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ATIVIDADE DAS ECTO-ENZIMAS EM PLAQUETAS DE RATOS COM HIPERCOLESTEROLEMIA INDUZIDA TRATADOS COM CURCUMINA E SUBMETIDOS AO EXERCÍCIO FÍSICO

DISSERTAÇÃO DE MESTRADO

Josiane Bizzi Schlemmer

Santa Maria, RS, Brasil
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por

Josiane Bizzi Schlemmer

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

Orientadora: Daniela Bitencourt Rosa Leal
Co-orientadora: Maria Rosa Chitolina Schetinger

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elaborada por
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como requisito parcial para obtenção do grau de
Mestre em Ciências Biológicas: Bioquímica Toxicológica

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"Procuro semear otimismo e plantar sementes de paz e justiça. Digo o que penso, com esperança. Penso no que faço, com fé. Faço o que devo fazer, com amor. Eu me esforço para ser cada dia melhor, pois bondade também se aprende. Mesmo quando tudo parece desabar, cabe a mim decidir entre rir ou chorar, ir ou ficar, desistir ou lutar; porque descobri, no caminho incerto da vida,que o mais importante é o decidir."

Ana Lins dos Guimarães Peixoto Brefas

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RESUMO

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

ATIVIDADE DAS ECTO-ENZIMAS EM PLAQUETAS DE RATOS COM HIPERCOLESTEROLEMIA INDUZIDA TRATADOS COM CURCUMINA E SUBMETIDOS AO EXERCÍCIO FÍSICO

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Data e Local da Defesa: Santa Maria, 31 de julho de 2012.

A hipercolesterolemia é um fator de risco para o surgimento da aterosclerose, sendo esta uma doença inflamatória crônica caracterizada por acúmulo de plaquetas nas paredes das artérias e também por um processo inflamatório. As plaquetas acumuladas dentro das lesões ateroscleróticas, podem recrutar plaquetas adicionais para formar um trombo. Os nucleotídeos e nucleosídeos extracelulares como ATP, ADP, AMP e adenosina têm funções importantes, incluindo a regulação do trombo, exercendo uma variedade de efeitos sobre as plaquetas. A família de ectoenzimas (E-NTPDase, E-5'-nucleotidase, E-NPP, E-ADA) é que regula a concentração destes nucleotídeos e nucleosídeos. Estudos demonstram que o exercício físico, pode provocar alterações no perfil lipídico. Dentre os exercícios físicos, o nado tem sido utilizado em ratos para avaliação de adaptações cardiovasculares ao exercício, podendo ser uma terapia adjuvante para a prevenção da progressão da aterosclerose. Além do exercício físico, produtos naturais tem sido utilizado para o controle de doenças cardiovasculares, como é o caso da curcumina, um composto fenólico natural, que tem propriedades antioxidantes e hipocolesterolêmicas. Diante disso, o objetivo deste trabalho foi avaliar a atividade das ectoenzimas E-NTPDase, E-5'-nucleotidase, E-NPP e E-ADA em plaquetas de ratos com hipercolesterolemia induzida tratados com curcumina e submetidos ao exercício físico. Os ratos utilizados no estudo foram divididos em 8 grupos: dieta padrão (C), dieta padrão mais curcumina (CC), dieta padrão mais exercício físico (PE), dieta padrão mais exercício físico e curcumina (CCPE), dieta hipercolesterolêmica (H), dieta hipercolesterolêmica mais curcumina (HCC), dieta hipercolesterolêmica mais exercício físico (HPE), e dieta hipercolesterolêmica mais exercício físico e curcumina (HCCPE). A atividade da E-NTPDase, E-NPP, E-5'-NT e E-ADA foram medidos em plaquetas isoladas. A curcumina foi administrada por gavagem na dose de 25 mg/kg/dia e os ratos foram submetidos ao nado

forçado. Foi observado um aumento na hidrólise do ATP e ADP nos ratos com hipercolesterolemia induzida. Quando esses ratos receberam tratamento com curcumina e exercício físico, o exercício físico por si só foi capaz de aumentar a hidrólise de ATP. Na hidrólise de ADP, observou-se uma diminuição na atividade da E-NTPDase quando os ratos hipercolesterolêmicos foram tratados com curcumina e exercício físico por si só, bem como quando foram tratados com curcumina e exercício físico concomitante. A atividade da E-5'nucleotidase, E-NPP, E-ADA e agregação plaquetária não mostraram diferenças significativas. Estes resultados sugerem que a dieta hipercolesterolêmica altera a atividade das ecto-enzimas em plaquetas, e que o tratamento com a curcumina e exercício físico pode ser capaz de modular a hidrólise de nucleotídeos e nucleosídeos de adenina nesta condição experimental.

Palavras chave: Hipercolesterolemia, Curcumina, Exercício físico, Sistema purinérgico

ABSTRACT

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

ACTIVITIES OF ECTOENZYMES IN PLATELETS IN RATS WITH INDUCED HYPERCHOLESTEROLEMIA TRARTED WITH CURCUMIN AND SUBMITTED TO PHYSICAL EXERCISE

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Place and Date: Santa Maria, july 31th, 2012.

Hypercholesterolemia is a risk factor for the development of atherosclerosis, which is a chronic inflammatory disease characterized by accumulation of platelets on the walls of arteries and also by an inflammatory process. Platelets accumulated within atherosclerotic lesions, may recruit additional platelets to form a thrombus. Extracellular nucleotides and nucleosides such as ATP, ADP, AMP and adenosine have important functions, including the regulation of the thrombus, causing a variety of effects on platelets. The family of ecto-enzymes (E-NTPDase, E-5'-nucleotidase, E-NPP, E-ADA) that regulates the concentration of these nucleotides and nucleosides. Studies show that physical exercise may cause changes in lipid profile. Among the exercises, swimming in rats has been used to evaluate cardiovascular adaptations to exercise, may be an adjunct therapy to prevent progression of atherosclerosis. In the exercise, natural products have been used for the control of cardiovascular diseases, such as curcumin, a natural phenolic compound, which has antioxidant and hypocholesterolemic. Therefore, the objective of this study was to evaluate the activity of ecto-enzymes E-NTPDase, E-5'-nucleotidase, E-NPP and E- ADA in platelets of rats with induced hypercholesterolemia treated with curcumin and submitted to physical exercise. The rats used in the study were divided into eight groups: standard diet (C), standard diet along with curcumin (CC), standard diet along with physical exercise (PE), standard diet along with curcumin and physical exercise (CCPE), hypercholesterolemic diet (H), hypercholesterolemic diet along with curcumin (HCC), hypercholesterolemic diet along with physical exercise (HPE), and hypercholesterolemic diet along with curcumin and physical exercise (HCCPE). The activity of E-NTPDase, E-NPP, E-5'-NT and E-ADA were measured in isolated platelets. Curcumin was administered by gavage at a dose of 25 mg / kg / day, the rats were submitted to forced swimming. There was an increase in the

hydrolysis of ATP and ADP in rats with hypercholesterolaemia induced. When these mice were treated with curcumin and physical exercise, physical exercise *per se* was able to further enhance the hydrolysis of ATP. In the hydrolysis of ADP, it was observed a decrease in activity of the E- NTPDase when hypercholesterolemic rats were treated with curcumin and physical exercise *per se,* well as when it was associated with curcumin and physical exercise. The activities of E-5'nucleotidase, E-NPP and E-ADA and platelet aggregation, did not show any significant. These results suggest that the hypercholesterolemic diet alters the activity of ecto-enzyme in platelets, and that treatment with curcumin and physical exercise may be able to modulate hydrolysis of nucleotides and nucleosides of adenine this experimental condition.

Keywords: Hypercholesterolemia, Curcumin, Physical exercise, Purinergic system.

LISTA DE ABREVIATURAS

ACRs: regiões conservadas da apirase

ADP: adenosina difosfato

AMP: adenosina monofosfato

ATP: adenosina trifosfato

Apo B-100: Apolipoproteína da LDL

ADA: adenosina desaminase

CTP: Citidina trifosfato

CT: Colesterol Total

CRP: proteína C- reativa

E-NTPDase: ecto-nucleosídeo trifosfato difosfoidrolase

E-NPP: ectonucleotídeo pirofosfatase/fosfodiesterase

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

E-ADA: ecto-adenosina desaminase

Fator de necrose- κβ: Fator de necrose- kappa beta

GTP: Guanosina trifosfato

HDL: Lipoproteína de alta densidade

Km: Constante de Michaelis Menten

ICAM-1: Molécula de adesão intercelular-1

IL-1: Interleucina-1

IL-6: Interleucina-6

IL-10: Interleucina-10

IL-18: Interleucina-18

IL-1RA: receptor da IL-1

LDL: (Low Density Lipoproteins) Lipoproteína de baixa densidade

NO: Óxido nítrico

NPC1L1: Transportador de colesterol na membrana celular

PAI-1: Inibidor do ativador de plasminogênio –1

TTP: Tiamina trifosfato

Treg: Células T reguladoras

TNF: fator de necrose tumoral

UTP: Uridina trifosfato

VCAM-1: Molécula de adesão celular-vascular-1

LISTA DE FIGURAS

INTRODUÇÃO

MANUSCRITO

LISTA DE TABELAS

MANUSCRITO

LISTA DE ANEXOS

SUMÁRIO

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-se 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: 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 a ingestão de grande quantidade de açúcar e gordura (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 particular, o consumo de dietas ocidentalizadas, as quais são ricas em gordura saturada e açúcar refinado e pobres em frutas e verduras, são fatores relevantes na etiologia de doenças crônicas (BLOCK et al., 1988; GOLDBART 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 aí situadas (IV DIRETRIZ SOBRE DISLIPIDEMIAS E PREVENÇÃO DA ATEROSCLEROSE, 2007).

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 & TURLEY, 2004).

Os conteúdos alimentares de gorduras saturadas e de colesterol influenciam diferentemente os 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. A absorção de gordura saturada, no entanto, não é limitada e, por isso, sua ingestão promove efeito mais intenso sobre a colesterolemia (IV DIRETRIZ SOBRE DISLIPIDEMIAS E PREVENÇÃO DA ATEROSCLEROSE, 2007).

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; SENNA, 2002).

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).

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 apo B-100 constitui a parte proteica da lipoproteína LDL, e é o componente que liga as partículas da LDL à receptores específicos, chamados de receptores B, E, situados na superfície da membrana plasmática celular (GOLDSTEIN et al., 1979).

A hipercolesterolemia está epidemiologicamente ligada ao desenvolvimento de doenças cardiovasculares, sendo um importante fator de risco e determinante no surgimento da aterosclerose (HANSSON, 2005; LUSIS, 2000; ROCHA e LIBBY, 2009; ROSS, 1999). A aterosclerose é uma enfermidade crônica multifatorial, lenta e progressiva, resultante de uma série de respostas celulares e moleculares altamente específicas (HACKAM & ANAND, 2003). Nesta enfermidade, ocorre retenção de lipídeos, células inflamatórias e elementos fibrosos na parede das artérias os quais são responsáveis pela formação de placas ou estrias gordurosas, que geralmente ocasionam a obstrução das mesmas e, consequentemente, a deterioração da função vascular (LIBBY, 2002).

A aterosclerose é caracterizada 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; LIBBY, 2009).

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 chamado de íntima, a camada do meio é conhecida como média e a camada mais externa compreende a adventícia arterial. Estas três camadas são demarcadas por camadas concêntricas de elastina, conhecida como a lâmina elástica interna que separa a íntima da média, e a lâmina elástica externa que separa a média da adventícia (Figura 1) (STOCKER; KEANEY, 2004). Na aterosclerose a lipoproteína LDL torna-se

aprisionada no espaço subendotelial, onde está sujeita a 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 endotelial ou disfunção (DIAZ et al.,1997) (Fig. 2A), que é caracterizada por permeabilidade endotelial reforçada, deposição da lipoproteína da 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 de leucócitos ao longo da parede arterial com a migração das células musculares lisas da íntima. Finalmente na aterosclerose avançada (Fig. 2C) é caracterizada pela acumulação de macrófagos, formação de capa fibrosa e necrose no núcleo da lesão (STOCKER; KEANEY, 2004).

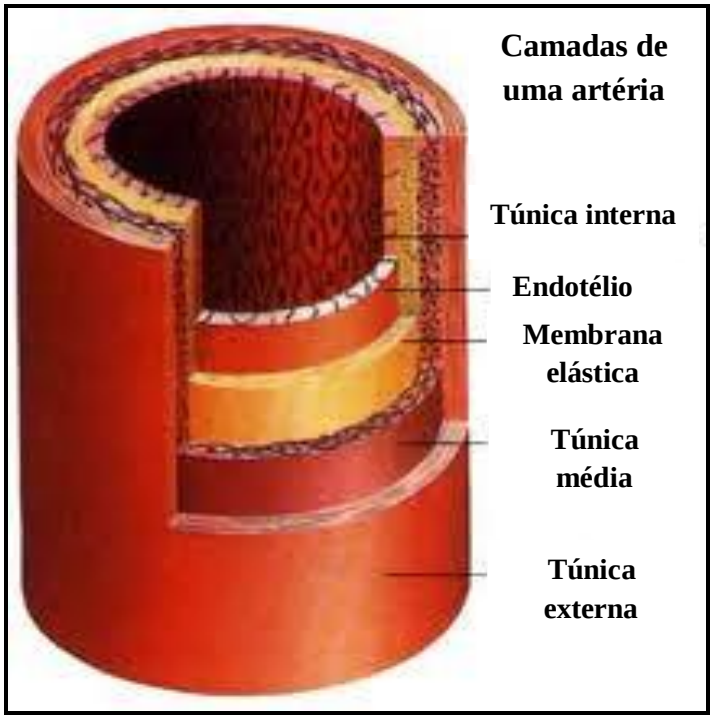

Figura 1: Morfologia de uma artéria (http://pt.wikipedia.org/wiki/Arteria)

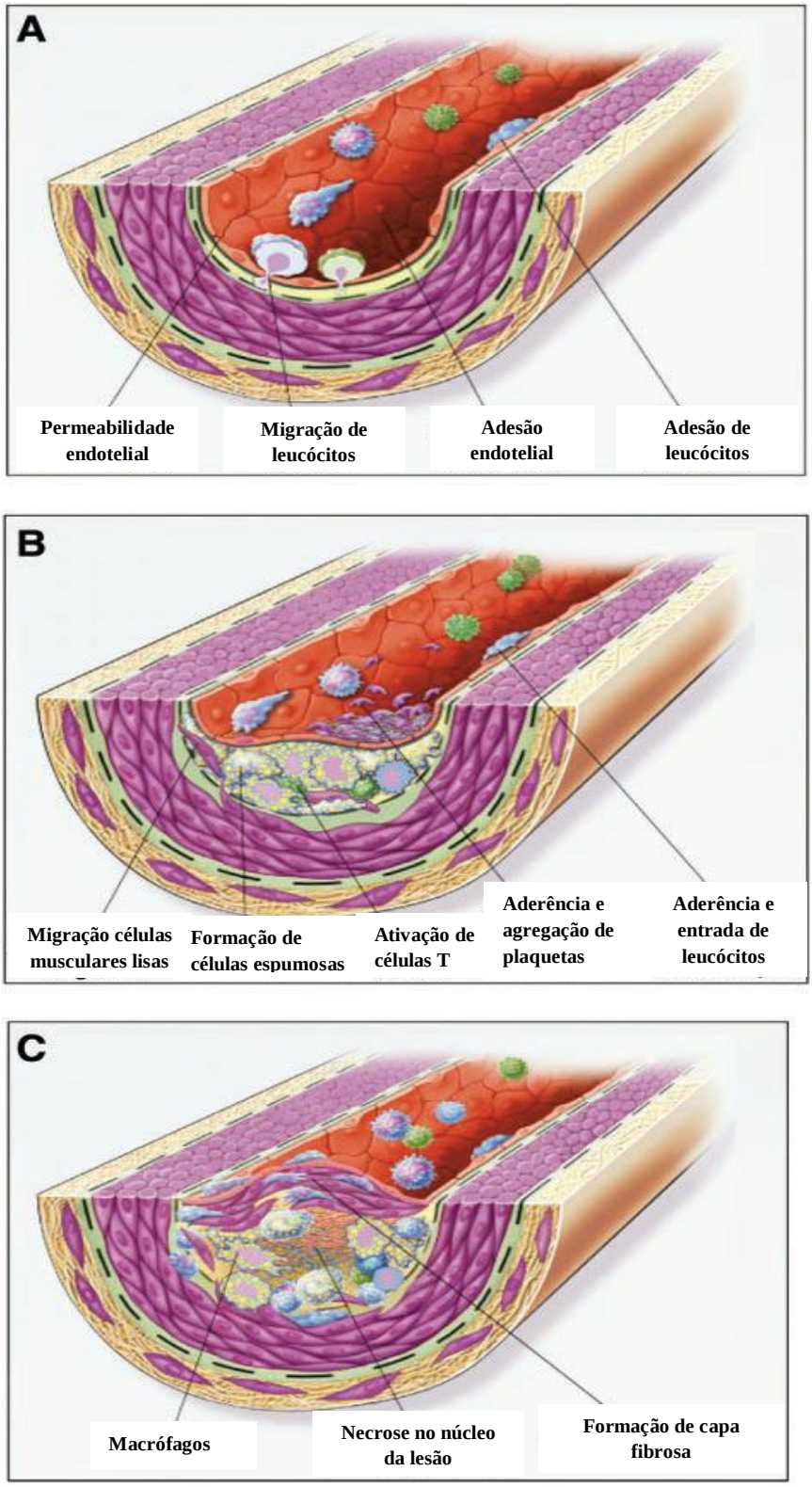

Figura 2: Variações dos estágios da aterosclerose (Adaptado por Stocker; Keaney, 2004).

O desenvolvimento e a progressão da placa aterosclerótica estão associados com alterações inflamatórias de fases iniciais, tanto localmente na parede arterial ou

sistemicamente, sendo este último identificado por um aumento precoce dos níveis séricos de marcadores inflamatórios (BALANESCU et al., 2010).

Os marcadores inflamatórios associados ao processo aterosclerótico podem ser divididos em categorias: fatores de ativação endotelial (VCAM-1 e ICAM-1); fatores pró-inflamatórios que induzem síntese hepática de proteínas de fase aguda (TNF, IL-1, IL-6 e IL-18); proteínas de fase aguda sintetizadas pelo fígado (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).

Entre os fatores de risco da aterosclerose influenciados pela dieta destacam-se: concentrações plasmáticas aumentadas de CT, LDL, hipertensão, diabetes e baixas concentrações plasmáticas de HDL e de antioxidantes (BARÓ et al., 2003). A hipercolesterolemia, por sua vez, propicia o espessamento e acúmulo de lipídeos na camada íntima de vasos, que produzem estrias gordurosas e placas de ateroma (NAPOLI et al., 2000).

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; GLYNN; HENNEKENS, 1998).

A aterosclerose também é caracterizada pela acumulação de plaquetas nas paredes das artérias (HANSSON, 2005; ROCHA; LIBBY, 2009).

As plaquetas exercem um papel fundamental na hemostasia, onde desempenham atividade mecânica e bioquímica (DANIEL et al., 1998), intervindo rapidamente na presença de lesões endoteliais (FROJMOVIC; PANJWANI, 1976). As plaquetas são células discóides, anucleadas e possuem um importante papel na manutenção da integridade endotelial (LORENZI et al., 2003). Possuem aproximadamente 3 μm de diâmetro, com forma e tamanho variável (FROJMOVIC; PANJWANI, 1976).

As plaquetas circulantes são produzidas a partir dos megacariócitos na medula óssea. Apresentam-se heterogêneas sob aspectos morfológicos, como tamanho e densidade (AUSTIN, 2008). A membrana celular de uma plaqueta é lipoproteica composta por fosfolipídeos, contendo muitos receptores responsáveis pelo desencadeamento da ativação plaquetária e de reações de adesão-agregação (ZAGO et al., 2004). Os demais componentes

estruturais das plaquetas consistem, essencialmente, em citoesqueleto, sistema tubular denso, sistema canalicular aberto e múltiplos grânulos de secreção, denominados grânulos alfa e grânulos delta (Figura 3) (LORENZI et al., 2003).

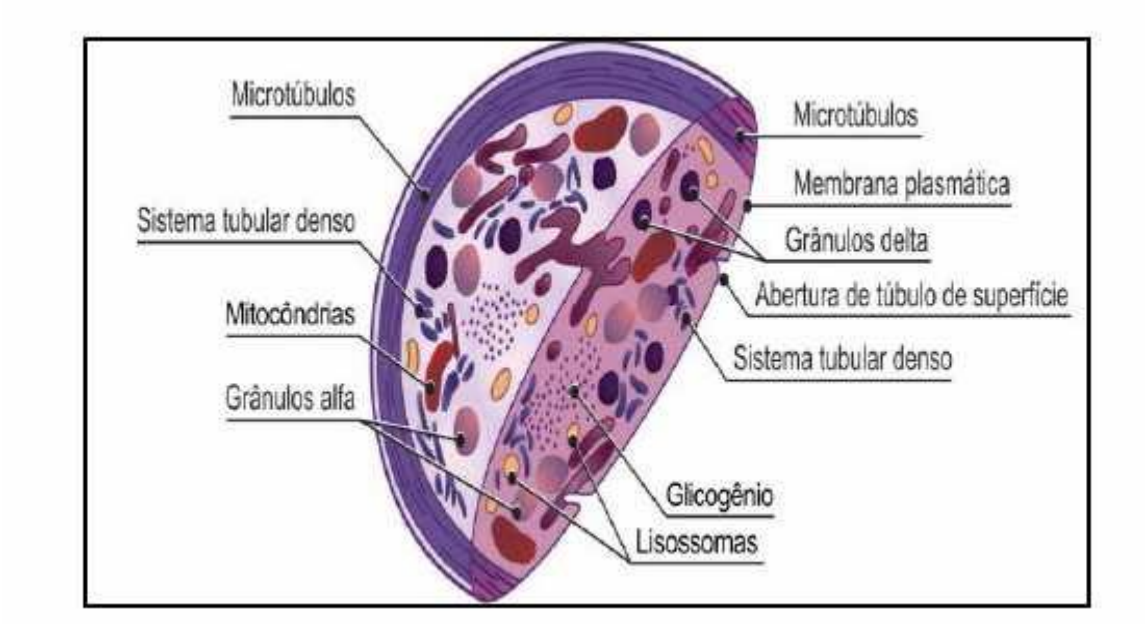

Figura 3 – Representação de uma plaqueta (LORENZI et al., 2003).

As três principais funções plaquetária são: adesão, ativação e agregação (Figura 4) (VANNI, 2007). A palavra "adesão" descreve a interação entre as plaquetas e qualquer outro tipo celular diferente, enquanto que "agregação" se refere exclusivamente à interação entre duas plaquetas (LOPEZ-FARRE, 2001). A agregação ocorre após a adesão e é definida como uma reação de plaquetas ativadas entre si (MESA & ALFONSO, 2000).

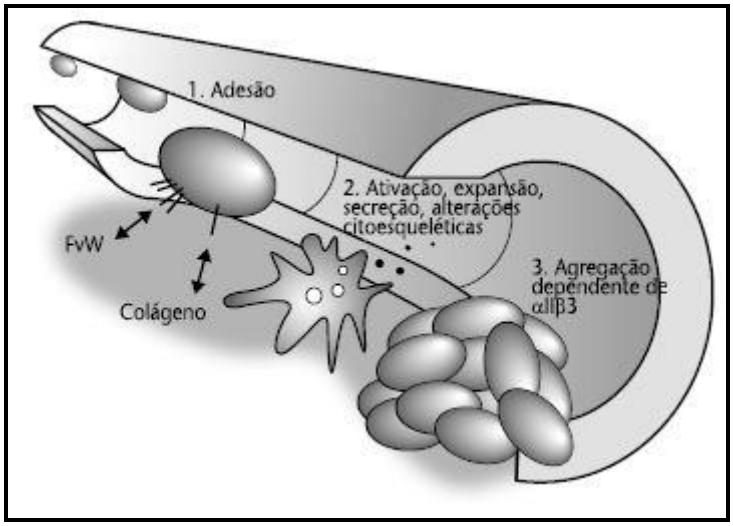

Figura 4: Funções plaquetária (VANNI, 2007).

As plaquetas acumuladas dentro das lesões ateroscleróticas, podem recrutar plaquetas adicionais para formar um trombo, indicando que a parede arterial pode assumir tanto uma inflamação como um fenótipo protrombogênico quando os níveis de colesterol no sangue estão elevados (WAGNER; BURGER, 2003).

As plaquetas são um dos mais importantes componentes do sangue que participam e regulam a formação de trombos, liberando substâncias ativas, tais como ADP (MARCUS et al., 2003). As plaquetas são ativadas quando entram em contato com o colágeno, trombina e ADP (DANIEL et al., 1998). Sob condições fisiológicas normais, as plaquetas circulam em estreito contato com a mucosa das células endoteliais da parede dos vasos sanguíneos e, apenas quando há uma lesão, elas aderem ao local da lesão (SALLES et al., 2008). Porém, quando o vaso sofre dano, elas são ativadas e aderem aos sítios danificados (SANTOS et al., 2008). Geralmente, a ativação plaquetária, é iniciada pela exposição a um agonista plaquetário que se liga a receptores de superfície, desencadeando uma cascata de eventos bioquímicos (COLMAN, 1990).

Massberg e colaboradores (2002) sugerem a participação das plaquetas na resposta inflamatória, uma vez que há ativação de plaquetas em doenças com componente inflamatório agindo na vasculatura. As plaquetas regulam uma variedade de respostas inflamatórias e possuem importante papel na aterotrombose, tornando-se claro sua atuação na hemostasia e na trombose (GAWAZ, 2006).

Tendo em vista, que gorduras saturadas presentes nas dietas causam alterações na composição lipídica (SILVA et al., 2002), levando à proliferação de certos tipos de células para o interior da parede arterial (STOCKER; KEANEY, 2004), modificações no estilo de vida, como o exercício físico, podem provocar alterações benéficas no perfil lipídico (FAN et al., 2009; HUSSEIN; NICHOLLS, 2010).

A prática de exercício físico tem sido adotada como uma estratégia no tratamento da hipercolesterolemia, por apresentar uma ação anti-aterogênica na circulação lipídica e de apolipoproteínas e aumentar a aptidão cardiorrespiratória (SUPERKO, 1998). A prática regular do exercício físico constitui medida auxiliar para o controle das dislipidemias, promovendo redução dos níveis plasmáticos de triglicerídeos, aumento dos níveis de HDL, porém sem alterações significativas sobre as concentrações de LDL (IV DIRETRIZ SOBRE DISLIPIDEMIAS E PREVENÇÃO DA ATEROSCLEROSE, 2007). Também está associada com níveis mais baixos de citocinas inflamatórias (EISENSTEIN et al., 2002), além de aumentar a sensibilidade à insulina, reduzir a pressão arterial de repouso e colaborar no

controle do peso corporal (IV DIRETRIZ SOBRE DISLIPIDEMIAS E PREVENÇÃO DA ATEROSCLEROSE, 2007).

O exercício físico é uma importante alternativa para restaurar a função endotelial, por apresentar ações sobre o sistema cardiovascular. A elevação do débito cardíaco e o aumento da pressão de perfusão durante o exercício físico aumentam a força que o sangue exerce sobre a parede vascular, sendo responsável pelo aumento da produção de óxido nítrico (NO), resultando no relaxamento do músculo vascular, com consequente vasodilatação (FISHER et al., 2002). Na hipercolesterolemia a atividade da óxido nítrico sintase (NOS) é reduzida, provocando alteração na produção de NO (NIEBAUER et al., 1999). Entretanto, a normalização dos níveis de LDL melhora a resposta vasodilatadora (ANDERSON et al., 1995).

Na aterosclerose, há um maior número de moléculas de adesão por células endoteliais, o que acarretam na exacerbação da resposta inflamatória, com conseqüente aumento da lesão tecidual (BITTENCOURT JÚNIOR; SENNA, 2002). O estado inflamatório é indicado por níveis elevados na circulação de marcadores de inflamação, tais como a IL-6, TNF e a CRP. É importante ressaltar que a inatividade física e o sedentarismo também aumentam o risco destas condições, como mostra a figura abaixo (Figura 5) (ROOK; DALGLEISH; 2011).

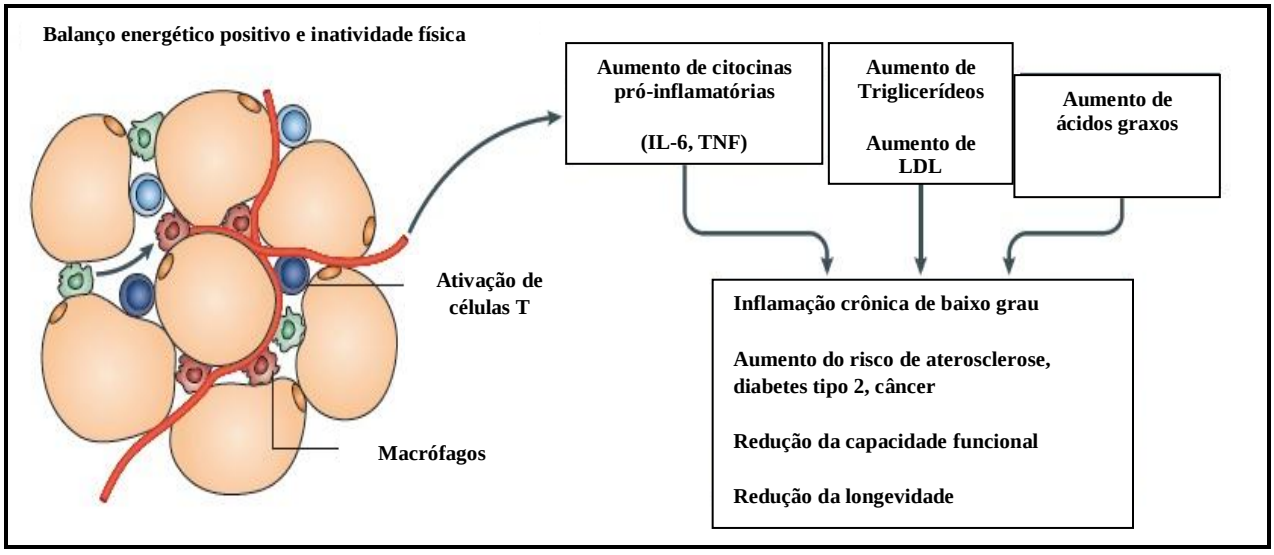

Fig. 5: Balanço energético positivo e inatividade física (ROOK; DALGLEISH; 2011).

Um estilo de vida sedentário leva ao acúmulo de gordura visceral, e isto é acompanhado por infiltração de tecido adiposo por células pró-inflamatórias do sistema imunológico, liberação aumentada de adipocinas e desenvolvimento de um baixo grau do estado inflamatório sistêmico (OUCHI et al., 2011).

O ATP é a fonte primária de energia para as células, e uma suplementação pode aumentar a capacidade de manter a rotatividade elevada de ATP durante um exercício de alta intensidade (BURNSTOCK, G., 2006). O exercício físico aumenta o gasto de ATP, queimando a gordura corporal que, de outra forma iria acumular-se nos indivíduos que consomem mais energia do que necessita. Neste sentido, o exercício reduz o risco de desenvolver obesidade e adiposidade excessiva. Promove também a saúde cardiovascular, uma vez que melhora o perfil lipídico sanguíneo (KRAUS et al., 2002). Estas alterações benéficas nos níveis dos lípidos plasmáticos limita o desenvolvimento da aterosclerose. O efeito protetor de um estilo de vida fisicamente ativa está associada contra doenças inflamatórias crônicas , isto pode ser atribuída a um efeito anti-inflamatório do exercício (KASAPIS, THOMPSON, 2005; MATHUR, PEDERSEN, 2008).

O exercício físico regular reduz o risco de doença metabólica crônica e doenças cardiorrespiratórias, em parte porque o exercício exerce efeitos anti-inflamatórios. Estes efeitos podem ser mediados por redução na massa de gordura visceral (com uma diminuição da liberação subsequente de adipocinas) e indução de um ambiente anti-inflamatório com cada sessão de exercícios (GLEESON et al., 2011).

Vários mecanismos podem contribuir para a geração deste ambiente anti-inflamatório do exercício físico. A ativação do eixo hipotálamo-hipófise-adrenal e sistema nervoso simpático leva à liberação de cortisol e adrenalina, do córtex-adrenal e medula, respectivamente. Estes hormônios inibem a secreção do TNF pelos monócitos. A IL-6 produzida pela contração do músculo esquelético também diminui a produção de TNF e pode estimular mais a liberação de cortisol. Elevações agudas de IL-6 estimulam a produção do antagonista do receptor de IL-1 (IL-1RA), aumentando assim as concentrações desta citocina anti-inflamatória na circulação. O exercício físico regular mobiliza as células T reguladoras (Treg) (que são uma fonte importante de citocinas anti-inflamatórias IL-10). O exercício físico aumenta as concentrações plasmáticas de células imunológicas (quimiocinas inflamatórias); sendo que elevações repetidas de quimiocinas pode conduzir a uma baixa regulação dos seus receptores celulares, resultando em uma reduzida infiltração de tecido. Uma redução na massa de tecido adiposo e tamanho dos adipócitos, juntamente com redução da infiltração de macrófagos, pode contribuir para uma redução na liberação de citocinas pró-inflamatórias (tais como IL-6 e TNF) e um aumento na liberação de citocinas anti-inflamatórias (tais como adiponectina e IL-10) a partir de tecido adiposo (Fig. 6) (GLEESON et al.; 2011).

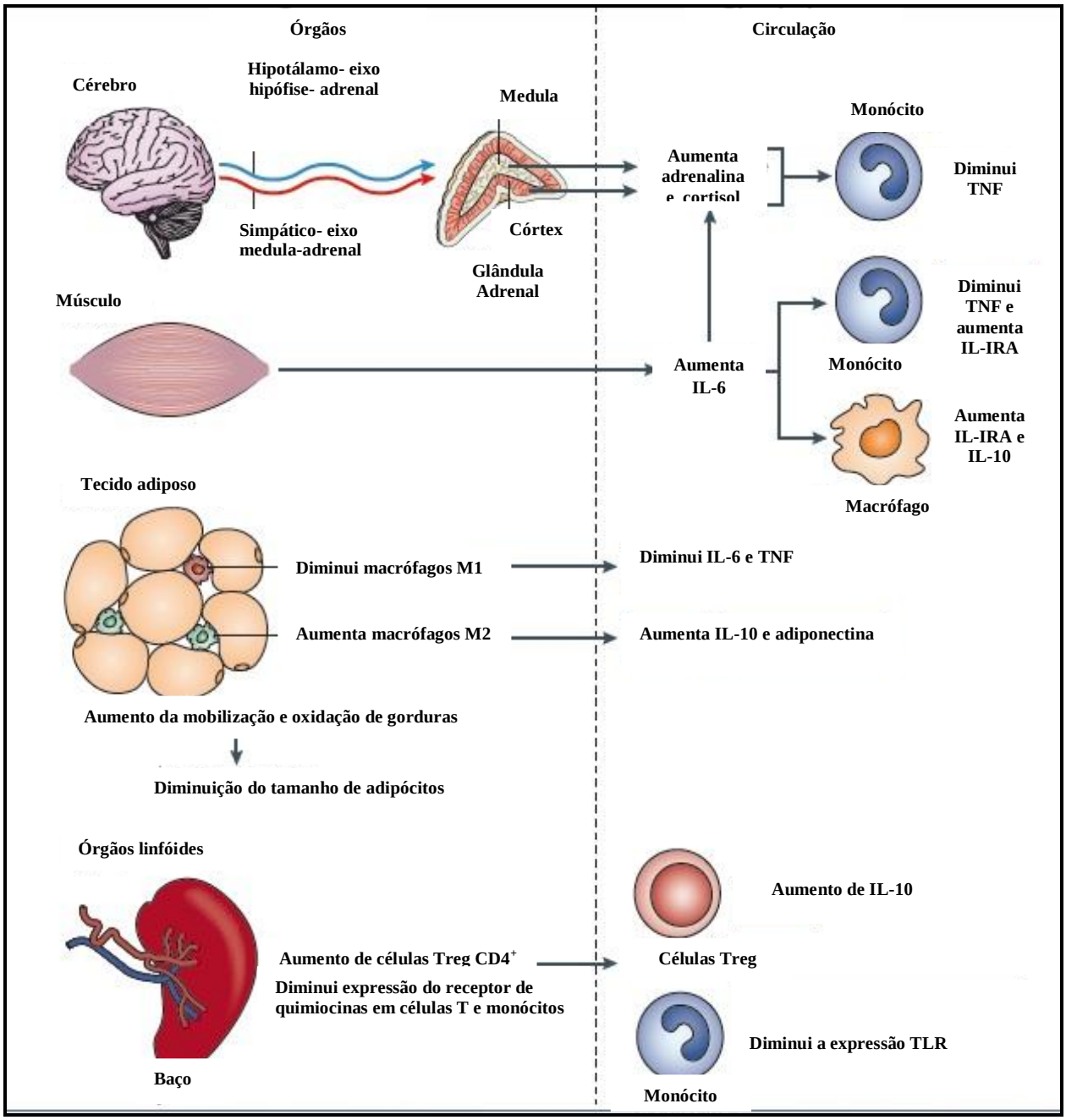

Fig. 6: Mecanismos potenciais contribuintes para os efeitos anti-inflamatórios do exercício físico (Adaptado por GLEESON et al., 2011).

Gleeson et al. (2011) sugerem que os diferentes mecanismos anti-inflamatórios provávelmente dependem do modo de frequência, intensidade e duração do exercício realizado. Pode-se esperar que a IL-6 assuma uma maior importância quando o exercício é prolongado e o glicogênio reduzido, enquanto as catecolaminas tendem a assumir efeitos com maior importância nos exercícios com duração mais curta, o exercício de alta intensidade. O treinamento com altas cargas podem ser necessária para aumentar o número de células Treg

circulantes e maximizar os efeitos anti-inflamatórios, mas possivelmente com um custo de um pequeno aumento no risco de infecção.

Dentre os exercícios físicos, o nado tem sido utilizado como modelo de exercícios físicos em ratos para avaliação de adaptações cardiovasculares ao exercício. Esse tipo de exercício parece gerar adaptações na performance dos animais (GEENEN; BUTTRICK; SCHEURER, 1988). O nado é considerado um exercício aeróbico tão eficaz como andar de bicicleta ou caminhar, e o exercício de natação pode ser uma terapia adjuvante para a prevenção da progressão da aterosclerose (PELLEGRIN et al., 2009).

Além do exercício físico, produtos naturais com ação terapêutica tem sido utilizado no tratamento para o controle de doenças cardiovasculares, como é o caso da curcumina, um polifenol presente no rizoma da planta *Curcuma longa*, que possui entre outras funções, efeito hipocolesterolêmico (RAO et al., 1970; KIM, 2010; FENG, et al., 2010) e antiinflamatório (ARAÚJO & LEON, 2001; SEEHOFER et al., 2009). A partir do processo de expansão da indústria de alimentos a espécie passou a ter grande apelo no mercado internacional, sendo principalmente utilizado como corante natural (ANTUNES & ARAÚJO, 2000; CECÍLIO-FILHO et al., 2000).

A cúrcuma (*Curcuma longa* L.) é uma planta originária do sudeste da Ásia, mais precisamente das florestas tropicais da Índia, país detentor da maior produção mundial e local onde ocorre a máxima diversidade genética (SASIKUMAR, 2005). Trata-se de uma planta do tipo herbácea e perene, embora se comporte como anual em algumas condições edafoclimáticas (CECÍLIO-FILHO et al., 2000). É uma planta monocotilêdonea pertencente à família Zingiberaceae, é conhecida popularmente no Brasil como açafroeira, açafrão-da-terra, açafrão-da-Índia, batatinha amarela, gengibre dourada e mangarataia (MAIA et al., 1995).

O comércio da cúrcuma pode ser realizado por meio do pó, obtido após secagem e moagem dos rizomas, bem como na forma de óleo resinas e extrato de curcumina purificado, podendo esta última apresentar concentrações de até 98% de corante (MARTINS & RUSIG, 1992). A cúrcuma é conhecida no mercado internacional por *turmeric*, sendo considerada uma preciosa especiaria, por ter sua importância econômica devida às peculiaridades características de seus rizomas. É utilizada desde a antiguidade como condimento no preparo e conservação de alimentos. Em diversos países asiáticos, trata-se de um componente indispensável no preparo de diversos pratos e temperos, como no caso do *curry* (CECÍLIO-FILHO et al., 2000).

Um dos princípios ativos da cúrcuma é o composto fenólico curcumina (Figura 7) (SRINIVASAN *et al*., 2006), que confere a coloração amarelo-alaranjada característica dos

rizomas. A qualidade do poder corante dos rizomas é usualmente avaliada pelo teor de curcumina, o qual varia entre 2,8 e 8% dependendo da variedade cultivada (GOVINDARAJAM, 1980).

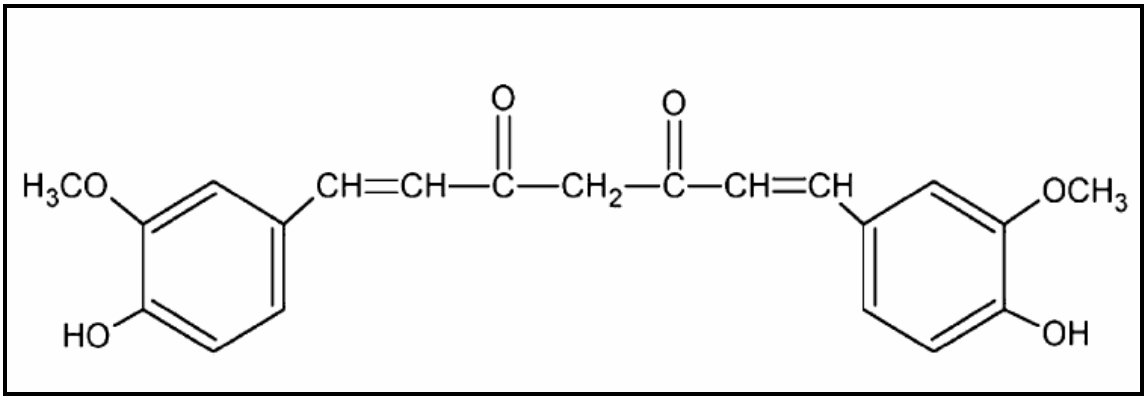

Figura 7: Estrutura da curcumina (SRINIVASAN *et al*., 2006).

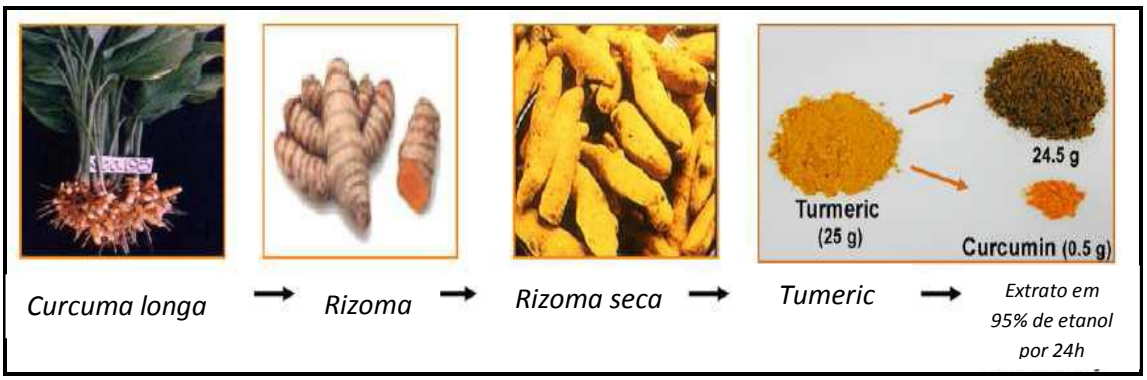

Figura 8 – Isolamento, extração e estrutura da curcumina. Adaptado de Aggarwal et al. (2010).

A curcumina (Figura 8) é extensivamente usada como uma especiaria e também como erva medicinal para doenças inflamatórias (CAO et al., 2006).

Os benefícios da curcumina incluem atividades farmacológicas, tais como: anti-hipercolesterolemica, antioxidante, antimicrobianos, antiinflamatória, anticancerígena, antimutagênico, anti-HIV (FILHO et al., 2000; SHARMA; GESCHER; STEWARD, 2005), proteção contra úlceras gástricas, disfunções hepáticas, desordens endócrinas, doenças pulmonares, desordens neurodegenerativas, doenças de pele (AGGARWAL; HARIKUMAR; 2009),e também evita o acúmulo de triglicerídeo no fígado e tecido adiposo (ASSAI; MIYAZAWA., 2001). Possui também forte capacidade de impedir a peroxidação lipídica, estabilizar membranas celulares e inibir a agregação plaquetária, todos fatores importantes na aterosclerose (AKHILENDER; THIPPESWAMY, 2002).

A curcumina tem apresentado múltiplas propriedades. Sabe-se que a curcumina se liga a uma variedade de proteínas e inibe a atividade de diversas quinases. Modulando a ação de diversos fatores de transcrição a curcumina regula a expressão de diversos fatores inflamatórios, citocinas e moléculas de adesão (AGGARWAL et al., 2010).

Uma das propriedades mais bem estudadas da curcumina é a sua característica antiinflamatória, tendo sido investigada em uma ampla variedade de doenças como infecção pelo *Helicobacter pylori* (KOOSIRIRAT et al., 2010), retinopatia diabética (KOWLURU & KANWAR, 2007), fibrose e inflamação hepática induzida por compostos químicos (WU et al., 2010), obesidade induzida pela inflamação (AGGARWAL, 2010), dentre outras. A propriedade antiinflamatória da curcumina vem sendo relacionada à inibição de citocinas, recrutamento tecidual de neutrófilos e a ativação do fator de necrose-κB (KIM et al., 2009; ILBEY et al., 2009).

De acordo com Feng e colaboradores (2010), o efeito hipocolesterolêmico da curcumina se deve à inibição da expressão de NPC1L1, o transportador chave do colesterol na membrana celular. Kang e Chen (2009) concluíram que a curcumina suprime a expressão do receptor da lipoproteína de baixa densidade, levando à inibição de LDL induzida por ativação das células hepáticas, assim possuindo efeito hipocolesterolêmico.

Estudos têm demonstrado as propriedades antioxidantes da curcumina, agindo como *scavenger* de espécies reativas de oxigênio, prevenindo a peroxidação lipídica e a redução do DNA modificado oxidativamente (VENKATESAN et al., 2000; KOWLURU & KANWAR, 2007; SEEHOFER et al., 2009).

Jaques e col. (2011) demostraram que há uma interação positiva entre o composto curcumina e a sinalização purinérgica. O sistema purinérgico 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 & KNIGHT, 2004).

O sistema purinérgico é composto por três importantes componentes: nucleotídeos e nucleosídeos extracelulares, receptores purinérgicos e as ecto-enzimas (ATKINSON et al., 2006). Diferentes tipos celulares, como plaquetas, linfócitos, células endoteliais entre outros, expressam distintos conjuntos de componentes de sinalização purinérgica, permitindo a formação de complexos personalizados de sinalização purinérgica (Figura 9) (JUNGER, 2011).

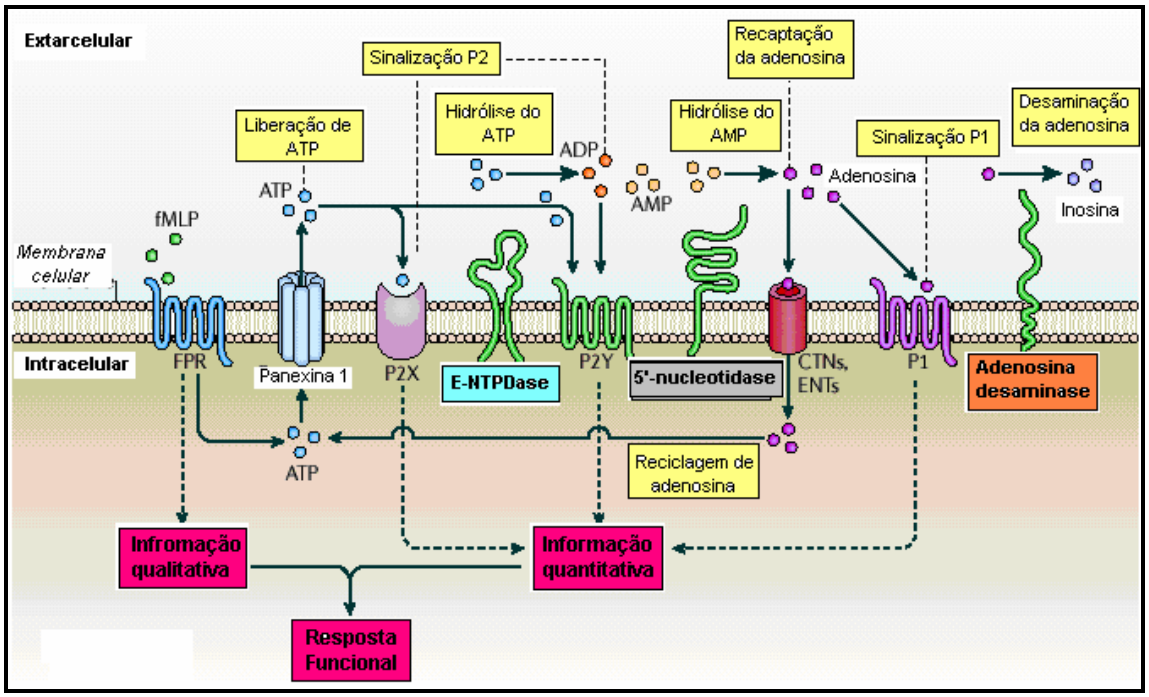

Figura 9 – Representação dos componentes do sistema purinérgico (Adaptado de JUNGER, 2011).

Os nucleosídeos são moléculas resultantes da união de uma base púrica ou pirimídica a uma pentose, um exemplo desta molécula é a adenosina. Uma vez que os nucleosídeos são fosforilados por quinases específicas ocorre a formação de um nucleotídeo. Os nucleotídeos desempenham funções importantes tanto no transporte quanto na transformação de energia celular. Os principais nucleotídeos que exercem funções biológicas são: a adenosina trifosfato (ATP), a adenosina difosfato (ADP) e a adenosina monofosfato (AMP) (ATKINSON et al., 2006).

Em condições fisiológicas, os nucleotídeos são encontrados no meio extracelular em baixas concentrações (DI VIRGILIO, 2001), isto devido a vários fatores como a quantidade liberada, os mecanismos de recaptação, situações de lise celular e a presença de enzimas como as ectonucleotidases (RATHBONE et al., 1999).

O ATP é um nucleotídeo presente em praticamente todas as células vivas e tem um papel fundamental no metabolismo energético (AGTERESCH et al., 1999). o ATP está envolvido em funções pró-inflamatórias, como a estimulação e proliferação de linfócitos e liberação de citocinas (TRAUTMANN, 2009). 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 e ainda estimular a proliferação de linfócitos (BOURS et al., 2006).

Juntamente com o ADP, o ATP também é liberado dos grânulos plaquetários no momento em que estas células sofrem o processo de ativação. O ATP desempenha um duplo efeito sobre a agregação das plaquetas: em baixas concentrações ele induz a agregação plaquetária, enquanto que em altas concentrações ele provoca a inibição deste fenômeno (SOSLAU & YOUNGPRAPAKORN, 1997).

O nucleotídeo ADP é o primeiro produto gerado na hidrólise do ATP, sendo conhecido por induzir a agregação plaquetária, alterar a forma das plaquetas, aumentar o cálcio citosólico e inibir a adenilato ciclase ativada (PARK & HOURANI, 1999). O ADP é importante por regular a ativação e o 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). Já o ADP em linfócitos não possui um papel definido (DI VIRGILIO et al, 2001).

O AMP é um metabólito intermediário da hidrólise do ATP (BARSOTTI & IPATA, 2004) que exerce a função de sinalizador em situações de desequilíbrio no metabolismo, servindo também como substrato para a formação da adenosina (CUNHA, 2001; LATINI & PEDATA, 2001). A ação do AMP em linfócitos é pouco definida, mas a adenosina acumulada em excesso no meio extracelular pode contribuir para a imunodeficiência, pois exerce um efeito tóxico no desenvolvimento dessas células (LUTHJE, 1989; RESTTA et al., 1997).

O nucleosídeo adenosina pode ser liberado no meio extracelular como resultado da degradação do ATP e do 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). É reconhecido por possuir propriedades anti-inflamatórias (CRONSTEIN, 1994), vasodilatadoras, neuroprotetoras (JACOBSON et al., 2006) e imunossupressoras (SPYCHALA et al., 1997), além de atuar como um potente inibidor da agregação plaquetária (BOROWIEC et al., 2006).

A sinalização induzida por nucleotídeos e nucleosídeos correlaciona-se diretamente à atividade de enzimas localizadas na superfície da membrana celular, as quais pertencem à família das ectonucleotidases, na qual regula as concentrações dos nucleotídeos extracelulares nos tecidos (ZIMMERMAN et al, 2012). Mas, antes dos nucleotídeos serem metabolizados pelas ectonucleotidases, eles devem interagir com receptores específicos da membrana plasmática, os receptores purinérgicos (ZANINI, 2006).

Os nucleotídeos e nucleosídeo de adenina podem exercer seus efeitos através da ativação de receptores purinérgicos, que foram divididos conforme suas propriedades farmacológicas e estruturais em três principais famílias (RALEVIC & BURNSTOCK, 1998): receptores P2X, receptores P2Y e receptores P1. Os purinoreceptores do tipo 1 são mais eficientemente ativados por adenosina, enquanto os receptores P2, principalmente por ATP (Figura 10) (Ralevic & Burnstock , 1998).

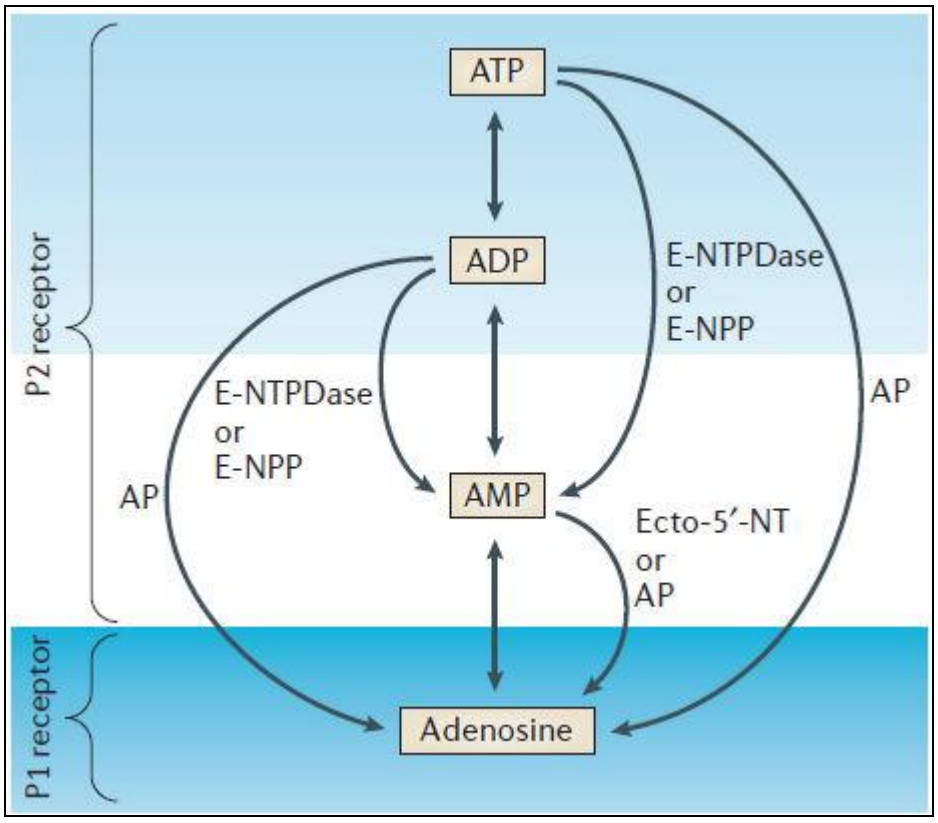

Figura 10- Receptores purinérgicos ligam o ATP extracelular e os produtos resultantes da hidrólise enzimática do ATP por ectonucleotidases (Adaptado de Fields & Burnstock, 2006).

Os receptores P2X, que são específicos para o ATP, são caracterizados por serem acoplados a canais iônicos e apresentarem seus domínios carboxi e amino terminal voltados para o meio intracelular, sendo que já foram descritos sete subtipos deste receptor (P2X1-7) (DI VIRGILIIO, 2001). Os receptores P2Y são receptores acoplados a proteína G, sendo que 14 subtipos deste receptor foram identificados (P2Y1-14). Os receptores P2Y1, P2Y11, P2Y12 e P2Y13 respondem principalmente aos nucleotídeos de adenina, ATP e ADP (YEGUTKIN, 2008).

A adenosina, por sua vez, medeia seus efeitos através de receptores de adenosina acoplados à proteína G. Existem quatro tipos de receptores: A_{2A}, A_{2B}, A1 e A3, os quais são

proteínas transmembrana acopladas a proteína G, os dois primeiros ativam a adenilato ciclase, enquanto os últimos a inibem (Figura 11).

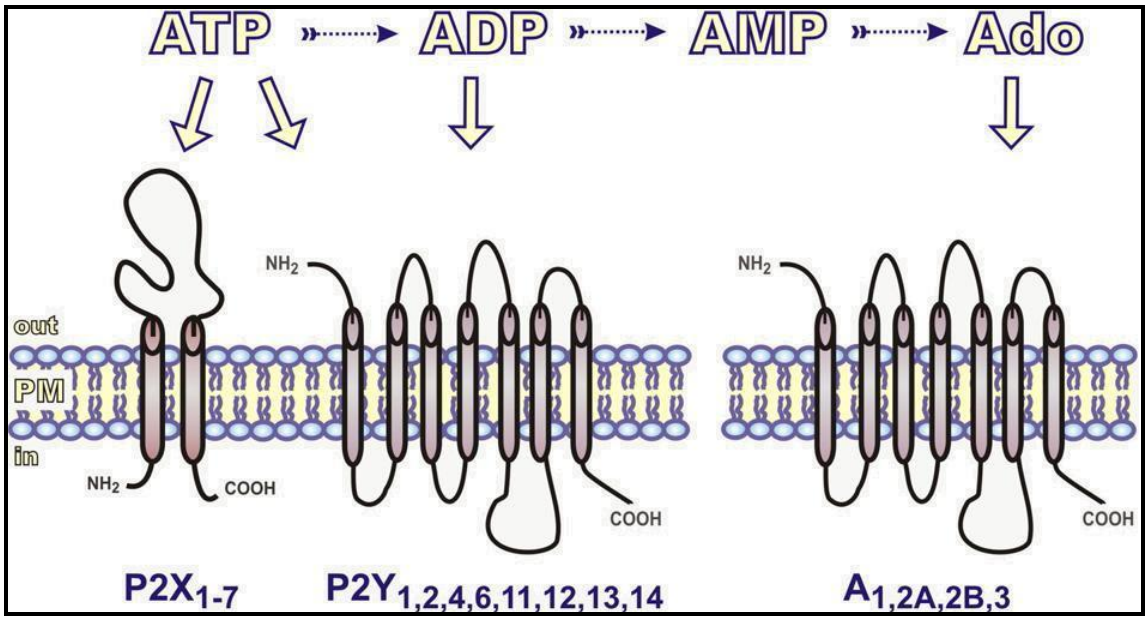

Figura 11: Tipos de receptores para nucleotídeos e nucleosídeos de adenina (Yegutkin, 2008).

As concentrações de ATP, ADP e adenosina no compartimento extracelular são controladas por enzimas que catalisam a sua conversão (Zimmermann et al., 2012). Estas enzimas, chamadas ecto-enzimas, localizam-se ancoradas na membrana ou solúveis no meio intersticial e agem sequencialmente formando uma cadeia enzimática. Várias famílias de ectonucleotidases podem degradar os nucleotídeos extracelulares, dentre os quais podemos citar as E-NTPDases (Ecto-Nucleosídeo Trifosfato Difosfohidrolase), a família das E-NPPs (Ecto- Nucleotídeo Pirofosfatase/ Fosfodiesterase), 5'-nucleotidase e a adenosina deaminase (ADA) (ROBSON et al., 2006 e YEGUTKIN, 2008) (Figura 12).

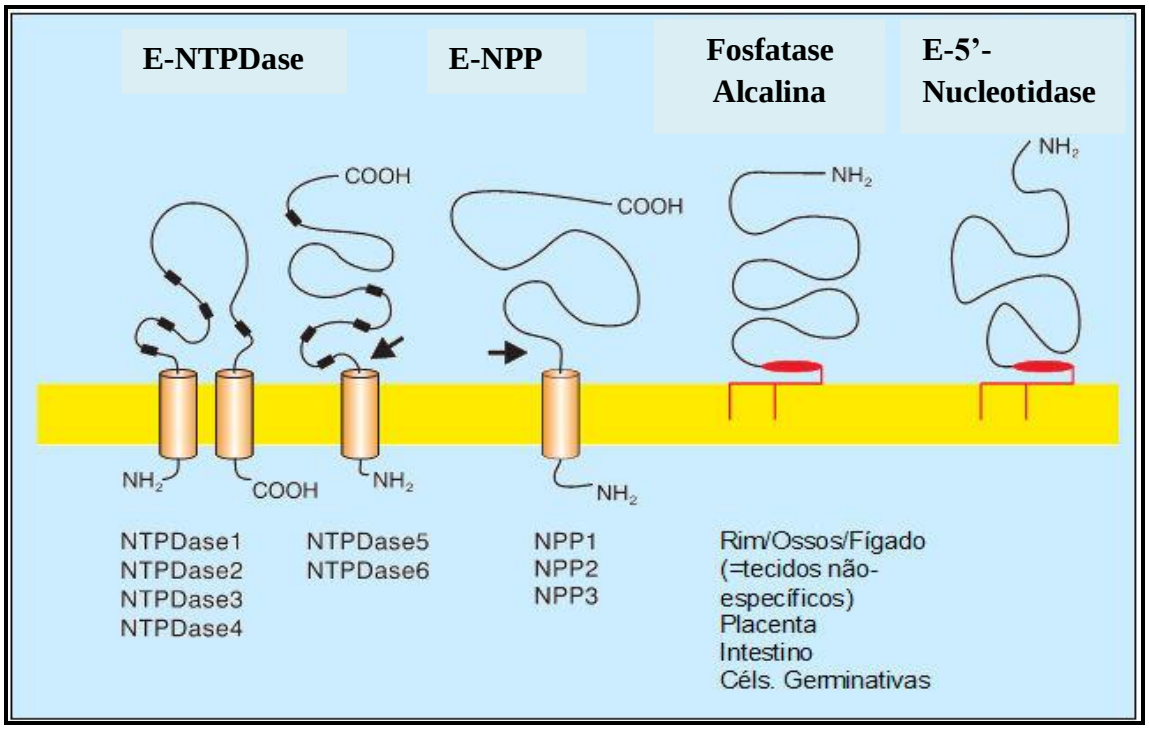

Figura 12. Atividade catalítica e topografia de membrana para a família das ectonucleotidases (Adaptado de Zimmermann, 2001).

Estas enzimas atuam em conjunto, formando uma cadeia enzimática que tem início com a ação da E-NTPDase e da E-NPP, as quais catalisam a hidrólise do ATP e do ADP formando AMP. A seguir a enzima 5'-nucleotidase hidrolisa a molécula do AMP formando adenosina, que por fim é desaminada pela ADA gerando inosina (GODING, 2000; ZIMMERMANN, 2001).

E-NTPDases (Ecto-nucleosídeo trifosfato difosfoidrolase, CD39, apirase, ATP-difosfoidrolase, E.C 3.6.1.5) é o termo usado para designar uma família de enzimas responsáveis pela hidrólise de nucleotídeos tri e difosfatados (PAPANIKOLAOU et al., 2005). Estas enzimas requerem concentrações milimolares de Ca^{2+} ou Mg^{2+} para exercerem suas atividades (ZIMMERMANN, 2001).

Essa classe de enzimas inclui oito membros (NTPDases 1-8) os quais são diferenciáveis através da preferência por um ou outro substrato, pela presença de cátions divalentes e também pelo tipo de produto formado. As NTPDases 1,2,3 e 8 são enzimas localizadas na superfície celular, com sítio catalítico localizado extracelularmente, enquanto nas NTPDases 4, 5, 6 e 7 este parece estar localizado intracelularmente (Figura 13) (ROBSON et al., 2006).

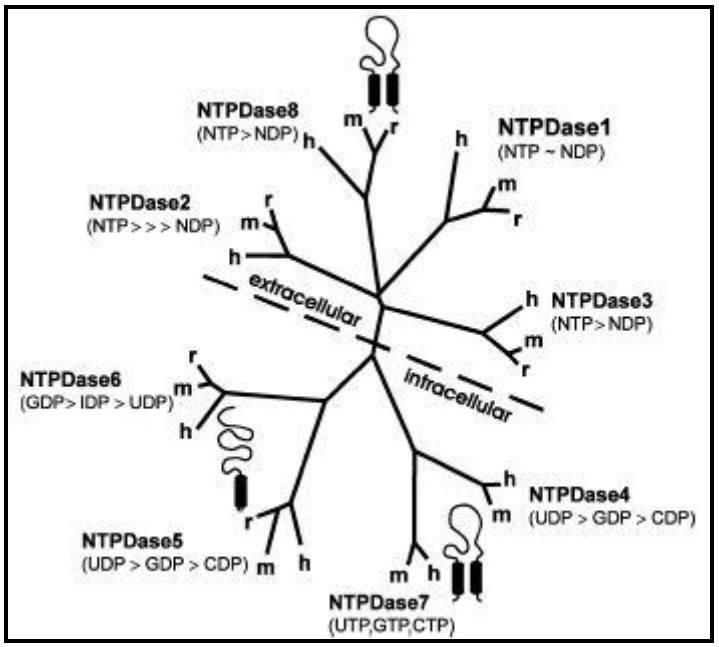

Figura 13: Membros da Família das E-NTPDases (Robson et al., 2006)

Estruturalmente, os membros desta família possuem cinco regiões denominadas regiões conservadas da apirase (ACRs), que apresentam grande similaridade na sequência de aminoácidos e estão envolvidas no reconhecimento do substrato, na ligação e na hidrólise (ZIMMERMANN, 2001). Existem evidências sugerindo que as diferenças na preferência pelo substrato, nessa família de enzimas, pode ser atribuída a pequenas diferenças na estrutura protéica que afetaria a ligação do substrato (ZIMMERMANN, 2001; ROBSON et al., 2006).

A NTPDase-1 foi primariamente identificada como um antígeno de ativação (CD39), que está ancorada à membrana via dois domínios transmembrana e que hidrolisa os nucleotídeos ATP e ADP em proporções semelhantes. É a principal ectonucleotidase presente na rede vascular, podendo também estar presente a NTPDase-2 (ENJYOJI et al., 1999; ROBSON et al., 2006). Encontrada também linfócitos, plaquetas e as células endoteliais, tendo um papel importante na regulação do fluxo sangüíneo e da trombogênese (PILLA et al., 1996; ZIMMERMANN, 2001; LEAL et al., 2005). Desempenha papel importante no sistema hemostático, uma vez que ela controla os efeitos pró-trombóticos e pró-inflamatórios de nucleotídeos como o ATP e o ADP (YEGUTKIN, 2008). A NTPDase-1, presente nas células endoteliais e membrana de plaquetas, converte o ATP em ADP e subseqüentemente o ADP, um nucleotídeo promotor da agregação, em AMP.

Dentre as principais funções imunoreguladoras mediadas pela atividade da NTPDase-1 estão a expressão de citocinas, a adesão célula-célula, a proliferação celular, a apoptose via

modulação dos níveis de ATP, e a produção de adenosina, que possui efeitos imunossupressores (MIZUMOTO et al., 2002; ROBSON et al., 2006).

A enzima 5'-nucleotidase (E.C 3.1.3.5, CD73) é uma glicoproteína ancorada à membrana, sendo responsável pela hidrólise de nucleotídeos monofosfato formando adenosina (Figura 14) (ZIMMERMANN, 2001; COLGAN et al., 2006).

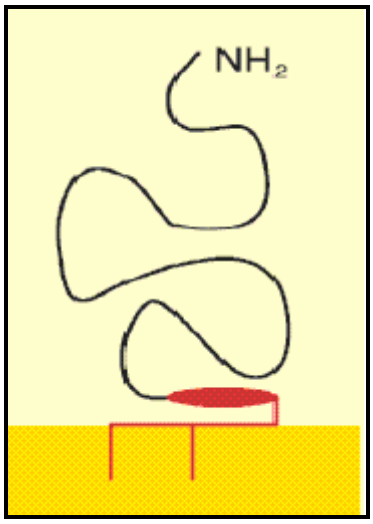

Figura 14 - Estrutura da ecto-5'-nucleotidase ancorada à membrana (ZIMMERMANN, 2001).

Amplamente distribuída em bactérias, plantas e animais, a enzima catalisa a hidrólise da ligação fosfodiéster de vários nucleosídeos 5'- monofosfatados a seus respectivos nucleosídeos (ZIMMERMANN, 1996). É expressa em vários tecidos, como por exemplo, no tecido nervoso, o renal e o hepático; e em diferentes tipos celulares entre os quais as plaquetas e linfócitos (ZIMMERMANN et al., 1992).

Sete membros já foram caracterizados apresentando diferentes localizações: cinco estão localizadas no citosol, uma na matriz mitocondrial e uma ancorada na membrana plasmática. Todas as formas citoplasmáticas são dependentes de Mg^{2+} (BOROWIEC et al., 2006). Elas diferem entre si por suas propriedades moleculares e cinéticas bem como pela especificidade com o substrato. Sabe-se que a adenosina, o produto da ação catalítica da enzima 5'-nucleotidase, exerce diversos efeitos em vários tecidos os quais são consequência de sua ligação aos diferentes tipos de receptores encontrados nestes locais (BOROWIEC et al., 2006).

Embora a 5'-nucleotidase hidrolize uma variedade de nucleosídeos 5'-monofosfatados, foi demonstrado que possui maior afinidade por AMP, com valores de K*m* na faixa de micromolaridade, sendo por isto considerada a principal enzima responsável pela formação de adenosina (ZIMMERMANN, 1996; ZIMMERMANN, 2001).

Assim, de acordo com a sua localização tecidual, ela desempenha importantes funções como, por exemplo, no controle da agregação plaquetária, na regulação do tônus vascular e também na neuromodulação e neuroproteção do sistema nervoso central (ZIMMERMANN et al., 1998; KAWASHIMA et al., 2000; DUNWIDDIE & MASINO, 2001).

A família das NPPs (nucleotídeo pirofosfatase/ fosfodiesterase, E.C. 3.1.4.1) inclui sete membros (NPP1-7), localizadas na superfície celular, expressas como ectoenzimas transmembranas ou como enzimas secretadas (Figura 15). As E-NPPs desempenham vários papéis fisiológicos dentre os quais destacam-se: reciclagem de nucleotídeos, regulação dos níveis extracelulares de pirofosfato e o estímulo da motilidade celular. Também desempenham funções como a formação dos ossos, a motilidade celular, as metástases tumorais e a resistência à insulina em diabetes do tipo II (GODING et al., 2003; STEFAN et al., 2006).

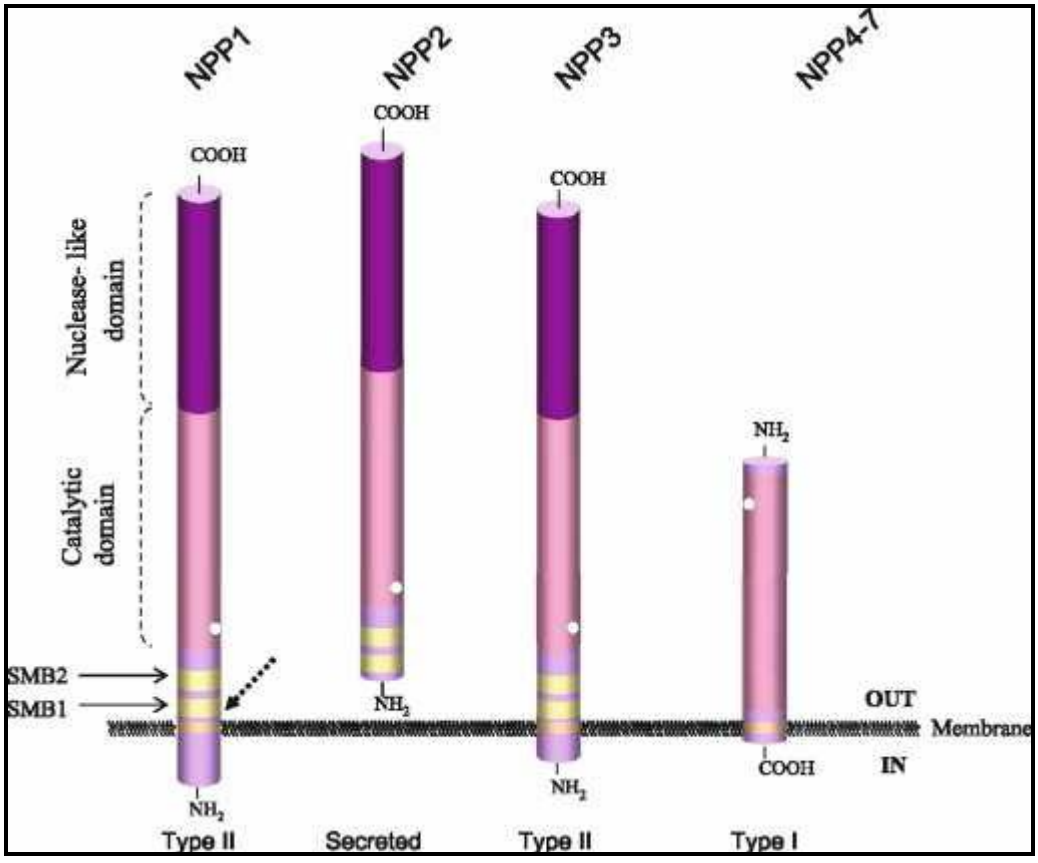

Figura 15 – Representação estrutural da família E-NPP (STEFAN et al., 2006).

As E-NPPs hidrolisam ligações pirofosfato ou fosfodiéster em uma ampla variedade de substratos, como nucleotídeos, ácidos nucléicos, fosfoésteres de colina e fosfolipídeos, resultando na liberação de 5'-monofosfatos (BOLLEN et al., 2000; GODING et al., 2003;

STEFAN et al., 2005), porém as diferentes isoformas apresentam especificidade por determinados substratos (STEFAN et al., 2006).

As NPP1-3 estão envolvidas na hidrólise de nucleotídeos e as NPPs 6-7 hidrolisam ligações fosfodiéster em fosfolipídeos e fosfoésteres de colina. Notavelmente a NPP-2 hidrolisa tanto fosfolipídeos quanto nucleotídeos (STEFAN et al., 2006). Relata-se também a existência das NPPs 4 e 5, porém pouco se sabe sobre sua atividade catalítica (GODING et al., 2003). Embora essa família de enzimas esteja envolvida na hidrólise de nucleotídeos de adenina, elas também podem hidrolisar GTP, TTP, CTP e UTP com eficiência similar (VOLLMAYER et al., 2003).

Exceto a NPP2, todas as demais NPPs têm um único domínio transmembrana. As NPPs 1 e 3 têm uma orientação transmembrana do tipo II, com sua porção amino-terminal voltada para o meio intracelular, enquanto que as NPPs 4-7 têm um orientação do tipo I, com sua porção amino-terminal voltada para o meio extracelular. A NPP2 só existe como uma proteína solúvel (STEFAN et al., 2005; STEFAN et al., 2006).

Todas as NPPs têm voltado para o meio extracelular, um domínio catalítico, com 60% de identidade de aminoácidos entre as diferentes isoformas da enzima (STEFAN et al., 2005; STEFAN et al., 2006). Esse domínio catalítico é fixado à membrana por uma "haste" que consiste de porções ricas em cisteína (GODING, 2000). Devido ao fato de seu sítio catalítico ser voltado para o meio extracelular essa família de enzimas é denominada ecto-NPPs (E-NPPs) e funcionam, *in vitro*, em pH alcalino entre 8.5-8.9 (GODING, 2000).

As NPPs são metaloenzimas e sua atividade pode ser bloqueada por quelantes de metais, sendo que a atividade pode ser restaurada pela adição de cátions divalentes como Mg^{2+}, Ca^{2+}, Mn^{2+} e Zn^{2+} (BOLLEN et al., 2000).

Em vista de esta enzima ter sido caracterizada na membrana plaquetária e, devido ao fato de apresentar propriedades catalíticas semelhantes à E-NTPDase, pressupõem-se que haja uma co-expressão da E-NTPDase, da E-NPP e da 5'-nucleotidase nesta célula. Com isso, pode-se dizer que elas constituem uma cadeia enzimática responsável pela hidrólise extracelular de nucleotídeos (FÜRSTENAU et al., 2006).

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 na circulação. A ADA é responsável pela desaminação da adenosina com a consequente produção de inosina regulando, com isso, as concentrações pericelulares e locais deste nucleosídeo (RESTA et al., 1998; ROBSON et al., 2006).

A ADA apresenta uma localização citosólica, mas pode também estar localizada na superfície da membrana celular, como uma ectoenzima (YEGUTKIN, 2008). Esta enzima é encontrada em praticamente todos os vertebrados. Em humanos, já foram descritas duas formas moleculares da ADA. São elas a ADA1 e a ADA2, que se diferenciam por características como peso molecular, propriedades cinéticas e distribuição tecidual (HIRSCHHORN & RATECH, 1980; UNEGERER et al., 1992).

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

A ADA2 é a isoenzima predominante no soro e representa a menor parte da atividade da ADA total em tecidos (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). A ADA2 representa a menor parte da atividade da ADA total em tecidos. A maioria das células humanas contém pequena quantidade de ADA2 e provavelmente sua maior fonte seja o sistema monócito-macrófago (GAKIS, 1996). 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 ecto-ADA por ser principalmente encontrada em linfócitos de sangue periférico de humanos, desempenha um papel chave no sistema imune, uma vez que está diretamente relacionada com a proliferação, a maturação e a função destas células (GORELL et al., 2001).

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

à AMP pela adenosina quinase ou desaminação à inosina pela ADA citosólica (HASKÓ & CRONSTEIN, 2004).

Diversos estudos garantem que mudanças na atividade da ADA refletem alterações na imunidade (ADAMS et al., 1976; FISCHER et al., 1976; UNGERER et al., 1992). Ela tem sido aceita como uma importante enzima na maturação e função dos linfócitos T, relacionando-se com proliferação e diferenciação linfocítica (GALANTI et al., 1981).

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 do sistema purinérgico, torna-se relevante e de interesse científico investigar a atividade dessas enzimas participantes da degradação de nucleotídeos e nucleosídeos da adenina em plaquetas de ratos induzidos à hipercolesterolemia tratados com curcumina e submetidos ao exercício físico.

2 OBJETIVOS

2.1 Objetivo geral

Avaliar a atividade das ecto-enzimas do sistema purinérgico em ratos com hipercolesterolemia induzida, tratados com curcumina e submetidos ao exercício físico.

2.2 Objetivos específicos

Em ratos normais e com hipercolesterolemia induzida, tratados com curcumina e submetidos ao exercício físico:

- Avaliar a atividade das enzimas E-NTPDase e E-5'-nucleotidase em plaquetas.
- Analisar a atividade da enzima E-NPP em plaquetas.
- Determinar a atividade da enzima adenosina desaminase (E-ADA) em plaquetas.
- Avaliar o perfil de agregação plaquetária.

3 MANUSCRITO

Os resultados que fazem parte desta dissertação estão apresentados sob a forma de 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.

EFFECT OF CURCUMIN AND PHYSICAL EXERCISE IN MODULATING THE PURINERGIC SYSTEM IN RATS WITH INDUCED HYPERCHOLESTEROLEMIA

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Abstract

Introduction: Hypercholesterolemia is a metabolic disorder, as risk factor for the atherosclerosis. Platelets accumulated within atherosclerotic lesions, can recruit additional platelets to form a thrombus. Studies show that physical exercise can cause changes in lipid profile, as well as curcumin, a natural phenolic compound, which has anti-inflammatory and hypocholesterolemic properties. Extracellular nucleotides have functions including thrombus regulation by exerting of effects on platelets. The ecto-enzymes (E-NTPDase, E-5'-nucleotidase, E-NPP, E-ADA) regulates the concentration of extracellular adenine nucleotides and nucleoside.

Objective: This study objective investigated the effect of curcumin and physical exercise in modulating the purinergic system in rats with induced hypercholesterolemia.

Materials and methods: The rats were divided in 8 groups: standard diet, standard diet more curcumin, standard diet more physical exercise, standard diet more physical exercise and curcumin, hypercholesterolemic diet, hypercholesterolemic diet more curcumin, hypercholesterolemic diet more physical exercise, and hypercholesterolemic diet more physical exercise and curcumin. The activities of ecto-enzymes were measured in platelets these rats.

Results: It was observed an increase in ATP and ADP hydrolysis of rats induced to hypercholesterolemia. When these rats were treated with curcumin and submitted to physical exercise, the physical exercise *per se* was able to further enhance the hydrolysis of ATP. In the hydrolysis of ADP, it was observed a decrease in E-NTPDase activity when hypercholesterolemic rats were treated with curcumin and physical exercise *per se*, well as when it was associated with curcumin and physical exercise. The activities of E-5'-nucleotidase, E-NPP and E-ADA and platelet aggregation, did not revealed statistical difference between the groups with standard diet and hypercholesterolemic groups.

Conclusion: Results demonstrate that this hypercholesterolemic diet changes the purinergic signaling in platelets and that treatment with curcumin and physical exercise was able to modulate hydrolysis of nucleosides and nucleotides adenine this experimental condition.

Key words: Hypercholesterolemia, platelets, curcumin, physical exercise, ecto-enzymes.

1. Introduction

Hypercholesterolemia is a metabolic disorder characterized by high levels of serum low density lipoprotein and blood cholesterol [1]. It is epidemiologically linked to cardiovascular disease, as a major risk factor and determinant for the development of atherosclerosis. Atherosclerosis is a chronic inflammatory disease characterized by the presence of lipid abnormalities, endothelial dysfunction, inflammation and also accumulation of platelets on the artery walls [2,3,4].

Platelets accumulated within atherosclerotic lesions, can recruit additional platelets to form a thrombus, indicating that the arterial wall can assume both an inflammatory and prothrombogenic phenotype when blood cholesterol levels are elevated [5]. Platelets are one of the most important blood components that participate and regulate thrombus formation by releasing active substances, such as ADP [6]. The nucleotide ATP is involved in inflammatory processes and plays an important role in platelet aggregation, depending on its concentration and binding to specific receptors [7]. High concentrations of ATP inhibit platelet aggregation induced by ADP. AMP, a breakdown product of ATP and ADP, is hydrolyzed to adenosine, which has a potent inhibitory activity on platelet aggregation [8].

ATP, ADP and AMP are member of an important class of extracellular molecules, that interact with the purine receptors in cell surface, that mediate biological effects, such as platelet aggregation [9]. Extracellular nucleotides have important functions including tissue development, blood flow, secretion, inflammation [10], and regulate the vascular response to endothelial damage by exerting a variety of effects on platelets [11]. The signal induced by these molecules is directly correlated to enzyme activity on the surface of the cell membrane. These enzymes belong to the family of ecto-enzymes, which regulates the concentration of extracellular nucleotides in the tissue [12].

E-NTPDase (CD39, ecto-apyrase, ATP diphosphohydrolase, EC 3.6.1.5) is a glycosylated, membrane-bound enzyme that hydrolyzes ATP and ADP to AMP, which is subsequently converted to adenosine by E-5’-nucleotidase (EC 3.1.3.5, CD73) [12,13]. E-NTPDase and E-5’-nucleotidase play an important role in the regulation of blood flow and thrombogenesis by regulating ADP catabolism [14]. The family of NPP (nucleotide pyrophosphatase/phosphodiesterase) hydrolyzes ATP and ADP, and other nucleotides to AMP [12,15]. Besides these enzymes, there is adenosine deaminase (ADA), which can also be located on the surface of the membrane, as ecto-enzymes [15], which catalyzes the irreversible deamination of adenosine in to inosine [16].

Considering that saturated fats present in the diet cause alterations in lipid composition of plasma membranes, affecting the activity of enzymes [17], changes in lifestyle, such as physical exercise, can cause changes in the lipid profile [18,19]. Physical Exercise increases the expense of ATP, which is a primary source of energy for the cell, and also burning body fat that would otherwise accumulate in individuals who consume more energy than it needs. In this sense, the exercise promoting cardiovascular health, as it limits the development of atherosclerosis. The protective effect of a physically active lifestyle is associated with chronic inflammatory diseases; this can be attributed to an anti-inflammatory effect of exercise [20]. Among the physical exercises, swimming has been used as a model of physical exercise in rats to evaluate cardiovascular adaptations to exercise [21].

In addition to physical exercise, another therapeutic option for hypercholesterolemia that has been studied is curcumin, a polyphenol present in the rhizome of *Curcuma longa* plant, which has antioxidant, anti-inflammatory [22,23] and hypocholesterolemic effects [24,25] and prevents the accumulation of triglycerides in the liver and adipose tissue [26]. It also has strong ability to prevent lipid peroxidation, stabilize cell membranes and inhibit platelet aggregation, all important in atherosclerosis [27].

The hypercholesterolemia it is a condition associated with inflammatory disorders and also adhesion and recruitment of platelets, these being important in maintaining homeostasis, preventing the endothelial dysfunction and consequent generation of injury. Based on this fact, this study aimed to evaluate the effect of curcumin and physical exercise in modulating the purinergic system in 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), 5'-monophosphate sodium salt (AMP), thymidine 5'-monophosphate p-nitrophenyl ester sodium salt (p-Nph-5'-TMP), adenosine, as well as bovine serum albumin, Trizma base, HEPES and Coomassie Brilliant Blue G were obtained from Sigma-Aldrich (St. Louis, MO, USA). K_2HPO_4 was purchased from Reagen and Tetrabutylammonium chloride from Merck (Darmstadt, Germany). All the other chemicals used in this experiment were of analytical grade and of the highest purity.

2.2 Animals

Male Wistar rats (approximately 200g), obtained from the Central Animal Laboratory of the Universidad Federal de 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. The animals were kept in a temperature range of 21-22^{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: 121/2010).

2.3 Diet

The rats used in this study were randomly divided into eight groups, namely, a standard diet (C), standard diet along with curcumin (CC), standard diet along with physical exercise (PE), standard diet along with physical exercise and curcumin (CCPE), diet hypercholesterolemic (H), hypercholesterolemic diet along with curcumin (HCC), hypercholesterolemic diet along with physical exercise (HPE), and hypercholesterolemic diet along with physical exercise and curcumin (HCCPE).

The standard diet was performed according to the publication Nutrient Requirements of Laboratory Animals [28], while the hypercholesterolemic diet was performed with some modification, as shown in Table 1. According with this study, the normal concentration of fat in the diet to rats range between 20% and 30% of the total content of macronutrients (carbohydrate, fat and protein). A diet with a fat percentage above it is already considered a high lipid content.

Table 1: Composition of diet.

Ingredients	**Standard 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 e minerals	5%	5%
TOTAL	100%	100%

The animals were fed the diets for a period of one month to induce hypercholesterolemia. After induction, continued receiving the diet for another month where the treatment was performed. Tests were conducted to check for total cholesterol and its fractions, by cardiac puncture, to verify the induction of hypercholesterolemia. After being

detected hypercholesterolemia animals continued on their respective diets and to start the treatment in hypercholesterolemic rats. The measurement of diet fed per day per rat was 10% of body weight according to Harkness and Wagner [29].

2.4 Treatment with curcumin

Curcumin was administered by gavage once a day, five days week, for a period of 1 month. It was diluted in corn oil (1mL/kg), in a dose of 25 mg/kg/day, and administered after the detection of hypercholesterolemia. The dose 25 mg/kg/day was used previously in our group and prevented changes in the purinergic system caused by exposure to cigarette smoke [30]. Curcumin was acquired from Sigma Chemical Co. (St. Louis, MO).

2.5 Practice of physical exercise

The swimming protocol was followed according to the proposed by Orenstein et al [31] with some training modifications. It started with 10 min/d initially, and increased by an additional 10 min/day until the mice were swimming continuously for 40 min /d. Swim frequency was 5 day/week for a total duration of one month. The rats swam in a 60cm-deep tub with water temperature maintained at 35^0C in groups of seven animals, and were toweled dry after each session. The study protocol was performed after the detection of hypercholesterolemia and lasted one month and during this period, the rats continued receiving hypercholesterolemic diet.

2.6 Preparation of biological samples

At the end of the experiment, the rats were anesthetized with isoflurane and whole blood was collected by cardiac puncture after thoracotomy and transferred to the vacuum tubes with sodium citrate 3.5% for the tests.

2.7 Platelet preparation and Cellular integrity

Platelets were prepared by the method of Pilla et al. [13] modified by Lunkes et al. [32]. Briefly, blood was collected into 0.129 M sodium citrate and centrifuged 160x g for 15 min. The platelet-rich plasma was centrifuged at 1400 x g for 30 min and washed twice with 3.5 mM HEPES isosmolar buffer containing 142 mM NaCl, 2.5 mM KCl and 5.5 mM glucose. The washed platelets were resuspended in HEPES isosmolar buffer and protein was adjusted to 0.4–0.6 mg/mL and used to determine enzymatic activities. The integrity of the platelet preparation was confirmed by determining the lactate dehydrogenase (LDH) activity

using the kinetic method of the Labquest apparatus (Diagnostics Gold Analyzer). The procedure was repeated before and after the incubation period. The protocol was carried out according to the manufacturer's instructions. Triton X-100 (1%, final concentration) was used to disrupt the platelet 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 E-NTPDase and E-5'-nucleotidase activity determination

The E-NTPDase enzymatic assay was carried out in a reaction medium containing 5 mM $CaCl_2$, 100 mM NaCl, 4 mM KCl, 5 mM glucose and 50 mM tris- HCl buffer, pH 7.4, at a final volume of 200 μL as describe by Lunkes et al [32]. For AMP hydrolysis, the 5'-nucleotidase activity was carried out as previously described, except that the 5 mM $CaCl_2$ was replaced by 10 mM $MgCl_2$. Twenty microliters of the enzyme preparation (8-12 μg of protein) was added to the reaction mixture and pre-incubation proceeded for 10 min at 37ºC. The reaction was initiated by the addition of ATP or ADP at a final concentration of 1.0 mM, and AMP at a final concentration of 2 mM, and the time of incubation was 60 min. Both enzyme assays were stopped by the addition of 200 μL of 10% trichloroacetic acid (TCA) to provide a final concentration of 5%. Subsequently, the tubes were chilled on ice for 10 min. Released inorganic phosphate (Pi) was assayed by method of Chan et al. [33], using malachite green as the colorimetric reagent and KH_2PO_4 as standard. Controls were carried out to correct for nonenzymatic hydrolyses of nucleotides by adding enzyme preparation after TCA addition. All samples were run in triplicate. Enzyme-specific activities are reported as nmol Pi released/min/mg of protein.

2.9 E-NPP activity determination in platelets

The E-NPP activity from platelets was assessed using p-nitrophenyl 5'- thymidine monophosphate (p-Nph-5'-TMP) as substrate as described by Fürstenau et al [34]. The reaction medium containing 50 mM Tris-HCl buffer, 120 mM NaCl, 5.0 mM KCl, 60 mM glucose and 5.0 mM $CaCl_2$, pH 8.9, was preincubated with approximately 20 μg per tube of platelet protein for 10 min at 37 ºC to a final volume of 200 μL. The enzyme reaction was started by the addition of p-Nph-5'-TMP at a final concentration of 0.5 mM. After 80 min of incubation, 200 μL NaOH 0.2N was added to the medium to stop the reaction. The amount of p-nitrophenol released from the substrate was measured at 400 nm using a molar extinction coefficient of $18.8x\ 10^{-3}$/M/cm. Controls to correct nonenzymatic substrate hydrolysis were performed by adding platelet preparations after the reaction had been

stopped. All samples were performed in triplicates. Enzyme activities were expressed as nanomole of p-nitrophenol released per minute per milligram of protein (nmol p-nitrophenol released/min/mg protein). On the platelet surface, E-NPP has catalytic activity in an optimum alkaline pH between pH 8.5 and 9.0, differently from E-NTPDase, which presents catalytic activity in an optimum pH between pH 7.5 and 8 [35]. Therefore, a Tris–HCl buffering system (pH 8.9) was used to perform the E-NPP activity assay, since the hydrolysis of the nucleodylated intermediate occurs at this pH and at lower pH values, the specific activity is decreased, as previously defined by Fürstenau et al. [34].

2.10 Adenosine deaminase activity determination (E-ADA)

E-ADA activity from platelets was determined according to Giusti and Galanti [36], which is based on the direct measurement of the formation of ammonia, produced when adenosine deaminase acts in excess of adenosine. Briefly, 50 μL of platelets reacted with 21 mmol/L of adenosine, pH 6.5, and was incubated at 37 ºC for 60 min. The protein content used for the platelet experiment was adjusted to between 0.7 and 0.9 mg/mL. Results were expressed in units per liter (U/L). One unit (1 U) of 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 Platelet aggregation

Platelet aggregation was measured by the method of Born and Cross [37], by turbidimetric measurement with a Chrono-log optical aggregometer, with AGGRO/LINK® Model 810-CA software for Windows version 5.1. The preparation of platelet rich plasma (PRP) was obtained by centrifugation of blood for 20 min at 1000 rpm and the preparation of platelet poor plasma (PPP) was obtained by centrifugation of the sample by 3700 rpm for 30 minutes. After calibration of the aggregometer, data concerning the assays and reagents were entered on a computer coupled to the equipment, and the patient's test was then performed. Aggregation was measured at 37°C and expressed as the maximal percent change in light transmittance from baseline at 5 min after the addition of the agonist ADP at concentration of 10 μM, with platelet poor plasma as a reference. The results were expressed as percentage of aggregation.

2.12 Biochemical analysis

Serum total cholesterol, HDL-cholesterol and triglyceride were determined through Diagnostic kit Labtest through SA, colorimetric mode. The serum AST, ALT were performed through of kit Labtest Diagnostica SA, mode kinetic UV-IFCC. The Alkaline phosphatase activity also was performed through the kit Labtest Diagnostic SA, mode colorimetric (modified Roy). LDL-cholesterol level was calculated by formula of Friendewald et al. [38].

2.13 Protein determination

Protein content was measured by the Coomassie Blue method according to Bradford [39], using bovine serum albumin as the standard. This assay is based on the binding of the dye Coomassie Blue G-250 to protein, and this binding is accompanied by measuring the absorbance maximum of the solution at 595 nm.

2.14 Statistical analysis

The statistical analysis was performed using Student's t test for independent sample to demonstrate the induction of hypercholesterolemia and of total cholesterol, LDL cholesterol, HDL cholesterol and triglycerides, before and after treatment. One-way ANOVA, Dunnett's Multiple Comparison Test, to evaluate the food consumption, body weight, AST, ALT and Alkaline phosphatase. Three-way ANOVA, followed by the Duncan multiple range test to evaluate the ecto-enzymes. $P < 0.05$ was considered to represent a significant difference among the analyses used. All data were expressed as mean ± SEM and were performed using the Statistical Analysis System (SAS) version 9.0 software.

3. Results

3.1 Cellular integrity

LDH activity measurement showed that approximately 5% of the platelets of both groups was disrupted, indicating that the preparation was predominantly intact after the isolation procedure.

3.1 Hypercholesterolemia induced

The results of total cholesterol, LDL cholesterol, HDL cholesterol and triglycerides tests to demonstrate the induction of hypercholesterolemia after one month of hypercholesterolemic diet, are shown in Table 2. A significant increase in the levels of total

cholesterol and LDL and decreased HDL were observed in hypercholesterolemic rats when compared to control rats (P<0.05; n=28). Triglycerides measurement showed no significant difference in hypercholesterolemic rats when compared to control rats (P>0.05; n=28).

3.2 Food consuption and body weight

The results obtained for the food consumption and body weight are presented in Tables 3 and 4 respectively. As can be observed, the food consumption was not significantly different when compared to the control group (P>0.05; n= 7). As body weight was significantly increased in groups: curcumin *per se* (CC), hypercholesterolemic group (H) and hypercholesterolemic group receiving treatment with curcumin (HCC), when compared with the control group (P<0.05; n= 7).

3.3 Biochemical analysis

Determinations of total cholesterol, HDL-cholesterol, LDL-cholesterol and triglyceride from control rats and rats hypercholesterolemic, before and after treatment are demonstrated in Table 5. The results shows a significant reduction of total cholesterol and LDL cholesterol and a significant increase in HDL cholesterol levels in HCC, HPE and HCCPE groups after receiving treatment with curcumin, physical exercise and curcumin and physical exercise together, when compared before treatment. There was also a decrease in LDL cholesterol in control rats along with curcumin (CC) after treatment, when compared before treatment (P<0.05; n=7).

The activities of AST, ALT and alkaline phosphatase in serum are demonstrated in Table 6, where no significant differences were observed in hypercholesterolemic rats when compared the control group (P>0.05; n=7).

3.4 E-NTPDase activity

The ATP and ADP hydrolysis by E-NTPDase in platelets are showed in Fig.1. Statistical analysis demonstrated an increase in ATP hydrolysis in the group receiving hypercholesterolemic diet (H) (22,9±4,2; n=7; P<0.05) as compared with the group receiving standard diet control (C) (10,6±2,7; n=7; P<0.05). In the group HPE, ATP hydrolysis was significantly increased (38,6±12,3; n=7; P<0.05)when compared to the H group (22,9±4,2; n=7; P<0.05), thus showing the physical exercise that was able to further enhance the hydrolysis in rats with hypercholesterolemia, compared to control. In the other

groups [HCC (25,02±2,1; n=7; P>0.05), HCCPE (31,2±8,4; n=7; P>0.05)] there were no significant differences when compared to the H group (22,9±4,2; n=7; P>0.05). The treatment with CC (15,9±4,5; n=7; P>0.05) PE (11,6±2,5; n=7; P>0.05) or CCPE *per se* (15,7±2,4; n=7; P>0.05) in the standard diet group did not improved the ATP hydrolysis when compared to the C group (10,6±2,7; n=7; P>0.05) (Fig. 1A). Statistical analysis for ADP hydrolysis showed a significant increase in ADP hydrolysis in the H group (22,2±3,04; n=7; P<0.05) when compared to the C group (14,9±2,01; n=7; P<0.05). In the HCC (12,02±2,1; n=7; P<0.05), HPE (14,6±0,3; n=7; P<0.05) and HCCPE (15,2±4,1; n=7; P<0.05) groups with the high-fat diet show a decrease significant difference in the ADP hydrolysis when compared to the H group 22,2±3,04; n=7; P<0.05). In the group with standard diet the use of PE (20,5±4,2; n=7; P<0.05) and CCPE *per se* (23,2±1,04; n=7; P<0.05) increased significantly the ADP hydrolysis when compared to the C group (14,9±2,01; n=7; P<0.05) (Fig. 1B).

3.5 E-5'-nucleotidase activity

The AMP hydrolysis in platelets is showed in Fig.2. Statistical analysis for AMP hydrolysis did not revealed statistical difference between the groups with a standard diet and hypercholesterolemic groups (P>0.05, n=7).

3.6 E-NPP activity

Results obtained for the E-NPP activity are shown in Fig. 3. The results obtained did not revealed statistical difference between the groups with a standard diet and hypercholesterolemic groups (P>0,05, n=7).

3.7 E-ADA activity

E-ADA activity in the platelets is shown in Fig. 4. The results obtained did not revealed statistical difference between the groups with a standard diet and hypercholesterolemic groups (P>0.05, n=7).

3.8 Platelet aggregation

Fig. 5 presents the results obtained for platelet aggregation using ADP 10µM as an agonist. The results obtained did not revealed statistical difference between the groups with a standard diet and hypercholesterolemic groups (P>0.05, n=7).

4. Discussion

Hypercholesterolemia is widely accepted as one of the major risk factors for the development of atherosclerosis [40]. The inflammatory process induced by hypercholesterolemia is not limited to large arteries. Low-density lipoprotein 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 [41]. Changes in the activity of ecto-enzymes, such as NTPDase were observed in some diseases such as multiple sclerosis [42], rheumatoid arthritis [43], ischemic heart disease [44] and lung cancer [45], indicating that NTPDase is an important enzyme of nucleotide hydrolysis in many pathologies, including hypercholesterolemia.

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 curcumin and physical exercise in hyperclesterolemics rats on the enzyme activity of the purinergic system in platelets these rats. First, it was observed that curcumin *per se* was not able to alter enzyme activity when administered to rats with standard diet. These results are in accordance with the study of Jaques et al. [30], in which curcumin *per se* was not able to alter the activity of ecto-enzymes E-NTPDase, E-5-nucleotidase and E-ADA in platelets from cigarette smoke-exposed rats.

When we evaluated the physical exercise *per se* and curcumin and physical exercise concurrently in the activity of ecto-enzymes, we observed an increase in the hydrolysis of ADP, possibly indicating that physical exercise is decreasing the ADP extracellular concentration. The regular moderate exercise could be an important factor in health, promoting physiological changes induced by the short-term or acute stress response [46]. Corroborating with the results Roque et al. [47], which also found an increased ADP hydrolysis in serum and sarcolemmal fraction of rats that were trained (swimming). In the vascular system, ADP is the major agonist involved in platelet aggregation [6], suggesting that physical exercise would decrease the levels of ADP extracellular, preventing platelet aggregation.

Although excessive platelet aggregation can occur as a result of inflammation [48] our results showed no statistical difference between the groups with a standard diet and hypercholesterolemic groups in platelet aggregation induced by ADP. Agreeing with the

results, Coppola et al. [49] also found no significant difference when evaluated platelet aggregation induced by ADP in subjects sedentary and physical exercise practitioners.

ATP and ADP hydrolysis were increased in platelets from rats with induced hypercholesterolemia. Corroborating with these results, Duarte et al. [50], demonstrated an increased E-NTPDase activity in platelets from patients with hypercholesterolemia, suggesting a beneficial role by preventing thrombus formation. Probably, high cholesterol levels induce an increase in platelet ATP and ADP hydrolysis as a compensatory mechanism to inhibit platelet aggregation and limit thrombus formation, showing a positive correlation between cholesterol levels and activity of NTPDase. Literature data indicate that adenine nucleotides and adenosine are important modulators of atherosclerosis [51], and since ATP stimulates the release of inflammatory cytokines by cultured macrophages, dendritic cells, or both [52], the purinergic system may be involved in the development of inflammatory reactions. Based on this, it is plausible that the increased total cholesterol and LDL cholesterol levels (Table 5) in the rats submitted to hypercholesterolemic diet, can lead to an inflammatory process, contributing to the alterations in the E-NTPDase activity observed in this study.

In relation to the treatment with curcumin and physical exercise, our results demonstrated a reduction of total cholesterol, LDL cholesterol and an increase in HDL levels in rats submitted to hypercholesterolemic diet and treated with curcumin and physical exercise when compared with the group hypercholesterolemic fat diet alone. These results are similar with the study of Folwarcznaeon et al. [53] that also demonstrated that ovariectomized rats receiving curcumin (10 mg/kg, podaily) decreased serum total cholesterol level. It was demonstrated that [24] that also demonstrated that curcumin supplemented diet significantly lowered the atherogenic index by 48% as compared to control group. Guerra et al. [54] also demonstrated that moderate continuous exercise (both 5 and 2 consecutive days/week) can result in positive adaptations in adipocyte area and lipid parameters in normo and hypercholesterolemic adult male rats, while Amin-Shokravi et al. [55] showed that physical exercise program could decrease levels of blood lipids such as total cholesterol and increase levels of HDL-C. Taken together, these findings showed that curcumin and physical exercise supplementation interfere with the cholesterol metabolism in diets rich in fat.

However, when hypercholesterolemic rats were treated with curcumin, they showed a decrease in ADP hydrolysis, returning to basal levels, as well when treated with curcumin and physical exercise concomitant, with that reversing the changes caused by

hypercholesterolemia. Differently found by Jaques et al. [30], curcumin was effective in preventing the reduction of E-NTPDase activity using ADP as a substrate in rats exposed to cigarette smoke.

The hypercholesterolemic rats that received the treatment of physical exercise presented an increase in ATP hydrolysis and a decrease in ADP hydrolysis, which probably would lead to an elevated ADP concentration in the bloodstream. It is well known that the enhanced level of ADP is related to an increased platelet aggregation [56]. Although an excessive platelet aggregation can occur in damaged vasculature as a consequence of inflammation, the platelet aggregation in this study showed no significant difference, suggesting that ADP excess may be reversed to ATP by the enzyme ecto-nucleoside diphosphate kinase (NDPK) [12]. As proposed by Yegutkin et al. [15], an opposite via the recovery of adenine nucleotides, which are essential to have effects on tromborregulation, such as ATP and ADP released from platelets granules that occurs when platelets are activated [57].

Continuing the purinergic cascade, E-5'-NT generates important intermediates to regulate the platelet aggregation, such as adenosine, from AMP substrate. However, when assessed the activity of this enzyme in platelets of rats with induced hypercholesterolemia no changes were observed in AMP hydrolysis. And adding this to the fact, there was an increased ADP hydrolysis these group, it may suggest high AMP levels found in extracellular environment. This increased in the ADP hydrolysis contributing in regulating the levels this nucleotide, since this it is capable of promoting the recruitment of platelets to the site of vascular injury, promoting platelet aggregation and thrombus formation. In regard the activity of E-ADA, no changes were observed between the groups, which indicate that this nucleoside is present in physiological levels in the extracellular medium, which would be related to normal platelet aggregation.

Since platelets express different sets of ecto-enzymes and purinergic receptors, which regulate thromboembolic process induced by vascular injuries [34] it was also evaluated the E-NPP activity, responsible for the direct ATP hydrolysis to extracellular AMP, but no change in this ecto-enzyme activity was observed in hypercholesterolemic rats with and without treatment, indicating that changes would occur only in E-NTPDase activity.

From the compiled results, we suggest that curcumin and physical exercise were shown to have a beneficial effect on health could be a potential treatment for cardiovascular diseases such as hypercholesterolemia. Also demonstrated, that the purinergic system has

been modified due to the action of diet on the activities of ecto-enzymes, suggesting a positive correlation between cholesterol levels and enzyme activities.

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Figure Legends

Figure 1: E-NTPDase activity in platelets of rats using ATP (A) and ADP (B) as substrate (nmol of Pi/min/mg of protein). Groups: C (control), CC (curcumin), PE (physical exercise), CCPE (curcumin+physical exercise), H (hypercholesterolemic), HCC (hypercholesterolemic+curcumin), HPE (hypercholesterolemic+physical exercise) and HCCPE (hypercholesterolemic+curcumin+physical exercise). Bars represent means±SEM. Groups with different letters are statistically different ($P<0.05$; n=7).

Figure 2: E-5'-nucleotidase activity in platelets of rats using AMP as substrate (nmol of Pi/min/mg of protein). Groups: C (control), CC (curcumin), PE (physical exercise), CCPE (curcumin+physical exercise), H (hypercholesterolemic), HCC (hypercholesterolemic+curcumin), HPE (hypercholesterolemic+physical exercise) and HCCPE (hypercholesterolemic+curcumin+physical exercise). Bars represent means±SEM. Groups with different letters are statistically different ($P> 0.05$; n=7).

Figure 3: E-NPP activity in platelets of rats using p-Nph-5 TMP as substrate (nmol p-nitrophenol released). Groups: C (control), CC (curcumin), PE (physical exercise), CCPE (curcumin+physical exercise), H (hypercholesterolemic), HCC (hypercholesterolemic+curcumin), HPE (hypercholesterolemic+physical exercise) and HCCPE (hypercholesterolemic+curcumin+physical exercise). Bars represent means±SEM. Groups with different letters are statistically different ($P>0.05$; n=7).

Figure 4: E-ADA activity in platelets of rats (U/L). Groups: C (control), CC (curcumin), PE (physical exercise), CCPE (curcumin+physical exercise), H (hypercholesterolemic), HCC (hypercholesterolemic+curcumin), HPE (hypercholesterolemic+physical exercise) and HCCPE (hypercholesterolemic+curcumin+physical exercise). Bars represent means±SEM. Groups with different letters are statistically different ($P>0.05$; n=7).

Figure 5: Platelet aggregation (%) in platelets of rats. Groups: C (control), CC (curcumin), PE (physical exercise), CCPE (curcumin+physical exercise), H (hypercholesterolemic), HCC (hypercholesterolemic+curcumin), HPE (hypercholesterolemic+physical exercise) and HCCPE (hypercholesterolemic+curcumin+physical exercise). Bars represent means±SEM. Groups with different letters are statistically different ($P>0.05$; n=7).

Table 2: Total cholesterol (TC), high-density lipoprotein (HDL-C), low-density lipoprotein (LDL-C), triglyceride (TRI) levels of rats after induction of cholesterol without treatment (mg/dL).

Groups	**TC**	**HDL-C**	**LDL-C**	**TRI**
Control rats	115±4.2	55±4.3	49.9 ± 5.6	60.35 ± 5.27
Hypercholesterolemic rats	185.8±12.4*	22.6±0.3*	152.4±7.82*	64.06 ± 6.76

Values are expressed as mean ± S.E.M *Significant difference between groups (*P<0.05, n= 28). Student's t test.

Table 3: Food consumption daily of controls rats (C), control+curcumin (CC), control+physical exercise (PE), control+curcumin+physical exercise (CCPE), hypercholesterolemic (H), hypercholesterolemic+curcumin (HCC), hypercholesterolemic+Physical exercise (HPE) and hypercholesterolemic+Curcumin + physical exercise (HCCPE), after of treatment.

Groups	Food Consumption (g)
C	51,07 ± 5,462
CC	54,31 ± 7,610
PE	48,23 ± 6,870
CCPE	54,6 ± 10,311
H	46,10 ± 3,627
HCC	45,06 ± 4,367
HPE	47,45 ± 3,422
HCCPE	43,90 ± 2,384

Values are expressed as mean ± S.E.M ($P>0.05$, n= 7), ANOVA-Dunnett's Test.

Table 4: Weight of controls (C), control+curcumin (CC), control+physical exercise (PE), control+curcumin+physical exercise (CCPE), hypercholesterolemic (H), hypercholesterolemic+curcumin (HCC), hypercholesterolemic+physical exercise (HPE) and hypercholesterolemic+Curcumin + physical exercise (HCCPE), before and after treatment.

	Weight (g)	
	Before treatment	**After treatment**
C	274,4 ± 9,594	321,9 ± 5,373
CC	300,0 ± 10,33	350,0 ± 4,788*
PE	288,0 ± 11,85	323,3 ± 4,269
CCPE	296,2 ± 9,375	342,6 ± 9,971
H	300,2 ± 8,870	347,6 ± 4,276*
HCC	302,2 ± 14,49	353,4 ± 10,68*
HPE	290,3 ± 10,52	331,6 ± 2,405
HCCPE	280,8 ± 10,22	323,4 ± 3,659

Values are expressed as mean ± S.E.M *Indicates significant difference when compared to the control group (*P<0.05, n= 7). ANOVA-Dunnett's Test.

Table 5: Total Cholesterol (TC), LDL Cholesterol (LDL-C), HDL Cholesterol (HDL-C), Triglyceride (TRI) levels of controls rats (C), contol+curcumin (CC), control+physical exercise (PE), control+curcumin+physical exercise (CCPE), hypercholesterolemic (H), hypercholesterolemic+curcumin (HCC), hypercholesterolemic+physical exercise (HPE) and hypercholesterolemic+curcum+physical exercise (HCCPE), before and after treatment (mg/dL).

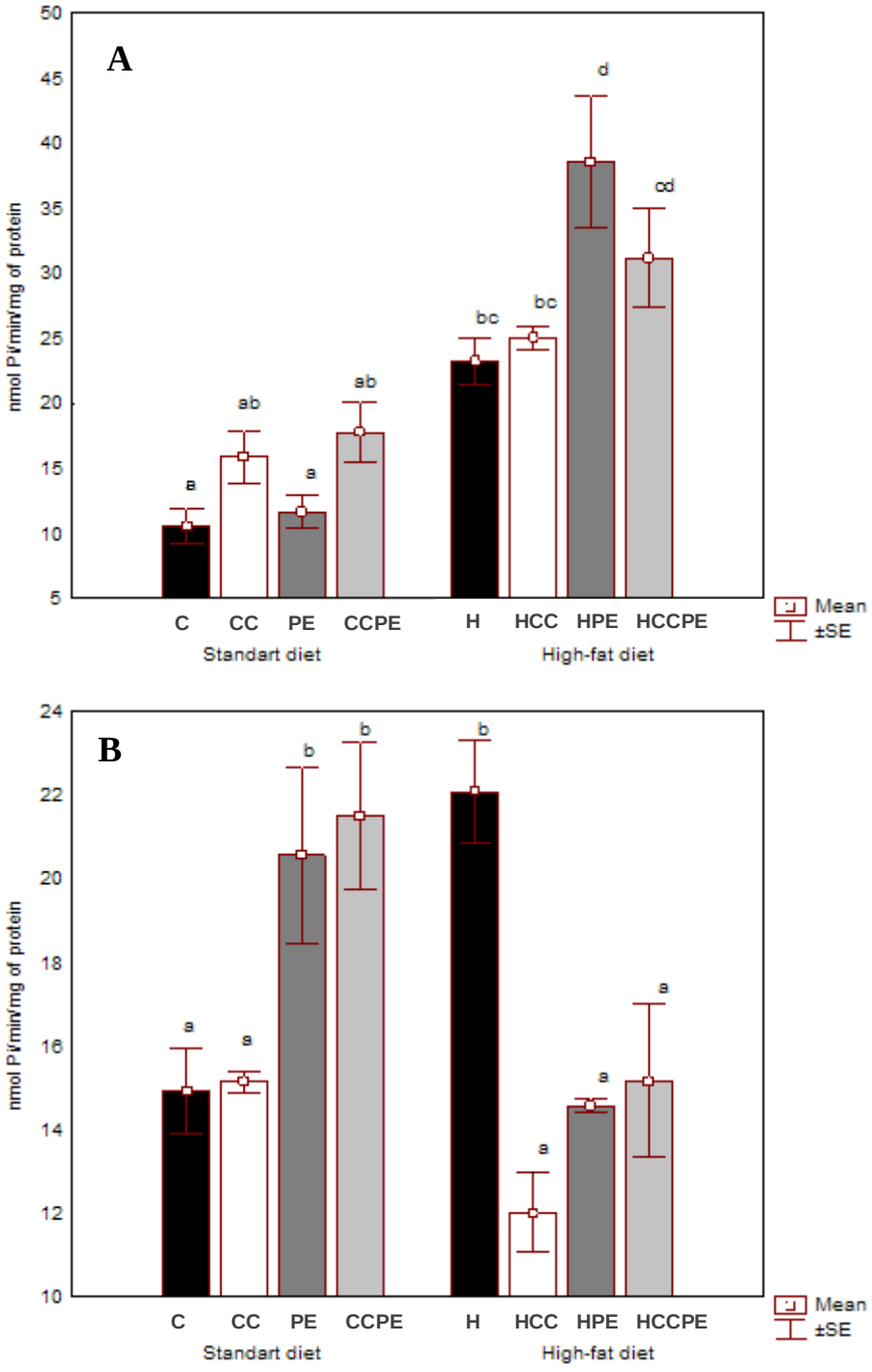
Groups	**TC**		**LDL-C**		**HDL-C**		**TRI**	
	Before	**After**	**Before**	**After**	**Before**	**After**	**Before**	**After**
C	120±2.4	127±4.8	57±4,1	63.4±3.6	52±4.7	54±2.7	56±3.2	53±6.6
CC	112±4.3	108±1.2	50.8±7.4	34±4.6*	46±3.2	59±7.3	76.2±8.6	76.2±8.6
PE	114±5	116±4.2	52±6.3	48±3	48±9.1	57±4.3	70.1±23.3	59.7±3.5
CCPE	112±3.1	120±4.6	55.6±3.1	62±3	42±3.8	48.3±4	72.4±2	50.7±13
H	146±1.3	153.4±2.4	107±3.5	119±9.1	26.3±8.2	19±9.5	65±7.7	75.6±11.9
HCC	160±2.1	112±6.4*	121.9±3	55.6±6*	21±2.1	45.1±3*	85.7±12	56.8±18.5
HPE	200±6.6	120.2±8*	166±6	73±4.1*	19±6	33±1*	72.6±9.1	70.2±15.6
HCCPE	196±4	133.6±2.7*	129±5.1	64.3±6*	22±6.5	57±9.6*	55.4±10.7	58.75±7.1

Values are expressed as mean ± S.E. *Indicates significant difference between groups (*P<0.05, n= 7). Student's t test.

Table 6: Liver enzyme of control (C), control+curcumin (CC), control+physical exercise (PE), control+curcumin+physical exercise (CCPE), hypercholesterolemic (H), hypercholesterolemic+curcumin (HCC), hypercholesterolemic+physical exercise (HPE) and hypercholesterolemic+Curcumin + physical exercise (HCCPE) (U/L).

Liver enzymes	AST	ALT	Alkaline phosphatase
C	30,83 ± 7,442	20,50 ± 1,655	455,1 ± 44,09
CC	35,60 ± 4,321	18,96 ± 1,843	428,1 ± 19,94
PE	49,56 ± 6,945	25,14 ± 3,300	331,2 ± 40,74
CCPE	52,63 ± 7,505	20,65 ± 1,873	485,8 ± 41,82
H	49,45 ± 5,828	32,73 ± 3,023	503,7 ± 57,64
HCC	48,50 ± 5,652	30,83 ± 3,815	545,9 ± 51,56
HPE	53,65 ± 9,898	32,30 ± 2,600	623,6 ± 68,16
HCCPE	27,90 ± 8,700	22,70 ± 1,700	637,3 ± 39,38

Values are expressed as mean ± S.E. Difference no when compared to the control group ($P>0.05$, n= 7). ANOVA-Dunnett's Test.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Standart diet
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Figure 1

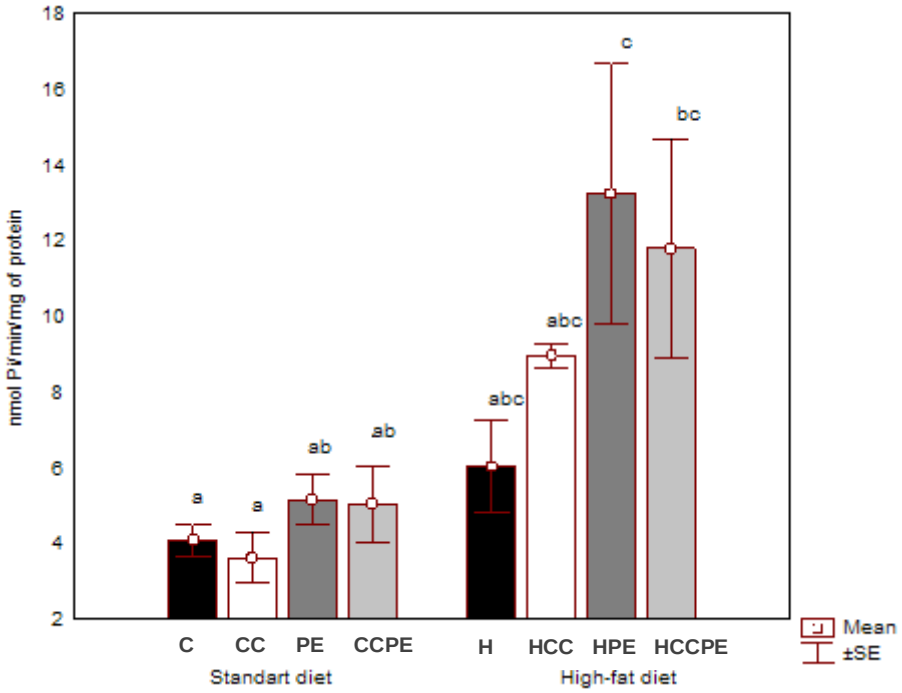
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Figure 2

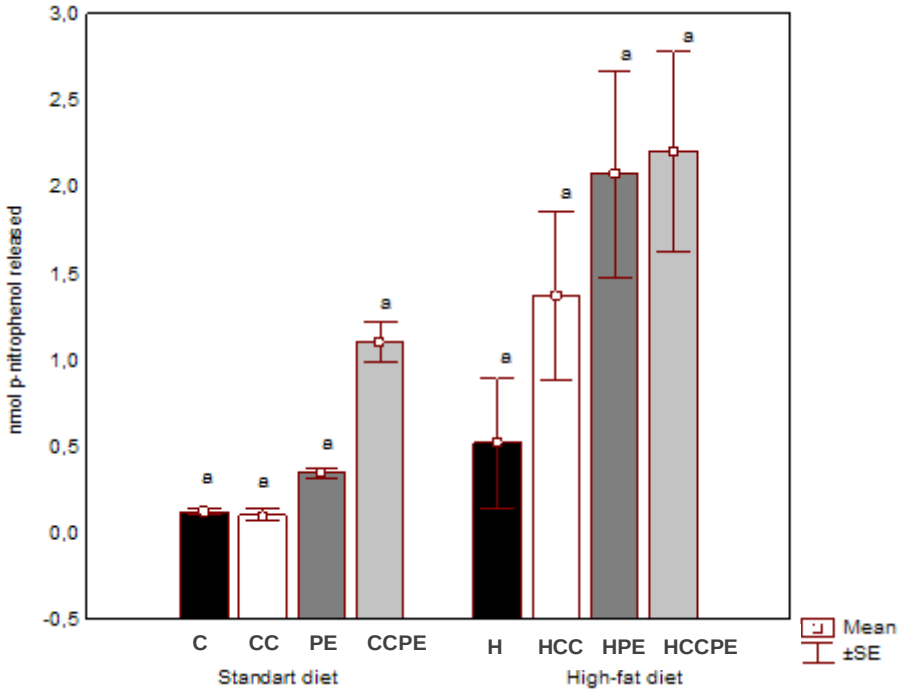
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Figure 3

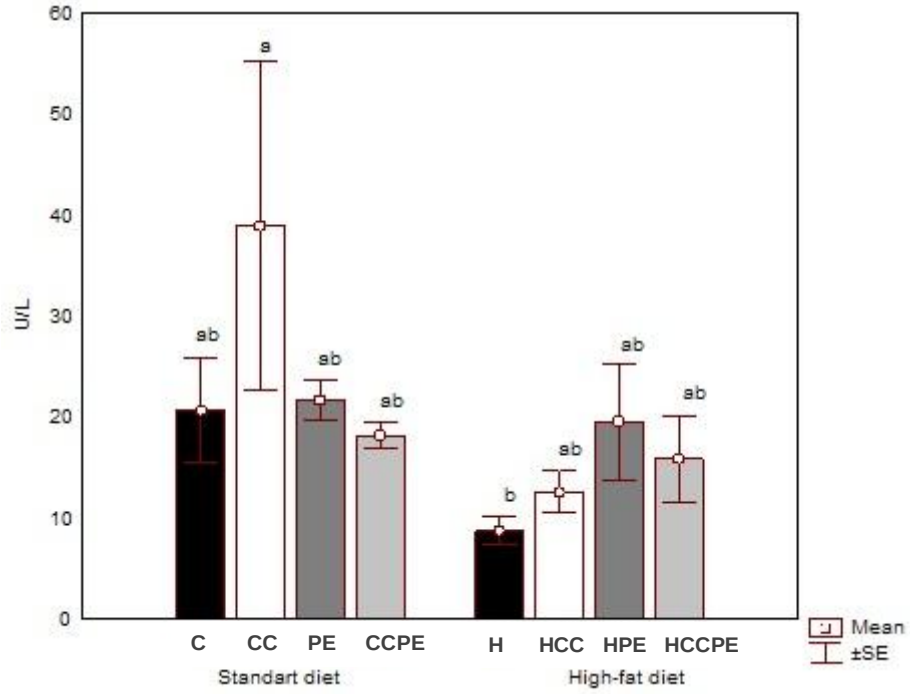
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Figure 4

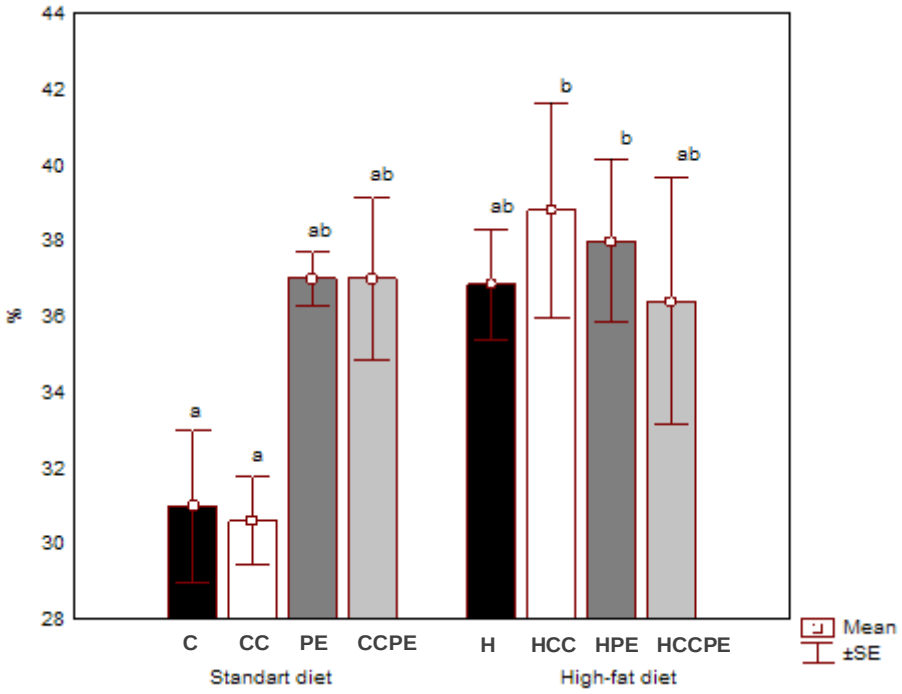
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Figure 5

4 CONCLUSÃO

- A curcumina e o exercício físico mostraram ter efeito benéfico sobre a saúde, pois houve redução do colesterol total e LDL-colesterol, e aumento do HDL-colesterol, indicando ser um possível tratamento contra doenças cardiovasculares, como por exemplo a hipercolesterolemia.

- A atividade da NTPDase foi modificada nos ratos hipercolesterolêmicos, demonstrando influência da dieta hipercolesterolêmica sobre o sistema purinérgico. Com isso, possivelmente protegendo o organismo contra a formação de trombos e agregação plaquetária, presentes na hipercolesterolemia.

- O tratamento com curcumina e exercício físico demonstrou efeito sobre a hidrólise do ADP em ratos hipercolesterolêmicos, indicando que o nucleotídeo retornou aos seus níveis basais.

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ANEXO 1

UNIVERSIDADE FEDERAL DE SANTA MARIA
PRÓ-REITORIA DE PÓS-GRADUAÇÃO E PESQUISA
COMITÊ INTERNO DE ÉTICA EM EXPERIMENTAÇÃO ANIMAL-UFSM

CARTA DE APROVAÇÃO

O Comitê Interno de Ética em Experimentação Animal-UFSM, analisou o protocolo de pesquisa:

Título do Projeto: Atividade das ecto-enzimas e perfil oxidativo em ratos com hipercolesterolemia induzida tratados com curcumina e submetidos à atividade física.

Numero do Parecer: 121/2010

Pesquisador Responsável: 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.

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

DATA DA REUNIÃO DE APROVAÇÃO:

Santa Maria, 18 de janeiro de 2011.

Marta Lizandra do Rêgo Leal
Coordenador do Comitê Interno de Ética em Experimentação
Animal-UFSM

Comitê Interno de Ética em Experimentação Animal - UFSM - Av. Roraima, 1000 – Prédio da Reitoria - 2º andar - Campus Universitário 97105-900 – Santa Maria – RS - - Tel: 0 xx 55 3220 9362

ANEXO 2

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