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EFEITO DO GUARANÁ (*Paullinia cupana*) NO METABOLISMO DE NUCLEOTÍDEOS E NUCLEOSÍDEO DE ADENINA EM UM MODELO EXPERIMENTAL DE HIPERCOLESTEROLEMIA

DISSERTAÇÃO DE MESTRADO

Jader Betsch Ruchel

Santa Maria, RS, Brasil

2013

EFEITO DO GUARANÁ (*Paullinia cupana*) NO METABOLISMO DE NUCLEOTÍDEOS E NUCLEOSÍDEO DE ADENINA EM UM MODELO EXPERIMENTAL DE HIPERCOLESTEROLEMIA

por

Jader Betsch Ruchel

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como requisito parcial para obtenção do grau de
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Santa Maria, 09 de agosto de 2013

"Cada sonho que você deixa pra trás,
é um pedaço do seu futuro que deixa de existir."

Steve Jobs

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RESUMO

Dissertação de Mestrado
Programa de Pós-Graduação em Ciências Biológicas: Bioquímica Toxicológica
Universidade Federal de Santa Maria, RS, Brasil

EFEITO DO GUARANÁ (*Paullinia cupana*) NO METABOLISMO DE NUCLEOTÍDEOS E NUCLEOSÍDEO DE ADENINA EM UM MODELO EXPERIMENTAL DE HIPERCOLESTEROLEMIA

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Data e Local da Defesa: Santa Maria, 09 de agosto de 2013.

A hipercolesterolemia é uma doença metabólica caracterizada por elevados níveis séricos de lipoproteínas de baixa densidade e de colesterol total, associada com desordens inflamatórias, com a migração de monócitos e linfócitos T e com disfunção endotelial. O sistema de sinalização purinérgica desempenha um papel importante na modulação das respostas inflamatórias e imunes através de biomoléculas extracelulares, como os nucleotídeos de adenina e seu derivado, o nucleosídeo adenosina, que são indispensáveis para a iniciação e manutenção de respostas inflamatórias. Os efeitos de tais moléculas são promovidos pela ação dos receptores purinérgicos específicos e controlados por ectoenzimas, na superfície das células. Com base nestes princípios, este estudo investigou o efeito do guaraná sobre a atividade da E-NTPDase e da E-ADA nos linfócitos, assim como analisar os parâmetros bioquímicos, de ratos com hipercolesterolemia induzida por dieta. Os ratos foram divididos em oito grupos, quatro foram induzidos à hipercolesterolemia e os outros quatro grupos receberam dieta normal. Os animais de cada dieta foram subdivididos de acordo com o tratamento, sendo utilizado solução salina, guaraná 12.5, 25 e 50 mg/kg/dia via oral, durante 30 dias. Observou-se um aumento em 74% na hidrólise de ATP na atividade da E-NTPDases de ratos com hipercolesterolemia, tratados com doses de 25 e 50 mg/kg/dia, quando comparados com todos os outros grupos. Contudo, a atividade da E-NTPDase não foi alterada quando utilizado ADP como substrato, para os ratos tratados nas três doses. No entanto, o grupo hipercolesterolêmico tratado com a maior concentração, apresentou maior diminuição na atividade da E-ADA, cerca de 70%, quando comparado com os outros grupos que receberam dieta normal. Também foi observado que o guaraná foi capaz de reduzir aos níveis basais, o colesterol total e LDL-C em ratos hipercolesterolêmicos. Em conclusão, o guaraná em concentrações elevadas quando associado com a dieta hipercolesterolêmica, foi capaz de aumentar a hidrólise do ATP e diminuir a atividade da E-ADA nos linfócitos, contribuindo para a redução do o processo inflamatório. Embora careça de maiores estudos, o guaraná poderia ser utilizado como uma terapia complementar em benefício de pessoas com hipercolesterolemia.

Palavras-chave: ectoenzimas, guaraná, hipercolesterolemia, linfócitos, sistema purinérgico.

ABSTRACT

Dissertation of Master's Degree
Post-Graduating Program in Biological Sciences (Toxicological Biochemistry) Federal University of Santa Maria, RS, Brazil

EFFECT OF GUARANÁ (*Paullinia cupana*) ON METABOLISM OF ADENINE NUCLEOTIDES AND NUCLEOSIDE IN EXPERIMENTAL MODEL OF HYPERCHOLESTEROLEMIA

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Place and Date: Santa Maria, august 09th, 2013.

Hypercholesterolemia is a metabolic disease characterized by high levels of serum low density lipoproteins and total cholesterol, associated with the inflammatory disorders, the migration of monocytes and T cells and with an endothelial dysfunction. The purinergic signaling system plays an important role in the modulation of inflammatory and immune response by extracellular biomolecules such as adenine nucleotides and their derived nucleoside adenosine, which are essential for the initiation and maintenance of response inflammatory. The effects of these molecules are promoted by the action of purinergic receptors specifics and controlled by ectoenzymes, in cell surface. Based on these principles, this study investigated the effect of guaraná on the activity of the E-NTPDase and E-ADA in lymphocytes, as well as analyze the biochemical parameters in rats with induced hypercholesterolemia by diet. The rats were divided in eight groups, of which four were induced hypercholesterolemia and the other four groups received normal diet. The animals in each diet were subdivided according treatment, saline, guaraná 12.5, 25 and 50 mg/kg/day orally for 30 days. There was observed an increase in 74% in ATP hydrolysis by E-NTPDase activity in lymphocytes of rats with hypercholesterolemia treated at doses of 25 and 50 mg/kg/day when compared with all other groups. However, E-NTPDase activity was not altered when used ADP as substrate for these three doses. Moreover, the hypercholesterolemic group treated with the higher concentration showed higher decreased of about 70% in E-ADA activity than the other normal diet groups. Was also observed that the guaraná was able to reduce to basal levels, total cholesterol and LDL-C in hypercholesterolemic rats. In addition, the guaraná in high concentrations when associated with hypercholesterolemic diet was able to increase the ATP hydrolysis and decrease the E-ADA activity in lymphocytes, contributing to reducing the inflammatory process. Although requiring further study, guaraná could be used as a complementary therapy for the benefit of people with hypercholesterolemia.

Keywords: ectoenzymes, guaraná, hypercholesterolemia, lymphocytes, purinergic system.

LISTA DE ABREVIATURAS

ACRs: Regiões conservadas da apirase

ADP: Adenosina difosfato

AMP: Adenosina monofosfato

ATP: Adenosina trifosfato

Apo A-I e II: Apolipoproteínas do HDL

Apo B-100: Apolipoproteína do LDL

CT: Colesterol Total

CRP: Proteína C-reativa

E-NTPDase: Ectonucleosídeo trifosfato difosfoidrolase

E-NPP: Ectonucleotídeo pirofosfatase/fosfodiesterase

E-5-NT: ecto-5'-nucleotidase

E-ADA: Ecto-adenosina desaminase

HDL: Lipoproteína de alta densidade

ICAM-1: Molécula de adesão intercelular-1

IFN-γ: Interferon γ

IL-1: Interleucina-1

IL-1β: Interleucina-1β

IL-2: Interleucina-2

IL-6: Interleucina-6

IL-18: Interleucina-18

LDL: Lipoproteína de baixa densidade

LRP: Receptor de lipoproteína de baixa densidade

MCP-1: Receptor de proteína quimiotática de monócito-1

MHC-I: Complexo principal de histocampatibilidade-I

OMS: Organização Mundial da Saúde

PAI-1: Fator endotelial trombótico–1

SRA- I e II: Receptor scavenger de macrófago classe A- I e II

TG: Triglicerídeo

TNF-α: Fator de necrose tumoral-α

TNF-β: Fator de necrose tumoral-β

VLDL: Lipoproteína de densidade muito baixa

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APRESENTAÇÃO

Esta dissertação está organizada na seguinte forma: primeiramente é apresentada a introdução. A seguir, os resultados, discussão e conclusões são apresentados na forma de manuscrito, o qual foi escrito, seguindo as normas do periódico ao qual o mesmo será submetido. As referências bibliográficas apresentadas no final da dissertação referem-se às citações que aparecem no item introdução.

Manuscrito: será submetido à revista Biochimie.

1 INTRODUÇÃO

De acordo com a Organização Mundial de Saúde (OMS), 56% da população brasileira sofre com o excesso de peso. Estima-se que o aumento das doenças provocadas pelo sobrepeso não esteja relacionado apenas com a ausência de exercício físico, mas também com o estilo de vida contemporâneo. Segundo estudos, o excesso de peso dos brasileiros está relacionado ao baixo consumo de alimentos saudáveis, ao aumento do consumo de alimentos industrializados e à ingestão de grande quantidade de açúcares e gorduras (MINISTÉRIO DA SAÚDE, 2011).

A etiologia da obesidade é multifatorial, onde predisposições genéticas interagem com o estilo de vida do indivíduo, como por exemplo, os padrões de dieta (ROBERTS et al., 2002). Em suma, o consumo de dietas ocidentalizadas, as quais são ricas em gordura saturada, açúcar refinado, pobres em frutas e verduras, são fatores relevantes na etiologia de doenças crônicas (GOLDBART et al., 2006).

Apesar de sua importância biológica, há muito tempo tem sido demonstrado que o aumento do consumo de gordura está associado à elevação da concentração plasmática de colesterol (IV DIRETRIZ SOBRE DISLIPIDEMIAS E PREVENÇÃO DA ATEROSCLEROSE, 2007). O nível de colesterol é um importante fator para o desenvolvimento da aterosclerose em seres humanos (DIETSCHY e TURLEY, 2004).

Os conteúdos alimentares de gorduras saturadas e de colesterol influenciam de forma diferente nos níveis lipídicos plasmáticos, em especial a colesterolemia. A maioria da população absorve aproximadamente metade do colesterol presente na luz intestinal, enquanto uma minoria é hiperresponsiva, ou seja, absorve maior quantidade. (IV DIRETRIZ SOBRE DISLIPIDEMIAS E PREVENÇÃO DA ATEROSCLEROSE, 2007).

No fígado, o conteúdo de colesterol é regulado por três mecanismos principais: a) síntese intracelular do colesterol; b) armazenamento após esterificação; c) excreção pela bile. Na luz intestinal, o colesterol é excretado na forma de metabólitos ou como ácidos biliares. Metade do colesterol biliar e aproximadamente 95% dos ácidos biliares são reabsorvidos e retornam ao fígado pelo sistema porta (ciclo entero-hepático) (COOPER, 1997; KAMOUN et al., 2006). O colesterol é precursor dos hormônios esteróides, dos ácidos biliares e da vitamina D. Além disso, como constituinte das membranas celulares, o colesterol atua na fluidez destas e na ativação de enzimas nelas situadas (QIN et al., 2006).

O desequilíbrio no metabolismo lipídico parece predispor ao desenvolvimento da

aterosclerose, visto que os fatores dietéticos, como dietas ricas em gordura saturada, trans ou colesterol, desempenham um papel importante, pois podem proporcionar a progressão da doença (BITTENCOURT JUNIOR e SENNA, 2002).

As lipoproteínas são responsáveis pelo transporte dos lipídios no plasma e são compostas por lipídeos, que são substâncias geralmente hidrofóbicas no meio aquoso plasmático, e proteínas, formando as chamadas apolipoproteínas (apos). Existem quatro grandes classes de lipoproteínas separadas em dois grupos: (1) as ricas em triglicerídeos (TG), maiores e menos densas, representadas pelos quilomícrons, de origem intestinal, e pelas lipoproteínas de densidade muito baixa (VLDL), de origem hepática; (2) e as ricas em colesterol de densidade baixa (LDL) e de densidade alta (HDL) (COOPER, 1997; KAMOUN et al., 2006).

Os quilomícrons são os responsáveis pelo transporte dos lipídeos da dieta e da circulação entero-hepática. O transporte de lipídeos de origem hepática ocorre por meio da VLDL e LDL, que caracteristicamente contém apolipoproteína B-100 (via endógena). A apo B-100 constitui a parte proteica da lipoproteína LDL, e é o componente que liga as partículas da LDL a receptores específicos, chamados de receptores B, E, situados na superfície da membrana plasmática celular (GOLDSTEIN et al., 1979). Os triglicerídeos das VLDL, assim como os dos quilomícrons, são hidrolisados pela lipase lipoprotéica (GOLDBERG, 1996). Os ácidos graxos são liberados para os tecidos e metabolizados. Os quilomícrons se transformam em remanescentes que são removidos pelo fígado por receptores específicos, sendo que o mais aparente é o receptor da LDL (COOPER, 1997).

O processo de catabolismo continua, envolvendo a ação da lipase hepática e resultando nas LDL, que permanecem por longo tempo no plasma. Tanto as VLDL como as LDL serão removidas no fígado por intermédio de ligação com receptores específicos (BROWN e GOLDSTEIN, 1986). As partículas de HDL são formadas no fígado, no intestino e na circulação e seu principal conteúdo proteico é representado pelas apos A-I e A-II A HDL carrega o colesterol até o fígado onde este será eliminado no chamado transporte reverso do colesterol (FIELDING e FIELDING, 1995).

O acúmulo de lipoproteínas ricas em colesterol como a LDL no compartimento plasmático resulta em hipercolesterolemia. Este acúmulo pode ocorrer por doenças monogênicas, em particular, por defeito no gene do receptor de LDL ou no gene da apolipoproteína (apo) B-100 (IV DIRETRIZ SOBRE DISLIPIDEMIAS E PREVENÇÃO DA ATEROSCLEROSE, 2007). Centenas de mutações do receptor de LDL foram

detectadas em portadores de hipercolesterolemia familiar, algumas causando redução de sua expressão na membrana, outras, deformações na sua estrutura e função (GOLDSTEIN et al., 1995). A mutação no gene que codifica a apo B100 pode também causar hipercolesterolemia através da deficiência no acoplamento da LDL ao receptor celular (DEFESCHE et al., 1993).

A lipoproteína LDL é o principal transportador de colesterol no plasma humano. Cerca de 70% do colesterol plasmático é transportado por esta lipoproteína para o fígado e para tecidos periféricos, onde é utilizado em diversos processos metabólicos, como por exemplo, na síntese de hormônios (estrógeno, progesterona, testosterona, etc) (GRIFFIN, 1999; JONES, 2001).

A hipercolesterolemia é caracterizada pelo acúmulo de colesterol total (CT) sérico acompanhado por um aumento nos níveis de LDL (OTUNOLA et al., 2010) e está epidemiologicamente ligada ao desenvolvimento de doenças cardiovasculares, incluindo angina e infarto do miocárdio, sendo um importante fator de risco e determinante no surgimento da aterosclerose (ROSS, 1999; LUSIS, 2000; HANSSON, 2005; ROCHA e LIBBY, 2009).

A aterosclerose é uma enfermidade crônica multifatorial, lenta e progressiva, resultante de uma série de respostas celulares e moleculares altamente específicas (HACKAM e ANAND, 2003), principalmente em virtude da agressão ao endotélio vascular com a elevação de lipoproteínas aterogênicas (LDL, VLDL, remanescentes de quilomícrons), hipertensão arterial ou tabagismo (ROSS, 1999; MONTECUCCO e MACH, 2009). Existe uma correlação direta entre a incidência e a gravidade de lesões ateromatosas e a concentração plasmática de CT, e em especial a LDL, que é o principal fator de risco para o processo aterosclerótico (ROSS, 1986). Por outro lado, a concentração aumentada de HDL no plasma correlaciona-se negativamente com a incidência de aterosclerose (RIDKER et al., 1998).

O processo aterosclerótico é caracterizado pela presença de anormalidades lipídicas, disfunção endotelial, acúmulo de leucócitos e plaquetas nas paredes das artérias e também por um processo inflamatório (ROSS, 1999; LUSIS, 2000; HANSSON, 2005; ROCHA e LIBBY, 2009). Ocorre também a adesão de elementos fibrosos na parede das artérias, como o colágeno e os proteoglicanos, além de algumas células musculares lisas, os quais contribuem para a formação de placas ou estrias

gordurosas, que geralmente ocasionam a obstrução das mesmas e, consequentemente, a deterioração da função vascular (LIBBY, 2002).

A artéria é constituída por três definidas camadas que cercam o lúmen arterial, cada um dos quais tem uma composição diferente de células e matriz extracelular. A camada imediatamente adjacente ao lúmen é chamada de túnica íntima, a camada intermediaria é conhecida como túnica média e a camada mais externa compreende a túnica adventícia. Essas três camadas são demarcadas por camadas concêntricas de elastina, conhecida como a lâmina elástica interna que separa as túnicas, íntima da média, e a lâmina elástica externa que separa a média da adventícia (Figura 1) (STOCKER e KEANEY, 2004).

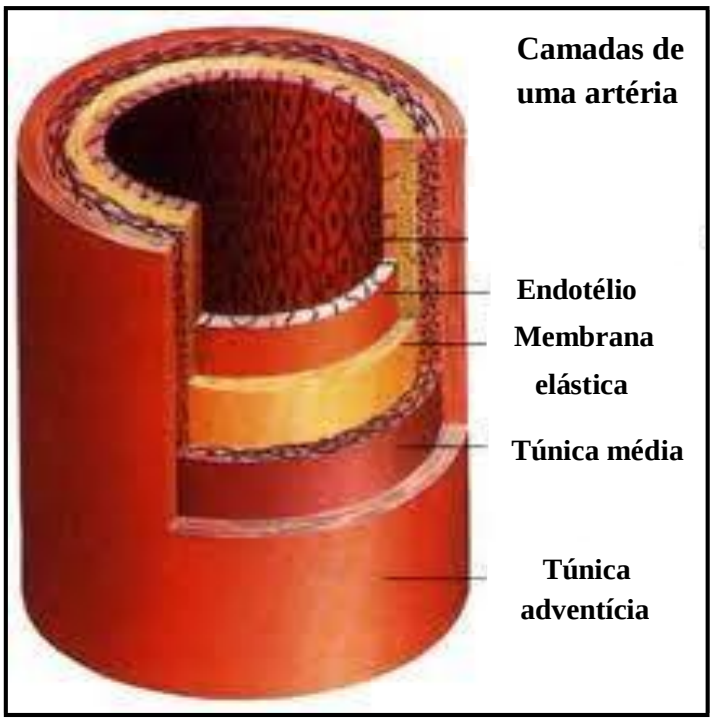

Figura 1: Morfologia de uma artéria (http://pt.wikipedia.org/wiki/Arteria)

Na aterosclerose a lipoproteína LDL torna-se aprisionada no espaço subendotelial, onde está sujeita à modificação oxidativa. A LDL oxidada estimula a migração de monócitos e formação de células espumosas. Uma vez formada, a LDL oxidada resulta em uma lesão ou disfunção endotelial (DIAZ et al., 1997) (Fig. 2A), que é caracterizada por permeabilidade endotelial reforçada, deposição da lipoproteína LDL no espaço subendotelial, adesão de leucócitos e transmigração através do endotélio. Nas fases intermédiarias da aterosclerose (Fig. 2B), há a formação de células espumosas e uma

resposta inflamatória que induz ativação das células T, a aderência e a agregação de plaquetas, e entrada de mais leucócitos ao longo da parede arterial com a migração das células musculares lisas da íntima. Finalmente a aterosclerose avançada (Fig. 2C) é caracterizada pela acumulação de macrófagos, formação de capa fibrosa e necrose no núcleo da lesão (STOCKER e KEANEY, 2004).

O depósito de LDL oxidado, na parede arterial, é o processo-chave no início da aterogênese, (HOLVOET et al., 1998; HARRISON et al., 2003). A oxidação do LDL é induzida pelos radicais livres produzidos pelos macrófagos, células endoteliais ou células musculares lisas (HEINECKE, 1998; HARRISON et al., 2003; ARMSTRONG et al., 2006 ; GAUTIER et al., 2009). A presença do LDL oxidado provoca outra manifestação da disfunção endotelial, representada pelo surgimento de moléculas de adesão leucocitária na superfície endotelial. As moléculas de adesão são responsáveis pela atração de monócitos e linfócitos para a parede arterial, estimulando a liberação de interleucina-1β (IL-1β) e interleucina-6 (IL-6) (LIBBY, 2000; LEVITAN et al., 2010). O LDL oxidado induz as células endoteliais a expressar a molécula de adesão intercelular - 1 (ICAM-1) e a molécula de adesão da célula vascular–1 (VCAM-1), permitindo que os monócitos e linfócitos T possam se aderir às células endoteliais, através de seus receptores da proteína quimiotática de monócitos–1 (MCP-1), (SUDHAHAR et al., 2007).

Induzidos por proteínas quimiotáticas, os monócitos migram para o espaço subendotelial onde se diferenciam em macrófagos, que por sua vez captam LDL oxidado através dos receptores específicos. Os receptores de varredura (*scavenger*), (SRA-I, SRA-II, LRP e CD36), localizados na superfície dos macrófagos, reconhecem LDL oxidado e este é internalizado pelos macrófagos, formando as células espumosas. Os macrófagos processam o auto-antígeno, promovendo a ativação dos linfócitos T e a secreção de citocinas (KOUNNAS et al., 1992; QIN et al., 2006; EMMANUEL et al., 2009). Os macrófagos ativados secretam citocinas pro-inflamatórias como interferon-γ (IFN-γ), fator de necrose tumoral-α (TNF-α) e IL-6, as quais podem potencializar a expressão dos receptores *scavenger* a induzirem fatores pró-coagulantes, fibrinolíticos e aumentar as propriedades adesivas das células endoteliais (PERSSON et al., 2006).

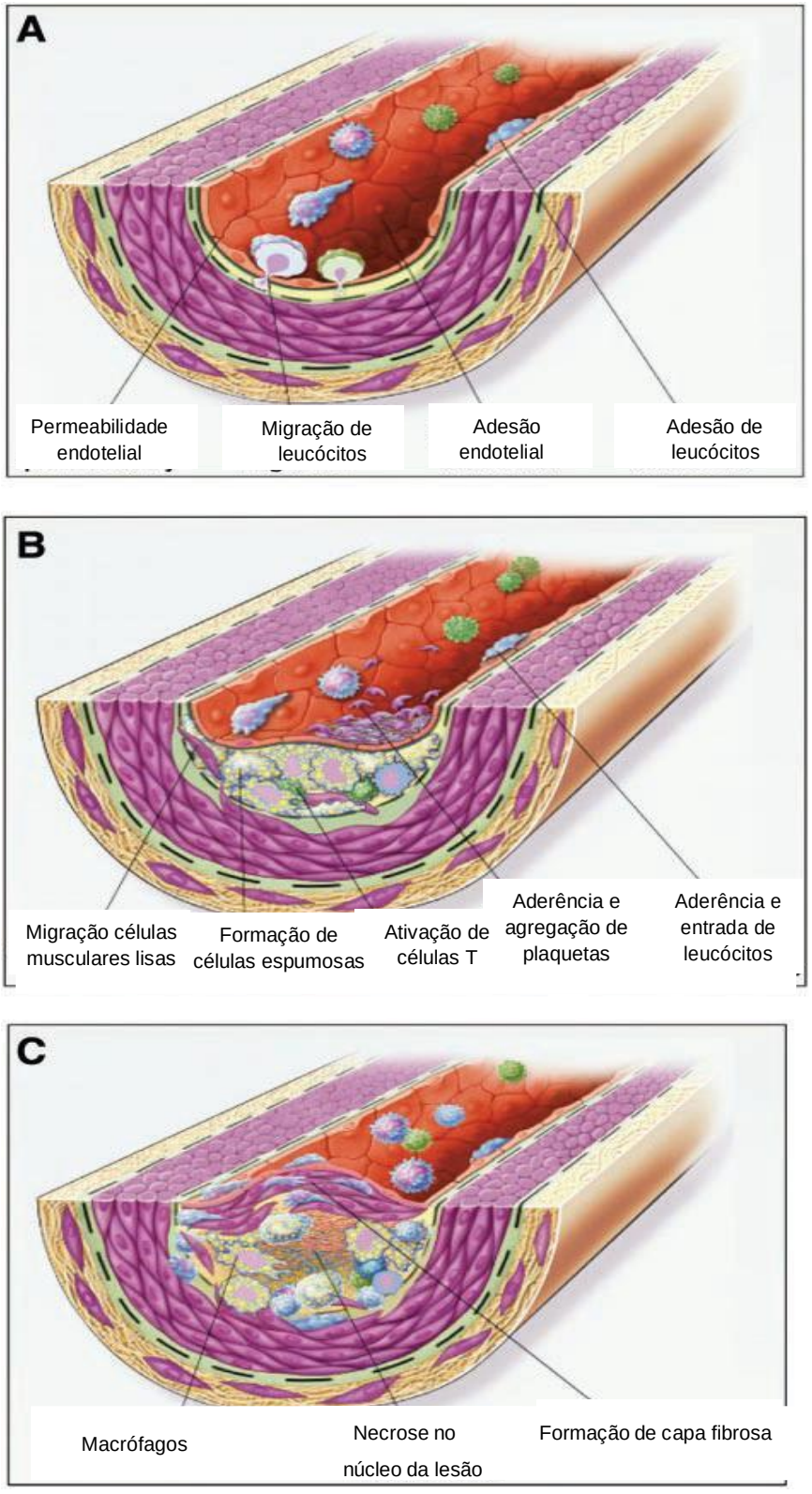

Figura 2: Variações dos estágios da aterosclerose (Adaptado de Stocker; Keaney, 2004).

Na resposta inflamatória ativada, os linfócitos T ($CD4^+$) ativados proliferam e passam também a secretar as citocinas pró-inflamatórias como, TNF-α e β, Interleucina-2 (IL-2) e IFN-γ, que causam uma maior ativação de macrófagos, ativação vascular e inflamação (CHARAKIDA et al., 2009). O IFN-γ·, TNF-α e IL-1 estimulam a proliferação das células musculares lisas e a síntese de colágeno, as quais se agregam às células espumosas, completando a formação da placa ateromatosa (LIBBY, 2002; BERG et al., 2009).

Os linfócitos T ($CD8^+$), que também participam da resposta imune, causam um ataque citotóxico nas células musculares lisas, quando as mesmas estão apresentando fragmentos lipídicos associados a proteínas de classe I do Complexo Principal de Histocompatibilidade (MHC- I), levando à apoptose dessas células (GENG et al., 1997). Os linfócitos B podem ocorrer na camada adventícia e no tecido conectivo e são os responsáveis pela síntese dos anticorpos anti-LDL oxidado (HANSSON, 2005; POLI et al., 2009). Os anti-LDL oxidado desempenham uma função imunoprotetora contra o desenvolvimento da arterosclerose, pois neutralizam e catabolizam o LDL oxidado (FUKUMOTO et al., 2000; MATSUURA et al., 2006).

Durante o processo inflamatório, fatores pró-inflamatórios (TNF, IL-1, IL-6 e IL-18) induzem a expressão dos genes hepáticos a sintetizarem proteínas de fase aguda (proteína C reativa, amilóide A sérica e fibrinogênio); fatores endoteliais trombóticos (PAI-1); e fatores específicos associados com doenças auto-imunes, tais como o anticorpo anti-cardiolipina. Alguns fatores inflamatórios são sintetizados localmente por células mononucleares que invadem a parede do vaso ou são produzidos pelos hepatócitos, estimulados por citocinas como a IL-6 liberada pelos macrófagos ativados (BALANESCU et al., 2010).

A fim de evitar, ou reduzir as alterações lipídicas causadas por gorduras saturadas presentes nas dietas (SILVA et al., 2002), muitos agentes terapêuticos estão disponíveis para o tratamento de pacientes hipercolesterolêmicos e muitos estudos têm demonstrado que o uso de drogas hipolipemiantes pode reduzir o número de eventos cardiovasculares e a mortalidade por parte de doenças coronárias (ARONOW, 2008). Além disso, uma dieta restrita em alimentos ricos em colesterol e exercícios físicos regulares são propostas que também devem fazer parte do tratamento destes pacientes, contribuindo significantemente para os cuidados primários de saúde (DE LORGERIL et al., 1999). Entretanto, devido a certa resistência a restrição dietética e o uso prolongado de drogas hipolipemiantes, muitos

indivíduos tem buscado por tratamentos alternativos para o controle de níveis de colesterol. Muitos desses tratamentos têm sido usados empiricamente, carecendo de estudos científicos que permitam conclusões mais confiáveis (DICKEL et al., 2007).

Dentre os tratamentos alternativos para o controle do colesterol, destaca-se a utilização do guaraná (Figura 3). De nome científico *Paullinia cupana*, o guaraná é uma planta arbustiva que apresenta duas variedades: a *Paullinia cupana*, variedade *typica,* que ocorre principalmente na Venezuela e na Colômbia e a *Paullinia cupana* variedade *sorbillis*, que ocorre na flora amazônica e é popularmente denominada guaraná. O seu consumo é restrito às sementes, a qual é torrada e moída para a obtenção de um pó (HENMAN, 1982; CORREIA, 1984). Apesar de ser consumido como planta medicinal pelos povos indígenas, o guaraná que é produzido comercialmente exclusivamente no Brasil é considerado e consumido como um alimento ou suplemento alimentar (BRASIL, 2001).

Figura 3. Guaraná em pó, semente e fruto, respectivamente (https://pt.wikipedia.org/wiki/Guaraná).

As propriedades funcionais do guaraná têm sido investigadas em estudos envolvendo cultura de células, modelos experimentais animais e ensaios clínicos em seres humanos (BARBOSA et al., 2004). As propriedades do guaraná se devem ao fato da semente possuir as principais enzimas da rota central da biossíntese de flavonóides (ANGELO et al., 2008), assim como a presença de compostos bioativos na sua composição, como metil xantinas, tais como a cafeína (FOUNI et al., 2007), teobromina (BELLIARDO et al., 1985) e teofilina, além de conter também taninos, saponinas, catequinas, epicatequinas, pró-antocianinas e outros compostos em menor quantidade (CARLSON e TOMPSON, 1998; SOMBRA et al., 2005).

A cafeína está presente em diversas plantas, assim como no guaraná, que possui três vezes mais cafeína que o próprio café. Além disso, é completamente absorvida pelo trato

gastrointestinal sendo distribuída para todos os tecidos corporais uma vez que tem a capacidade de atravessar as barreiras hemato-encefálica, testicular e placentária (ANDREWS et al., 2007). A teofilina, também encontrada no chá verde (*Cammelia sinensis*) permite o relaxamento da musculatura lisa dos brônquios, a estimulação do sistema nervoso central, a estimulação do músculo cardíaco e a diurese. A teobromina, possui atividade vasodilatadora e pode ser utilizada como anti-asmático e cardioestimulante.

Há poucos anos foi realizado um estudo que apontou grande similaridade entre as propriedades funcionais do guaraná como o café, o chá (verde e preto) e o chocolate (ANGELO et al., 2008). A identificação dos compostos bioativos do guaraná e a evidência de que a cafeína presente no guaraná é absorvida no intestino de modo similar a cafeína livre (BEMPONG e HOUGHTON, 1992) ampliou os horizontes para estudos sobre suas propriedades funcionais.

Nas últimas décadas, as investigações foram intensificadas e uma série de propriedades funcionais começou a ser observada a partir de estudos independentes, como: as propriedades cardiotônica e cardioprotetora, por evitar a agregação plaquetária, prevenindo a aterosclerose (BYDLOWSKI et al., 1988; BYDLOWSKI et al., 1991; PONTIERI et al., 2007; RAVI SUBBIAH e YUNKER, 2008), propriedade energética (BEMPONG e HOUGHTON, 1992), atividade anti-bacteriana (FONSECA et al., 1994; BASILE et al., 2005), melhora cognitiva (ESPINOLA et al., 1997; KENNEDY et al., 2004; HASKELL et al., 2007; KENNEDY et al., 2008), propriedades antioxidantes, uma vez que diminui a peroxidação lipídica causada por agentes pró-oxidantes (MATTEI et al., 1998; BASILE et al., 2005; RAY et al., 2006; YAMAGUTI-SASAKI et al., 2007), propriedades anti-inflamatórias (KREUER et al., 2012), efeito anti-obesidade, termogênico, no aumento da taxa metabólica (ANDERSEN et al., 2001; BOOZER et al., 2001; BERUBE-PARENT et al., 2005; ROBERTS et al., 2005; RAVI SUBBIAH e YUNKER, 2008), atividade gastroprotetora (CAMPOS et a.l, 2003), quimiopreventivo na hepatocarcinogênese já que o guaraná exerce efeito protetor contra dano no DNA (FUKUMASU et al., 2006), anti-depressivo (FONSECA et al., 1994) e anti-mutagênico (FUKUMASU et al., 2008).

Pesquisas sobre o efeito do guaraná na obesidade e obesogênese têm sido conduzidas (MORELLI e ZOOROB, 2000). Entretanto, as primeiras evidências anti-obesogênicas mostraram uma perda significativa de peso e gordura corporal nos indivíduos

tratados com um composto contendo guaraná e a erva chinesa Mahuang (BOOZER et al., 2001). Além disso, foi observado um aumento de velocidade de esvaziamento gástrico e diminuição de peso em indivíduos que utilizaram o guaraná (OPALA et al., 2006). Os efeitos antiobesogênicos e o aumento no gasto calórico de indivíduos que ingerem guaraná têm sido atribuídos especialmente à cafeína e às catequinas da sua composição (BERUBÉ-PARENT et al., 2005).

Outra abordagem avaliou o efeito do guaraná no metabolismo lipídico, onde se observou melhorias no metabolismo do tecido adiposo em ratos principalmente os treinados com atividade física, através do aumento da lipólise por ativação dos receptores de adenosina A1. Os autores sugerem que esta propriedade estaria associada às metilxantinas presentes no guaraná (LIMA et al., 2005). Evidências científicas também sugerem que o guaraná tem ação no metabolismo, devido à diminuição dos níveis pressóricos, de LDL e colesterol total, relacionados com menor prevalência de alguns distúrbios metabólicos como obesidade e aterosclerose (KREWER et al., 2011).

Sabe-se que síndromes metabólicas, como a aterosclerose geram um processo inflamatório. Este, por sua vez, é mediado por moléculas sinalizadoras, os nucleotídeos extracelulares, que são essenciais para o início e para a manutenção das reações inflamatórias. Os nucleotídeos de adenina, adenosina trifosfato (ATP), adenosina difosfato (ADP) e adenosina monofosfato (AMP) e seu derivado nucleosídeo adenosina, são secretados por leucócitos, plaquetas e células endoteliais danificadas e representam uma importante classe de moléculas extracelulares que desempenham um papel importante na modulação da resposta imune (ZIMMERMANN, 2000). Essas moléculas interagem com receptores purinérgicos presentes na superfície celular e desencadeiam cascatas de eventos que modulam diversos efeitos biológicos (RALEVIC e BURNSTOCK, 2003).

O sistema purinérgico envolve três principais componentes: (1) nucleotídeos e nucleosídeos extracelulares, mediadores da sinalização; (2) receptores, através dos quais esses nucleotídeos e nucleosídeos exercem seus efeitos e, (3) as ectoenzimas, responsáveis pelo controle dos níveis extracelulares destas moléculas (YEGUTKIN, 2008). É caracterizado por ser uma via de sinalização importante em diversos tecidos, desencadeando múltiplos efeitos celulares, incluindo resposta imune, inflamação, dor, agregação plaquetária, vasodilatação mediada pelo endotélio, proliferação e morte celular (BURNSTOCK e KNIGHT, 2004).

Diferentes tipos celulares, como plaquetas, linfócitos, células endoteliais entre outros, expressam distintos conjuntos de componentes de sinalização purinérgica descritos acima, permitindo a formação de complexos personalizados de sinalização purinérgica (Figura 4) (JUNGER, 2011).

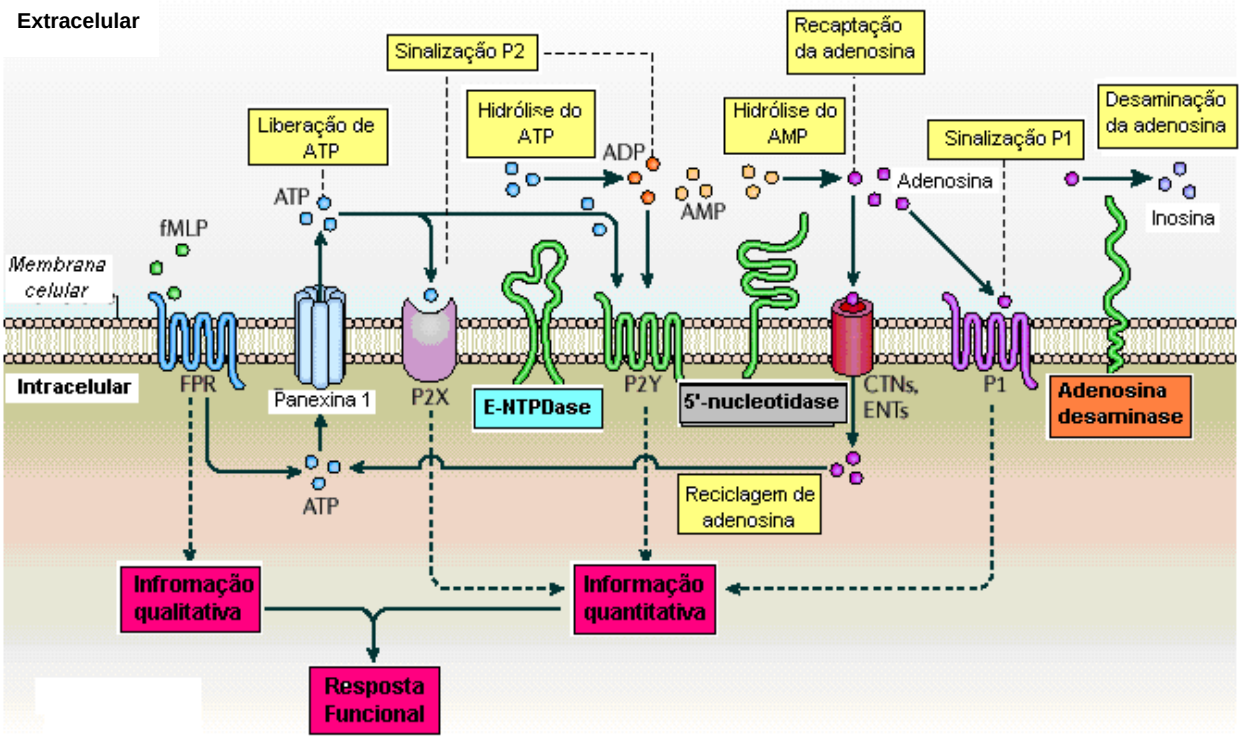

Figura 4 - Representação dos componentes do sistema purinérgico (adaptado de JUNGER, 2011).

Os nucleosídeos são moléculas resultantes da união de uma base nitrogenada (púrica ou pirimídica) a uma pentose. Exemplos destas moléculas incluem a citidina, a uridina, a guanosina, a timina, a inosina e a adenosina. Quando os nucleosídeos são fosforilados por quinases específicas formam moléculas denominadas de nucleotídeos (ATKINSON et al., 2008).

Os nucleotídeos da adenina tais como o ATP, ADP e AMP, e seu derivado nucleosídeo adenosina são liberados para o meio extracelular por células sanguíneas e vasculares, como eritrócitos, plaquetas, linfócitos e células endoteliais (WOCHENSCHR, 1989; DUBYAK e EL-MOATASSIM, 1993). Também podem ser liberados frente a um dano celular, nos sítios inflamatórios ou de estresse oxidativo, onde há um aumento da liberação de nucleotídeos. Já a adenosina pode ser liberada no meio extracelular como resultado da degradação do ATP e ADP por enzimas específicas (HUNSUCKER et al., 2005; YEGUTKIN, 2008), ou através de transportadores na membrana das células que

transportam a adenosina de dentro das células para o meio extracelular (BOROWIEC et al., 2006).

Em condições fisiológicas, os nucleotídeos são encontrados no meio extracelular em baixas concentrações (DI VIRGILIO et al., 2001). Já em altas concentrações, podem atuar como uma molécula citotóxica e levar à morte celular, pela formação de grandes poros na membrana plasmática (PODACK et al., 1985; YOUNG et al., 1986). Essas variações de concentração se dão devido a vários fatores como a quantidade de nucleotídeos liberada, os mecanismos de recaptação, situações de lise celular e a presença de enzimas como as ectonucleotidases (BURNSTOCK, 2007).

O ATP é um nucleotídeo presente em praticamente todas as células vivas e tem um papel fundamental no metabolismo energético (AGTERESCH et al., 1999). Também possui diversas funções fisiológicas, como a neurotransmissão, a inibição da agregação plaquetária, e induz a secreção de importantes mediadores por parte dos linfócitos T como interferon-γ (INF-γ) e interleucina-2 (IL-2) que estão envolvidos na indução da resposta imune. Também apresenta outros efeitos como contração do músculo liso, inflamação e dor (RALEVIC; BURNSTOCK, 1998). O ATP pode funcionar como uma molécula sinalizadora no controle da inflamação e da resposta imune (DI VIRGILIO et al., 2001). A modulação do processo inflamatório e da resposta imune pelo ATP extracelular é complexa e resulta de efeitos específicos sobre uma grande variedade de células imunes e não imunes. O ATP é capaz de desencadear funções pró-inflamatórias nos neutrófilos, estimular a produção de citocinas inflamatórias como IL-1 e TNF-α e ainda estimular a proliferação de linfócitos (BOURS et al., 2006; TRAUTMANN, 2009).

O nucleotídeo ADP é o produto gerado na hidrólise do ATP e não possui um papel definido nos linfócitos (DI VIRGILIO et al., 2001). O ADP é conhecido por induzir a agregação plaquetária, alterar a forma das plaquetas, aumentar o cálcio citosólico e inibir a adenilato ciclase ativada (PARK e HOURANI, 1999). O ADP é importante sinalizador da regulação, ativação e recrutamento plaquetário (MARCUS et al., 1997). Em situações de disfunção ou dano vascular, o ADP é liberado do interior de grânulos existentes nas plaquetas, sendo então considerado o agonista mais importante do recrutamento plaquetário e o indutor da formação de trombos no interior de vasos (MARCUS et al., 2003).

O AMP é um metabólito intermediário da hidrólise do ATP (BARSOTTI e IPATA, 2004) que exerce a função de sinalizador em situações de desequilíbrio no metabolismo,

servindo também como substrato para a formação da adenosina (CUNHA, 2001; LATINI; PEDATA, 2001). Já a adenosina, a qual é formada a partir do precursor ATP nos espaços intra e extracelulares (BARSOTTI; IPATA, 2004) desempenha um papel importante como agente anti-inflamatório endógeno (CRONSTEIN, 1994), vasodilatadora, neuroprotetora (JACOBSON et al., 2006) e imunossupressora (SPYCHALA et al., 1997), através da inibição da liberação de citocinas, da adesão de células imune e do funcionamento de linfócitos citotóxicos (CRONSTEIN et al., 1983). A adenosina também atua como um potente inibidor da agregação plaquetária (BOROWIEC et al., 2006).

Os nucleotídeos da adenina e o nucleosídeo adenosina realizam suas ações biológicas através da ativação de receptores específicos presentes na superfície celular, denominados receptores purinérgicos (DI VIRGÍLIO et al., 2001). Os receptores purinérgicos se dividem em duas famílias, P1 e P2, presentes na superfície de diversas células cujos membros são ativados pela adenosina e por ATP e ADP respectivamente (BURNSTOCK, 2007). Os purinoreceptores P2 podem ainda ser divididos em duas subclasses: acoplados à proteína G (metabotrópicos), chamados de P2Y e os ligados a canais iônicos, designados P2X, que são específicos para o ATP (DI VIRGÍLIO et al., 2001). Em mamíferos já foram identificados oito subtipos de receptores P2Y (P2Y1, P2Y2, P2Y4, P2Y6, P2Y11, P2Y12, P2Y13 e P2Y14), sete P2X (P2X1-7) e quatro subtipos de receptores P1 (A1, A2A, A2B e A3) que foram clonados e caracterizados farmacologicamente (RALEVIC e BURNSTOCK, 1998).

Os receptores P1 reconhecem a adenosina e também são metabotrópicos (BURNSTOCK, 2007). Os receptores subtipos A2A e A2B estão acoplados a proteínas estimulatórias G (Gs) e tipicamente suprimem as respostas celulares por aumentar os níveis de AMPc intracelulares. Enquanto, os receptores subtipos A1 e A3 estão acoplados a proteínas Gi/0 ou Gq/11 e promovem a ativação celular (JUNGER, 2011).

Portanto, o ATP liberado no meio extracelular exercerá seus efeitos ao se ligar a receptores P2X ou a P2Y, e consequentemente será metabolizado à adenosina por ectoenzimas localizadas na superfície da membrana celular, que controlam seus níveis extracelulares. A adenosina formada, por sua vez, exercerá seus efeitos biológicos através da ativação de receptores P1 (Figura 5) (HASKÓ et al., 2005).

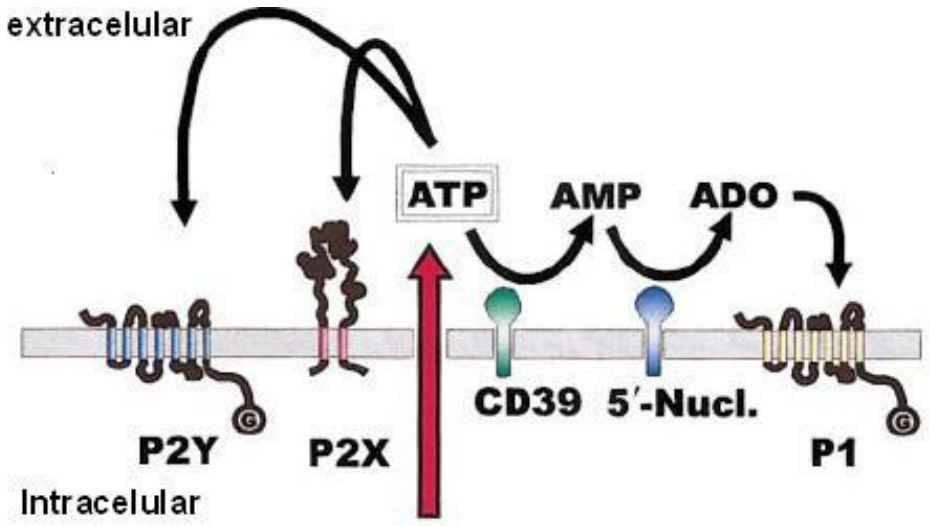

Figura 5 - Liberação de ATP no meio extracelular (DI VIRGILIO et al., 2001).

O controle dos níveis extracelulares dos nucleotídeos da adenina e adenosina, bem como a consequente sinalização purinérgica por eles induzida através dos receptores, é fundamental na manutenção dos processos fisiológicos de sinalização purinérgica como secreção, inflamação, fluxo sanguíneo, dentre outros (ROBSON et al., 2006). Os nucleotídeos após desempenhar suas funções orgânicas, devem ser degradados de modo a manter seus níveis extracelulares em concentrações fisiológicas. Para isto, existe este sistema responsável pelo controle dos seus níveis extracelulares que é realizado por uma variedade de enzimas ancoradas à superfície celular ou localizadas no meio intersticial de forma solúvel, sendo conhecidas como ectonucleotidases (ZIMMERMANN et al., 2007).

As ectonucleotidases são ectoenzimas responsáveis pela hidrólise dos nucleotídeos da adenina (ATP, ADP e AMP) e incluem diversos membros das seguintes famílias: Ecto-nucleosídeo trifosfato difosfohidrolase (E-NTPDases), Ecto-nucleotídeo pirofosfatases/ fosfodiesterases (E-NPPs), Fosfatase Alcalina e Ecto-5’-nucleotidase (Figura 6). Outra ectoenzima também importante no metabolismo purinérgico é a adenosina desaminase (E-ADA), responsável pela desaminação do nucleosídeo adenosina (ZIMMERMANN, 2001; ZIMMERMANN et al., 2012).

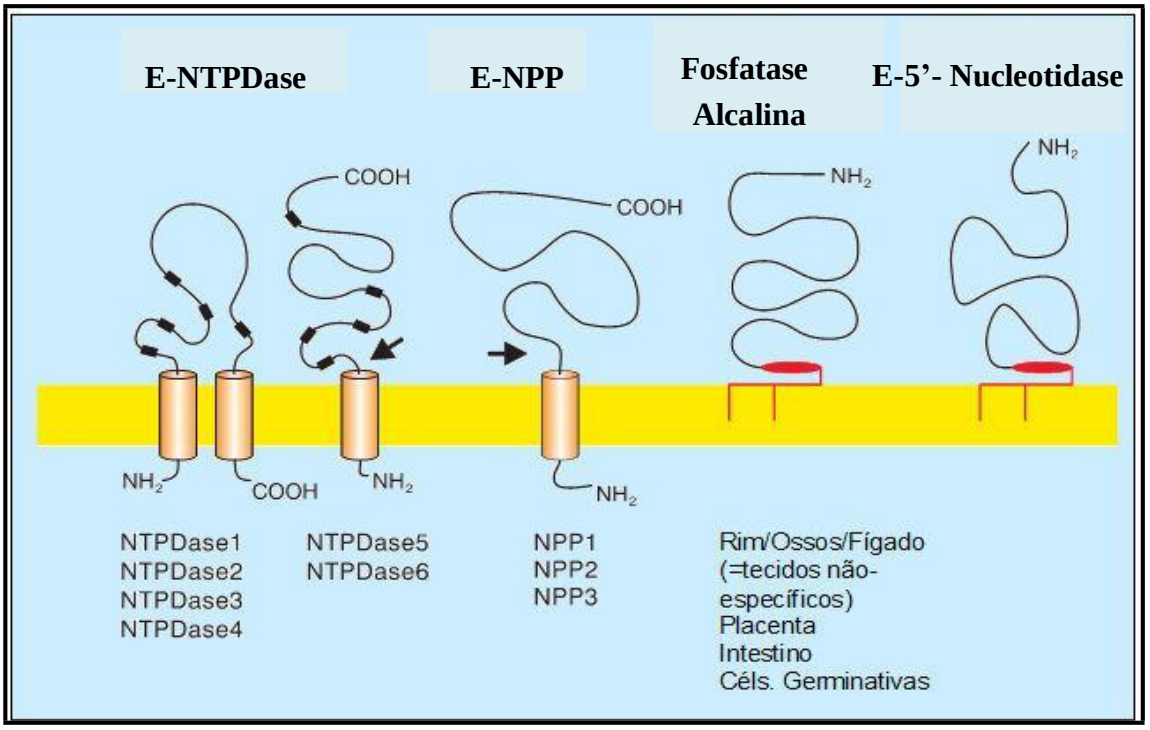

Figura 6 - Atividade catalítica e topografia de membrana para a família das ectonucleotidases (Adaptado de Zimmermann, 2001).

O conjunto de ações destas enzimas forma uma cadeia enzimática que tem início com a ação da E-NTPDase e da E-NPP, as quais catalisam a hidrólise do ATP e ADP formando AMP (ZIMMERMANN et al., 2007). A seguir a enzima E-5'-nucleotidase hidrolisa a molécula do AMP formando adenosina, a qual posteriormente é degradada pela ação da E-ADA gerando inosina (Figura 7) (YEGUTKIN, 2008).

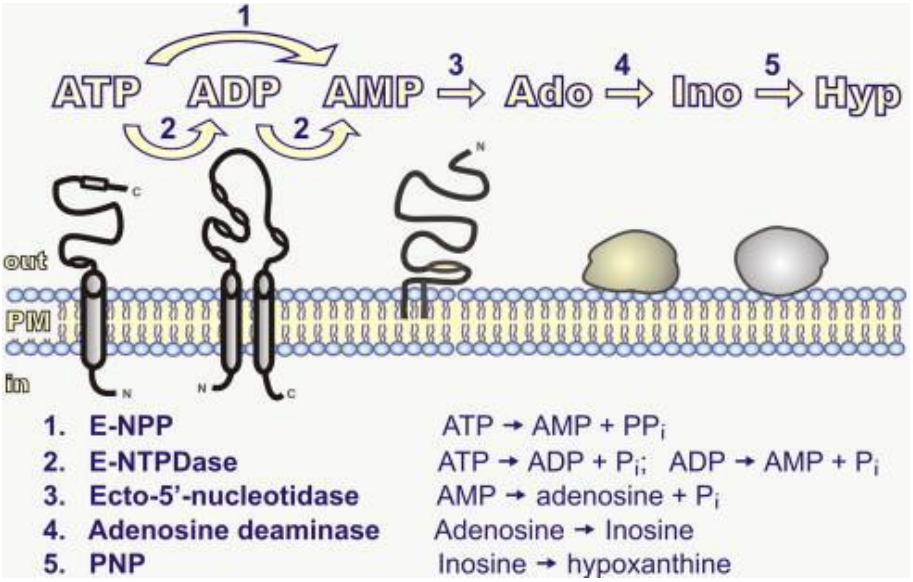

Figura 7 - Enzimas envolvidas na degradação extracelular de nucleotídeos e nucleosídeo da adenina (Adaptado de YEGUTKIN, 2008).

As E-NTPDases (CD39; EC 3.6.1.5) são uma família de enzimas responsáveis pela hidrólise de nucleotídeos di e trifosfatados a seus monofosfonucleotídeos correspondentes (ZIMMERMANN et al., 2007). As enzimas da família das NTPDases são expressas pelos genes Entpd, sendo que oito membros desta família já foram identificados (Figura 8) e diferem quanto a especificidade de substratos, distribuição tecidual e localização celular (BIGONNESSE et al., 2004; SHI et al., 2001; ZIMMERMANN, 2001).

Quatro destes membros estão localizados na membrana celular com o sítio catalítico voltado para o meio extracelular (E-NTPDase 1, 2, 3, 8) e requerem Ca^{2+} ou Mg^{2+} para sua máxima atividade, sendo inativas na ausência destes cátions; e quatro exibem uma localização intracelular (NTPDase 4,5,6,7) (KUKULSKI et al., 2005; ROBSON et al., 2006; ZIMMERMANN, 2001).

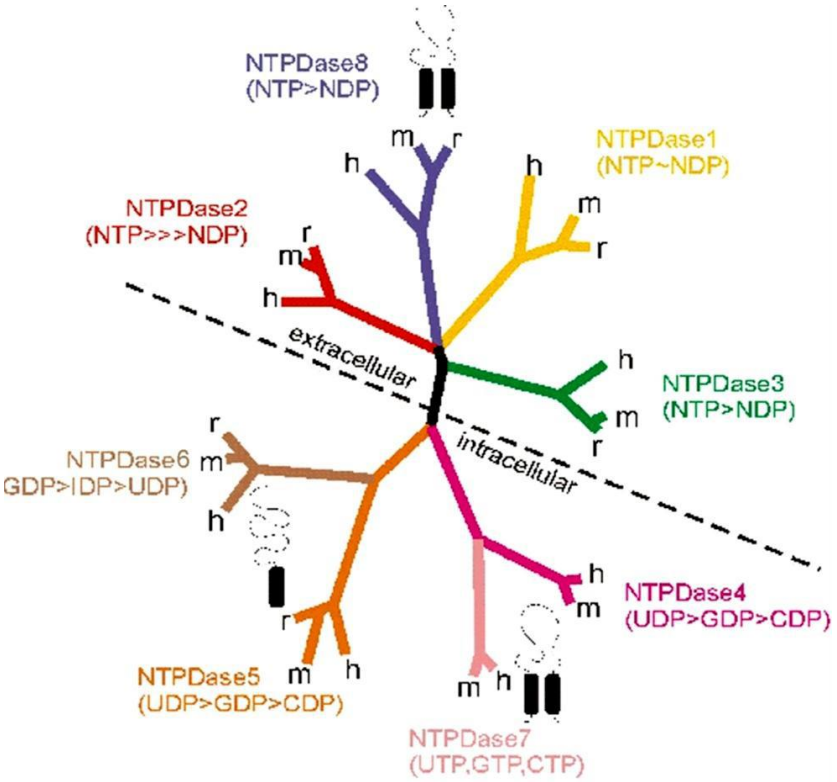

Figura 8 - Membros da família das NTPDases (1 - 8) (Adaptado de ROBSON et al., 2006).

Estruturalmente, os membros desta família possuem 5 regiões denominadas regiões conservadas da apirase (ACRs), que são locais que apresentam grande similaridade na sequência de aminoácidos (ZIMMERMANN, 2001). Tais regiões estão envolvidas no reconhecimento do substrato, ligação e hidrólise (KIRLEY et al., 2006). Algumas pesquisas sugerem que as diferenças na preferência pelo substrato, nessa família de

enzimas, podem ser atribuídas a pequenas diferenças na estrutura proteica que afetaria a ligação do substrato (ZIMMERMANN, 2001; ROBSON et al., 2006).

A primeira E-NTPDase identificada foi a E-NTPDase-1, como proteína CD39, que está ancorada à membrana via dois domínios transmembrana e que hidrolisa os nucleotídeos ATP e ADP em proporções semelhantes (ZIMMERMANN, 2001). A E-NTPDase-1 é um marcador de ativação de linfócitos, sendo também expressa em células natural killer, monócitos, células dendríticas e em um subconjunto de células T ativadas. Através da modulação da sinalização purinérgica a enzima desempenha um papel importante no controle da resposta imune celular (MIZUMOTO et al., 2002; ROBSON et al., 2006; DEAGLIO et al., 2007; DWYER et al., 2007). A E-NTPDase-2 é associada ao sistema nervoso central e periférico. A E-NTPDase-3 é associada com estruturas neuronais, agindo na regulação dos níveis de ATP pré-sinápticos (YEGUTKIN, 2008). Já as NTPDases 4, 5, 6 e 7 estão localizadas no meio intracelular (ZIMMERMANN, 2001).

Vários estudos têm mostrado uma atividade alterada da enzima E-NTPDase em pacientes com diferentes condições patológicas como o diabetes (LUNKES et al., 2003), a esclerose múltipla (SPANEVELLO et al., 2010), o infarto agudo do miocárdio (BAGATINI et al., 2008) e na síndrome da imunodeficiência adquirida (AIDS) (LEAL et al., 2005). A atividade da enzima também se encontra alterada em pacientes com hipercolesterolemia e processo inflamatório, onde a hidrólise do ATP e do ADP se encontra aumentada em plaquetas, assim como a expressão da CD39 na superfície da célula (DUARTE et al., 2007). Já em ratos hipercolesterolêmicos, a atividade da E-NTPDase se encontra diminuida em estruturas cerebrais (GUTIERRES et al., 2012)

Após a hidrólise do ATP e ADP pela E-NTPDase, a enzima ecto-5'-nucleotidase (eN, CD73, E.C 3.1.3.5) é responsável pela desfosforilação de ribo- e desoxiribonucleossídeos 5' monofosfatados como AMP, CMP, UMP, IMP e GMP, porém com uma maior afinidade pelo AMP, sendo por isto considerada a principal enzima responsável pela formação de adenosina (ZIMMERMANN et al., 2012).

A enzima adenosina desaminase (ADA, E C 3.5.4.4) também faz parte do conjunto de enzimas responsáveis pela degradação sequencial dos nucleotídeos e nucleosídeos da adenina (YEGUTKIN, 2008). A E-ADA é responsável pela desaminação irreversível da adenosina e 2'-deoxiadenosina em inosina e 2'-deoxinosina, respectivamente (RESTA et al., 1998; ROBSON et al., 2006). A primeira proteína de superfície celular capaz de ancorar a ecto-ADA à membrana plasmática foi identificada como CD26 por Kameoka e

cols. (1993), a qual se tornou conhecida como um marcador molecular de ativação de células T, pois quando estas células estão ativadas o nível de expressão da CD26 aumenta consideravelmente (FOX et al., 1984; FRANCO et al., 1997).

A E-ADA é uma enzima essencial para a proliferação e diferenciação dos linfócitos e monócitos-macrófagos no sistema imune, sendo usada para monitorar várias patologias imunológicas (HITOGLU et al., 2001; POURSHARIFI et al., 2008). Essa enzima é encontrada praticamente em todos os vertebrados. Em humanos, existe na forma de duas isoenzimas classificadas como ADA1 e ADA2, cada uma com suas próprias características, como peso molecular, propriedades cinéticas e distribuição tecidual (SHAROYAN et al., 2006).

A ADA1 está presente em todos os tecidos humanos, apresentando alta atividade em linfócitos e monócitos, e é responsável por grande parte do desaparecimento da adenosina circulante nesse meio. Apesar de sua localização intracelular, a ADA1 pode estar combinada com uma glicoproteína dimérica não específica, designada como proteína combinante (CP), formando o complexo ADA-CP que forma uma ecto-ADA, encontrada na superfície celular (TSUBOI et al., 1995). Aparentemente não existem diferenças, tanto catalíticas quanto moleculares, entre a enzima presente no citosol e a ecto-ADA. Esta evidência deve-se ao fato de que apenas 1 gene para a ADA foi encontrado, demonstrando que as sequências proteicas das duas enzimas são idênticas (FRANCO et al., 1997).

A ADA2 é a isoenzima predominante no soro e representa a menor parte da atividade da ADA total em tecidos (ZUKKERMAN et al., 1980). Diferentemente da ADA1, a ADA2 apresenta diferenças tanto estruturais quanto cinéticas e é encontrada predominantemente no soro de indivíduos normais (UNGERER et al., 1992). Dados recentes têm sugerido que ADA2 no plasma humano pode ser secretada por monócitos ativados em processos inflamatórios, tendo a habilidade de regular a proliferação celular (IWAKI-EGAWA et al., 2006).

A adenosina é liberada pelas células dependendo da sua concentração intracelular ou pode ser proveniente da degradação do ATP extracelular devido à ação das ectonucleotidases. O controle da sinalização adenosinérgica também pode ser exercido através da via de recuperação de adenosina por transportadores de nucleosídeos, seguida por fosforilação à AMP pela adenosina quinase ou desaminação à inosina pela ADA (Figura 9) (HASKÓ e CRONSTEIN, 2004).

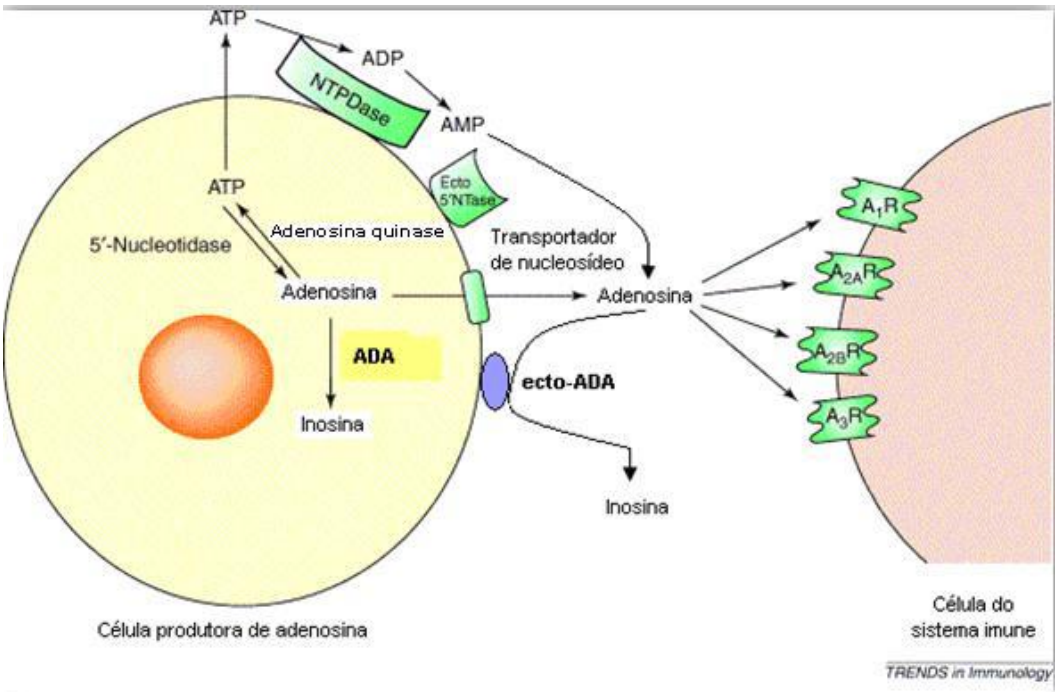

Figura 9 – Vias envolvidas no metabolismo da adenosina (Adaptado de HASKÓ e CRONSTEIN, 2004).

Além de possuírem importante atividade na regulação dos níveis de nucleotídeos e nucleosídeos da adenina, as ectoenzimas possuem ações extremamente importantes no sistema imunológico (SALAZAR-GONZALEZ et al., 1985; BENREZZAK et al., 1999).

Enzimas como a E-NTPDase e a E-ADA, estão presentes na membrana dos linfócitos desempenhando um importante papel na resposta inflamatória. Considerando que as respostas imune, inflamatória e vascular desencadeadas pela hipercolesterolemia, são moduladas por nucleotídeos e nucleosídeos, que se correlacionam diretamente com a atividade das ecto-nucleotidases e o guaraná por atuar como um potente modulador do metabolismo e com propriedades anti-inflamatórias, torna-se relevante e de interesse científico investigar o efeito do guaraná na atividade da E-NTPDase e da E-ADA em linfócitos de ratos com hipercolesterolemia induzida. Contribuindo assim, para a busca de novas terapias complementares que possam beneficiar pacientes com hipercolesterolemia.

2 OBJETIVOS

2.1 Objetivo geral

Avaliar o efeito do guaraná (*Paullinia cupana*) no metabolismo de nucleotídeos e nucleosídeo de adenina em linfócitos de ratos com hipercolesterolemia induzida por dieta.

2.2 Objetivos específicos

Em ratos com dieta normal e ratos com hipercolesterolemia induzida, tratados com guaraná:

- Avaliar a massa corporal dos animais durante o experimento.
- Determinar as concentrações de glicose, colesterol total e suas frações, albumina e ácido úrico no soro.
- Avaliar a atividade das enzimas marcadoras de dano hepático, a alanina aminotransferase (ALT), a aspartato aminotransferase (AST) e também a fosfatase alcalina em soro.
- Avaliar a atividade da enzima E-NTPDase em linfócitos.
- Determinar a atividade da enzima adenosina desaminase (E-ADA) em linfócitos.

3 MANUSCRITO

Os resultados que fazem parte desta dissertação estão apresentados sob a forma de um manuscrito. Os itens Materiais e Métodos, Resultados, Discussão e Referências Bibliográficas encontram-se compondo o próprio manuscrito e representam a íntegra deste estudo.

O manuscrito será submetido à revista Biochimie e encontra-se nas normas.

Effect of Guaraná (*Paullinia cupana*) on the metabolism of adenine nucleotides and nucleoside in lymphocytes an experimental model of hypercholesterolemia

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Abstract

This study investigated the effect of guaraná on the activity of the E-NTPDase and E-ADA in lymphocytes, as well as analyze the biochemical parameters in rats with induced hypercholesterolemia by diet. The rats were divided in eight groups, of which four were induced hypercholesterolemia and the other four groups received normal diet. The animals in each diet were subdivided according treatment, saline, guaraná 12.5, 25 and 50 mg/kg/day orally for 30 days. Was observed an increase of 74% in ATP hydrolysis by E-NTPDase activity in lymphocytes of rats with hypercholesterolemia treated at doses of 25 and 50 mg/kg/day when compared with all other groups. However, E-NTPDase activity was not altered when used ADP as substrate for these three doses. Moreover, the hypercholesterolemic group treated with the higher concentration showed higher decreased of about 70% in E-ADA activity than the other normal diet groups. Was also observed that the guaraná was able to reduce to basal levels, total cholesterol and LDL-C in hypercholesterolemic rats. In addition, the guaraná in high concentrations when associated with hypercholesterolemic diet was able to increase the ATP hydrolysis and decrease the E-ADA activity in lymphocytes, contributing to reducing the inflammatory process. Although requiring further study, guaraná could be used as a complementary therapy for the benefit of people with hypercholesterolemia.

Key words: Hypercholesterolemia, lymphocytes, guaraná, ecto-enzymes, purinergic system.

1 Introduction

Hypercholesterolemia is a metabolic disorder characterized by high levels of serum low density lipoprotein and blood cholesterol [1]. It is a major health problem with increased prevalence in many countries because of the close correlation between cardiovascular diseases and metabolic disorders [2,3]. Hypercholesterolemia is accepted as one of the major risk factors for the development of ischemic heart diseases, including angina and myocardial infarction, and the incidence of atherosclerosis increases [4]. Atherosclerosis is a chronic inflammatory condition characterized by the presence of lipid abnormalities, endothelial dysfunction, inflammation and also accumulation of platelets on the artery walls [5,6].

The lesions of atherosclerosis occur principally in large and medium-sized elastic and muscular arteries and can lead to ischemia of the heart, brain, or extremities, resulting in infarction. They may be present throughout a person's lifetime. In fact, is a pure inflammatory lesion, consisting only of monocyte-derived macrophages and T lymphocytes [7]. In persons with hypercholesterolemia, the influx of these cells is preceded by the extracellular deposition of amorphous and membranous lipids [8].

Extracellular nucleotides are essential molecules for the onset and maintenance of inflammatory reactions, whereas they are important signaling molecules [9]. The purinergic signaling system plays an important role in modulating the inflammatory and immune responses by extracellular biomolecules such as adenine nucleotides and their derived nucleoside adenosine [10]. Evidence indicates that high extracellular ATP level acts through specific cell surface receptors as a pro-inflammatory agent that potentiates the release of pro-inflammatory cytokines [11] from activated lymphocytes [12]. However, low extracellular ATP levels play an additional role as a negative modulator of immunity [13].

Extracellular ATP and adenosine levels as well as the ensuing purinergic signaling can be dynamically controlled during inflammation by the action of enzymes expressed in immune cells [11]. E-NTPDase (CD39) is the membrane-bound enzyme involved in the breakdown of ATP and ADP to AMP, which is sequentially hydrolyzed by E-5‘-nucleotidase to adenosine [14,15].

In studies with humans, was analyzed the NTPDase activity and expression in platelets from patients with hypercholesterolemia and results obtained demonstrated that the hydrolysis of ATP and ADP increases according to the increase of serum cholesterol levels, confirmed by an increase in CD39 expression on it surface. The increase in CD39 activity is possibly related to a compensatory response to the inflammatory state associated with hypercholesterolemia [16].

E-ADA is another important enzyme that catalyzes the irreversible deamination of adenosine and 2‘-deoxyadenosine to inosine and 2‘-deoxyinosine respectively, therefore contributing to the removal of adenosine from the extracellular compartment [17]. This enzyme has fundamental biological role in the immune system and inflammatory processes [18].

Previous experimental investigations have suggested that guaraná (*Paullinia cupana)* has positive effects on lipid metabolism [19] and in body weight loss [20]. Furthermore, studies suggested that guaraná exhibits a cardioprotective effect by inhibiting platelet aggregation [21]. These all positive effects contributed to reduce the risk factors for cardiovascular diseases including aterosclerosis. In addition, these effects can be attributed a high concentrations of xanthines, such as caffeine, theophylline and theobromine [22]. There is also a high concentration of polyphenols, such as tannins, flavonoids and catechins [23,24] substances that have antioxidant action [25].

Can be attributed to many other properties polyphenols, such as an anti-thrombogenic, anti-inflammatory, hypotensive and hypocholesterolemic action [26,27,28]. It is a rich source of caffeine, which stimulates the metabolism and can enhance thermogenesis, acting as an ergogenic agent, assisting in the degradation of lipids and atherosclerosis prevention [28,29,30].

Hypercholesterolemia is a condition associated with adhesion and recruitment of platelets, inflammatory disorders, with the migration of monocytes and T lymphocytes and endothelial dysfunction. For this reason, it is of clinical interest to investigate the therapeutic action of compounds with anti-inflammatory properties, such as guaraná. Until the moment there are few studies demonstrating the effect of guaraná on the metabolism of adenine nucleotides. Thus, it is relevant to investigate its effect on the activity of E-NTPDase and E-

ADA in lymphocytes, as well as biochemical parameters, of rats with induced hypercholesterolemia**.**

2 Materials and methods

2.1 Chemicals

The substrates adenosine 5'-triphosphate disodium salt (ATP), adenosine 5'- diphosphate sodium salt (ADP), adenosine, bovine serum albumin, Trizma base, HEPES and Coomassie Brilliant Blue G were obtained from Sigma-Aldrich (St. Louis, MO, USA). All the chemicals used in this experiment were of analytical grade and of the highest purity.

2.2 Animals

Male Wistar rats (approximately 250g), obtained from the Central Animal House of the Federal University of Santa Maria (UFSM) were used in this experiment. They were housed four/three per cage in a cycle of 12/12h light and dark maintained artificially (lights on at 19:00 and off at 7:00). At a temperature range of 21-23^{o}C and 60-65% relative humidity, with free access to water. All animal procedures were approved by the Ethics Committee on Animal Experiments of UFSM (protocol under number: 102-2012).

2.3 Diet

The rats used in this study were randomly divided into eight groups, namely, a normal diet plus saline (N+S), normal diet plus guaraná 12.5 mg/kg (N+12.5), normal diet plus guaraná 25 mg/kg (N+25), normal diet plus guaraná 50 mg/kg (N+50), hypercholesterolemic diet plus saline (H+S), hypercholesterolemic diet plus guaraná 12.5 mg/kg (N+12.5), hypercholesterolemic diet plus guaraná 25 mg/kg (N+25), hypercholesterolemic diet plus guaraná 50 mg/kg (N+50). The normal diet was performed according to the publication Nutrient Requirements of Laboratory Animals [31], while the hypercholesterolemic diet was performed with some modification, as shown in Table 1. According with this study, the normal concentration of fat in a diet to rats must stand between 20% and 30% of the total

content of macronutrients (carbohydrate, fat and protein). Thus, a diet with a higher percentage of fat is a high-fat diet.

The animals were fed with the diets for a period of one month to induce hypercholesterolemia. After induction, they continued receiving the diet for another month in which the treatment was performed. To certify the induction of hypercholesterolemia it was assessed the levels of total cholesterol and its fractions in a sample of animals which were killed. After the hypercholesterolemia was detected the other animals continued on their respective diets and started the treatments. The measurement of diet given *per* day *per* rat was 10% of body weight according to Harkness and Wagner [32].

2.4 Treatment with guaraná

Guaraná was administered by gavage once a day, 7 days a week, for a period of 1 month. It was diluted in saline (0.9% NaCl), at doses of 12.5, 25 and 50 mg/kg/day, and administered after the detection of hypercholesterolemia. Powdered *Paullinia cupana* seed produced and supplied by EMBRAPA Oriental (Agropecuary Research Brazillian Enterprise) located in Western Amazon in Maués, Amazonas-Brazil was used in experiment.

The detailed description and determination of mainly bioactive compounds presents guaraná used in this study is presented in Bittencourt et al. [33]. The mainly xanthines and catechins presented in guaraná extract were analyzed by chromatography and from this analysis was found caffeine = 12.240 mg/g, theobromine = 6.733 mg/g, total catechins = 4.336 mg/g, and condensed tannins = 22 mg/g.

2.5 Separation of blood serum

Rats were anesthetized with ketamine and xylazine and blood was collected by cardiac puncture. The blood samples were collected in tubes without anticoagulant and after the clot formation were centrifuged at 1400 g for 15 min at room temperature. The resultant serum samples were aliquotted in microtubes and kept on ice for subsequent measurements.

2.6 Biochemical parameters

Serum total cholesterol, high-density lipoprotein cholesterol (HDL-C), triglycerides (TRI), glucose, albumin, uric acid, aspartate transaminase (AST), alanine transaminase (ALT), alkaline phosphatase (ALP), were evaluated in a semi-automatic analyzer (TP Analyzer Plus®, Thermoplate) using commercial kits (Labtest® Diagnóstica S.A.). Tests were carried out in duplicate. Low-density lipoprotein (LDL-C) level was calculated by the formula of Friendewald et al. [40].

2.7 Isolation of lymphocytes from blood

Rats were anesthetized with ketamine and xylazine and blood was collected by cardiac puncture. Blood was collected with 7.2 mg dipotassium EDTA as anticoagulant and lymphocyte-rich mononuclear cell were isolated from blood collected with Ethylenediaminetetraacetic acid (EDTA) and separated on Ficoll-Histopaque density as previously described in Böyum [34]. The percentage of lymphocytes was superior to 93% as previously described in Jaques et al. [35]. The integrity of the lymphocytes preparation was confirmed by determining the lactate dehydrogenase (LDH) activity in intact and disrupted lymphocytes using the kinetic method of the Labquest apparatus (Diagnostics Gold Analyzer). The procedure was repeated before and after the incubation period. The protocol was carried out according to the manufacturer instructions. Triton X-100 (1%, final concentration) was used to disrupt the lymphocytes preparation. The enzymatic activity is expressed as units per liter, and one unit (1U) corresponds to 1 μmol of NADH formed per minute per liter.

2.8 Protein determination

Protein was measured by the Comassie Blue method according to Bradford [36] using serum albumin as standard. This assay is based on the binding of the dye Coomassie Brilliant Blue G-250 to protein, and this binding is accompanied by measuring the absorbance maximum of the solution at 595 nm.

2.9 **E-NTPDase activity determination**

After lymphocytes isolation, the E-NTPDase activity was determined as previously described by Leal et al. [37], in which the reaction medium contained

0.5 mM $CaCl_2$, 120 mM NaCl, 5 mM KCl, 60 mM glucose and 50 mM Tris-HCl buffer at pH 8.0, with a final volume of 200 µL. Twenty microliters of the intact mononuclear cells suspended in saline solution was added to the reaction medium (2-4 µg of protein), and pre-incubated for 10 min at 37^{o}C; incubation proceeded for 70 min. The reaction was initiated by the addition of substrate (ATP or ADP) at a final concentration of 2.0 mM and stopped with 200 µL of 10% trichloracetic acid (TCA). The released inorganic phosphate (Pi) was assayed by a method previously described by Chan et al. [38] using malachite green as colorimetric reagent and KH_2PO_4 as standard. Controls were carried out by adding the enzyme preparation after TCA addition to correct for non-enzymatic nucleotide hydrolysis. All samples were run in triplicate and the specific activity is reported as nmol of Pi released/min/mg of protein.

2.10 Ecto-Adenosine deaminase activity determination (E-ADA)

E-ADA activity was measured spectrophotometrically in lymphocytes by the method of Giusti and Galanti [39], which is based on the direct measurement of the formation of ammonia produced, when E-ADA acts in excess of adenosine. Briefly, 25 µL of lymphocytes reacted with 21 mM of the substrate (adenosine), pH 6.5, and incubation was carried out for 1 h at 37ºC. The reaction was stopped by adding 106 mM and 167.8 mM sodium nitroprussiate and hypochlorite solution and was again incubated for 30 min at 37°C. Ammonium sulfate of 75 µM was used as ammonium standard. All the experiments were performed in triplicate and the values were expressed in U/mg of protein for E-ADA activity. One unit (1U) of E-ADA is defined as the amount of enzyme required to release 1 mmol of ammonia per minute from adenosine at standard assay conditions.

2.11 Statistical analysis

The statistical analysis was performed using Student's t test to demonstrate the induction of hypercholesterolemia and of total cholesterol, LDL-C, HDL-C, TRI, glucose, albumin, ALT, AST and ALP before the treatment with guaraná. Two-way ANOVA, Newman-Keuls Multiple Comparison Test, was used to evaluate the food consumption, body weight, such as total cholesterol, LDL-C, HDL-C, TRI, glucose, albumin, ALT, AST, ALP, uric acid and the activities of ecto-enzymes, after the

treatment with guaraná. $P<0.05$ was considered to represent a significant difference among the analyses used. All data were expressed as mean ± Standard Error of the Mean (SEM).

3 Results

3.1 Cellular integrity

LDH activity measurement showed that approximately 5% of the lymphocytes of both groups was disrupted, indicating that the preparation was predominantly intact after the isolation procedure (data not shown).

3.2 Hypercholesterolemia induction

With the aim of confirm the induction of hypercholesterolemia were analyzed glucose, total cholesterol, LDL-C, HDL-C, TRI and albumin levels and ALT, AST and ALP activity. A significant increase on the levels of total cholesterol and in LDL-C was observed in hypercholesterolemic rats when compared to control, in 83.13% ($P < 0.001$; n=7) and 122.59% ($P < 0.01$; n=7), respectively. These tests demonstrate the induction of hypercholesterolemia after one month of hypercholesterolemic diet, as shown in Table 2. Glucose, HDL-C, TRI, albumin, ALT, AST and ALP measurement showed no significant difference in hypercholesterolemic rats when compared to control (data not shown).

3.3 Food consumption and body weight

Results obtained for the mean daily food consumption of the animals after two months of supply with hypercholesterolemic or normal diet treated with guaraná and body weight of rats are presented in Tables 3 and 4, respectively. As can be observed, the mean daily food consumption was significant decreased in 39.47% in hypercholesterolemic groups when compared to normal groups ($P<0.001$; n=7). However, body weight of rats had no significant difference in hypercholesterolemic and normal diet groups before and after the treatment (data not shown).

3.4 Biochemical analysis after treatment with guaraná

Determinations of total cholesterol, HDL-C, LDL-C and TRI from both normal and hypercholesterolemic diet rats after the treatment were demonstrated in Table 5. The results after treatment with guaraná showed a significant decrease of total cholesterol in 72.71% ($P< 0.001$; n = 7) and LDL-C in 159.41% ($P< 0.001$; n = 7) only in H+S group when compared with all the other groups.

Activities of hepatic enzymes AST and ALP in serum did not differ between hypercholesterolemic and normal diet groups after the treatment (data not shown). However, the activity of ALT in serum presented significantly increased in 82.06% in hypercholesterolemic group when compared with normal diet group ($P< 0.001$; n = 7)

Furthermore, others biochemical parameters as glucose, albumin and uric acid in serum did not differ between hypercholesterolemic and normal diet groups after the treatment (data not shown).

3.5 E-NTPDase activity

Figure 1 shows the effect of oral administration of guaraná on ATP and ADP hydrolysis by E-NTPDase activity in lymphocytes of rats submitted to an experimental model of hypercholesterolemia by diet. Results of E-NTPDase activity in lymphocytes with ATP as substrate are shown in Figure 1A. As can be observed, the hydrolysis of ATP was altered in H+25 group, demonstrating that ATP hydrolysis was significantly increased in 52.60% when compared with N+S group, the same occurred when compared the H+50 group with N+S group, was observed significantly increased in 74.43% in hydrolysis of ATP by E-NTPDase activity. However, the others groups did not presented significantly alterations in hydrolysis of ATP by E-NTPDase activity, when compared with among themselves.

In addition, results obtained for E-NTPDase activity in lymphocytes with ADP as substrate are shown in figure 1B, where the ADP hydrolysis was similar among all the groups.

3.6 Ecto-Adenosine deaminase activity determination (E-ADA)

Results obtained for adenosine hydrolysis by E-ADA activity in lymphocytes are shown in Figure 2. As can be observed, E-ADA activity was significantly decreased in about 70% in H+50 group when compared with normal diet groups. Also occurred significantly decreased in 35% in E-ADA activity in H+S groups when compared with N+S, N+12.5 and N+25 groups. However, N+50 group presented no significant alteration in the E-ADA activity when compared with hypercholesterolemic groups, except by H+50 group.

4 Discussion

Hypercholesterolemia is widely accepted as one of the major risk factors for the development of ischemic heart diseases and atherosclerosis [4]. The inflammatory process induced by hypercholesterolemia is not limited to large arteries. LDL-C is a major carrier of cholesterol in the circulation, and can play an important role in atherogenesis if it undergoes oxidative modification by endothelial cells, vascular smooth muscle, or macrophages within the arterial wall [5]. Guaraná exhibits a cardioprotective effect by inhibiting platelet aggregation and a high concentration of polyphenols, with properties anti-thrombogenic, anti-inflammatory, hypotensive and hypocholesterolemic action [26,27,28].

Changes in the activity of ecto-enzymes, such as E-NTPDase were observed in some diseases such as multiple sclerosis [41], rheumatoid arthritis [42], ischemic heart disease [43] and lung cancer [44], indicating that changes in the E-NTPDase activity is involved with the pathogenesis of many diseases, including hypercholesterolemia [16]. Data from the literature demonstrates that high-fat diet is associated with cardiovascular disease, endothelial dysfunction and inflammation. In line with this, the present study explored the effects of treatment with guaraná in hypercholesterolemic rats on the activities of purinergic system enzyme in lymphocytes as well as biochemical parameters.

First, the hypercholesterolemia was induced in animals by supply high-fat diet and it was confirmed through the measurement of increased levels of total cholesterol and LDL-C as in accordance with Otunola et al. [1]. After that, was analyzed the ability of guaraná to reverse this process. After 30 days of treatment

with guaraná, hypercholesterolemic rats, at all doses tested, returned the total cholesterol and LDL-C levels at baseline. Thus, confirming the efficacy of guaraná previously described as hypocholesterolemic properties because of polyphenols [26].

In relation to transaminase activity, we found ALT activity increased in all the hypercholesterolemic groups, while AST activity was not altered. Nevertheless, approximately 80% of AST in hepatocytes appear to be located in mitochondria, whereas ALT is thought to be predominantly nom mitochondrial and it has been postulated that in "mild" hepatocelular injury, when the hepatocytes plasmatic but not the mitochondrial membrane is damaged [45].

Previous studies show that the hypercholesterolemic state generates an inflammatory process, due to the presence of pro-inflammatory cytokines such as IL-1, IL-6, TNF-α [46,47]. Knowing this, we can suggest that in this study, the hypercholesterolemic diet increased levels of LDL-C and total cholesterol in the serum of rats, generating an inflammatory process.

Literature data indicate that adenine nucleotides and adenosine are important modulators of atherosclerosis [48], and since ATP stimulates the release of inflammatory cytokines by cultured macrophages, dendritic cells, or both [49]. In fact, the inflammatory process generated by hypercholesterolemia may lead to a significant increase in the levels of purine and pyrimidine nucleotides on the sites involved, probably contributing to the amplification of the inflammatory reaction [50]. Extracellular ATP can act as a damage-associated molecular patterns, given that it is normally confined to intracellular sites but can be released at high local levels following cell lysis, infection, or via regulated efflux. ATP released into the extracellular space can modulate the immune response through its capacity to bind and activate multiple nucleotide receptor family members [51,52].

While the enzymes E-NTPDase and E-ADA constitute a multiple system for extracellular nucleotide hydrolysis, the increase of these activities reflects an increased degradation of nucleotides as a compensatory organic response. The results of the present study show an increase in the E-NTPDase activity in rats supply with hypercholesterolemic diet and treated with guaraná at doses of 25 and 50 mg/kg/day when compared with all the other groups.

The increased activity of E-NTPDase lead the hydrolysis of the nucleotides ATP. This nucleotide, released to the extracellular environment at high concentrations activates the pro-inflammatory purinergic P2X7 receptors stimulate the Th1 immune response and contributes to tissue damage and inflammation [53].

For this reason, we suggest that the guaraná, at doses of 25 and 50 mg/kg acted modulating the enzyme activity to decrease excess extracellular ATP caused by hypercholesterolemic state and decreasing the activation of P2X7 receptor by increasing the rate of ATP hydrolysis. The extracellular ATP at low concentration possesses affinity for P2Y receptor (G protein coupled receptors) subtype on surfaces of lymphocytes, this purinergic receptor stimulates the Th2 immune response, leading to the production of anti-inflammatory cytokines [11].

In this study it was observed that there was no alteration in NTPDase activity in lymphocytes of rats with hypercholesterolemia. This result is opposed to data obtained in platelets from humans with hypercholesterolemia, where the activity and expression of NTPDase are increased [16]. However, alterations in NTPDase activity were not observed possibly due to the time of supplementation.

However, when hypercholesterolemic rats were treated with guaraná, they showed that neither the guaraná nor the hypercholesterolemia *per se* or in combination have promoted significant alterations in E-NTPDase activity using ADP as a substrate, keeping to basal levels. Knowing that these enzymes act in a cascade and the extracellular ATP is related to one of its degradations products, the nucleoside adenosine [15]. The ectoenzymes, E-NTPDase and E-ADA, control the extracellular concentration of ATP and adenosine [11]. The immunosuppressive actions of adenosine are triggered by activation of four receptor subtypes: A1, A2A, A2B and A3. These receptors are transmembrane glycoproteins coupled to protein G [54].

Moreover, with the increase of E-NTPDase activity in hypercholesterolemic groups treated with high doses of guaraná suggest a decreased of levels of ATP and increased ADP levels, generating a large amount of adenosine, which is converted to inosine by E-ADA. As large amounts of ATP were released from injured cells, the rapid hydrolyses of ATP and ADP (by E-NTPDase) favors the

production of adenosine, with possesses anti-inflammatory and analgesic properties [55].

In this study, hypercholesterolemia *per se* reduced the E-ADA activity when compared to the normal diet group, possibly as a compensatory mechanism to reduce the damage caused by excess extracellular ATP. These data corroborate with studies which bring that the inflammatory activity in hypercholesterolemic state depends on the balance between pro-inflammatory response, Th1-type and anti-inflammatory response, Th2-type [56].

The results of the present work demonstrated that the greater reduction in E-ADA activity was observed in hypercholesterolemic group treated with the higher concentration of guaraná when compared with the other groups. These results suggest that in addition to the balance of the inflammatory response, due to hypercholesterolemic state, guaraná acts by modulating the activity of the enzyme. It was due to high content of polyphenolic compounds in guaraná, which may act to prevent atherogenesis through a combination of effects, including the other positive effects of guaraná on lipid metabolism [19], inibition of platelet aggregation [21] and anti-inflammatory properties [27].

Adenosine elevated in extracellular environment could be binding to specific receptors expressed on the cell surface exercising its anti-inflammatory function include inhibition of pro-inflamatory cytokine realize, inhibition of adhesion of immune cells, cardioprotective effect, vasodilator and inhibition of proliferation of T cells trough the activation of A2A receptors [57,58].

In conclusion, our data demonstrates that the guaraná was able to reduce to basal levels the total cholesterol and LDL-C in hypercholesterolemic rats. In addition, the guaraná in high concentrations when associated with hipercholesterolemic diet was able to increase on the E-NTPDase activity and decrease the E-ADA activity in lymphocytes contributing to reducing the inflammatory process by increasing the concentration of extracellular adenosine. Although requiring further study, guaraná is a promising compound to be used as complementary therapy for the benefit of people with hypercholesterolemia.

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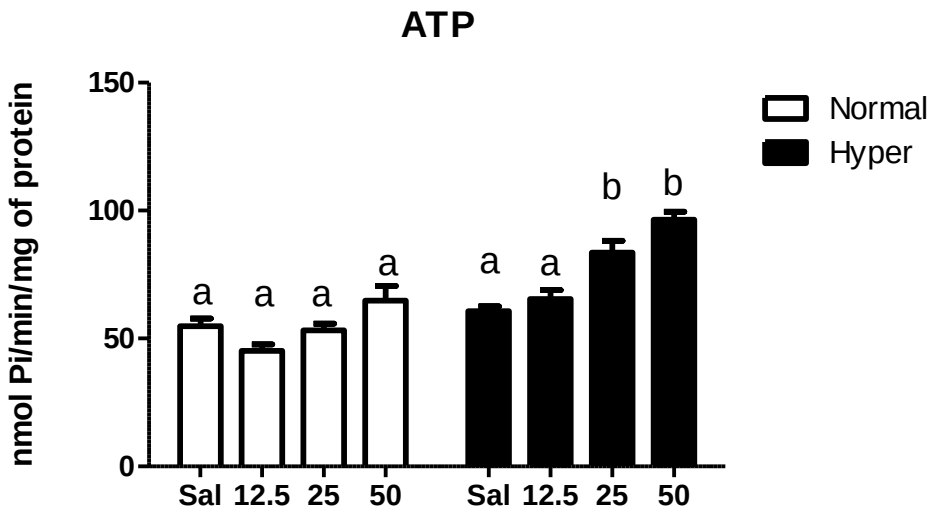
(A)



(B)

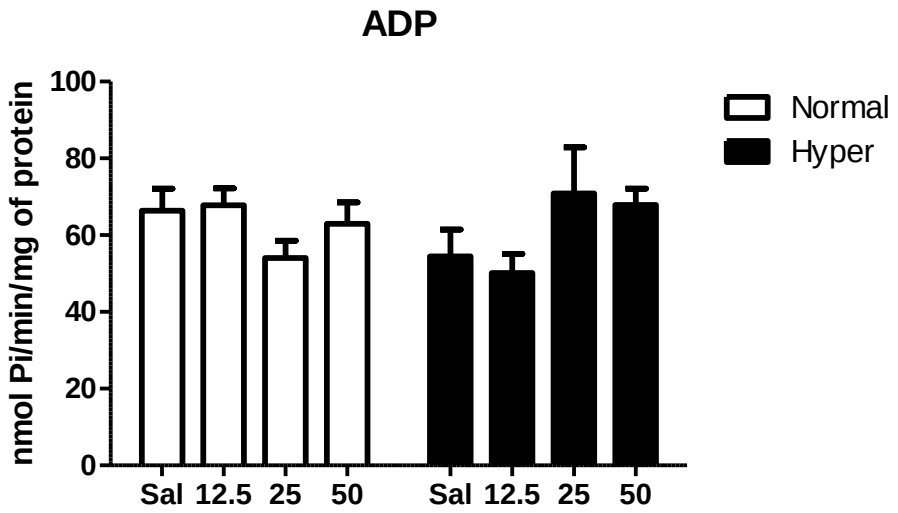


Fig. 1 E-NTPDase activity with ATP (A) and ADP (B) as substrate in lymphocytes of rats submitted to an experimental model of hypercholesterolemia treated with guaraná at doses of 12.5, 25 and 50 mg/kg/day for a period of 30 days. The results were analyzed using two-way ANOVA- Newman-Keuls multiple comparison test and expressed as mean ± S.E.M. Different letters confer significant statistical difference among groups (P< 0.001, n = 7).

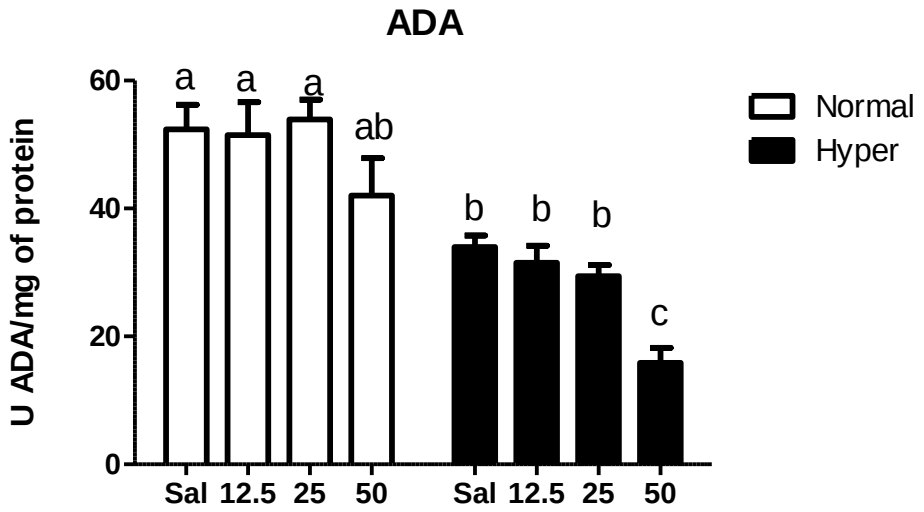


Fig. 2 E-ADA activity in lymphocytes of rats submitted to an experimental model of hypercholesterolemia treated with guaraná at doses of 12.5, 25 and 50 mg/kg/day for a period of 30 days. The results were analyzed using two-way ANOVA- Newman-Keuls multiple comparison test and expressed as mean ± S.E.M. Different letters confer significant statistical difference among groups ($P < 0.05$, n = 7).

Table 1: Composition of the diets

Ingredients	Normal diet	Hypercholesterolemic diet
Corn starch	15%	9%
Sugar	50%	18,5%
Albumin	20%	20%
Animal fat	--	38%
Vegetable fat	5%	5%
Fiber	5%	4,5%
Vitamins and mineral	5%	5%
TOTAL	100%	100%

Table 2: Lipid parameters after induction of hypercholesterolemia after one month of supply with hypercholesterolemic or normal diet.

Groups	TC	LDL-C
Control rats	64.60 ± 2.30	29.04 ± 3.30
Hypercholesterolemic rats	118.3 ± 2.30***	64.64 ± 10.46**

TC: total cholesterol; LDL-C: low-density lipoprotein-cholesterol (mg/dL). The results were analyzed using Student's t test and expressed as mean ± S.E.M. Significant difference between groups: (***$P < 0.001$; n = 7); (**$P < 0.01$; n = 7).

Table 3: Mean daily food consumption of the animals after two month of supply with hypercholesterolemic or normal diet treated with guaraná

Groups	Food Consumption (g)
N+S	21.46 ± 0.71^{a}
N+12.5	19.61 ± 0.63^{a}
N+25	21.31 ± 0.71^{a}
N+50	23.07 ± 0.74^{a}
H+S	12.99 ± 0.40^{b}
H+12.5	12.75 ± 0.33^{b}
H+25	13.61 ± 0.41^{b}
H+50	12.93 ± 0.44^{b}

N+S: normal food + Saline, N+12.5: normal food + guaraná 12.5 mg/kg, N+25: normal food + guaraná 25 mg/kg, N+50: normal food + guaraná 50 mg/kg, H+S: hypercholesterolemic food + Saline, H+12.5: hypercholesterolemic food + guaraná 12.5 mg/kg, H+25: hypercholesterolemic food + guaraná 25 mg/kg, H+50: hypercholesterolemic food + guaraná 50 mg/kg. The results were analyzed using two-way ANOVA- Newman-Keuls multiple comparison test and expressed as mean ± S.E.M. Different letters confer significant statistical difference among groups ($P < 0.001$, n = 7).

Table 4: Body weight of animals before and after treatment with guaraná

	Weight (g)		
	Before of diet supply	**Day 30**	**Day 60**
N+S	311.5 ± 14.15	393.4 ± 11.64	397.6 ± 14.22
N+12.5	281.7 ± 10.92	368.9 ± 9.65	389.1 ± 8,821
N+25	300.1 ± 12.19	380.6 ± 13.33	409.1 ± 18,32
N+50	313.1 ± 16.28	409.0 ± 15.02	419.1 ± 14,15
H+S	295.8 ± 14.55	375.9 ± 13.63	409.0 ± 11,07
H+12.5	308.6 ± 9.17	370.3 ± 11.47	399.0 ± 13,71
H+25	308.5 ± 9.05	379.6 ± 11.36	393.2 ± 9,59
H+50	315.9 ± 12.17	391.8 ± 11.07	400.0 ± 10,04

N+S: normal food + Saline, N+12.5: normal food + guaraná 12.5 mg/kg, N+25: normal food + guaraná 25 mg/kg, N+50: normal food + guaraná 50 mg/kg, H+S: hypercholesterolemic food + Saline, H+12.5: hypercholesterolemic food + guaraná 12.5 mg/kg, H+25: hypercholesterolemic food + guaraná 25 mg/kg, H+50: hypercholesterolemic food + guaraná 50 mg/kg. The results were analyzed using two-way ANOVA- Newman-Keuls multiple comparison test and expressed as mean ± S.E.M. ($P > 0.05$, n = 7).

Table 5: Lipid parameters after two month of supply with hypercholesterolemic or normal diet.

Groups	TC	TRI	HDL-C	LDL-C
N+S	79,21 ± 4,53[a]	64,23 ± 8,97	62,33 ± 3,52	26,73 ± 5,43[a]
N+12.5	81,95 ± 4,59[a]	71,86 ± 5,92	63,38 ± 3,93	13,32 ± 1,07[a]
N+25	86,72 ± 2,18[a]	66,56 ± 15,94	67,63 ± 2,10	15,84 ± 4,01[a]
N+50	72,32 ± 6,37[a]	76,60 ± 6,49	56,43 ± 4,65	20,22 ± 0,03[a]
H+S	136,8 ± 12,95[b]	64,18 ± 5,97	59,86 ± 2,99	69,34 ± 7,18[b]
H+12.5	68,64 ± 3,47[a]	64,84 ± 13,34	58,14 ± 5,29	29,30 ± 0,30[a]
H+25	75,91 ± 7,82[a]	56,79 ± 10,44	63,80 ± 6,29	17,95 ± 0,57[a]
H+50	82,35 ± 8,81[a]	47,49 ± 6,84	61,33 ± 7,04	18,29 ± 2,39[a]

TC: total cholesterol; LDL-C: low-density lipoprotein-cholesterol; HDL-C: high-density lipoprotein-cholesterol; TRI: triglycerides; N+S: normal food + Saline, N+12.5: normal food + guaraná 12.5 mg/kg, N+25: normal food + guaraná 25 mg/kg, N+50: normal food + guaraná 50 mg/kg, H+S: hypercholesterolemic food + Saline, H+12.5: hypercholesterolemic food + guaraná 12.5 mg/kg, H+25: hypercholesterolemic food + guaraná 25 mg/kg, H+50: hypercholesterolemic food + guaraná 50 mg/kg. The results were analyzed using two-way ANOVA-Newman-Keuls multiple comparison test and expressed as mean ± S.E.M. Different letters confer significant statistical difference among groups ($P < 0.0001$, n = 7).

Table 6: Activity of hepatic enzyme in serum after treatment with guaraná.

Liver enzyme	ALT
N+S	29.71 ± 1.28[a]
N+12.5	30.90 ± 1.86[a]
N+25	30.32 ± 1.63[a]
N+50	28.91 ± 3.10[a]
H+S	54.09 ± 6.90[b]
H+12.5	44.60 ± 2.57[b]
H+25	44.90 ± 3.93[b]
H+50	44.91 ± 6.18[b]

ALT: alanine aminotransferase (U/L), N+S: normal food + Saline, N+12.5: normal food + guaraná 12.5 mg/kg, N+25: normal food + guaraná 25 mg/kg, N+50: normal food + guaraná 50 mg/kg, H+S: hypercholesterolemic food + Saline, H+12.5: hypercholesterolemic food + guaraná 12.5 mg/kg, H+25: hypercholesterolemic food + guaraná 25 mg/kg, H+50: hypercholesterolemic food + guaraná 50 mg/kg. The results were analyzed using two-way ANOVA-Newman-Keuls multiple comparison test and expressed as mean ± S.E.M. Different letters confer significant statistical difference among groups ($P < 0.001$; n = 7).

4 CONCLUSÕES

- Apesar da dieta hipercolesterolêmica possuir um valor calórico superior ao da dieta normal, os animais deste grupo não apresentaram alterções significativas na massa corporal, possivelmente devido a uma diminuiçao da ingestão de dieta hipercolesterolêmica.
- O guaraná mostrou mais de uma vez ter efeito benéfico sobre a colesterolemia, pois houve redução do colesterol total e LDL-colesterol, indicando ser um possível tratamento contra distúrbios metabólicos, quando causados pelo padrão alimentar como, por exemplo, a hipercolesterolemia.
- A hipercolesterolemia foi capaz de gerar um aumento na atividade da enzima hepática alanina aminotrasnferase (ALT) possivelmente devido ao efeito pró-oxidante da dieta, gerando grandes quantidades de radicais livres.
- O guaraná *per se* não apresentou efeito sobre a atividade das enzimas E-NTPDase e E-ADA em linfócitos, demonstrando que ele sozinho não é capaz de atuar na modulação enzimática. Entretanto, em ratos hipercolesterolêmicos tratados com guaraná, houve alteração na atividade da E-NTPDase e E-ADA em linfócitos, indicando participação da sinalização purinérgica.

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ANEXO 1

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COMISSÃO DE ÉTICA NO USO DE ANIMAIS-UFSM

CARTA DE APROVAÇÃO

A Comissão de Ética no Uso de Animais-UFSM, analisou o protocolo de pesquisa:

Título do Projeto: "Efeito do extrato de guaraná (*Paullinia cupana*) no metabolismo de nucleotídeos e nucleosídeo de adenina e perfil oxidativo em modelo experimental de hipercolesterolemia".

Numero do Parecer: 102-2012-2013

Pesquisador Responsável: Profa. Dra. Daniela Bitencourt Rosa Leal

Este projeto foi **APROVADO** em seus aspectos éticos e metodológicos. Toda e qualquer alteração do Projeto, assim como os eventos adversos graves, deverão ser comunicados imediatamente a este Comitê.

OBS: Anualmente deve-se enviar à CEUA relatório parcial ou final deste projeto.

Os membros da CEUA-UFSM não participaram do processo de avaliação dos projetos onde constam como pesquisadores.

DATA DA REUNIÃO DE APROVAÇÃO: 06/06/2013

Santa Maria, 07 de junho de 2013.

Carlos Alberto Ceretta
Pró-reitor Adjunto de Pós-graduação e Pesquisa

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