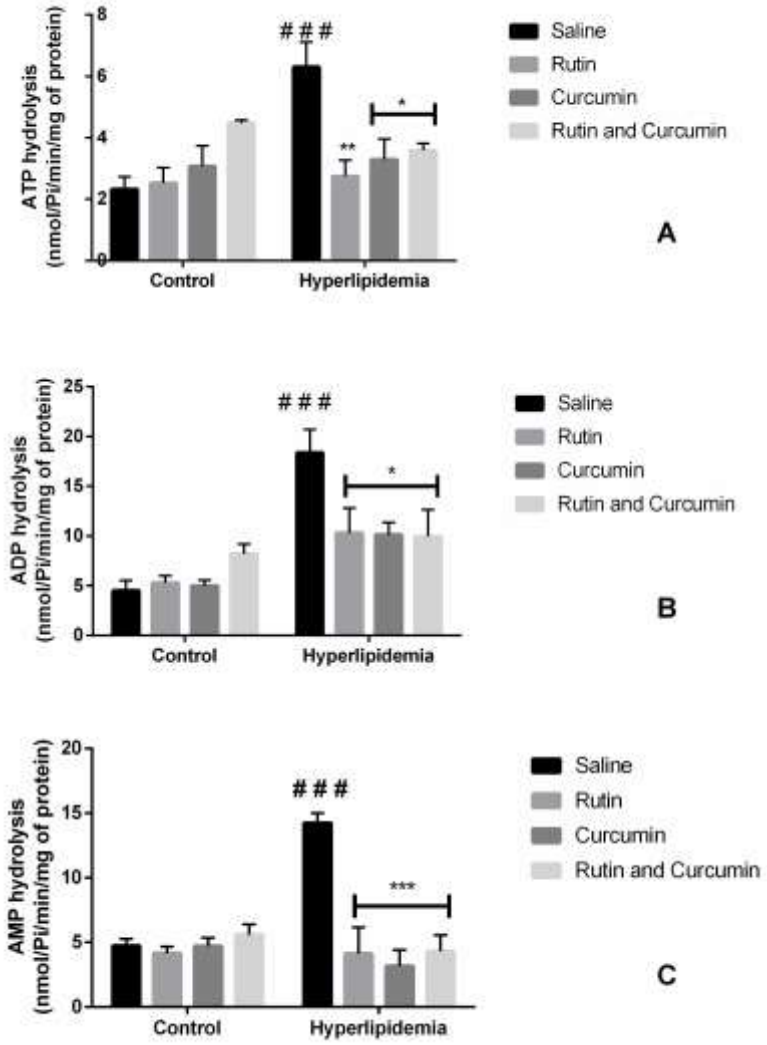

Figure 1: Hydrolysis of ATP (Fig 1A), ADP (Fig 1B) and AMP (Fig 1C) in platelets of rats with poloxamer-407-induced hyperlipidemia (n=5) and the control groups (n=5), both were pretreated with (rutin 50 mg/kg/day (n=6) curcumin 50 mg/kg/day (n=6) and rutin and curcumin 50 mg/kg/day (n=6)). The results were analyzed using the two-way ANOVA-Tukey multiple comparison test and expressed as mean ± SEM. ### The value is significantly different from the control group ($P<0.001$). * The value is significantly different from the hyperlipidemic group ($P<0.05$) *** The value is significantly different from the hyperlipidemic group ($P<0.001$).

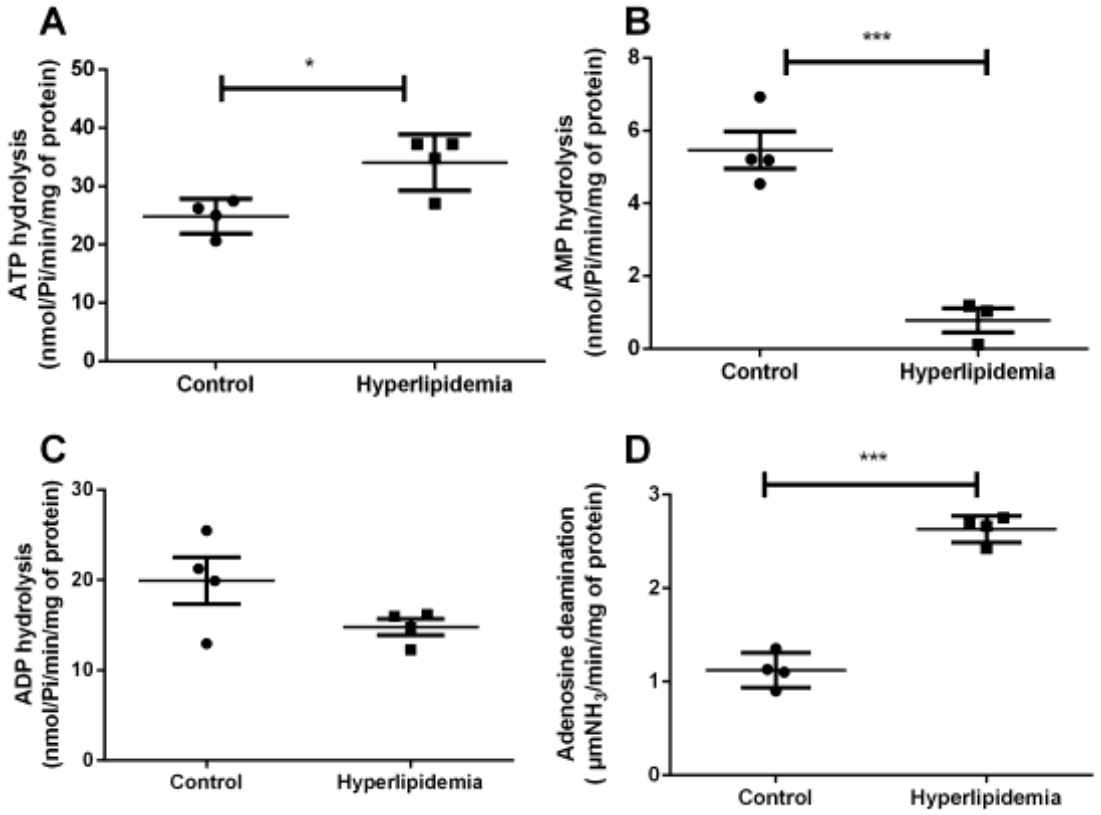

Figure 2: The hydrolysis of ATP (Fig 2A), ADP (Fig 2B), AMP (Fig 2C) and Adenosine (Fig 2D) was measured in membranes of the heart cells of rats with poloxamer-407-induced hyperlipidemia (n=4) and the control group (n=4). The activity of ATP, ADP, and AMP in measure in nmol/Pi/mg of protein. The results were analyzed using the t test and expressed as mean ± SEM. * The value is significantly different from the control group (P<0.05). *** The value is significantly different from the hyperlipidemic group (P<0.001).

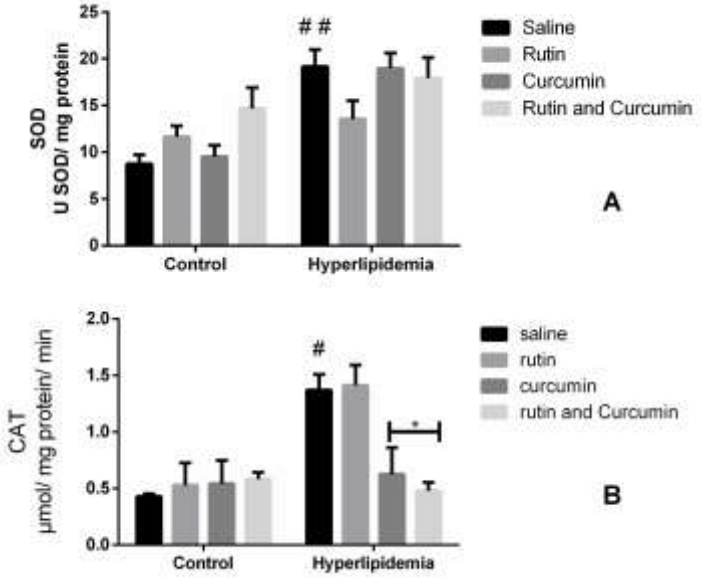

Figure 3: The antioxidant activity of SOD and CAT in heart homogenates of rats with poloxamer-407-induced hyperlipidemia (n=5) and the control groups (n=5), both were pre-treated with (rutin 50 mg/kg/day, n=6), (curcumin 50 mg/kg/day, n=6) and (rutin and curcumin 50 mg/kg/day, n=6). The results were analyzed using the two-way ANOVA-Tukey multiple comparison test and expressed as mean ± SEM. ## The value is significantly different from the control group (P<0.01). # The value is significantly different from the control group (P<0.05). * The value is significantly different from the control group (P<0.05).

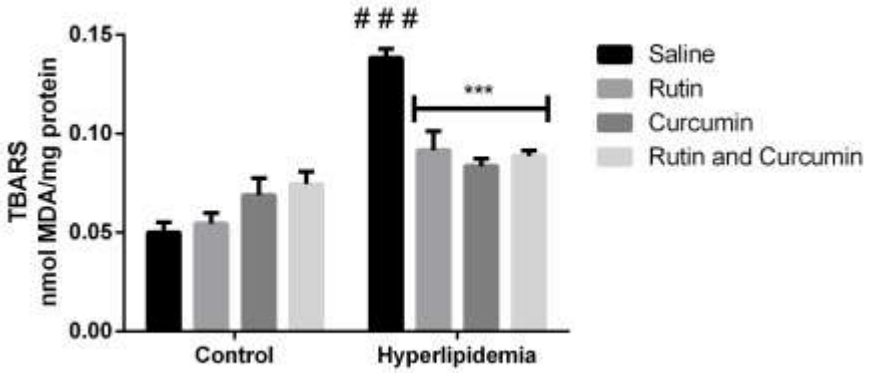

Figure 4: The lipid peroxidation in heart homogenates of rats with poloxamer-407-induced hyperlipidemia (n=5) and the control groups (n=5), both were pretreated with (rutin 50 mg/kg/day, n=6), (curcumin 50 mg/kg/day, n=6) and rutin and curcumin 50 mg/kg/day, n=6). The results were analyzed using the two-way ANOVA-Tukey multiple comparison test and expressed as mean ± SEM. ### The value is significantly different from the control group (P<0.001).*** The value is significantly different from the hyperlipidemic.

5- DISCUSSÃO

Este trabalho teve como objetivo avaliar sistemicamente os efeitos da hiperlipidemia, bem como os efeitos do pré-tratamento com rutina e/ou curcumina nas alterações decorrentes. No primeiro artigo foram descritas mudanças bioquímicas, hematológicas e em parâmetros de estresse oxidativo em órgãos como rim, fígado e baço. Já no segundo artigo foram avaliadas as atividades de enzimas purinérgicas e danos oxidativos em plaquetas e membranas de células cardíacas.

O modelo escolhido para a indução de hiperlipidemia está bem descrito na literatura. O poloxamer-407 é utilizado tanto para indução de hiperlipidemia, quanto de aterosclerose (JOHNSTON; KOROLENKO; SAHEBKAR, 2017). Os resultados de aumento de colesterol e triglicerídeos deste estudo, já foram publicados anteriormente e comprovam que o modelo foi eficaz em aumentar os níveis plasmáticos de lipídeos (MANZONI et al., 2019). A eutanásia foi feita após 36 horas da injeção de P407 via intraperitoneal pois de acordo com o estudo de PALMER 1998, os níveis séricos máximos desses lipídios ocorrem após este periodo, e a administração contínua desse composto leva ao desenvolvimento de aterosclerose (PALMER; EMESON; JOHNSTON, 1998).

Quanto aos parâmetros hematológicos, anteriormente nosso grupo de pesquisa demonstrou alterações significativas nas contagens de glóbulos brancos (neutrofilia e linfopenia), assim como nas razões entre as contagens de neutrófilos/linfócitos e plaquetas/linfócitos em modelo experimental de hiperlipidemia (MANZONI et al., 2019). A alteração destes marcadores tem sido observada em processos inflamatórios e em doenças onde há disfunção endotelial (SANTOS; FERNANDO; IZIDORO, 2018). Já em relação a rutina e a curcumina, ambas foram eficazes em prevenir as alterações hematológicas, sugerindo uma redução da resposta imune e do processo inflamatório.

Neste trabalho, foi demonstrado que a porcentagem de linfócitos e eosinófilos em relação aos leucócitos totais, foi menor em animais com hiperlipidemia, quando comparados com os animais controle. Estas alterações já foram descritas como sendo biomarcadores para doenças cardiovasculares (SHAH et al., 2016). A rutina e a curcumina já haviam mostrado seu papel preventivo em marcadores hematológicos em um estudo anterior (MANZONI et al., 2019).

Também foram testados os pre-tratamentos rutina e curcumina *per se*, no primeiro artigo deste trabalho, foram avaliados alguns marcadores de imunotoxicidade, com isso conseguimos verificamos que a utilização destes compostos se mostrou segura e não imunotóxica, utilizados isoladamente ou em combinação.

Ainda, modificações em parâmetros bioquímicos, como o aumento nos níveis séricos de albumina e ácido úrico foram encontradas nos animais hiperlipidêmicos, quando comparados aos grupos controles. O aumento nos níveis de albumina é considerado um marcador para doenças renais e cardiovasculares (DORNELLES et al., 2017). Ademais, elevados níveis de ácido úrico foram descritos como marcadores de risco para o surgimento de doenças como síndrome metabólica e hiperlipidemia (ONAT et al., 2006; YANG et al., 2012). Os pré-tratamentos com rutina e/ou curcumina foram capazes de prevenir o aumento nos níveis de albumina, porem somente a curcumina isolada foi capaz de prevenir os aumentos de ácido úrico.

Para medir os danos causados em órgãos, utilizamos marcadores de danos oxidativos, dentre eles se destacam os danos causados em proteínas e lipídeos, que foram avaliados pelas técnicas carbonil e TBARS, respectivamente (ARAUJO et al., 1995). No primeiro artigo, foram avaliados os danos oxidativos em fígado e rim, bem como os marcadores antioxidantes enzimáticos e não enzimáticos. Também foi avaliado dados referentes ao baço, aonde encontramos um aumento no peso do baço e também uma maior atividade da ADA em homogeneizado de baço, o que nos indica a presença de processo inflamatório bem como ativação imune neste órgão.

Na hiperlipidemia foi observado estresse oxidativo, demonstrado pelo aumento nos níveis de marcadores de dano oxidativo nos órgãos e redução nas defesas antioxidantes, colaborando com os achados da literatura que descrevem a lipotoxicidade causada pela hiperlipidemia, bem como os danos celular e tecidual associados (KIM et al., 2012). Com relação aos pré-tratamentos com rutina e/ou curcumina, eles foram eficazes em prevenir os danos oxidativos, porém somente a rutina foi capaz de prevenir o aumento nas defesas antioxidantes enzimáticas, e nenhum dos compostos testados isolados ou em associação preveniram a redução nos níveis de defesas antioxidantes não enzimáticas.

A hiperlipidemia se caracteriza pelo desenvolvimento de um processo inflamatório sendo este capaz de gerar dano oxidativo, e lesões teciduais que estão relacionadas às doenças cardiovasculares. Além disto, o acúmulo destes lipídeos em órgão causa lipotoxicidade, que está associada ao aparecimento de efeitos deletérios nos órgãos (HAUCK; BERNLOHR, 2016).

Com relação ao baço, foi observado um aumento no tamanho do baço, bem como aumento na atividade da ADA. A atividade desta enzima no soro já está bem descrita como um marcador não especifico de ativação celular e inflamação (SANDS, 2015; VINAPAMULA et al., 2015), assim como no baço (XIE et al., 2018). A rutina e a curcumina tanto isoladas como em associação foram capazes de prevenir o aumento da ADA e o aumento do tamanho do baço.

Quando avaliados os tratamentos rutina e/ou curcumina nos parâmetros testados no primeiro artigo, podemos notar que a curcumina e a rutina isoladas ou em combinação foram capazes de prevenir o aumento nos níveis de marcadores de dano oxidativos. Porém quando avaliamos o papel destes compostos na prevenção da redução dos marcadores antioxidantes a rutina isolada foi o composto que se mostrou com maior capacidade de prevenir a redução destas enzimas. A combinação de rutina e curcumina não mostrou o mesmo efeito, isso indica que neste marcador em especifico, a associação destes compostos não é benéfica e isso pode ter sido causado devido a ação antagonista de um composto com o outro. Outro trabalho já mostrou que a combinação de alguns flavonoides pode trazer efeitos antagônicos em marcadores anti-inflamatórios (HIDALGO; SÁNCHEZ-MORENO; DE PASCUAL-TERESA, 2010).

Com relação ao segundo artigo, foram encontradas alterações importantes na hidrólise dos nucleotídeos de adenina. Certos nucleotídeos como o ATP, são conhecidos por serem padrões associados ao dano celular (DAMP's). A ativação de células causa um aumento na liberação de ATP, o que consequentemente gera um aumento na atividade das enzimas do sistema purinérgico, que são responsáveis pela degradação desde nucleotídeo.

A atividade das enzimas responsáveis pela hidrólise do ATP em ADP e ADP em AMP, NTPDase e 5'-nucleotidase, estavam aumentadas em plaquetas de animais hiperlipidêmicos quando comparados com os grupos controles. Este aumento sugere que estes nucleotídeos estão sendo liberados em maior quantidade nos animais hiperlipidêmicos, podendo estar associado aos danos provocados a estas células, devido ao excesso de lipídeos. O pré-tratamento com rutina e/ou curcumina preveniu o aumento na atividade destas enzimas em plaquetas, sugerindo uma redução na resposta imune e redução no processo inflamatório. Com relação a atividade de enzimas purinérgicas em plaquetas, a rutina e a curcumina isoladas ou em associação foram capazes de prevenir o aumento da atividade das duas enzimas testadas.

A atividade da enzima ADA, que degrada a adenosina proveniente do metabolismo do ATP em inosina, pode estar elevada no soro e na superfície das células imunes, o que está associado à ativação imune e inflamação (BEYAZIT et al., 2012). Em membranas de células cardíacas foi observado um aumento na atividade da enzima NTPDase, juntamente com a redução na atividade da 5'-nucleotidase, o que nos sugere que os níveis de adenosina no tecido cardíaco estavam baixos, provavelmente devido a uma menor produção pela degradação do AMP e também relacionada a uma maior degradação devido a atividade da ADA. A adenosina é uma molécula considerada anti-inflamatória e protetora de danos, sendo sua redução uma

consequência preocupante para o desenvolvimento doenças imunes e inflamatórias (BOURS et al., 2006).

Em relação ao marcadorde lipoperoxidação, os níveis estavam aumentados no tecido cardíaco de animais hiperlipidêmicos, quando comparados ao controle. Em contraste, foi observado um aumento nas defesas antioxidantes do tecido cardíaco. Este aumento pode estar relacionado a uma tentativa de proteção contra os danos oxidativos gerados no coração. A curcumina e a rutina isoladas e em associação conseguiram prevenir o aumento dos níveis de TBARS no coração, porém quanto a atividade antioxidante somente a curcumina e a associação conseguiram prevenir o aumento da atividade da CAT no coração.

A lipotoxicidade causou danos não somente ao tecido endotelial, acompanhado por um processo inflamatório e presença de estresse oxidativo, mas também afetou diversos órgãos como o fígado, rim e baço de ratos hiperlipidêmicos. O aumento no estresse oxidativo provavelmente foi uma consequência da excessiva oxidação de lipídeos. Os pré-tratamentos com rutina e/ou curcumina foram eficazes em reduzir a maioria dos danos causados pela hiperlipidemia. Com isso, é possível sugerir que estes polifenois, tanto isolados como em associação, foram eficientes em prevenir alterações causadas pela hiperlipidemia. Entretanto, mais estudos são necessários, para avaliar a interação desses compostos em cada órgão e parâmetros avaliados, para determinar se a utilização em conjunto desses compostos tem ação sinérgica ou antagônica em cada situação.

6- CONCLUSÕES

- Em animais submetidos a indução da hiperlipidemia, foram observadas alterações bioquímicas como o aumento de ácido úrico e de albumina.
- Os níveis de fosfatase alcalina, creatinina e a atividade da ALT e AST, não apresentaram diferença estatística nos diferentes grupos experimentais.
- As porcentagens de linfócitos e eosinófilos em relação aos leucócitos totais foi menor em animais com hiperlipidemia, quando comparados com os animais controle.
- A atividade das enzimas NTPDase e 5'-nucleotidase estavam aumentadas em plaquetas de animais hiperlipidêmicos, quando comparados com os grupos controles. Em membranas de células cardíacas foi observado um aumento na atividade da NTPDase e uma redução na atividade da 5'-nucleotidase, juntamente com um aumento na atividade da ADA.
- A lipotoxicidade causou danos não somente ao tecido endotelial, como também gerou um processo inflamatório e exacerbou a presença de estresse oxidativo, afetando órgãos como o fígado, rim, baço e coração de ratos hiperlipidêmicos.
- Os pré-tratamentos com rutina e/ou curcumina foram eficazes em reduzir os níveis de estresse oxidativo nos órgãos, prevenir o aumento das enzimas purinérgicas em plaquetas e normalizar os parâmetros testados em baço. A rutina isolada foi o composto que melhor preveniu a redução das defesas antioxidantes.
- A rutina e a curcumina se mostraram não imunotóxicas, evidenciando ainda mais sua ação. Assim podemos sugerir que estes polifenois, tanto isolados como em associação, foram eficientes em prevenir a maioria das alterações causadas pela hiperlipidemia.

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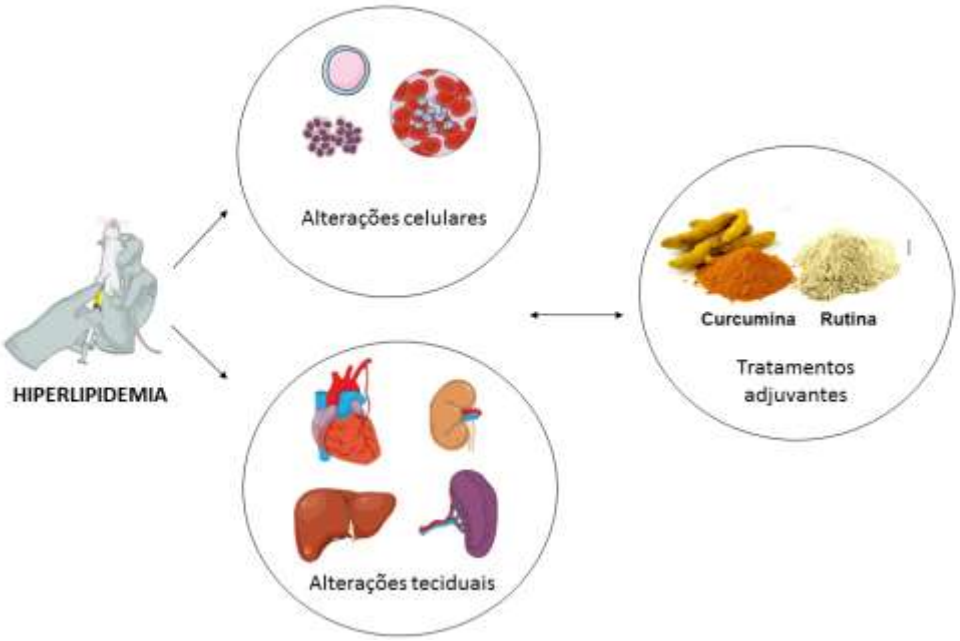
ANEXOS

1- Graphical Abstract



2- Artigo 1

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Hyperlipidemia-induced lipotoxicity and immune activation in rats are prevented by curcumin and rutin

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ABSTRACT

We assessed the effects of curcumin, rutin, and the association of rutin and curcumin in organs of hyperlipidemic rats. Rutin and curcumin have notable antioxidant and anti-inflammatory actions, so we hypothesized that their association would enhance their beneficial effects. Hyperlipidemia results in lipotoxicity and affects several organs. Lipotoxicity is not only an outcome of lipid accumulation in non-adipose tissues but also a result of the hyperlipidemia-associated inflammation and oxidative stress. Wistar rats were treated with rutin and curcumin for 30 days before the induction of acute hyperlipidemia by Poloxamer-407. After 36 h, the animals were euthanized for collection of blood and organs. Untreated hyperlipidemic rats showed higher uric acid and albumin levels in the serum and increased spleen size and ADA activity. Rutin, curcumin and the association reduced the spleen size by 20% and ADA activity by 23, 28, and 27%, respectively. Rats pretreated with rutin showed reduced lipid damage in the liver (40%) and the kidney (44%), and the protein damage was also reduced in the liver (75%). The lipid damage was decreased by 40% in the liver, and 56% in the kidney of rats pretreated with curcumin. The association reduced lipid damage by 50% and 36%, and protein damage by 77% and 64% in the liver and kidney, respectively. Rutin better prevented the decrease in the antioxidant defenses, increasing SOD by 34%, CAT by 246% and GST by 84% in the liver, as well as SOD by 119% and GST by 190% in the kidney. Also, analyses of blood and spleen parameters of untreated and pretreated non-hyperlipidemic rats showed no signs of immunotoxicity. Despite showing protective effects, the association did not perform better than the isolated compounds. Here, we showed that rutin and/or curcumin reestablished the immune homeostasis and redox balance disrupted by hyperlipidemia in peripheral organs of rats.

1. Introduction

Lipotoxicity is caused by the accumulation of lipids in non-adipose tissues, or as a consequence of inflammation and oxidative stress. Several organs, such as the liver, spleen, heart, and kidneys are affected by lipotoxicity [1,2]. Hyperlipidemia is a condition caused by elevated serum levels of total cholesterol, low-density lipoprotein cholesterol and triglycerides which accumulate in the body and may induce lipotoxicity affecting different organs and systems [3]. Hyperlipidemic individuals are not only more prone to develop cardiovascular disorders [4] but are also at risk of developing liver [5] and kidney diseases [1].

Lipid deposition in the arterial walls elicits immune activation and inflammation by LDL oxidation and formation of foam cells [6], changing the profile of circulating immune cells [7], and is likely to affect secondary lymphoid organs such as the spleen. The spleen maintains peripheral tolerance, modulates of both innate and adaptive immune responses and reacts to different pathological conditions [8,9].

Polyunsaturated lipids include cytotoxic lipids that impact on cellular homeostasis, even small changes in quantity, composition, or location of such lipids can have profound effects on cellular viability and function. Lipid antigens are recognized by splenic natural killer T (NKT) cells, initiating the adaptive response by releasing cytokines [10,11].

Due to the anatomic proximity, inflammatory mediators originated in the spleen may reach the liver and kidney, increasing toxicity [11,12]. The liver is a central organ in the metabolism of fatty acids, proteins, and glucose, and is sensitive to lipid toxicity and

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ORIGINAL ARTICLE

Purine Metabolism in Platelets and Heart Cells of Hyperlipidemic Rats

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Abstract

Purpose Hyperlipidemia, characterized by an increase in circulating lipid levels, doubles the chance of developing cardiovascular diseases. It prompts inflammation, immune activation, and oxidative stress in the bloodstream and organs of rats. Thus, we theorized that the metabolism of purines, an immunomodulatory mechanism, is altered in cells involved in the development of cardiovascular diseases.

Methods Therefore, we induced acute hyperlipidemia in Wistar rats with Poloxamer-407 and euthanized the animals 36 h later. The leucocyte differential, the rate of purine metabolism on the surface of platelets and heart cells, and markers of oxidative stress in the heart tissue were evaluated. These parameters were also assessed in animals pretreated for 30 days with curcumin and/or rutin.

Results Hyperlipidemia increased the hydrolyses of adenosine triphosphate (ATP), adenosine diphosphate (ADP), and adenosine monophosphate (AMP) in platelets. In heart cells, the metabolism of ATP and adenosine (ADO) were increased, while ADP hydrolysis was reduced. Additionally, lipid damage and antioxidant defenses were increased in heart homogenates. Hyperlipidemic rats also exhibited a reduced percentage of eosinophils and lymphocytes.

Conclusion Together, these findings are indicative of an increased risk of developing cardiovascular diseases in hyperlipidemic rats. The pretreatments with antioxidants reverted some of the changes prompted by hyperlipidemia preventing detrimental changes in the cells and tissues.

Keywords Hyperlipidemia · Purine metabolism · Platelets · Heart · Oxidative stress

Introduction

Hyperlipidemia, a condition defined as the elevation of circulating lipids, such as triglycerides (≥150 mg/dl), total cholesterol (≥ 200 mg/dl), and low-density lipoproteins (≥ 160 mg/dl), doubles the risk of cardiovascular diseases [1]. Lipid and inflammatory cell build-up in the arterial walls and oxidative stress are the most common effects of hyperlipidemia, leading to systemic immune activation and inflammation, increasing the risk of atherosclerosis, heart failure, and coronary disease [2]. Poloxamer-407 is a well-established method used to induce hyperlipidemia in murine models [3], causing an increase in TC, TG, and LDL levels [4]. P407 induces hyperlipidemia by inhibiting the enzymes involved in triglyceride hydrolysis, cholesterol synthesis, and bile acid synthesis [5].

Excessive levels of circulating lipids impair the transport of cholesterol in the bloodstream, causing it to be deposited in the arterial wall, leading to endothelial damage, and increasing the permeability of the endothelial cell membranes. Retained LDL particles oxidize and become immunogenic, aggravating the process of aggression to the vascular endothelium. In response, monocytes migrate to the subendothelial space where they differentiate into macrophages and capture the oxidized LDL. Macrophages are activated, along with other inflammatory cells, such as T cells, and lead to the progression of the atherosclerotic plaque and amplification of inflammation

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4- Carta de aprovação do CEUA.

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

CERTIFICADO

Certificamos que a proposta intitulada "AVALIAÇÃO DO EFEITO DA RUTINA E CURCUMINA NO SISTEMA PURINÉRGICO E PERFIL OXIDATIVO DE RATOS COM HIPERLIPIDEMIA INDUZIDA", protocolada sob o CEUA nº 1006200117, sob a responsabilidade de **Daniela Bitencourt Rosa Leal** - que envolve a produção, manutenção e/ou utilização de animais pertencentes ao filo Chordata, subfilo Vertebrata (exceto o homem), para fins de pesquisa científica ou ensino - está de acordo com os preceitos da Lei 11.794 de 8 de outubro de 2008, com o Decreto 6.899 de 15 de julho de 2009, bem como com as normas editadas pelo Conselho Nacional de Controle da Experimentação Animal (CONCEA), e foi **aprovada** pela Comissão de Ética no Uso de Animais da Universidade Federal de Santa Maria (CEUA/UFSM) na reunião de 08/03/2017.

We certify that the proposal "EVALUATION OF THE EFFECT OF RUTIN AND CURCUMIN IN THE PURINERGIC SYSTEM AND OXIDATIVE PROFILE OF RATS WITH INDUCED HYPERLIPIDEMIA", utilizing 56 Heterogenics rats (56 males), protocol number CEUA 1006200117, under the responsibility of **Daniela Bitencourt Rosa Leal** - which involves the production, maintenance and/or use of animals belonging to the phylum Chordata, subphylum Vertebrata (except human beings), for scientific research purposes or teaching - is in accordance with Law 11.794 of October 8, 2008, Decree 6899 of July 15, 2009, as well as with the rules issued by the National Council for Control of Animal Experimentation (CONCEA), and was **approved** by the Ethic Committee on Animal Use of the Federal University of Santa Maria (CEUA/UFSM) in the meeting of 03/08/2017.

Finalidade da Proposta: Pesquisa (Acadêmica)

Vigência da Proposta: de 03/2017 a 10/2018 Área: Microbiologia E Parasitologia

Origem:	Biotério Central UFSM						
Espécie:	Ratos heterogênicos	sexo:	Machos	idade:	60 a 90 dias	N:	56
Linhagem:	Wistar			Peso:	250 a 300 g		

Resumo: A hiperlipidemia é um fator de risco para o desenvolvimento da aterosclerose, que é caracterizada por anormalidades lipídicas, desenvolvimento de processos inflamatórios crônicos e consequente dano oxidativo. Durante estes processos, os nucleotídeos e nucleosídeos da adenina são mediadores capazes de modular a ação de linfócitos e plaquetas para o sítio inflamatório, os quais possuem seus níveis controlados por meio da ação de ecto-enzimas ancoradas na superfície de linfócitos e plaquetas. A curcumina e a rutina são flavonóides importantes que possuem ação antioxidante, antiinflamatória e potencial para redução dos níveis de colesterol. Sendo assim, o objetivo do presente estudo será avaliar a atividade de ecto-enzimas do sistema purinérgico em linfócitos e plaquetas e o perfil oxidativo em ratos com hiperlipidemia induzida, tratados com curcumina e rutina. Serão utilizados para o experimento ratos machos Wistar adultos. Os animais serão pré tratados com curcumina (50mg/kg) e rutina (50mg/kg) por gavagem, em dose equivalente para humanos, por um período de 30 dias. A hiperlipidemia será induzida, de forma aguda, mediante utilização de 500 mg/kg de Poloxamer-407 via intraperitoneal. Os linfócitos e plaquetas serão separados para a determinação da atividade das ecto-enzimas, e o soro será utilizado para quantificação dos nucleotídeos e nucleosídeo, bem como para avaliação dos parâmetros bioquímicos. Além disso, serão realizados testes para determinação de espécies reativas de oxigênio, tiós não proteicos e peroxidação lipídica. Espera-se assim, investigar o potencial efeito preventivo da curcumina e rutina frente a hiperlipidemia em ratos, a fim de contribuir para a elucidação dessa patologia, bem como buscar novas terapias adjuvantes que possam beneficiar pacientes com hiperlipidemia.

Local do experimento: Laboratório de Imunobiologia Experimental e Aplicada Predio 20 Sala 4229

Santa Maria, 07 de janeiro de 2019

Prof. Dr. Denis Broock Rosemberg
Coordenador da Comissão de Ética no Uso de Animais
Universidade Federal de Santa Maria

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