UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL
INSTITUTO DE CIÊNCIAS BÁSICAS DA SAÚDE
PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS BIOLÓGICAS:
BIOQUÍMICA

ENVOLVIMENTO DO ESTRESSE OXIDATIVO E HIPERCOLESTEROLEMIA NA ATEROSCLEROSE E AVALIAÇÃO DO EFEITO PROTETOR DO CAROTENÓIDE ASTAXANTINA

Tese de doutorado

PAULA ROSSINI AUGUSTI

Porto Alegre, RS
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Tese apresentada ao Programa de Pós-Graduação em Ciências Biológicas: Bioquímica do Instituto de Ciências Básicas da Saúde da Universidade Federal do Rio Grande do Sul como requisito parcial para obtenção do título de Doutor em Ciências Biológicas-Bioquímica.

Orientadora:

Profa. Dra. Tatiana Emanuelli

Porto Alegre, RS

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A Comissão Examinadora, abaixo assinada, aprova a Tese de Doutorado

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elaborada por

Paula Rossini Augusti

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Comissão examinadora:

Profª. Dra. Tatiana Emanuelli
(Presidente/Orientador)

Prof. Dr. José Cláudio Fonseca Moreira (UFRGS)

Profª. Dra. Andreza Fabro de Bem (UFSC)

Profa. Dra. Cristina Wayne Nogueira (UFSM)

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ÍNDICE

APRESENTAÇÃO

A presente Tese de Doutorado encontra-se organizada em três partes principais:

A **Parte I** representa a **Introdução**, a qual contém o referencial teórico utilizado para a construção das hipóteses investigadas nesta Tese. Em seguida, os **Objetivos** (Geral e Específicos) trazem o principal questionamento do trabalho realizado e as questões de pesquisa específicas que nortearam a realização desta Tese.

A **Parte II** está subdividida em três **Capítulos**. O **Capítulo I** traz um manuscrito em preparação para submissão à publicação; o **Capítulo II** contém um artigo já publicado; o **Capítulo III** contém um artigo submetido à publicação; As seções Materiais e Métodos, Resultados, Discussão e Referências Bibliográficas encontram-se nos próprios manuscritos e artigo e representam na íntegra este estudo.

A **Parte III** abrange **Discussão, Conclusões, Perspectivas e Referências Bibliográficas.** A **Discussão** representa uma interpretação geral dos dados obtidos em todos os trabalhos. As **Conclusões** contêm um resumo dos principais resultados da tese. Em seguida, está apresentada a seção **Perspectivas**, a qual sugere possíveis estudos futuros a partir dos resultados obtidos nesta ivestigação. A seção **Referências Bibliográficas** apresenta a bibliografia citada nas seções Introdução e Discussão desta Tese.

PARTE I

RESUMO

A aterosclerose consiste na formação de placas que reduzem o diâmetro dos vasos sanguíneos e é a principal causa das doenças cardiovasculares. O estresse oxidativo tem sido apontado como um importante mecanismo no processo de aterogênese induzido pela hipercolesterolemia. A astaxantina (ASX) é um carotenóide presente em algas e frutos-do-mar, amplamente estudado por suas propriedades antioxidantes. No presente trabalho, avaliou-se a ocorrência de estresse oxidativo em diferentes estágios de hipercolesterolemia em humanos e a relação entre o estresse oxidativo e o processo inflamatório nesses indivíduos. Adicionalmente, o potencial antiaterogênico da ASX foi avaliado em coelhos hipercolesterolêmicos. No primeiro capítulo demonstramos que a peroxidação lipídica e oxidação de proteínas aumentou em indivíduos com níveis de LDL considerados de alto risco para doenças cardiovasculares (>160mg/dL), enquanto os níveis de LDLox, LDLoxAB, do marcador de processo inflamatório hs-CRP e a atividade das enzimas TrxR-1 e SOD aumentaram em indivíduos com níveis de colesterol considerados altos, mas clinicamente aceitáveis (130-160mg/dL), persistindo este aumento nos sujeitos com LDL > 160mg/dL. A enzima PON1 não teve sua atividade modificada em nenhum dos estágios de hipercolesterolemia estudados. Os resultados apresentados no capítulo I permitem concluir que o estresse oxidativo ocorre concomitantemente ao processo inflamatório durante a hipercolesterolemia. Além disso, a utilização da atividade da enzima PON1 sérica como fator de risco independente para doenças cardiovasculares necessita maiores esclarecimentos. No segundo e terceiro capítulos, observamos que a adição de colesterol (1%) na dieta de coelhos (60 dias) induziu hiperlipidemia sérica, estresse oxidativo no soro e tecido aórtico e formação de ateroma no tecido aórtico. A suplementação de ASX (50-100mg%) concomitante com o colesterol atenuou o estresse oxidativo de maneira mais acentuada no tecido aórtico do que no soro, mas não preveniu o aumento dos lipídios séricos nem a formação de ateroma nos coelhos. Estes resultados indicam que apesar da ausência de efeito hipolipidêmico e antiaterogênico da ASX, esse carotenóide exerce efeitos antioxidantes no soro e tecido aórtico de coelhos hipercolesterolêmicos. Em conjunto, os dados desta Tese indicam a ocorrência de estresse oxidativo durante a hipercolesterolemia em humanos e a ocorrência de aterosclerose e estresse oxidativo em coelhos hipercolesterolêmicos. Apesar da ASX não ter apresentado efeito antiaterogênico neste trabalho, mais estudos são necessários antes de uma conclusão definitiva sobre o potencial deste carotenóide como adjunto na prevenção e tratamento de doenças cardiovasculares.

ABSTRACT

Atherosclerosis consists in the formation of plaques that reduces blood vassels diameter and it is the major cause of cardiovascular diseases. Oxidative stress has been pointed as an important mechanism in atherogenesis process induced by hypercholesterolemia. Astaxanthin (ASX) is a carotenoid present in algaes and seafoods, widely studied by its antioxidant properties. In the present study it was evaluated the ocurrence of oxidative stress at different stages of hypercholesterolemia in humans and the relathionship between oxidative stress and inflamatary process in these subjects. Additionally, the antiatherogenic potential of ASX was evaluated in hypercholesterolemic rabbits. In the first chapter, we demonstrated that lipid peroxidation and protein oxidation increased in subjects with LDL levels considered of high risk for cardiovascular diseases (>160mg/dL), while the levels of LDLox, LDLoxAB, the marker of inflamatory process hs-CRP and the activity of the enzymes TrxR-1 and SOD increased in subjects with LDL levels considered high, but clinically acceptable (130-160mg/dL), persisting this increase in subjects with LDL > 160mg/dL. The enzyme PON1 did not have its activity modified in none of the studied stages of hypercholesterolemia. The results presented in chapter I allow to conclude that oxidative stress ocurrs concomitantly to the inflamatory process during hipercholesterolemy. Besides, the use of enzyme PON1 activity in serum as an independent risk factor for cardiovascular diseases needs more clarification. In the second and third chapters, we observed that the addition of cholesterol (1%) in the diet of rabbits (60 days) induced serum hyperlipidemia, oxidative stress in serum and aortic tissue and atheroma formation in aortic tissue. ASX supplementation (50-100mg%) concomitant with cholesterol atenuated oxidative stress in a more pronounced way in aortic tissue than in serum, but did not prevent the increase in serum lipids nor atheroma formation in rabbits. These results indicate that despite the absence of hypolipidemic and antiatherogenic effect of ASX, this carotenoid exerts antioxidant effects in serum and aortic tissue of hypercholesterolemic rabbits. Taken together, the data of this thesis indicate the ocurrence of oxidative stress during hypercholesterolemia in humans and the ocurrence of atherosclerosis and oxidative stress in hypercholesterolemic rabbits. Despite ASX not having presented antiatherogenic effect in this work, more studies are necessary before a final conclusion about the potential of this carotenoid as an adjunct in prevention and therapy of cardiovascular diseases.

LISTA DE ABREVIATURAS

AG – Ácidos graxos

ANOVA – Análise de variância

ASX – Astaxantina

CAT – Catalase

CT – Colesterol total

DHA – Ácido docosahexaenóico

DTNB – Ácido 5,5'-ditio*bis*-2-nitrobenzóico

EPA – Ácido eicosapentaenóico

EROs – Espécies reativas de oxigênio

GSH – Glutationa

GR – Glutationa Redutase

GSH-Px – Glutationa peroxidase

HDL – Lipoproteína de alta densidade

Hs-CRP – Proteína C reativa

LDL– Lipoproteína de baixa densidade

LDLox – LDL oxidada

LDLoxAB – Anticorpos contra LDL oxidada

MCP-1 – proteína quimiotáxica para monócitos

M-CSF – fator estimulador das colônias de monócitos

MDA - Malondialdeído

MUFA – Ácidos graxos monoinsaturados

NADPH – Nicotinamida adenina dinucleotídeo

NPSH – Grupos tiólicos não-protéicos

PON1 – Paraoxonase 1

PUFA – Ácidos graxos poliinsaturados

SEM – Erro padrão da média

SH – Grupo sulfidrila

SOD – Superóxido dismutase

TBARS – Substâncias reativas ao ácido tiobarbitúrico

TG - Triglicerídeos

TrxR-1 – Tioredoxina redutase 1

Trx-1 – Tioredoxina 1

SFA – Ácidos graxos saturados

USFA- Ácidos graxos insaturados

LISTA DE FIGURAS

INTRODUÇÃO

1. Doenças cardiovasculares e aterosclerose

De acordo com dados da Organização Mundial da Saúde (WHO), as doenças cardiovasculares, tais como doença arterial coronariana, acidente vascular cerebral, doença arterial periférica, doença arterial congênita e falência cardíaca são a principal causa de morte ao redor do mundo, sendo responsáveis por 17,1 milhões de mortes no ano de 2004, ou seja, 29% do número de mortes mundial (WHO, 2009). Nessa estimativa, 7,2 milhões de mortes estão associadas a ataques cardíacos e 5,7 milhões de mortes estão associadas a acidente vascular cerebral. Em 2002 o Brasil apareceu em 9º lugar na lista dos países com maior número de mortes (números absolutos) por doenças cardíacas (139.601mortes) e em 6º em mortes por acidente vascular cerebral (129.172 mortes) (WHO, 2004). Nesse contexto, estima-se que em 2030 23,6 milhões de pessoas morram de doenças cardiovasculares (WHO, 2009).

A aterosclerose é definida como uma doença inflamatória crônica caracterizada pela formação de placas de constituição fibrosa e lipídica (ateromas) que diminuem progressivamente o diâmetro de vasos sanguíneos, podendo resultar na obstrução dos mesmos (Berliner et al., 1995). Essa patologia, induzida ou influenciada por acúmulo de lipídios, é a principal causa de infarto agudo do miocárdio, acidente vascular cerebral e demais doenças vasculares (Glass e Witztum, 2001) e sua prevenção passa pela identificação e controle do conjunto dos fatores de risco (Sociedade Brasileira de Cardiologia, 2001). Os fatores clássicos no desenvolvimento da arterosclerose são tabagismo, hipertensão arterial sistêmica, diabetes melito, idade, histórico familiar (Sociedade Brasileira de Cardiologia, 2001), níveis elevados de colesterol total (CT), lipoproteína de baixa densidade (LDL) e triglicerídeos (TG), níveis baixos de lipoproteína de alta densidade (HDL) (Brewer, 2004) e aumento dos níveis dos marcadores inflamatórios (Libby, 2002) e agregação plaquetária (Furman et al., 1998).

2. Hipercolesterolemia

A hipercolesterolemia, particularmente altos níveis de LDL, é um fator de risco independente para o desenvolvimento da aterosclerose, podendo acelerar o desenvolvimento e progressão da aterogênese (McKenney, 2001). Enquanto a HDL é constituída por 52% de proteínas e 48% de lipídios (Forti e Diament, 2006), a LDL é composta por 78% de lipídios (sendo 45% de colesterol livre ou esterificado) e 22% de proteínas (Figura 1) e constitui a principal forma de transporte de colesterol na circulação (Farmer e Gotto, 2002). Assim, um aumento na ingestão de colesterol na dieta implica diretamente em aumento pronunciado nos níveis de LDL.

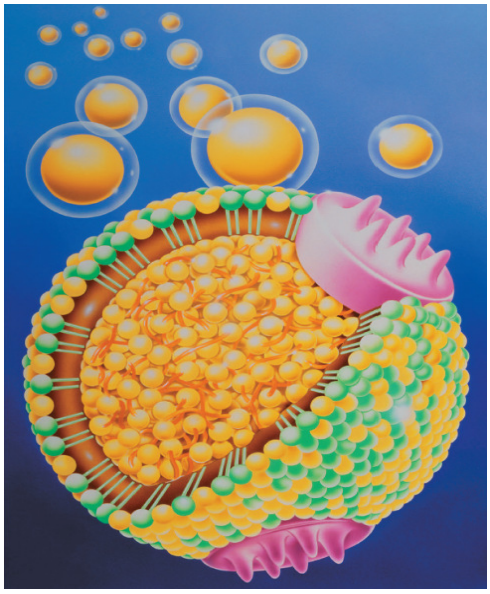

Figura 1: Ilustração da estrutura da LDL, onde observamos a fração protéica (apo-B, rosa), fosfolipídios (verde) e colesterol livre (amarelo). Extraído de Steinberg (2002).

3. Ácidos graxos

A quantificação de ácidos graxos (AG) circulantes no plasma seja na forma livre ou esterificada (TG, fosfolipídios e ésteres de colesterol) tem sido empregada como instrumento

na avaliação objetiva do tipo de gordura consumida por humanos (Andersen et al., 1999). Além do consumo via dieta, onde são transportados pelas lipoproteínas, os AG podem ser sintetizados endogenamente por processos de elongação e dessaturação, gerando AG monoinsaturados (MUFA) e AG poliinsaturados (PUFA) (Vaz et al., 2006). AG essenciais, como o ácido linolênico (18:3 n-3) e ácido linoléico (18:2n-6), não são sintetizados endogenamente nos seres humanos e em alguns animais, devido à carência de enzimas dessaturases e hidrogenases específicas. Esses AG são sintetizados, exclusivamente, pelo reino vegetal e obtidos através da dieta (Schaefer, 2002), podendo sofrer modificações no organismo humano. Portanto, os PUFAs ácido eicosapentaenóico (EPA, 20:5 n-3) e docosahexaenóico (DHA, 22:6 n-3), são produtos do metabolismo do ácido α-linolênico, assim como o PUFA araquidônico (20:4 n-6) é o produto do metabolismo do ácido linoléico (Schaefer, 2002). Após os processos de absorção, transporte intracelular e plasmático e síntese, os AG serão utilizados como fonte de energia, na estrutura de membranas e, no caso dos AG essenciais, como precursores de eicosanóides. Os AG também serão importantes em uma série de sistemas fisiológicos, e como mediadores das respostas imune e inflamatória (Vaz et al., 2006). Um exemplo disso é a relação positiva entre níveis circulantes adequados de PUFA, especialmente os da série n-3, e a função cardiovascular (Harris e Isley, 2001). Uma partícula de LDL típica contém 2700 moléculas de AG incorporadas nas várias classes de lipídios, sendo que 50% desses AG são PUFA (Keaney e Frei, 1994). Uma vez que os PUFAs são suscetíveis à processos de oxidação (Keaney e Frei, 1994), o comprometimento desses AG pode ter conseqüências significativas para a função do sistema cardiovascular.

4. Modificação oxidativa da LDL

As lipoproteínas atravessam as células endoteliais intactas por transporte vesicular (transcitose), o qual não requer receptores. A magnitude desse transporte é concentração-dependente; níveis elevados de LDL resultam em aumento da quantidade do composto que atinge a íntima, através de endotélio intacto (Schenke e Carew, 1989a; Schenke e Carew, 1989b). Níveis elevados de colesterol induzem um aumento na síntese de prostaglandinas pela via das ciclooxigenases, fazendo com que as células endoteliais e celulas musculares lisas liberem ânion superóxido durante o processo (Lee e Prasad, 2003).

Durante a síntese de prostaglandinas pela via enzimática, as ciclooxigenases incorporam oxigênio molecular aos ácidos graxos poliinasturados (PUFAs), presentes em grandes quantidades na LDL, gerando hidroperóxidos lipídicos. Os PUFAs com elétrons desemparelhados seguem a rota oxidativa em sucessivas reações em cadeia, desencadeando o processo de peroxidação lipídica (Steinbrecher et al., 1990). Dessa maneira, por ação do ânion superóxido e das ciclooxigenases, ocorre há a formação de LDL minimamente oxidada (LDL mmox), caracterizada pela oxidação dos componentes lipídicos da LDL, com poucas alterações na fração protéica (Berliner et al., 1995). A LDL mmox induz a produção, pelas células endoteliais, de potentes ativadores dos monócitos, como a proteína quimiotáxica para monócitos (MPC-1) e o fator estimulador das colônias de monócitos (M-CSF), resultando na ativação de monócitos circulantes que migram para o espaço subendotelial e são diferenciados até macrófagos. Os macrófagos secretam espécies reativas de oxigênio (EROs), causando a oxidação da fração protéica da LDL, originando a LDL altamente oxidada (LDLox) (Berliner et al., 1995). A LDLox deixa de ser reconhecida pelos receptores normais dos macrófagos, responsáveis pelo efluxo de colesterol e passa a ser reconhecidas por receptores específicos nos macrófagos, denominados receptores “scavenger” (Sparrow et al., 1989). Tais receptores não sofrem regulação e acumulam LDLox ilimitadamente em seu interior, originando as células espumosas. Essas células são características das estrias gordurosas presentes nas

lesões ateroscleróticas, que podem progredir até a ruptura, precipitando eventos clínicos como ataque cardíaco e derrames (Stocker e Keaney, 2004). A representação do processo de oxidação da LDL até a formação das estrias gordurosas pode ser visualizada na figura 2.

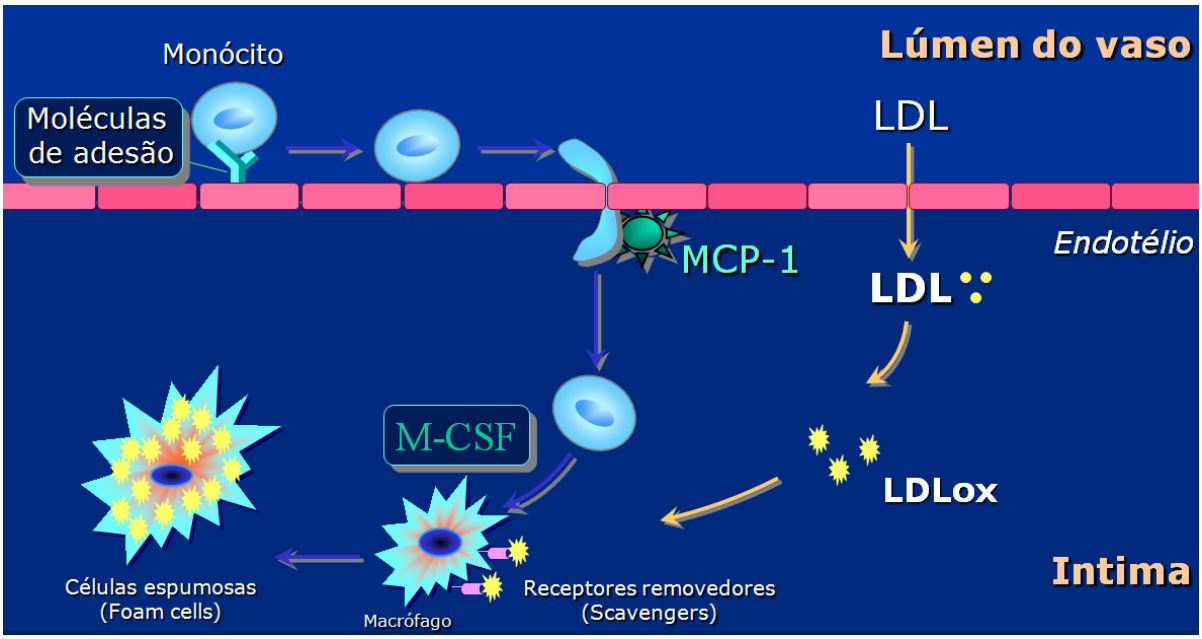

Figura 2: Ilustração do processo de oxidação da LDL e formação das células espumosas. Adaptado de Steinberg et al. (1989). LDLox = LDL oxidada; M-CSF = fator estimulador das colônias de monócitos; MCP-1 = proteína quimiotáxica para monócitos.

5. EROs, estresse oxidativo e aterosclerose

O estresse oxidativo pode ser definido como uma condição resultante do desequilíbrio entre a produção de espécies oxidantes (como as EROs) e os sistemas de remoção das espécies oxidantes, denominados sistemas antioxidantes (Sies, 2000). A produção de radicais livres e o estresse oxidativo têm sido apontados como causa do desenvolvimento da aterosclerose durante a hipercolesterolemia (Prasad e Kalra, 1993), uma vez que a modificação biológica mais relevante da partícula de LDL é causada por reações de oxidação (Morel et al., 1984). Tais reações são associadas a EROs como o ânion radical superóxido

[$^{\cdot}O_2^-$], radical hidroxil [$^{\cdot}OH$] e peróxido de hidrogênio [H_2O_2] (figura 3) (Morel et al., 1984). O radical superóxido é gerado em todas as células aeróbias a partir da redução parcial do oxigênio molecular por um elétron na cadeia de transporte de elétrons, particularmente entre os complexos I e III (Carreras, 2004). Adicionalmente, vários sistemas enzimáticos podem levar a geração de radical superóxido, participando assim de eventos oxidativos relevantes ao processo aterogênico. Um desses sistemas é o sistema NADPH oxidase, que quando ativado em células fagocíticas (como os macrófagos) origina o ânion superóxido, que será utilizado na formação de ácido hipocloroso, espécie reativa tóxica para microrganismos (Sies, 1985). O radical superóxido pode ser gerado também durante a síntese de prostaglandinas no retículo endoplasmático liso das células. Nesse processo de conversão do ácido araquidônico a prostaglandinas ocorre adição de oxigênio molecular em algumas etapas de reação, o que pode levar a geração de ânion superóxido se sua redução for incompleta (Nelson e Cox, 2000). Ambos os sistemas enzimáticos estão intimamente relacionados com o estresse oxidativo durante a aterosclerose (Madamanchi et al., 2005). Outro sistema enzimático importante na geração de ânion superóxido é o sistema da xantina oxidase, envolvido no catabolismo de purinas e formação de ácido úrico. Durante as reações desse sistema, há geração de radical superóxido (Murray et al., 1996). Tem sido descrita a ativação do sistema xantina oxidase em pacientes com doença arterial coronariana (Spiekermann et al., 2003). Independentemente do sistema gerador, a formação do radical superóxido parece ser um evento importante durante a aterosclerose induzida por hipercolesterolemia (Ohara et al., 1993).

A eliminação do radical superóxido pode ser feita pela enzima superóxido dismutase (SOD), resultando na formação de peróxido de hidrogênio (figura 3) (Halliwell e Gutteridge, 1990). De acordo com Stralin et al. (1995), a SOD na sua isoforma extracelular (ECSOD) é a isoforma majoritária na parede arterial, participando de forma ativa na remoção do ânion

superóxido gerado em eventos pré-ateroscleróticos. Assim, por ação da SOD, as vias geradoras de radical superóxido indiretamente contribuem para a formação de peróxido de hidrogênio. Entretanto, outros eventos como a oxidação de ácidos graxos nos peroxissomos celulares e a oxidação de flavoproteínas desidrogenases pelo oxigênio molecular também contribuem para a geração desta ERO (Nelson e Cox, 2000). Recentemente foi demonstrado que o peróxido de hidrogênio é um regulador da proliferação de células vasculares em humanos durante eventos ateroscleróticos (Panchenko et al., 2009).

O peróxido de hidrogênio pode ser removido pela enzima catalase (CAT), que atua na remoção do peróxido de hidrogênio gerando oxigênio e água (figura 3) (Hunt et al., 1998). Outro sistema removedor de peróxido de hidrogênio é composto pelo tripeptídeo glutationa (GSH) em conjunto com duas enzimas, a glutationa peroxidase (GSH-Px) e a glutationa redutase (GR). Esse sistema opera em ciclos, uma vez que a GSH reduz o H_2O_2 a H_2O em reação catalisada pela GSH-Px. Nesse processo a GSH é oxidada e, em um segundo momento, é regenerada pela GR utilizando equivalentes redutores da nicotinamida adenina dinucleotídeo (NADPH) (figura 3) (Flohé e Brand, 1969). Apesar de compartilhar o mesmo substrato (H_2O_2), ambos os sistemas enzimáticos funcionam de forma diferenciada. Enquanto a GSH-Px remove pequenas quantidades de H_2O_2 (Km da enzima é de 1 μM) (Flohé e Brand, 1969), a CAT age de forma mais significativa contra níveis elevados de H_2O_2 (Km da enzima é de 25 mM) (Aebi, 1984). Recentemente, a atividade reduzida da GSH-Px foi apontada como um fator de risco independente para eventos cardiovasculares em pacientes com doença arterial coronariana (Blankenberg et al., 2003).

O radical hidroxil [$HO^{\cdot}$] é a ERO mais deletéria ao organismo, pois devido a sua meia-vida muito curta dificilmente pode ser seqüestrado *in vivo*. Adicionalmente, mamíferos não possuem sistemas de detoxificação enzimáticos desse radical (Sies, 1985). Assim, antioxidantes não enzimáticos como a GSH, as vitaminas C e E, bem como flavonóides e

carotenóides têm sido apontados como os principais agentes removedores do radical hidroxil (Sies, 1993). Além disso, extratos de plantas contendo substâncias antioxidantes têm mostrado potencial na remoção desse radical (Gao et al., 1999; Silva et al., 2007). O principal efeito deletério do radical hidroxil reside na sua ação sobre lipídios de membrana, processo denominado peroxidação lipídica. O radical hidroxil ataca a cadeia lipídica em sítios susceptíveis como o grupo metilênico alílico, convertendo-o em novo centro de radical livre, desencadeando reações em cadeia que culminam com a formação de produtos de oxidação biologicamente ativos, como o malondialdeído (MDA) e o 4-hidroxinonenal (Barreiros e David, 2006).

6. Tioredoxina redutase

Em conjunto com as enzimas removedoras de H_2O_2 clássicas (CAT e GSH-Px), a enzima tioredoxina redutase (TrxR) também participa de forma ativa na remoção de H_2O_2 e outros peróxidos (figura 3). A TrxR é uma selenoflavoproteína que em conjunto com a proteína Trx e o NADPH forma o sistema da tioredoxina, um sistema efetivo para a redução de proteínas na forma dissulfeto (Holmgren & Björnstedt, 1995). O sistema da tioredoxina exerce diversas funções importantes em nível celular, como a regulação da apoptose e da resposta imune (Nordberg & Arnér, 2001). Adicionalmente, esse sistema atua como doador de elétrons durante a síntese de DNA (Powis & Montfort, 2001) e para a GSH-Px plasmática (Björnstedt et al., 1994) permitindo que essa enzima reduza hidroperóxidos mesmo com baixos níveis de GSH disponíveis (Wassmann et al., 2004).

Três isoformas das TrxR já foram identificadas incluindo a TrxR citosólica (TrxR-1), a mitocondrial (TrxR-2) e uma terceira isoforma isolada de mitocôndria de testículos de ratos (TrxR-3) (Nordberg & Arnér, 2001). Uma característica particular da TrxR-1 é a ampla especificidade de substratos, uma vez que a TrxR-1 é responsável pela redução não apenas da

Trx, mas também de hidroperóxidos (May et al., 2002), ácido lipóico, ubiquinona e deidroascorbato (Nordberg & Arnér, 2001). Assim, tem sido atribuído ao sistema tioredoxina uma função primordial na manutenção do estado redox da célula, podendo atuar no sistema de regulação da expressão de genes redox-sensíveis, através da ativação de fatores de transcrição (Nordberg & Arnér, 2001). Adicionalmente, sugere-se que a TrxR possa funcionar como um sensor redox celular (Sun et al., 1999).

Apesar do papel importante da enzima TrxR na defesa antioxidante e controle da da produção de EROs intracelular (Nordberg & Arnér, 2001), seu papel em eventos cardiovasculares ainda não está bem definido. A proteína Trx é muito expressada em células endoteliais e células musculares lisas de artérias normais (Okuda et al., 2001) e a expressão da enzima aumenta significativamente em placas ateroscleróticas de roedores, particularmente em células endoteliais e macrófagos (Takagi et al., 1998). Em concordância com o aumento da expressão da Trx em placas ateroscleróticas, Furman et al. (2004) demonstraram que a expressão da enzima TrxR-1 aumenta em macrófagos humanos oriundos de placas ateroscleróticas em resposta a LDLox. Adicionalmente, em um estudo de Hägg et al. (2006) a LDLox induziu um aumento na expressão da TrxR em macrófagos oriundos de ateromas humanos e em sistema *in vitro*. Em conjunto, essas evidências sugerem que Trx e TrxR cooperam mutuamente na defesa antioxidante durante o processo de aterogênese.

Apesar das evidências existentes sobre a cooperação da Trx e TrxR em nível celular, o funcionamento do sistema tioredoxina no ambiente extracelular ainda não foi totalmente elucidado. Sabe-se que os níveis de Trx aumentam no plasma em condições associadas a estresse oxidativo e inflamação, como a artrite reumatóide (Yoshida et al., 1999), infarto agudo do miocárdio (Miyamoto et al., 2003), angina (Hokamaki et al., 2005) e falência cardíaca crônica (Jekell et al., 2004). Esses relatos também sustentam a associação entre os níveis de Trx e doenças cardiovasculares. Entretanto, existem poucas evidências do

comportamento da enzima TrxR no plasma e sua relação com eventos cardiovasculares. Söderberg et al. (2000) demonstraram pela primeira vez a presença de TrxR no plasma de indivíduos saudáveis e a secreção da enzima parece ser realizada por monócitos circulantes como efeito protetor dessas células contra os radicais livres gerados durante a resposta inflamatória. Uma vez que os monócitos circulantes estão envolvidos na resposta contra a LDLox, é possível que a atividade da TrxR plasmática sofra alterações durante eventos cardiovasculares, tais como hipercolesterolemia e aterosclerose.

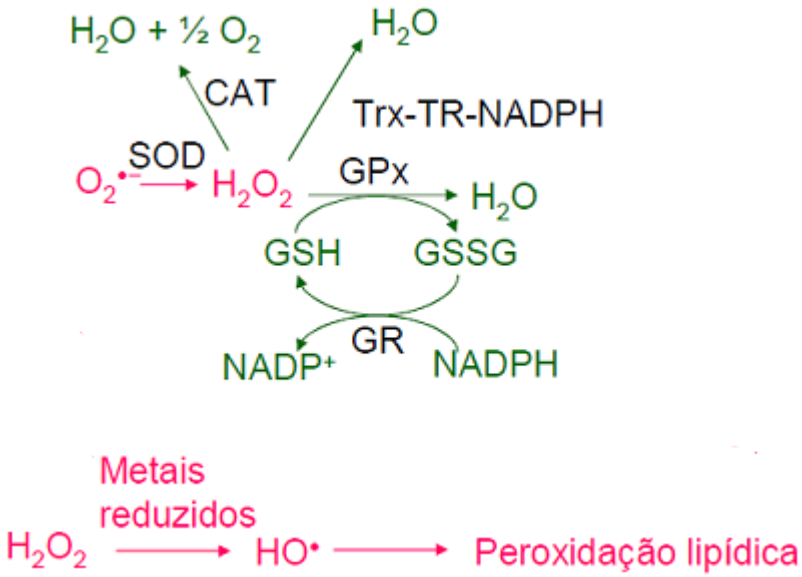

Figura 3: Principais EROs e reações de remoção de EROs pelas enzimas antioxidantes celulares. $O_2^{\cdot -}$ = ânion superóxido; H_2O_2 = peróxido de hidrogênio; $HO^{\cdot}$ = radical hidroxila; SOD = superóxido dismutase; CAT = catalase; GPX = glutationa peroxidase; Trx = tioredoxina; TrxR = tioredoxina redutase; NADPH = nicotinamida adenina dinucleotídeo; GSH = glutationa; GSSG = glutationa dissulfeto; GR = glutationa redutase. Inspirado em imagem do sítio na internet www2.iq.usp.br/redoxoma (acessado em 10/07/2010).

7. Ações da LDLox

A LDL-ox induz a formação de anticorpos específicos (LDLoxAB) e ambos têm sido encontrados em lesões ateroscleróticas e no sangue de pacientes hipercolesterolêmicos e portadores de doenças cardiovasculares (Palinski et al. 1990; Yla-Herttuala et al. 1994; Duarte et al., 2007; Itabe e Ueda 2007). A presença de LDLoxAB em lesões ateroscleróticas é apontada como um mecanismo preventivo, uma vez que tais anticorpos bloqueiam a captação da LDLox pelos macrófagos, prevenindo a formação das células espumosas (Shaw et al., 2001; Matssura et al., 2006).

Além da secreção de anticorpos específicos, a oxidação da LDL induz a produção de mediadores inflamatórios como a proteína C reativa (hs-CRP), um marcador de fase aguda de processos inflamatórios presente na doença arterial coronariana (Toss et al.,1997) e na hipercolesterolemia (Duarte et al., 2009). Em populações sem antecedentes cardiovasculares e de forma independente dos fatores de risco normalmente avaliados, níveis elevados de PCR são preditores de infarto do miocárdio e acidente vascular encefálico (Esporcatte et al., 2004). A presença de altas concentrações de hs-CRP inibe a síntese do óxido nítrico nas células endoteliais, facilitando a apoptose destas células e bloqueia a angiogênese (Verma et al., 2002). Seus efeitos pró-aterogênicos são evidenciados pelo estímulo à migração e proliferação das células musculares lisas, bem como a produção de espécies reativas de oxigênio (EROs) (Wang et al., 2003; Verma et al., 2004).

8. Paraoxonase

Enquanto a presença de LDL em níveis elevados no sangue é apontada como o principal fator de risco para doenças cardiovasculares, estudos epidemiológicos indicam uma relação inversa entre os níveis sanguíneos de HDL e o risco de doença aterosclerótica

(Assmann e Gotto, 2004; Asheikh et al., 2005; Linsel-Nitschke e Tall, 2005). Classicamente, esse efeito protetor está relacionado à função primordial da HDL, o transporte do colesterol dos tecidos para o fígado, que faz a conversão desse colesterol em sais biliares (Fielding e Fielding, 1995). Entretanto, um mecanismo alternativo tem sido sugerido para o papel da HDL na prevenção da aterosclerose: a capacidade dessa lipoproteína em prevenir a oxidação da LDL via mecanismos enzimáticos (Durrington e Mackness, 2001). Embora muitas enzimas associadas a HDL tenham sido relacionadas à capacidade antioxidante dessa lipoproteína, numerosas evidências sugerem que a enzima paraoxonase (PON1) é o principal componente da HDL responsável pela inibição da oxidação da LDL (Mackness et al., 1997; Aviram et al., 1999). A PON1 é uma enzima sintetizada no fígado e carreada na corrente sanguínea, mais conhecida por sua habilidade em hidrolizar metabólitos ativos de muitos inseticidas organofosforados (Costa et. al, 2005). Adicionalmente, a PON1 possui a capacidade de hidrolizar hidroperóxidos lipídicos (Mackness et al., 1991a). Essa capacidade tem sido associada à atividade de esterase, peroxidase e fosfolipase, que permite a PON1 inibir a formação de lipídios oxidados proinflamatórios ou degradá-los se houver sua formação. Assim, a PON1 modula a oxidação lipídica tanto na HDL quanto na LDL (Costa et al., 2003).

9. Carotenóides

Considerando que o estresse oxidativo está envolvido nos eventos que antecedem a patogênese da aterosclerose, manipulações na dieta com substâncias antioxidantes são descritas como alternativas de prevenção e controle da doença (Atkin et al., 2005). Os carotenóides são pigmentos naturais, sintetizados por plantas e microrganismos, conhecidos por suas propriedades antioxidantes. Devido ao fato de que a maioria dos carotenóides é transportada por lipoproteínas na corrente sanguínea, essas substâncias têm sido estudadas na

prevenção de doenças cardiovasculares (Agarwal e Rao, 2000). Osganian et al. (2003) observaram correlação inversa entre os níveis circulantes de β-caroteno e a ocorrência de doença arterial coronariana. Entretanto, tal correlação não foi observada para carotenóides como luteína, zeaxantina e β-criptoxantina. Além disso, o efeito protetor do carotenóide licopeno na prevenção de doenças cardiovasculares descrito por Fuhrman et al. (1997) ainda é controverso, uma vez que Frederiksen et al. (2007) relataram que a suplementação de coelhos hiperlipidêmicos com extrato de tomates rico em licopeno durante 24 semanas não foi capaz de reduzir a incidência da aterosclerose nesses animais. Apesar da controvérsia, já está disponível no mercado um medicamento a base de licopeno, o Ateronon^{TM}, indicado para prevenção e tratamento de doenças cardiovasculares. Dessa maneira, o real papel dos carotenóides nas doenças cardiovasculares ainda necessita ser elucidado.

10. Astaxantina

A astaxantina (ASX) é um pigmento pertendente a classe dos carotenóides oxigenados ou xantofilas, sintetizado principalmente por algas (*Haematococcus pluvialis*) e fungos (*Phaffia rizoma*) (Kurashige et al., 1990). A ASX é o principal pigmento em aves como o flamingo, peixes como o salmão e crustáceos como camarão e lagosta, que acumulam esse carotenóide após o consumo de algas produtoras. Por essa razão, a ASX é muito utilizada na aquicultura de salmão, camarão e lagosta a fim de conferir a coloração laranja adequada à comercialização dessas espécies (Higuera-Ciapara, 2006).

A estrututura química da ASX pode ser descrita como uma longa cadeia hidrocarbonada (40 átomos de carbono) com duplas ligações conjugadas e um anel aromático

contendo um grupo hidroxil e um grupo carbonil em cada extremidade da cadeia (Goto et al., 2001) (Figura 4).

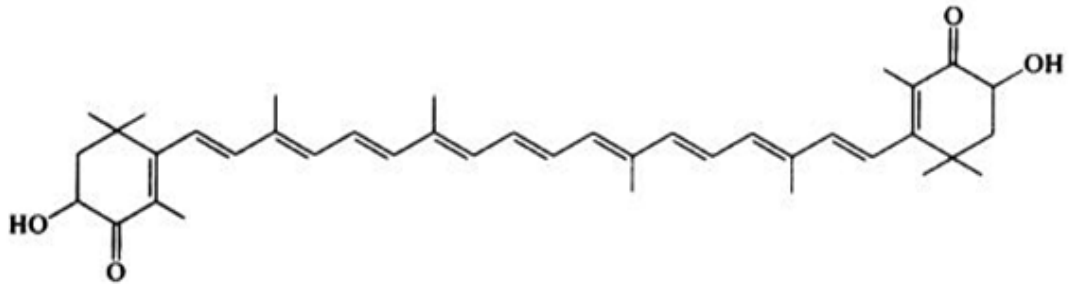

Figura 4: Estrutura química da ASX. Extraído de Goto et al., (2001).

Embora não possua atividade pró-vitamina A, a ASX possui grande potencial antioxidante, sendo 100-500 vezes mais potente que o α-tocoferol (Naguib et al., 2000), além de combater eficientemente o radical hiroxil e o oxigênio singlete (Wu et al., 2006). Adicionalmente, a síntese de ASX em fungos é estimulada por ânion superóxido, peróxido de hidrogênio e oxigênio singlete, provavelmente como resposta de defesa contra essas EROs (Schroeder e Johnson, 1993; Schroeder e Johnson, 1995; Liu e Wu, 2006). Essa elevada atividade antioxidante é associada à estrutura química da ASX, onde os grupos hidroxil dos anéis polares removeriam radicais livres na superfície da membrana e a cadeia hidrocarbonada agiria como antioxidante no interior hidrofóbico da membrana (Goto et al., 2001).

Apesar do elevado potencial antioxidante da ASX e da participação do estresse oxidativo no início e manutenção da aterosclerose, existem poucos estudos avaliando o potencial antiaterogênico deste carotenóide. Em um estudo de Li et al. (2004), a ASX conferiu estabilidade às placas ateromatosas por diminuir a infiltração de macrófagos e a apoptose das células, enquanto Iwamoto et al. (2000) relataram a inibição da oxidação de LDL humana *in vitro* pela ASX**.** Adicionalmente, Spiller et al. (2006) demonstraram que a

suplementação de indivíduos saudáveis com 4 mg/dia de ASX durante 8 semanas causou uma redução nos níveis circulantes de hs-CRP, sugerindo que a ASX diminuiu a ocorrência do processo inflamatório nestes indivíduos. Entretanto, Jacobsson et al. (2004) observaram que a suplementação de coelhos hiperlipidêmicos com ASX durante 16 semanas não inibiu a oxidação da LDL ou a ocorrência de aterosclerose nesses animais. Adicionalmente, um estudo recente tem apontado a ASX como um novo potencial tratamento para o estresse oxidativo e inflamação em doenças cardiovasculares, particularmente na injúria causada por isquemia-reperfusão e na oclusão trombótica vascular (Paskow et al., 2008). Apesar da existência de vários estudos comprovando o potencial antioxidante da ASX, seu uso na prevenção e tratamento de doenças cardiovasculares permanece controverso, provavelmente devido às diferenças entre os estudos no que diz respeito aos modelos utilizados (*in vitro*, animais, humanos). No Brasil, a ASX é disponível para uso na aqüicultura por importação de empresas estrangeiras, o que causa um aumento nos custos de produção de pescados e frutos-do-mar. Além disso, existem poucos suplementos nutracêuticos à base deste carotenóide para consumo humano, sendo freqüentemente comercializados como uma mistura de carotenóides (Calibex, 2008). Desta maneira, a elucidação do real potencial desse carotenóide contra doenças cardiovasculares, pode estimular um aumento na produção de nutracêuticos à base de ASX e introduzir o Brasil no mercado produtor de ASX para uso na aqüicultura.

OBJETIVOS

1. Objetivo geral

Verificar a participação do estresse oxidativo e hipercolesterolemia no processo de aterogênese em animais e humanos, bem como verificar o potencial antioxidante, hipolipidêmico e antiaterogênico do carotenóide ASX.

2. Objetivos específicos

- Avaliar parâmetros de estresse oxidativo no soro de indivíduos com diferentes níveis de LDL, correlacionando com os níveis do indicador de processo inflamatório hs-CRP.

- Avaliar o comportamento da enzima TrxR-1 no soro e tecido aórtico de coelhos hipercolesterolêmicos bem como no soro de indivíduos com diferentes níveis de LDL.

- Avaliar a atividade da enzima PON1 no soro de coelhos hipercolesterolêmicos e de indivíduos com diferentes níveis de LDL.

- Avaliar o potencial hipolipidêmico, antiaterogênico e antioxidante da ASX em coelhos hipercolesterolêmicos.

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PARTE II

CAPÍTULO I

Thioredoxin reductase and paraoxonase activities in subjects with different low density lipoprotein levels: relationship with oxidative and inflammatory processes.

Paula R. Augusti, Greicy M.M. Conterato, Juliana T. Vicentini, Sabrina Somacal, Andréia Quatrin, Amanda R. Ruviaro, Marta M.M.F. Duarte, Tatiana Emanuelli

Manuscrito em preparação para submissão ao periódico *Clinical Biochemistry*

Thioredoxin reductase and paraoxonase activities in subjects with different low density lipoprotein levels: relationship with oxidative and inflammatory processes.

Paula R. Augusti[1, 2], Greicy M.M. Conterato [3], Juliana T. Vicentini[3], Sabrina Somacal[3], Andréia Quatrin[3], Amanda R. Ruviaro[3], Marta M.M.F. Duarte[4], Tatiana Emanuelli[3*].

[1] Graduate Program on Biological Sciences/Biochemistry, Department of Biochemistry, Institute of Health Basic Sciences, Federal University of Rio Grande do Sul, Porto Alegre, RS, Brazil;

[2]Center for Agricultural Sciences, Foundation Federal University of Pampa, Itaqui, RS, Brazil.

[3]Integrated Center for Laboratory Analysis Development (NIDAL), Department of Food Technology and Science, Center of Rural Sciences, Federal University of Santa Maria, Santa Maria, RS, Brazil.

[4]Departament of Health Sciences, Lutheran University of Brazil, Santa Maria, RS, Brazil.

*Corresponding author: tatiemanuelli@gmail.com (Tatiana Emanuelli)

Tel.: +55 55 3220 8547; fax: +55 55 3220 8353.

Abstract

Objectives: This study aimed at assessing paraoxonase (PON1) and thioredoxin reductase (TrxR-1) activities and their relationship with lipids, oxidative stress and inflammation in subjects with different low-density lipoprotein (LDL) levels.

Design and Methods: Serum lipids, high-sensitivity C-reactive protein (hs-CRP), lipids and proteins oxidation, oxidized LDL (LDLox) and LDLox autoantibodies (LDLoxAB) levels, PON1, TrxR-1 and superoxide dismutase (SOD) activities were measured in group I (N=42, LDL levels <130 mg/dL), group II (N=34, LDL levels between 130 and 160 mg/dL) and group III (N=41, LDL levels >160 mg/dL).

Results: Lipid and protein oxidation increased in group III, while LDLox, LDLoxAB and hs-CRP levels as well as TrxR-1 and SOD activities increased equally in groups II and III. PON1 activity did not change among groups.

Conclusion: Inflammatory and oxidative events initiate when LDL levels are clinically acceptable and the meaning of TrxR-1 and PON1 behaviors in this study needs to be elucidated.

Keywords: Hypercholesterolemia; Protein carbonyl content; Thiobarbituric acid reactive substances; Oxidized low density lipoprotein.

1. Introduction

Atherosclerosis is the main underlying mechanism of leading causes of death, such as heart and brain disorders [1]. Hypercholesterolemia (HC), especially high levels of low density lipoprotein-cholesterol (LDL), seems to be an important risk factor accounting for severe atherosclerotic diseases, since LDL enters into vessels by a concentration-dependent mechanism [2]. Once into the endothelium, LDL suffers oxidative attack by reactive oxygen species (ROS) on its lipid and protein components, generating oxidized LDL (LDLox) [3]. Additionally, LDLox can initiate and enhance the inflammatory process, which plays a pivotal role in the development of atherosclerotic changes [4]. Accordingly, the levels of highly sensitive C-reactive protein (hs-CRP), an inflammation marker, are enhanced in patients with high levels of total cholesterol [5].

It has been suggested that high density lipoprotein (HDL) protects LDL particles from the oxidative process and subsequent inflammation [6]. This protective action of HDL is due to its associated enzyme paraoxonase1 (PON1) [6]. Although PON1 protects against atherosclerosis in both *in vitro* and *in vivo* models [7] and PON1 activity is inversely associated to the incidence of coronary artery disease [8], there is no data on the behavior of PON1 during different stages of HC.

Another line of defense against oxidative stress during HC set on enzymatic defenses such as superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GSH-Px), which play an important role in alleviating negative effects from ROS generation. Thioredoxin reductase (TrxR-1) is a redox-active selenoprotein that efficiently regenerates oxidized thioredoxin protein (Trx-1) to its reduced form [9]. Reduced Trx-1 acts as antioxidant maintaining the reduced state of many proteins [9]. Furthermore, TrxR-1 is overexpressed and released during oxidative stress and has been recently detected in plasma [10]. Although elevated Trx-1 levels have been found during heart failure, fulminant

myocarditis and coronary vasospasm in humans [11-13], few reports are available on TrxR-1 participation during coronary events and/or in patients with major risk factors, such as HC.

Considering the growing evidences about reduced PON1 activity as a predictive factor for cardiovascular diseases in humans and the link between HC, oxidative stress and inflammation in atherogenesis, the aim of this study was to evaluate the behavior of PON1 activity at different stages in the development of HC and the association between PON1 activity, oxidative stress and inflammation markers and circulating lipids in the blood of subjects with different LDL levels. We also investigated the behavior of TrxR-1 activity at different stages of HC and its relationship with the oxidative and inflammatory processes. Results will contribute to clarify the role of PON1 and TrxR-1 in atherosclerosis.

2. Material and methods

2.1. Study population

Population studied consisted of patients from LABIMED (Santa Maria, RS, Brazil). All subjects gave written informed consent to participate in the study. The protocol was approved by the Research Ethics Committee of the Federal University of Santa Maria (Protocol number: 23081.019182/2007-10). Subjects were divided into three groups according to serum LDL levels, as follows: group I, LDL levels <130 mg/dL (3.4 mmol/L); group II, LDL levels ranging from 130 to 160 mg/dL (3.4-4.15 mmol/L); and group III, LDL levels > 160 mg/dL (4.15 mmol/L). These LDL ranges were selected to correspond approximately to the actual clinical interval criterion for cardiovascular risk factors, where LDL levels < 130mg/dL are considered safe; between 130 to 160 mg/dL are clinically acceptable, but close to the limit; and LDL levels > 160 mg/dL are considered to be well above the safe limit for risk of cardiovascular disease. Smokers, patients with blood glucose

levels >95 mg/dL and patients undergoing hypolipemic or anti-inflammatory treatment were excluded from the study.

2.2. Sample collection and analysis

Blood samples were collected after a 12-h overnight fasting by venous puncture into Vacutainers® (BD Diagnostics, Plymouth, UK) tubes with no anticoagulant. Blood samples were routinely centrifuged within 1 h of collection for 15 min at 2500 x g, and aliquots of serum samples were immediately used to assess TBARS levels and TrxR-1 activity. Then, serum samples were stored at −20°C for a maximum of 4 weeks before remaining measurements. Total cholesterol (TC) and triglyceride (TG) concentrations were measured by standard enzymatic methods using Ortho-Clinical Diagnostics® reagents on a fully automated analyzer (Vitros 950® dry chemistry system; Johnson & Johnson, Rochester, NY, USA). HDL cholesterol was measured after precipitation of apolipoprotein B-containing lipoproteins with dextran sulfate and magnesium chloride, as previously described [14]. LDL was estimated with the Friedewald equation [15]. Hs-CRP was measured by nephelometry (Dade Behring, Newark, DE, USA), while LDLox was determined by a capture ELISA according to the manufacturer instructions (Mercodia AB, Uppsala, Sweden) and as described before [16]. LDLoxAB were determined using ELISA as described by Wu and Lefvert [17]. Lipid peroxidation, measured as TBARS levels, was assessed after the addition of 7.2 mM of butylated hydroxytoluene to prevent further oxidation, reaction with thiobarbituric acid and extraction with *n*-butanol as previously described [18]. The reaction product was determined at 535 nm using a standard curve of 1,1,3,3-tetraethoxypropane. Protein oxidation was assessed as protein carbonyl content based on the reaction of the carbonyl groups with 2,4-dinitrophenylhydrazine to form 2,4-dinitrophenylhydrazone [19]. Samples were read at 370 nm and carbonyl content was calculated using the molar absorption coefficient for aliphatic

hydrazones (22,000 M^{-1} cm^{-1}). SOD activity was determined at 480 nm using 50 mM glycine buffer, pH 10.2, and 1 mM epinephrine at 30°C [20]. SOD activity was expressed as the amount of enzyme that inhibits the auto-oxidation of epinephrine to adrenochrome by 50%, which is equal to 1 unit. TrxR-1 activity was determined using 5,5′-dithiobis (2-nitrobenzoic acid) (DTNB) and adenine dinucleotide phosphate reduced. The method is based on the reduction of DTNB, which is indicated by an increase in absorbance at 412 nm [21]. PON1 activity was assessed by measuring the rate of paraoxon hydrolysis to yield p-nitrophenol, at 412 nm and 25°C [22]. The amount of p-nitrophenol generated was calculated using the molar extinction coefficient 17,000 M^{-1} cm^{-1} and 1 U of PON1 activity is defined as 1 nmol p-nitrophenol generated per minute [22].

2.3. Statistical analysis

Data were analyzed by one-way analysis of variance (ANOVA) followed by Duncan's test when appropriate. Data that did not exhibit a normal distribution were transformed (log or square root transformation) in order to meet ANOVA assumptions before analysis. When a variable was found not following normal distribution even after log or square root transformation, it was analyzed by the nonparametric Kruskal-Wallis ANOVA followed by the *post hoc* Dunn´s test when appropriate. The associations between variables were evaluated by Pearson's correlation for variables that had a normal distribution and by Spearman's rank order correlation for variables that did not exhibit a normal distribution. Results were considered significant when $p<0.05$.

3. Results

Population characteristics are shown in Table 1. No significant differences were found between groups concerning age ($p>0.05$). TC levels increased significantly along with the

increase in LDL levels among groups ($p<0.05$). TG levels were also higher in the high-LDL group when compared to the low-LDL group ($p<0.05$), but no differences were observed between the low and intermediate LDL groups. In contrast, HDL values of the low-LDL group were similar to those of the two other groups, but the intermediate-LDL group had higher HDL levels than the high-LDL group ($p>0.05$).

One-way ANOVA revealed that patients from high-LDL group showed significantly increased TBARS levels and protein carbonyl content when compared to the low and intermediate-LDL groups ($p<0.05$, table 2). The nonparametric Kruskal-Wallis test revealed that LDLox levels and the levels of the inflammatory marker hs-CRP increased in groups along with the increase in LDL levels ($p<0.05$, table 2), while LDLoxAB levels of patients from the intermediate and high-LDL groups were significantly higher than those from low-LDL groups ($p<0.05$, table 2).

SOD and TrxR-1 activity were significantly higher in patients from the intermediate and high-LDL groups compared to the low-LDL group ($p<0.05$), but no significant differences were found between the intermediate and high LDL groups (figures 1A e 1B). No significant differences were observed in PON1 activity among groups ($p>0.05$, figure 1C).

LDL levels were positively correlated to TC, TBARS levels, protein carbonyl content, LDLox, LDLoxAB and hs-CRP levels as well as SOD activity ($p<0.05$, table 3). In contrast, LDL levels had no significant correlation with age, HDL levels, TG levels, TrxR-1 or PON1 activities ($p>0.05$, table 3). TrxR-1 activity was positively correlated with SOD activity ($p<0.05$, figure 2A), while hs-CRP was positively correlated with LDLox as well as LDLoxAB ($p<0.05$, figure 2B and 2C). In addition, a positive correlation between TBARS and LDLox levels was also found ($p<0.05$, figure 2D).

4. Discussion

Cardiovascular diseases (CVD) are clearly the major cause of death in the Western world and there is no doubt that elevated plasma cholesterol levels play a dominant role in these diseases [23]. This can be evidenced by the occurrence of atheroma in aorta of hypercholesterolemic rabbits [24]. Thus, the control of LDL levels, which is the major cholesterol carrier in circulation, is an important step in the prevention of CVD [23]. The injury theory of atherosclerosis holds that circulating LDL accumulates at susceptible sites where they undergo modifications including oxidation of its protein and lipid components, generating LDLox [25]. LDLox, in turn, participates in the inflammatory processes, contributing to lesion progression [25]. Our results corroborate this theory, since elevated concentrations of TBARS and protein carbonyl groups, products of lipid and protein oxidation respectively, were found in patients from the high-LDL group. These results indicate that LDL levels above the safe limit induce serum lipid and protein oxidation. Accordingly, oxidation in serum lipids and proteins has been observed in hypercholesterolemic rabbits [26]. However, increased oxidation of LDL was found in the intermediate-LDL group even without lipid or protein oxidation. This can be explained because TBARS and protein carbonyl assays evaluated lipid and protein oxidation in the whole serum, not only LDL oxidation, which would render them less sensitive to oxidative changes in LDL. In addition, TBARS values mainly reflect malondialdehyde (MDA), an aldehyde derived from the oxidation of unsaturated fatty acids, providing little information about the extent of oxidation in other lipoprotein lipids, such as cholesterol [27]. Despite this, we observed a positive correlation between TBARS and LDLox, reinforcing that TBARS levels are at least partially associated to LDLox levels. Concerning the protein oxidation process, sometimes amino groups from LDL are attacked by oxidant species and proteins are converted to non-carbonyl species [28]. Thus, the protein carbonyl content assay may not reflect the whole extension of protein oxidation. Furthermore, increased LDLox levels in the

intermediate-LDL group suggest that LDL oxidation takes place even when LDL levels are considered clinically acceptable. We also observed that LDLox increased in the intermediate-LDL group accompanied by LDLoxAB elevation, probably as an attempt to prevent LDLox citotoxicity. In agreement, it has been demonstrated that LDLoxAB blocks the uptake of oxidized LDL by macrophages [29]. However, while LDLox levels increased in the high-LDL group when compared to the intermediate-LDL group, this increase was not accompanied by LDLoxAB elevation. This may indicate that the immune system plays a modifying important role in atherosclerosis under more "normal" conditions, as previously suggested [30]. Thus, in severely proatherogenic conditions, such as hypercholesterolemic situations, this role may be overshadowed by the proatherogenic lipoproteins [30]. This explanation is supported by the fact that mouse lacking both B and T cells (RAG-1 knockout) and apoE develop significantly less atherosclerosis than apoE single knockout mice when fed standard chow, but not when mice are fed a cholesterol-rich diet [31]. Although we found a positive correlation between LDL and LDLoxAB levels, we can not rule out that the immune response in the high-LDL group has been eclipsed by high LDL levels. All these data, along with the correlations between LDL levels and TBARS levels, protein carbonyl content, LDLox, LDLoxAB indicate that oxidative stress is an early event in the evolution of hyperlipidemia, as previously suggested [32].

Plasma lipoproteins are protected against oxidative modification by the antioxidant defense system of the organism, constituted by the enzymes SOD, glutathione peroxidase and catalase, as well as non-enzymatic hydrophilic antioxidants such as ascorbate, reduced glutathione and urate. All these molecules react with ROS or block free radical chain reactions [33]. The enzyme TrxR-1 along with protein Trx-1 has been recognized as an essential component for cellular redox control and antioxidant defense [34,35]. In this study, we demonstrated for the first time the behavior of serum TrxR-1 activity during different

stages of HC in humans, although an elevation in serum Trx-1 has already been described in patients with HC [36]. Additionally, results from our research group revealed an increase in serum TrxR-1 activity in hypercholesterolemic rabbits (unpublished data). We observed that TrxR-1 activity increased in the intermediate and high-LDL groups when compared to the low-LDL group. Thus, LDL levels close to limit, but still clinically acceptable may induce an increase of TrxR-1 activity, in order to prevent oxidative events. This hypothesis is corroborated by increased TrxR-1 expression in atherosclerotic plaques when compared to health tissue from the same patients [37]. The thioredoxin system (TrxR-1 and Trx-1) acts as a hydrogen peroxide (H_2O_2) scavenging system, reducing H_2O_2 to H_2O [38]. SOD activity had a behavior similar to that of TrxR-1 along the LDL-groups, which may indicate that TrxR-1 activity was increased to remove H_2O_2 produced by SOD. In fact, we found a weak, but significant correlation between TrxR-1 and SOD activities. The elevation of SOD activity in this study occurred most likely to counteract superoxide anion overproduction caused by HC [39]. Besides scavenging H_2O_2, we can speculate that TrxR-1 activity increased in the intermediate-LDL group in order to prevent protein oxidation in this group, by regenerating oxidized Trx-1 to its reduced form [9]. Thus, reduced Trx-1 can act as antioxidant maintaining the reduced state of many proteins [9]. Accordingly, we did not observe protein oxidation in the intermediate-LDL group when compared to the low-LDL group. Interestingly, SOD and TrxR-1 activities did not differ between the intermediate and high-LDL groups, which suggests that both enzymes may have an important role in early hypercholesterolemic events. Moreover, SOD and TrxR-1 activities are susceptible to oxidative stress [40, 41] and we can not rule out that the lack of changes in both enzymes in the high-LDL group when compared to the intermediate-LDL group may reflect a balance state between induction and inactivation process. Furthermore, we observed increased protein oxidation in the high-LDL group when compared to the low-LDL group, which probably is a

result from mild TrxR-1 inhibition process. Despite the interesting changes in TrxR-1 activity in this study, no correlation was observed between LDL levels and TrxR-1 activity.

Similar to TrxR-1, the behavior of PON1 activity at different stages of HC has not been demonstrated yet. PON1 is believed to be responsible for the antioxidant effects of HDL [6] and its activity is inversely associated with the progression of atherosclerosis and the incidence of coronary artery disease [42]. In agreement, previous results from our group revealed diminished serum PON1 activity along with atherosclerotic plaque in the aorta of hypercholesterolemic rabbits (unpublished data). Moreover, a large prospective study pointed low serum PON1 activity as an independent risk factor for coronary events in men at high risk of coronary heart disease [43]. However, we did not found differences in PON1 activity among the three studied groups or significant correlations between LDL levels and PON1 activity. This could be explained by an established balance between PON1 inactivation by oxidant species [43] and PON1 elevation in order to provide a protecting mechanism against oxidant species and atherosclerotic plaque progression [45]. In fact, PON has previously been shown to accumulate in the artery wall during atherosclerosis, with an increase in PON immunoreactivity with the progression of disease from fatty streaks to advanced plaques [45]. Also, these differences in PON1 activity between previous studies and ours can be due the fact that studies concerning PON1 and cardiovascular diseases usually evaluated the relationship between serum enzyme activity and plaque formation [46] or cardiovascular events such as myocardial infarction [43]. Here, we evaluated the relationship between PON1 activity and another known predicting factor for cardiovascular diseases, the LDL levels. Interestingly, despite the known inhibitory effect of LDLox on PON1 activity [47], we found no association between PON1 activity and LDLox levels. In fact, the lack of associations between LDLox and PON1 activity has already been described in conditions such as type 2 diabetes [48] and familial HC [49]. Moreover, PON1 seems not play a role in early

atherosclerosis, although it may play a role in a later stage of cardiovascular diseases [49]. Since the atheroma formation was not evaluated in this study, we can not rule out that unchanged PON1 activity and the lack of associations between PON1, LDL and LDLox levels are most likely because LDL levels evaluated in this study corresponded to early stages of atherosclerosis. Thus, the involvement of serum PON1 in subclinical vascular events, such as HC, needs further investigation before a final statement.

Oxidative stress leads to an inflammatory process as well as the inflammatory process induces continued oxidative stress [50]. Accordingly, we observed a significant correlation between LDLox and hs-CRP, a marker of inflammatory process. The level of hs-CRP in the serum of patients with different levels of TC and its relationship with LDLox has already been demonstrated [51]. Moreover, those authors found that serum hs-CRP increased only in the group with the highest levels of TC and with LDL levels similar of those of the high-LDL group of the present study. In contrast, we observed increased serum hs-CRP also in the intermediate-LDL group, which indicates that inflammation process takes place even when LDL levels are considered safe.

In conclusion, the present study demonstrated that inflammatory and oxidative events initiate even when LDL levels are clinically acceptable. Moreover, important changes in TrxR-1 activity in patients with intermediate and high-LDL levels were found in this study and need further investigations. Also, the actual role of serum PON1 as a predictor of cardiovascular events remains controversial.

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Table 1: Characteristics of the studied groups

	Groups (LDL-cholesterol levels)		
	I (< 130 mg/dL)	II (130-159 mg/dL)	III (> 160 mg/dL)
N	42	34	41
Age (years)	$52.7_{\pm}1.9$ (28.0-77.0)	$54.8_{\pm}2.3$ (29.0-87.0)	$55.6_{\pm}2.5$ (31.0-75.0)
Sex (male/female)	23/19	17/17	16/25
TC (mg/dL)	$160.4_{\pm}5.6^{a}$ (72.0-243.0)	$222.0_{\pm}2.7^{b}$ (198.0-270.0)	$275.3_{\pm}6.3^{c}$ (226.0-449.0)
TG (mg/dL)	$115.8_{\pm}11.0^{a}$ (20.0-331.0)	$138.8_{\pm}16.2^{ab}$ (46.0-430.0)	$148.9_{\pm}9.1^{b}$ (67.0-300.0)
LDL (mg/dL)	$91.5_{\pm}3.9^{a}$ (24.0-129.6)	$145.5_{\pm}1.8^{b}$ (131.6-187.4)	$203.8_{\pm}6.0^{c}$ (162.0-341.8)
HDL (mg/dL)	$44.0_{\pm}2.2^{ab}$ (14.0-75.0)	$48.8_{\pm}2.4^{a}$ (33.0-95.0)	$41.7_{\pm}2.0^{b}$ (20.0-91.0)

Results are mean $\pm$ SEM (minimum – maximum). [a,b]Values within the same line that do not share a common superscript letter are significantly different ($P<0.05$). TC=total cholesterol; TG=triglycerides; LDL=low-density lipoprotein; HDL=high-density lipoprotein.

Table 2: Oxidative stress and inflammatory markers in patients with different LDL levels

	Groups (LDL-cholesterol levels)		
	I (< 130 mg/dL)	II (130-159 mg/dL)	III (> 160 mg/dL)
TBARS (nmol MDA/mL)	$4.9_{\pm}0.2^{a}$ (2.4-8.5)	$5.1_{\pm}0.2^{a}$ (2.8-7.0)	$6.3_{\pm}0.3^{b}$ (4.0-13.3)
Protein carbonyl content (nmol/mg of protein)	$1.3_{\pm}0.1^{a}$ (0.2-4.9)	$1.3_{\pm}0.1^{a}$ (0.7-2.3)	$1.9_{\pm}0.2^{b}$ (0.5-6.7)
LDLox (mg/L)	$0.28_{\pm}0.04^{a}$ (0.0-1.1)	$0.42_{\pm}0.05^{b}$ (0.06-1.7)	$0.71_{\pm}0.07^{c}$ (0.03-1.9)
LDLoxAB (mg/L)	$11.6_{\pm}1.7^{a}$ (0.7-36.0)	$26.9_{\pm}1.1^{b}$ (16.7-46.4)	$32.1_{\pm}1.4^{b}$ (9.2-54.7)
hs-CRP (mg/L)	$0.4_{\pm}0.04^{a}$ (0.1-1.2)	$1.05_{\pm}0.32^{b}$ (0.1-7.9)	$1.5_{\pm}0.26^{c}$ (0.1-7.9)

Results are mean $\pm$ SEM (minimum – maximum). N= 34-42, as shown in Table 1. ^{a,b}Values within the same line that do not share a common superscript letter are significantly different ($p<0.05$). TBARS=thiobarbituric acid reactive substances; MDA=malondialdehyde; LDLox= oxidized LDL; LDLoxAB= LDLox autoantibodies; hs-CRP= highly sensitive C-reactive protein.

Table 3. Correlations of serum LDL with age, lipid levels, PON1 activity and oxidative stress and inflammation markers.

Parameters	LDL correlations coefficient
Age (years)	0.05
TC (mg/dL)	0.95*
HDL (mg/dL)	-0.04
TG (mg/dL)	0.18
TrxR- 1 (U/L)	0.1
Protein carbonyl (nmol/mg of protein)	0.25*
TBARS (nmol MDA/mL)	0.31*
SOD (U/L)	0.38*
PON1 (U/mL)	-0.016
LDLox (mg/L)	0.51*
LDLoxAB (mg/L)	0.64*
Hs-CRP (mg/L)	0.43*

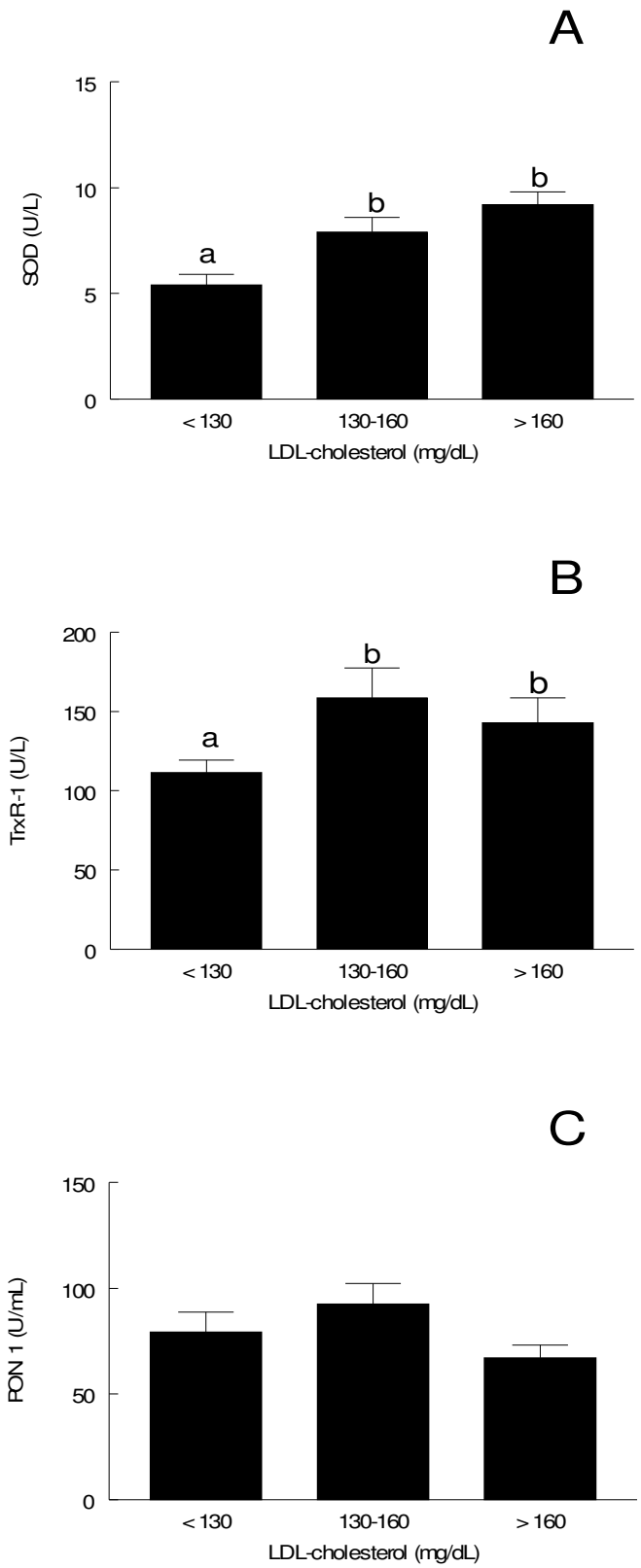
The associations between LDL versus TC, HDL, TG, TrxR-1, carbonyl groups, TBARS and SOD were evaluated by Pearson's correlation, while the associations between LDL and PON 1, LDLox, LDLoxAB and hs-CRP were evaluated by Spearman's rank order correlation. Correlations were considered significant when $p<0.05$. TC=total cholesterol; TG=triglycerides; TBARS=thiobarbituric acid reactive substances; MDA= malondialdehyde; SOD= superoxide dismutase; TrxR-1= thioredoxin reductase 1; PON1= Paraoxonase 1; LDLox= oxidized LDL; LDLoxAB= LDLox autoantibodies; hs-CRP= highly sensitive C-reactive protein.

Figures legends:

Figure 1. SOD (A), TrxR-1 (B) and PON1 (C) activities of patients with different LDL levels. Results are mean ± SEM (n = 34-42, as shown in Table 1). Different letters indicate a significant difference at $p < 0.05$ between groups. SOD= superoxide dismutase; TrxR-1= thioredoxin reductase 1; PON1= Paraoxonase 1.

Figure 2. Significant correlations between (A) TrxR-1 and SOD, (B) hs-CRP and LDLox, (C) hs-CRP and LDLoxAB and (D) TBARS and LDLox. TBARS= thiobarbituric acid reactive substances; MDA= malondialdehyde; SOD= superoxide dismutase; TrxR-1= thioredoxin reductase 1; LDLox= oxidized LDL; LDLoxAB= LDLox autoantibodies; hs-CRP= highly sensitive C-reactive protein.

Figure 1


A
SOD (U/L)
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a
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130-160
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LDL-cholesterol (mg/dL)
C
PON 1 (U/mL)
150
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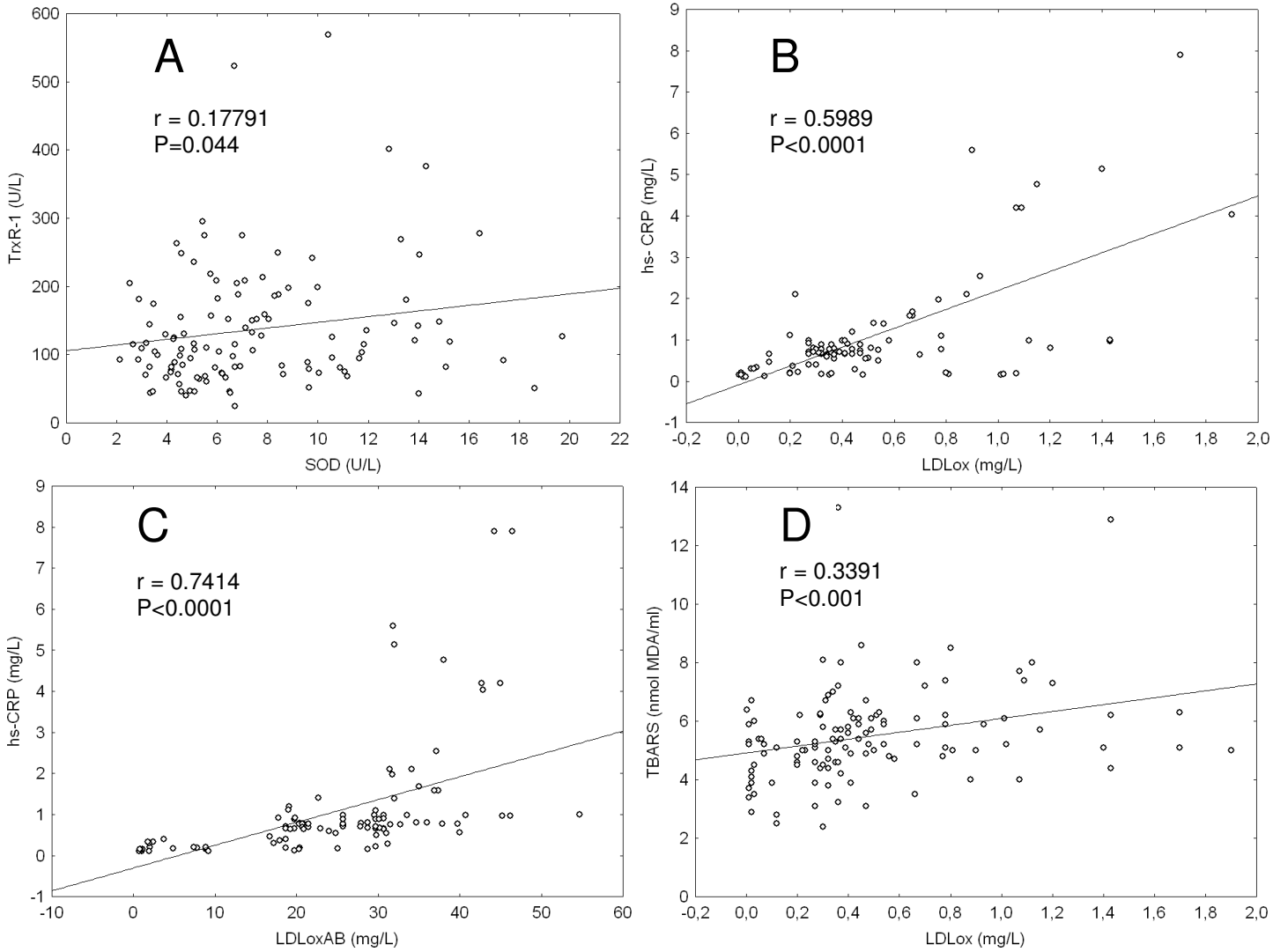
< 130
130-160
> 160
LDL-cholesterol (mg/dL)

Figure 2


A
r = 0.17791
P=0.044
TrxR-1 (U/L)
SOD (U/L)
B
r = 0.5989
P<0.0001
hs- CRP (mg/L)
LDLox (mg/L)
C
r = 0.7414
P<0.0001
hs-CRP (mg/L)
LDLoxAB (mg/L)
D
r = 0.3391
P<0.001
TBARS (nmol MDA/ml)
LDLox (mg/L)

CAPÍTULO II

Astaxanthin reduces oxidative stress, but not aortic damage in atherosclerotic rabbits

Paula R. Augusti, Greicy M.M. Conterato, Sabrina Somacal, Rocheli Sobieski, Andréia Quatrin, Luana Maurer, Marta P. Rocha, Ione T. Denardin, Tatiana Emanuelli

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Astaxanthin Reduces Oxidative Stress, but not Aortic Damage in Atherosclerotic Rabbits

Paula R. Augusti, MSc, Greicy M. M. Conterato, MSc, Sabrina Somacal, BSc, Rocheli Sobieski, BSc, Andréia Quatrin, Luana Maurer, Marta P. Rocha, MD, Ione T. Denardin, MSc, and Tatiana Emanuelli, PhD

We evaluated whether carotenoid astaxanthin (ASX) could prevent oxidative and atherosclerotic damage in rabbits. Rabbits received regular chow (control) or an atherogenic diet (1% cholesterol) alone or supplemented with 50, 100, and 500 mg% ASX for 60 days (n = 5-9 per group). The atherogenic diet increased the serum cholesterol levels and the ratio of the intima/media area in the aortic arch. These changes were not prevented by ASX. Atherosclerotic rabbits showed increased aortic lipid peroxidation and nonprotein thiol group (NPSH) levels along with inhibition of glutathione peroxidase (GSH-Px). All ASX doses attenuated lipid peroxidation and the increase in NPSH but not the inhibition of GSH-Px. Aortic superoxide dismutase (SOD), catalase (CAT), and thioredoxin reductase (TrxR) activities were enhanced in atherosclerotic rabbits. Although all ASX doses prevented the increase in SOD activity, only 100 and 500 mg% ASX prevented the increase in CAT activity. Furthermore, these same doses partially prevented the increase in TrxR activity, while 50 mg% ASX completely prevented the effects of the atherogenic diet on this enzyme. However, ASX did not attenuate the hypercholesterolemia or the atherosclerotic lesions caused by the atherogenic diet at any of the doses evaluated. Our results indicate that although ASX did not prevent hypercholesterolemia or atherosclerotic lesions, it could play a beneficial role by preventing lipid peroxidation and changes in antioxidant enzyme activities.

Keywords: antioxidant enzymes; carotenoids; intima/media ratio; thiobarbituric acid reactive substances

From the Graduate Program in Biological Sciences/Biochemistry, Department of Biochemistry, Institute of Health Basic Sciences, Federal University of Rio Grande do Sul, Porto Alegre, RS, Brazil (PRA); Graduate Program in Toxicological Biochemistry, Center of Natural and Exact Sciences (GMMC), Integrated Center for Laboratory Analysis Development (NIDAL), Department of Alimentary Technology and Science, Center of Rural Sciences (SS, RS, AQ, LM), Department of Pathology, Center of Health Sciences (MPR), Polytechnical School, Center of Rural Sciences (ITD), and Department of Food Technology and Science (IE), Federal University of Santa Maria, Santa Maria, RS, Brazil.

The work was carried out at the Integrated Center for Laboratory Analysis Development (NIDAL), Department of Food Technology and Science, Center of Rural Sciences, Federal University of Santa Maria, Santa Maria, RS, Brazil.

All authors declare that there is no proprietary, financial, professional or other personal interest of any nature or kind that could influence the position presented in this manuscript.

Address correspondence to: Tatiana Emanuelli, Department of Food Technology and Science, Center of Rural Sciences, Federal University of Santa Maria, 97105-900, Santa Maria, RS, Brazil; e-mail: tatiemanuelli@smail.ufsm.br.

Introduction

Atherosclerosis is an inflammatory disease of the arterial wall that remains a major cause of death and disability all over the world. High plasma levels of low-density lipoprotein (LDL) and its oxidation followed by LDL accumulation into macrophages residing in the arterial wall are a central feature in the development of atherosclerosis.[1,2] Low-density lipoprotein oxidation in vivo affects both its lipid and protein components[1] and generates biologically active oxidation products. The oxidation of lipids can be assessed by measuring thiobarbituric acid reactive substances (TBARS) that are an index of oxidative

stress. The cellular uptake of oxidized LDL leads to the generation of reactive oxygen species (ROS), such as superoxide anion, hydrogen peroxide (H_2O_2), or hydroxyl radicals, which have been implicated in the development of atherosclerosis during hypercholesterolemia.[3,4] Antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GSH-Px), and thioredoxin reductase (TrxR) are able to protect cells against oxidative damage. In addition, nonenzymatic antioxidants such as glutathione (GSH), which is the major non protein thiol-containing compound in most tissues, may also prevent free radical-induced injury.[5] Due to the convincing evidence of the involvement of ROS in the atherogenic process, it would be worthwhile to find new antioxidant substances effective to counteract atherosclerosis.

Astaxanthin (ASX), a red carotenoid pigment without provitamin A activity, is a biological antioxidant that occurs naturally in a wide variety of living organisms such as plants, algae, and seafood.[6] Despite its antioxidant potential in lipid membranes,[7] there are few reports about the antiatherogenic potential of ASX. Astaxanthin inhibits human LDL oxidation in vitro and ex vivo[8] and stabilizes atherosclerotic plaques.[9] Moreover, ASX has been suggested as a novel potential treatment of oxidative stress and inflammation in cardiovascular disease, especially in ischemia-reperfusion injury and in vascular thrombotic occlusion.[6] However, supplementation with ASX for 24 weeks did not protect Watanabe heritable hyperlipidemic rabbits against LDL oxidation and atherosclerosis.[10]

The objective of this investigation is to clarify whether dietary supplementation with ASX attenuates oxidative damage and atherosclerosis by evaluating serum total cholesterol levels along with histopathological damage and markers of oxidative stress in the aortic tissue of atherogenic rabbits.

Methods and Materials

This study was approved by the Ethics and Animal Welfare Committee of the Federal University of Santa Maria (23081.019182/2007-10). Eight-week-old male New Zealand white rabbits were housed in individual cages, under a 12:12-hour light:dark cycle with access to 100 g chow (SUPRA, São Leopoldo, Brazil) per day and water ad libitum.

After 1 week of adaptation, the rabbits weighing 1.7 ± 0.03 kg were randomly divided into 5 experimental groups. The control group was fed a standard diet chow (SUPRA); the atherosclerotic group was fed the standard diet chow enriched with 1% cholesterol (Vetec, Brazil) to induce atherosclerotic lesions; ASX 50, ASX 100, and ASX 500 groups were fed the same cholesterol-rich diet plus 50, 100, and 500 mg of natural ASX (2% powder; Fuji Chemical Industry Co Ltd, Toyama, Japan) per 100 g chow, respectively. Cholesterol and ASX were dissolved in diethyl ether and sprayed on the standard chow to yield the cholesterol-enriched and ASX-supplemented diets. The standard chow of the control group was sprayed with diethyl ether (vehicle). The standard and supplemented chows were left to allow the ether to evaporate before storage protected from light and under refrigeration. Chows were prepared once a week. All animals received a daily portion of 100 g chow and their body weight was checked once a week.

The amount of cholesterol chosen for treating the animals was based on previous studies[11,12] and on a pilot study, which demonstrated that the dietary supplementation with 1% cholesterol for 60 days induced atherosclerotic lesions. Astaxanthin concentrations in the diets were chosen based on previous studies[9,10] and on the doses considered safe for humans.[13]

After 60 days of dietary treatment, the rabbits were food-deprived for 1 night and were killed with an intravenous overdose of thiopental. Blood samples were collected into tubes without additives and centrifuged at 3000*g* for 10 minutes. Serum was immediately removed and stored at −20°C until analysis of cholesterol levels. Aortas (between the origin and the bifurcation of iliac arteries) were immediately excised and cleaned of gross adventitial tissue. Approximately 2 cm of the aortic arch was removed for histopathological examinations, and the remaining tissue was homogenized in 10 volumes of 150 mmol/L NaCl. One part of the homogenate was used to assess the levels of nonprotein thiol groups (NPSH) and TBARS. Another part of the homogenate was centrifuged at 3000*g* at 4°C for 10 minutes to yield a low-speed supernatant that was used to determine antioxidant enzyme activities.

Serum Cholesterol Analyses

Serum total cholesterol was determined using commercial kit reagents (Doles, Goiania-GO, Brazil).

The assay consisted of an enzymatic method that uses cholesterol esterase, cholesterol oxidase, and peroxidase in the presence of 4-aminoantipyrine/phenol to form a quinoneimine that is measured at 510 nm.

Histopathological Analyses of Aortic Atherosclerotic Lesions

For histopathological analysis, a 10% formalin-fixed aortic segment was embedded in paraffin. Sections of 5 μm were obtained with a standard microtome and were stained with hematoxylin and eosin. The sections were examined by a pathologist without the knowledge of the experimental groups who measured the intima layer/media layer ratio. This ratio was used as an index of atherosclerosis as described before.[14] Because the lesions were not uniform, the results of each sample were expressed as the mean of 5 different measurements along the aortic section.

Lipid Peroxidation

After the addition of 7.2 mmol/L butylated hydroxytoluene to prevent further oxidation, the homogenate was used for the determination of TBARS.[15] Samples were extracted with *n*-butanol, and the reaction product was determined at 535 nm using a standard curve of 1,1,3,3-tetraethoxypropane.

Nonprotein Thiol Groups

The homogenate fraction was mixed with 10% trichloroacetic acid (1:1 v/v), followed by centrifugation. Nonprotein thiol groups were immediately determined as described by Ellman[16] using a standard curve of cysteine.

Antioxidant Enzymes

Superoxide dismutase activity was determined based on its ability to inhibit the autoxidation of adrenaline to adrenochrome at an alkaline pH.[17]

Catalase activity was determined using H_2O_2 as the substrate.[18] The pseudo-first order reaction constant (k) of the decrease in H_2O_2 absorption at 25°C was determined, and specific activity was expressed as k/g protein.

Glutathione peroxidase activity was determined using GSH reductase and nicotinamide adenine dinucleotide phosphate reduced (NADPH). The method is based on the oxidation of NADPH, which is indicated by a decrease in absorbance at 340 nm.[19]

Thioredoxin reductase-1 (TrxR-1) activity was determined using 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) and NADPH.[20] The method is based on the reduction of DTNB, which is indicated by an increase in absorbance at 412 nm.

Glutathione S-transferase (GST) activity was determined by measuring the increase in absorbance at 340 nm using 1-chloro-2,4-dinitrobenzene as the substrate.[21]

Glutathione reductase (GR) activity was determined using oxidized GSH and NADPH.[22] The method is based on the oxidation of NADPH, which is indicated by a decrease in absorbance at 340 nm.

Protein Quantification

Protein was measured using bovine serum albumin as standard.[23]

Statistical Analysis

Data were analyzed using 1-way analysis of variance (ANOVA) followed by post hoc analyses with Duncan multiple range test when necessary. All variables were checked for normality. When a variable was found that did not follow normal distribution, it was logarithmically transformed before analysis. Data on GST activity and histopathological data on the intima layer/media layer ratio did not follow normal distribution even after log transformation and therefore were analyzed by the nonparametric Kruskal-Wallis test followed by the post hoc Nemenyi test. Results were expressed as the mean ± standard error (SEM), and differences were considered statistically significant when $P < .05$.

Results

No differences were found among the experimental groups with respect to the weight gain during the experimental period (data not shown). At the beginning of the experiment, the average body weight of the animals was 1.7 ± 0.03 kg and the animals grew to a final body weight of 2.7 ± 0.03 kg.

Analysis of variance revealed a significant increase in the serum total cholesterol levels of all cholesterol-fed groups when compared to the control group ($P < .05$), but no difference was found among the cholesterol groups fed with different levels of ASX (0-500 mg%; Figure 1A). The nonparametric Kruskal-Wallis test revealed an increase in the ratio

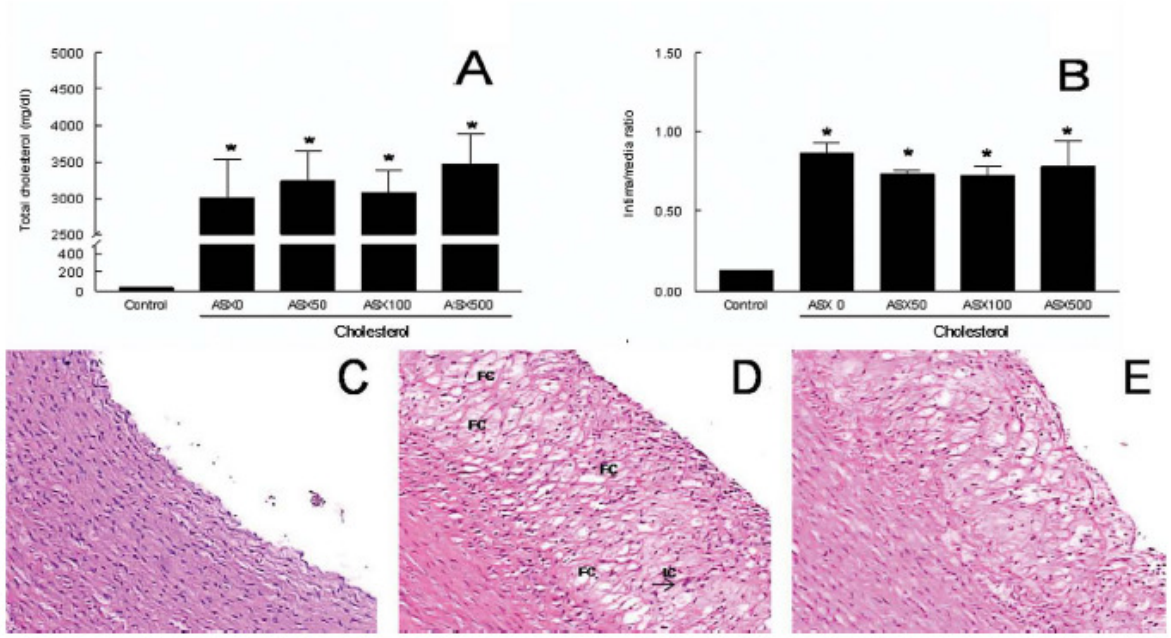

Figure 1. Serum cholesterol levels (A), intima layer/media layer ratio (B, magnification of ×40) and representative histology (C-E, magnification of ×100) of aortic arch of rabbits fed with cholesterol and/or astaxanthin (ASX)-enriched diet. Tissues were fixed in 10% formalin, embedded in paraffin, sectioned at 5 μm, and stained with hematoxylin and eosin. C, control group. D, ASX 0 group. E, ASX 500 group. Data are means ± SEM. In (A), n = 9 for control and ASX 0 groups, n = 8 for ASX 100 and 500 groups, and n = 7 for ASX 50 group. In (B), n = 5 for all groups. *Different from control group ($P < .05$). Observe the preserved architecture of the intima layer with regular endothelial cells and the absence of foam cells in the control group (C). In the ASX 0 group (D), there is loss of endothelial architecture with high subintimal deposits of foam cells (FC) and inflammatory cells (IC) showing the extension and thickening of the aortic arch. In ASX 500 group (E), there are features identical to those observed in D, indicating the lack of effect of ASX. ASX 0, cholesterol-enriched diet; ASX 50, cholesterol-enriched diet plus 50 mg% ASX; ASX 100, cholesterol-enriched diet plus 100 mg% ASX; ASX 500, cholesterol-enriched diet plus 500 mg% ASX.

of the intima layer/media layer area in the aorta of all cholesterol-fed groups when compared to the control group ($P < .05$; Figure 1B-1E). These results confirm that the cholesterol-enriched diet induced hypercholesterolemia and caused atherosclerotic lesions. Dietary supplementation with ASX did not prevent these effects at any of the doses evaluated.

The intake of an atherogenic diet for 60 days significantly increased TBARS and NPSH content in the aortic tissue ($P < .05$; Figure 2), and ASX did prevent these effects at the doses evaluated.

Analysis of variance revealed a significant effect of the atherogenic diet on the activity of all antioxidant enzymes evaluated (Figure 3). Post hoc comparisons demonstrated that SOD, CAT, and TrxR-1 activities were increased in the aortic tissue of atherosclerotic rabbits when compared to the control group ($P < .05$; Figure 3A, 3B, and 3D). The increase in SOD activity was prevented by all ASX doses, while only 100 and 500 mg% ASX prevented the increase in CAT activity. Only 50 mg% ASX was able to completely prevent the increase in TrxR-1 activity caused by the atherogenic diet, while 100 and 500 mg% ASX partially prevented this effect. Post hoc comparisons also demonstrated that GSH-Px activity decreased in the aortic tissue of animals that received a cholesterol-enriched diet when compared to the control group ($P < .05$; Figure 3C), and no dose of ASX prevented GSH-Px inhibition. Neither the atherogenic diet nor the ASX-supplemented diet changed the GR or GST activities in the aortic tissue (data not shown).

Discussion

Male New Zealand white rabbits fed with a high cholesterol diet were used in the current study because

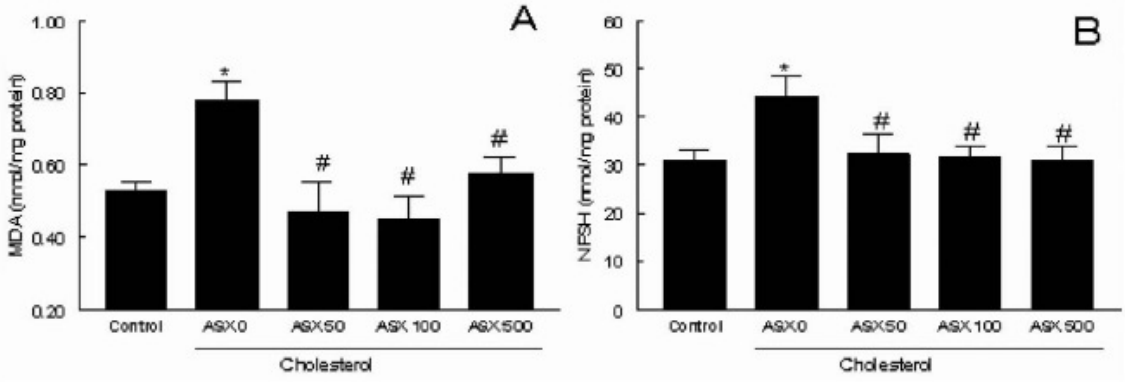

Figure 2. TBARS (A) and NPSH levels (B) of aorta from rabbits fed a cholesterol and/or astaxanthin (ASX)-enriched diet. Data are means ± SEM. In (A), n = 7 for control and ASX 0 groups, n = 6 for ASX 100 group, and n = 5 for ASX 50 and 500 groups. In (B), n = 7 for ASX 50 group, n = 5 for control, ASX 0, and ASX 100 groups, and n = 6 for ASX 500 group. *Different from control group ($P < .05$). #Different from ASX 0 group ($P < .05$). ASX 0 indicates cholesterol-enriched diet; ASX 50, cholesterol-enriched diet plus 50 mg% ASX; ASX 100, cholesterol-enriched diet plus 100 mg% ASX; ASX 500, cholesterol-enriched diet plus 500 mg% ASX. MDA, malondialdehyde; NPSH, nonprotein thiol groups; TBARS, thiobarbituric acid reactive substances.

this species is well known for their tendency to develop severe hypercholesterolemia when the dietary cholesterol level is increased.[24] In addition, there is a strong relationship between the levels of plasma cholesterol and the degree of aortic lesions in this species,[24] and the distribution of atherosclerotic lesions in the rabbit model resembles atherosclerosis in humans to a certain degree.[25] Accordingly, we observed severe atherosclerotic damage along with hypercholesterolemia in animals receiving a cholesterol-enriched diet for 60 days.

In our study, the intake of the atherogenic diet significantly increased the NPSH content in the aortic tissue. This increase has been reported before[26] and seems to be related to the increased expression of GR, which was previously reported in human macrophages exposed to oxidized LDL in vitro.[27] Although GR activity was unchanged in atherosclerotic rabbits in our study, we observed decreased GSH-Px activity, which could also explain the increased NPSH levels. This enzyme uses GSH (GSH, the major NPSH) as substrate and its inhibition would result in higher GSH levels. The inhibition of this enzyme is in agreement with data from human atherosclerotic plaques, indicating the susceptibility of GSH-Px to oxidant species.[28] In fact, the deficient GSH-Px activity of the atherosclerotic tissue may significantly weaken its antioxidant potential favoring oxidative stress and atherogenic processes, even in the presence of an apparently adequate amount of low-molecular-weight scavenging antioxidants.[29] Additionally, the lack of GSH-Px activity in atherosclerotic lesions seems to be associated with a more severe expression of atherosclerosis in humans.[28] Interestingly, atherosclerotic rabbits presented increased SOD, CAT, and TrxR-1 activities despite GSH-Px inhibition. This increase is in agreement with the increased expression of SOD, CAT, and TrxR in macrophages responding to oxidized LDL in vitro and in human atherosclerotic plaques.[27] Additionally, an increase in SOD and CAT activities in the aortic tissue of atherosclerotic rabbits has been described before.[30] The increase of antioxidant enzymes activities, as well as the rise in NPSH levels probably occurred as a defense response against free radicals. In fact, hypercholesterolemia increases superoxide anion production in the aortic tissue,[31] and SOD is one of the primary enzymes involved in arterial wall protection.[27] It is able to remove the superoxide anion by converting it to H_2O_2. The latter can be additionally removed by CAT, GSH-Px, or TrxR.

The enhanced activity of TrxR-1 may indicate that this enzyme plays a role in the decomposition of H_2O_2 generated by SOD. Thioredoxin reductase is the only class of enzymes known to reduce oxidized thioredoxin, which is involved in scavenging oxidant species such as H_2O_2 and alkyl peroxides.[32] In fact, the upregulation of TrxR in human macrophages has been described as a cellular defense against oxidized LDL and possibly modulates the development of atherosclerosis.[27] In addition, this is the first study

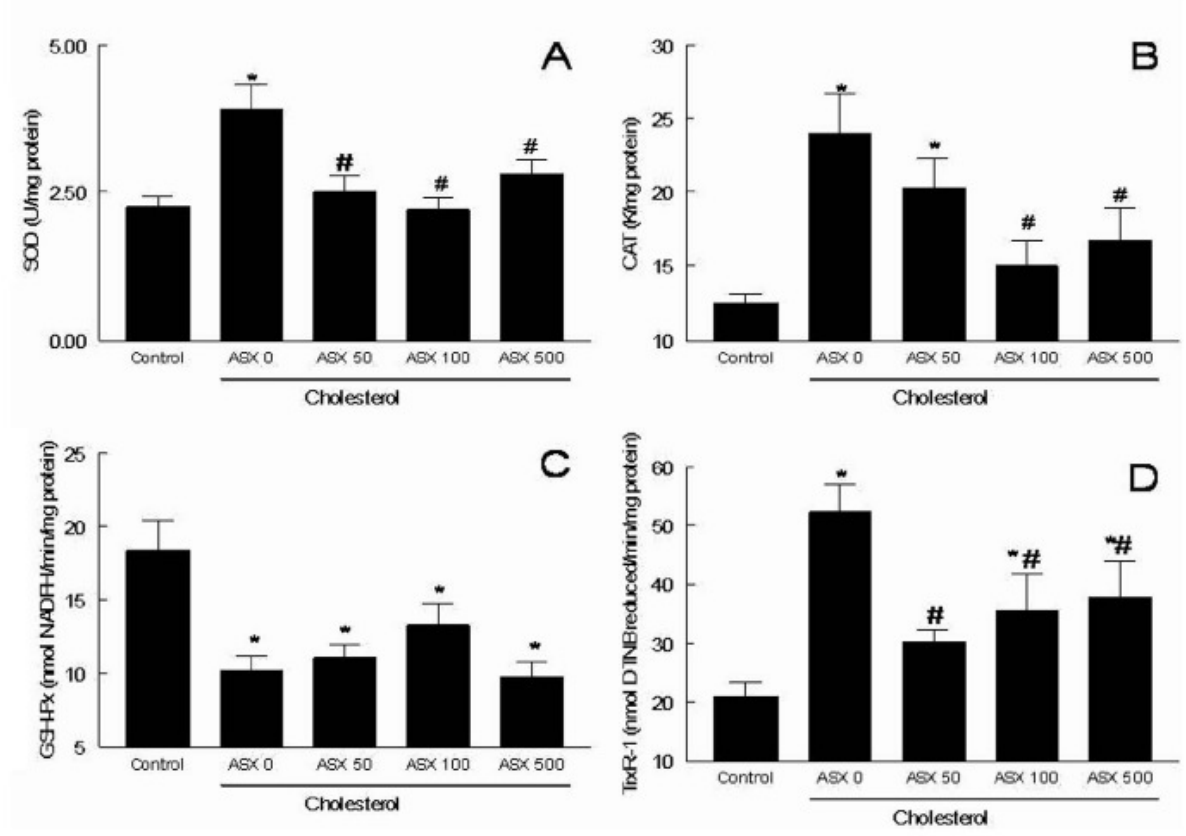

Figure 3. Superoxide dismutase (A), CAT (B), GSH-Px (C), and TrxR-1 (D) activities from aorta of rabbits fed cholesterol- and/or astaxanthin (ASX)-enriched diet. Data are expressed as means ± S.E.M. In (A), n = 8 for control group, n = 7 for ASX 0 group, n = 6 for ASX 50 group, and n = 5 for ASX 100 and 500 groups. In (B), n = 6 for control, ASX 0 and ASX 500 groups, and n = 5 for ASX 50 and 100 groups. In (C), n = 7 for ASX 0 and 50 groups, n = 6 for control group and n = 5 for ASX 100 and 500 groups. In (D), n = 8 for control group, n = 7 for ASX 0 group, n = 6 for ASX 50 group, and n = 5 for ASX 100 and 500 groups. *Different from control group ($P < .05$). #Different from ASX 0 group ($P < .05$). ASX 0 indicates cholesterol-enriched diet; ASX 50, cholesterol-enriched diet plus 50 mg% ASX; ASX 100, cholesterol-enriched diet plus 100 mg% ASX; ASX 500, cholesterol-enriched diet plus 500 mg% ASX; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); CAT, catalase; GSH-Px, glutathione peroxidase; SOD, superoxide dismutase; TrxR-1, thioredoxin reductase.

evaluating TrxR-1 activity in atherosclerotic rabbits. Because this is a key enzyme in redox regulation, it may play an important protective role against oxidative stress in atherosclerosis.

Despite the rise in NPSH content and antioxidant enzyme activities, we observed the occurrence of lipid peroxidation in atherosclerotic rabbits. This indicates that the free radicals generated by hypercholesterolemia exceeded the endogenous antioxidant activity and induced tissue damage. In fact, lipid peroxidation is a fundamental event in hypercholesterolemic atherosclerosis. Lipoproteins are modified by free radical-mediated reactions, leading to alterations that are a starting point for the first events in the outcome and development of the atherogenic process.[33]

Astaxanthin, a reddish-colored carotenoid, is a powerful antioxidant that acts as a chain-breaking scavenger of free radicals.[6] A water-dispersible ASX derivative was approved as an animal food additive in 1987, and natural microalgae or shrimp shell extracts containing esterified ASX have been approved by the Food and Drug Administration (FDA) as nutraceuticals.[6] Furthermore, the derivatives of ASX are being developed for pharmaceutical applications in human disorders where oxidative stress-mediated damage may be important, including

cardiovascular diseases.[6] A human trial is currently under development to investigate whether the antioxidant and anti-inflammatory potential of ASX could be helpful in renal transplant patients, because cardiovascular disease is the major cause of morbidity in these patients.[34]

Some recent reports have described the protective effect of ASX against oxidative stress by maintaining mitochondria in the reduced state and protecting its functional integrity against oxidative stress in vitro.[35] Protection of mitochondrial function and antioxidation properties are also associated with the neuroprotective potential of ASX in vitro.[36,37] In addition, ASX attenuated oxidative stress and DNA damage caused by the anticancer drug cyclophosphamide in mouse liver.[38] In the current study, we found that all ASX doses prevented lipid peroxidation and the increase in NPSH in atherosclerotic rabbits. Lipid peroxidation proceeds as a chain reaction into the arterial wall. This reaction may be interrupted only by suitable antioxidants within LDLs themselves.[39] In fact, ASX has been demonstrated to protect against lipid oxidation at the surface and inside the phospholipid membrane in vitro by scavenging lipid radicals and destroying peroxides.[40] This effectiveness can be explained by the location of ASX inside membranes, where the polar ring of ASX most likely scavenges ROS near the membrane surface, while the polyene chain inhibits the radical chain reaction into the membrane.[40] Although all doses of ASX prevented the increase in SOD activity, only 100 and 500 mg% ASX prevented the increase in CAT activity. Furthermore, these same doses partially prevented the increase in TrxR-1 activity, while 50 mg% ASX completely prevented the effects of an atherogenic diet on this enzyme. This effect could be attributed to the high scavenging potential of ASX, protecting aortic tissue against free radicals generated by hypercholesterolemia and sparing antioxidant defenses. This protective effect of ASX against changes in antioxidant enzymes is in agreement with previous reports, which demonstrated that ASX prevented changes in the activities of SOD, GSH-Px, and CAT in animal models of gastric ulcer.[41,42] Moreover, ASX attenuated changes in antioxidant enzyme activities induced by inorganic mercury in kidneys.[43] To the best of our knowledge, this is the first report about the protective effect of ASX against changes in antioxidant enzymes in an atherosclerotic model. However, no ASX dose prevented the impairment in GSH-Px activity.

The different effects of the varied doses of ASX on the antioxidant enzymes could be due to the distinct role of these enzymes in oxidative processes.[44] In fact, SOD catalyzes the conversion of the superoxide anion to H_2O_2, and this can be removed by CAT, GSH-Px, and TrxR-1. However, H_2O_2 can also be generated by the prostaglandin pathway, which is enhanced by the presence of high cholesterol levels.[45] Additionally, GSH-Px seems to be more sensitive to inhibition by oxidant species than other enzymes.[28] Thus, we can speculate that the inability of ASX to protect against GSH-Px inhibition is either due to a higher production of H_2O_2 associated to enzyme sensitivity and/or due to a limited capacity of ASX to prevent such production. In addition, it is possible that the location of antioxidant enzymes in the cells may influence the modulatory effect of ASX.[44] Thus, the location of CAT in peroxisomes could hamper the access of low doses of ASX. This is in agreement with β-carotene modulation of the increase of SOD induced by a high fat diet, along with the absence of effect on CAT activity.[46]

Despite its protection against oxidative damage, ASX did not prevent the increase in serum cholesterol levels nor did it protect against atherosclerotic lesions in this study. These results are in agreement with a previous study, which revealed a lack of hypocholesterolemic and antiatherogenic effects of ASX in Watanabe heritable hyperlipidemic rabbits.[10] This finding suggests that mechanisms other than oxidative damage may also contribute to the artery injury induced by hypercholesterolemia, and that these mechanisms could not be completely controlled by ASX. Alternatively, some protective effect of ASX against aortic damage may have been undetected in the current study. In fact, previous studies in Watanabe heritable hyperlipidemic rabbits revealed that ASX decreased macrophage infiltration, apoptosis, and atheroma vulnerability without an improvement in LDL oxidizability or in the macroscopic lesion size.[9,10] These findings were interpreted as a beneficial stabilizing effect of ASX on the atherosclerotic plaques that would possibly contribute to reduce the risk of rupture and thrombus formation.[9] Because plaque composition and stability were not evaluated in the current study, we cannot rule out the possibility that ASX could have had such an effect in our experimental model.

Concerning the antioxidative effects of ASX in atherosclerotic models, there are conflicting data showing the lack of ASX protection against LDL oxidation in Watanabe heritable hyperlipidemic rabbits[10] along with protection against human LDL oxidation in vitro and ex vivo.[8] These controversies could be explained by differences among studies in relation to animal species and experimental periods. Hence, there are still many controversies regarding ASX potential as an antiatherogenic agent.

In conclusion, we demonstrated that, although ASX did not prevent the increase of serum cholesterol levels or the aortic damage induced by a hypercholesterolemic diet, protection against lipid peroxidation and changes in antioxidant defenses may be achieved through the use of ASX. However, there is a need for further studies before a conclusive statement may be made on the potential usefulness of ASX as a therapeutic agent in cardiovascular diseases.

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CAPÍTULO III

Dietary astaxanthin improves paraoxonase activity and oxidative status in hypercholesterolemic rabbits: a lipid-lowering independent effect

Paula R. Augusti, Greicy M.M. Conterato, Sabrina Somacal, Rocheli Sobieski, Andréia Quatrin, Amanda R. Ruviaro, Luana H. Maurer, Marta F. F. Duarte, Tatiana Emanuelli

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Dietary astaxanthin improves paraoxonase activity and oxidative status in hypercholesterolemic rabbits: a lipid-lowering independent effect

Paula R. Augusti[1,2], Greicy M.M. Conterato[3], Sabrina Somacal[3], Rocheli Sobieski[3], Andréia Quatrin[3], Amanda R. Ruviaro[3], Luana H. Maurer[3], Marta F. F. Duarte[4], Tatiana Emanuelli[3*]

[1] Graduate Program in Biological Sciences/Biochemistry, Department of Biochemistry, Institute of Health Basic Sciences, Federal University of Rio Grande do Sul, 90035-003, Porto Alegre, RS, Brazil;

[2]Center for Agricultural Sciences, Foundation Federal University of Pampa, 97650-000, Itaqui, RS, Brazil.

[3]Integrated Center for Laboratory Analysis Development (NIDAL), Department of Food Technology and Science, Center of Rural Sciences, Federal University of Santa Maria, 97105-900, Santa Maria, RS, Brazil.

[4]Departament of Health Sciences, Lutheran University of Brazil, 97020-001, Santa Maria, RS, Brazil.

Running title: Astaxanthin in hypercholesterolemia

*Corresponding author: T. Emanuelli, Tel.: +55 55 3220 8547; fax: +55 55 3220 8353, e-mail tatiemanuelli@gmail.com.

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Abstract

Oxidative stress plays an important role in hypercholesterolemia and atherosclerosis. This study explored the effects of the antioxidant astaxanthin on the lipid profile, oxidative stress and paraoxonase activity in hypercholesterolemic rabbits. Male New Zealand white rabbits were fed a standard or a hypercholesterolemic diet (1 % cholesterol) alone or supplemented with 50, 100 or 500 mg % of astaxanthin for 60 days. Serum levels of total cholesterol, triglycerides, oxidised LDL and oxidised LDL antibodies were measured. Thiobarbituric acid reactive substances levels and carbonyl content in proteins were also measured as lipid and protein oxidation indicators. Paraoxonase and antioxidant enzymes activities as well as fatty acids composition were evaluated in serum. The hypercholesterolemic diet increased serum total cholesterol and triglycerides levels. This increase in serum lipids was accompanied by elevated oxidised LDL and oxidised LDL antibodies, as well as lipid and protein oxidation. In addition, hypercholesterolemia decreased SFA and increased unsaturated fatty acids levels in the serum. Astaxanthin (100 and 500 mg %) attenuated protein oxidation in hypercholesterolemic rabbits, whereas 500 mg % of astaxanthin reduced protein oxidation *per se*. In addition, the activities of antioxidant enzymes superoxide dismutase and thioredoxin reductase were enhanced, while paraoxonase activity was inhibited in hypercholesterolemic rabbits. Although all astaxanthin doses prevented changes in thioredoxin reductase and paraoxonase activities, the change in superoxide dismutase activity was not improved by astaxanthin. Our study suggests that astaxanthin could play a beneficial role in hypercholesterolemia by preventing protein oxidation as well as changes in thioredoxin reductase and paraoxonase activities.

Introduction

Hypercholesterolemia is widely accepted as one of the major risk factors for the development of atherosclerosis[1]. LDL, which is the major carrier of cholesterol in the circulation, can undergo oxidative modification by endothelial cells, vascular smooth muscle or macrophages within the arterial wall. The cellular uptake of oxidised LDL (LDLox) also leads to the generation of reactive oxygen species (ROS)[2]. Accordingly, increased levels of LDLox and oxidised LDL antibodies (LDLoxAB) have been found in the serum of hypercholesterolemic (HC) subjects[3].

The imbalance between ROS production and antioxidant mechanisms in the arterial wall and circulating cells leads to oxidative stress, which appears to play an important role in hypercholesterolemia and atherosclerosis[4]. LDL oxidation in vivo affects both its lipid and protein components and can be assessed by measuring thiobarbituric acid reactive substances (TBARS) and protein carbonyl contents[5]. In addition, LDL size and oxidisability seem to be related to its fatty acid composition[6]. However, the relationships between LDL oxidisability, fatty acid composition and oxidant/antioxidant status in hypercholesterolemia are still not clear.

As LDL oxidation can initiate atherogenesis, antioxidant status should have a major impact not only on the rate of LDL oxidation but also on the development of atherosclerosis[4]. Thus, the administration of antioxidant substances could make LDL less sensitive to oxidation. Another line of defence against oxidative stress might be the hydrolysis of specific oxidative products by hydrolases such as paraoxonase (PON1). PON 1 is a circulating enzyme that is attached to HDL. It protects plasma lipoproteins (including LDL) from oxidative modification by hydrolysing bioactive lipid hydroperoxides and reducing peroxides to their respective hydroxides[7]. In humans, low PON1 activity is an independent predictor of acute coronary events[8].

Supplementation with antioxidant-containing foodstuffs such as polyphenol-[9,10] and carotenoid-rich[11-12] fruits showed a beneficial effect against cardiovascular diseases. Astaxanthin (ASX) is a naturally occurring carotenoid with no pro-vitamin A activity and strong antioxidant properties. This red pigment is synthesised by plants and algae and is distributed mainly in marine seafood[13]. Although ASX has been suggested as a novel potential treatment for oxidative stress and inflammation in cardiovascular disease[14], there are still many controversies about the protective potential of ASX in cardiovascular events. ASX had no protective effect against LDL oxidation and atherosclerotic lesion formation in Watanabe heritable hyperlipidemic (WHHL) rabbits[15] or against aortic damage in atherosclerotic New Zealand rabbits[16]. Moreover, no studies have examined the role of ASX in PON1 activity during experimental hypercholesterolemia.

100 The objective of the present investigation is to determine the effects of ASX on
101 hypercholesterolemia by evaluating lipid levels, PON1 activity, oxidative status and fatty acid
102 composition in the serum of HC rabbits.

103 **Experimental methods**

104 *Animals and diets*

105 This study was approved by the Ethics and Animal Welfare Committee of the Federal University of
106 Santa Maria (23081.019182/2007-10) and experimental procedures followed the Institutional and
107 National guidelines for the care and use of animals. Eight-week-old male New Zealand white
108 rabbits were housed in individual cages, under a 12:12 hour light:dark cycle with access to 100 g of
109 chow per day and water ad libitum.

110 After 1 week of adaptation, the rabbits weighing 1.83 ± 0.33 kg were randomly divided into
111 eight experimental groups, each one with six animals. The control group was fed a standard diet
112 chow (SUPRA, São Leopoldo, Brazil); the HC group was fed a diet enriched with 1 % cholesterol
113 (Vetec, Brazil); and the supplemented groups were fed natural ASX (2% powder, Fuji Chemical
114 Industry Co. Ltd., Toyama, Japan) at 50, 100 and 500 mg % in standard or HC diets. The standard
115 diet chow had (g/kg) 120 moisture, 170 crude protein, 20 ether extract, 140 ash, 130 crude fibre, 12
116 phosphorus, and 6 calcium. Cholesterol and ASX were dissolved in diethyl ether and sprayed on the
117 standard chow to yield the cholesterol-enriched and ASX-supplemented diets. The standard chow of
118 the control group was sprayed with diethyl ether (vehicle). The standard and supplemented chows
119 were left to allow the ether to evaporate before storage, protected from light and under refrigeration.
120 Chows were prepared once a week. All animals received a daily portion of 100 g chow, and their
121 body weight was checked once a week.

122 The cholesterol dose was chosen based on a previous study, where dietary supplementation
123 with 1 % of cholesterol for 60 days increased serum total cholesterol (TC), LDL and triglycerides
124 (TG) levels[17]. Furthermore, we conducted a pilot experiment in order to corroborate this
125 experimental model. ASX concentrations in diets were based on a previous study[15] and on doses
126 considered safe for humans[18]. Blood samples were collected from rabbits fasted for twelve hours
127 by cardiac puncture before (baseline) and after 60 days of treatment. At the end of the experimental
128 period, animals were sacrificed with an intravenous overdose of thiopental. Blood samples were
129 collected into tubes without additives and centrifuged at 3,000 *g* for 10 min. Serum was removed
130 immediately and stored at -20°C until biochemical analyses.

131

132 *Serum lipids*

Total cholesterol (TC) and triglycerides (TG) levels were determined by enzymatic methods, using commercial kit reagents (Doles, Goiania-GO, Brazil).

Lipid peroxidation (LPO)

After the addition of 7.2 mM butylated hydroxytoluene to prevent further oxidation, LPO was estimated by the measurement of TBARS in serum samples as previously described[19]. Samples were extracted with *n*-butanol and the reaction product was determined at 535 nm using a standard curve of 1,1,3,3-tetraethoxypropane.

Protein oxidation

Protein oxidation was determined based on the reaction of the carbonyl groups with 2,4-dinitrophenylhydrazine (DNPH) to form 2,4-dinitrophenylhydrazone[20]. Samples were read at 370 nm and carbonyl content was calculated using the molar absorption coefficient for aliphatic hydrazones (22,000 M^{-1} cm^{-1}).

LDLox and LDLoxAB

LDLox was determined by a capture ELISA according to the manufacturer's instructions (Mercodia AB, Uppsala, Sweden)[21]. LDLoxAB were determined using ELISA as previously described[22].

PON1 activity

PON1 activity was assessed by measuring the rate of paraoxon hydrolysis to yield p-nitrophenol, at 412 nm and 25°C. The amount of p-nitrophenol generated was calculated using the molar extinction coefficient 17,000 M^{-1} cm^{-1} and 1 U of PON1 activity was defined as 1 nmol p-nitrophenol generated per min[23].

Antioxidant enzymes

Superoxide dismutase (SOD) activity was determined based on its ability to inhibit the autoxidation of epinephrine to adrenochrome at an alkaline pH[24]. SOD activity was expressed as the amount of enzyme that inhibits the oxidation of epinephrine by 50 %, which is equal to 1 unit.

Thioredoxin reductase-1 (TrxR-1) activity was determined using 5,5′-dithiobis (2-nitrobenzoic acid) (DTNB) and adenine dinucleotide phosphate reduced (NADPH). This method is based on the reduction of DTNB, which is indicated by an increase in absorbance at 412 nm[25].

Fatty acid composition

Fatty acids in serum were determined by a one-step reaction[26] with some modification. Serum (50 µl) was mixed with 1 ml methanol/isooctane (4:1, v/v) and 100 µl acetyl chloride and was incubated at 100°C for 60 min; then 60 g/l aqueous potassium carbonate containing 100 g/l sodium chloride was added. The mixture was shaken for 10 min at room temperature and centrifuged at 1,800 *g* for 5 min. The isooctane phase, containing the fatty acid methyl esters, was subjected directly to GLC using an Agilent Technologies gas chromatograph (HP 6890) fitted with a capillary column DB-23 (50 % cyanopropyl-methylpolysiloxane, 60 m × 0.25 mm × 0.25 µm) and flame ionisation detection. The temperature of the injector port and the detector was set at 250°C, and the carrier gas was nitrogen (0.6 ml/min). After injection (1 µl, split ratio 50:1), the oven temperature was held at 120°C for 5 min, increased to 240°C at a rate of 4°C min^{-1}, and held at this temperature for 10 min. Standard fatty acid methyl esters (37-component FAME Mix, Sigma, Saint Louis, MO, USA) were run under the same conditions and the subsequent retention times were used to identify the fatty acids. Fatty acids were expressed as percentage of the total fatty acids content.

Protein quantification

Protein was measured using bovine serum albumin as the standard, as described previously[27].

Statistical analysis

Data were analysed using two-way ANOVA (2 cholesterol doses x 4 ASX doses) followed by post hoc Duncan's multiple range test when necessary using the Statistica® 6.0 software system (Statsoft Inc., 2001). Results were expressed as the mean and standard error of the means (SEM) and differences were considered statistically significant when $p < 0.05$.

Results

No differences were found among the experimental groups with respect to weight gain during the experimental period (data not shown). At the beginning of the experiment, the average body weight of the animals was 1.86 ± 0.04 kg, and the animals grew to a final body weight of 2.68 ± 0.22 kg. Also, no differences were found among the experimental groups with respect to all evaluated parameters before the experimental period (baseline values, data not shown).

The profile of the major serum fatty acids in rabbits is presented in Table 1. Feeding rabbits a HC diet for 60 days increased the serum concentration of oleic acid (18 : 1*n*-9) along with an increase in total unsaturated fatty acids (UFA), MUFA and UFA/SFA ratio when compared to the no-ASX control group ($p<0.05$). Also, decreased levels of palmitic acid (16 : 0), estearic acid (18 : 0), arachidonic acid (20 : 4*n*-6) and total SFA were found in HC rabbits when compared to the no-

ASX control group ($p<0.05$). ASX did not affect changes in fatty acid composition induced by a HC diet at any of the evaluated doses ($p>0.05$). In addition, neither cholesterol nor ASX supplementation changed the content of linoleic (18 : 2*n*-6) and linolenic (18 : 3*n*-3) acids or total PUFA in serum of rabbits.

The cholesterol enriched diet increased TC and TG serum levels when compared to control groups (Fig. 1, $p<0.05$), which confirms that the cholesterol-enriched diet induced hyperlipidemia. Dietary supplementation with ASX did not prevent the increase in serum lipids at any of the doses evaluated (Fig. 1).

The HC diet significantly increased TBARS content, protein carbonyl content, as well as LDLox and LDLoxAB levels when compared to the control group (Fig. 2, $p<0.05$). Although the increased levels of TBARS, LDLox and LDLoxAB were not attenuated by any dose of ASX, 100 and 500 mg % of ASX did prevent the increase of protein carbonyl content. In addition, 500 mg % of ASX decreased protein carbonyl content *per se*.

The HC diet inhibited PON1 activity, but increased SOD and TrxR-1 activities when compared to the control group ($p<0.05$; Fig. 3). Although the increase in TrxR-1 activity and PON1 inhibition were attenuated by all ASX doses evaluated, no ASX doses prevented the increase in SOD activity.

Discussion

Cholesteryl esters are the major form of circulating cholesterol in the plasma of cholesterol-fed rabbits[28] and the esterification process requires NEFA, mainly MUFA[29]. Therefore, cholesterol-enriched diets induce MUFA synthesis[29]. Accordingly, we observed increased levels of serum 18 : 1*n*-9 and total MUFA after feeding rabbits with a HC diet for 60 days. Moreover, we found decreased levels of 16 : 0, 18 : 0 and total SFA, along with increased levels of total UFA and UFA/SFA ratio in these animals. In fact, the SFA 16 : 0 and 18 : 0 are consumed to synthesise MUFA as 18 : 1*n*-9[29]. Thus, induction of MUFA synthesis by way of a cholesterol enriched diet would increase plasma lipids and lipoproteins and may lead to cardiovascular diseases[29]. Accordingly, the HC diet increased levels of serum TC and TG along with changes in the fatty acid profile.

Hypercholesterolemia increases the synthesis of prostaglandins and leukotrienes from arachidonic acid and during this process ROS are produced[30]. For this reason, hypercholesterolemia is particularly associated with the increased susceptibility of LDL to oxidation[30]. Accordingly, in the present study we observed that hypercholesterolemia caused arachidonic acid depletion accompanied by LDL oxidation. In addition, we observed increased lipid

and protein oxidation in the serum of HC rabbits. These results indicate that free radicals generated by hypercholesterolemia caused LDL oxidation, particularly in its protein and lipid components. The oxidisability of LDL is dependent on the presence of oxidisable lipids, such as PUFA[31]. However, an increase in LDLox in HC rabbits was not accompanied by PUFA elevation, which can be a result of a balance between PUFA synthesis and degradation. In fact, PUFA may be lost by oxidation in salmon and oxidative events induce a compensatory overproduction of PUFA in order to replace this loss[32].

In our study, ASX supplementation did not prevent changes in the fatty acid composition in HC rabbits. This finding was expected because xanthophylls, such as ASX, are found in chylomicrons and in the serum of humans in a non-esterified free form[33]. Consequently, this carotenoid does not require fatty acids for its transport and does not interfere in fatty acid metabolism. In accordance with the lack of protective effect of ASX against changes in fatty acid composition, this carotenoid was also not able to reduce the increased lipid levels in the serum of HC rabbits. Although these results are in agreement with a previous study that revealed a lack of hypolipidemic effect of ASX in Watanabe heritable hyperlipidemic rabbits[15], this study is the first to evaluate the effects of ASX on serum fatty acid composition during experimental hypercholesterolemia.

LDL oxidation in vivo affects its lipid and protein components at different stages[5]. In the first stage, lipids are oxidised with minor protein oxidation. In the second stage, both lipids and proteins are attacked by free radicals[5]. Moreover, oxidative modifications in these molecules by free radical-mediated reactions are involved in the molecular mechanisms leading to endothelial dysfunction during atherosclerosis induced by hypercholesterolemia[34]. In agreement, HC rabbits showed increased serum lipid and protein oxidation along with high levels of LDLox. LDLox may damage membrane proteins and phospholipids, which can impair the endothelial function[35]. Oxidative modification of LDL induces the formation of immunogenic epitopes in the LDL molecule, leading to the formation of LDLoxAB, which can be detected in serum[35]. Moreover, LDLoxAB in atherosclerotic lesions have been shown to block the uptake of LDLox by macrophages, suggesting a possible role in preventing the formation of foam cells[36]. Accordingly, we observed increased LDLoxAB levels in serum of HC rabbits, which supports the idea that antibodies were secreted in order to combat LDLox toxicity.

ASX did not prevent LPO at any of the doses evaluated in the present study, however, protein oxidation was attenuated by 100 and 500 mg % of dietary ASX. Moreover, 500 mg % of ASX alone decreased protein oxidation, even in the absence of cholesterol in the diet. In fact, ASX is a potent scavenger of oxygen radicals and inhibits the oxidation of proteins[13, 37] alone as well as

269 in the presence of prooxidant treatment[37]. Due to its polarity, ASX is assumed to be attached to the
270 surface of the chylomicrons and lipoproteins as well as in association with serum albumin[38]. Thus,
271 we cannot rule out the thought that ASX would be in a favourable location to prevent protein
272 oxidation rather than lipid oxidation. In fact, LPO proceeds as a chain reaction, which may be
273 terminated only by suitable antioxidants within LDLs themselves[39]. Because ASX seems to be
274 located in the lipoprotein surface rather than in the lipid core, this could hamper its protective
275 ability against LPO. In addition, the lack of protection of ASX against LPO could be responsible for
276 its inability to restore LDLox and LDLoxAB levels. This study is the first to evaluate ASX
277 behaviour against LDLox and LDLoxAB levels.
278 Despite protection against protein oxidation, no ASX doses prevented the increase in SOD
279 activity induced by hypercholesterolemia. In our study, we measured SOD activity in serum, which
280 represents extracellular SOD. This SOD isoenzyme is found in the extracellular matrix of tissues
281 and in extracellular fluids, which allows superoxide anion removal in this specific location[40]. In
282 addition, extracellular SOD is mainly synthesised and secreted from vascular smooth muscle cells
283 and macrophages, playing a central role in cardiovascular antioxidant mechanisms[41]. Accordingly,
284 increased SOD activity in serum is in agreement with the increase in the activity of this enzyme in
285 aortic tissue[42]. SOD activity increased in HC rabbits most likely to remove superoxide anion
286 generated during hypercholesterolemia[42] and superoxide production seems to have exceeded the
287 antioxidant capacity of ASX. SOD catalyses the conversion of the superoxide anion to H_2O_2 and
288 this can be removed by other antioxidant enzymes, such as TrxR-1.
289 TrxR-1 is a redox-active selenoprotein that efficiently regenerates oxidised thioredoxin.
290 Reduced thioredoxin is responsible for maintaining proteins, such as peroxiredoxins, in their
291 reduced state in order to remove H_2O_2 and alkyl peroxides[43]. The increased TrxR-1 activity in HC
292 rabbits may indicate that TrxR-1 plays a protective role in protein oxidation induced by
293 hypercholesterolemia. These facts are supported by the overexpression and release of TrxR-1 in
294 oxidative stress events[44]. Moreover, it suggests a role of TrxR-1 in the decomposition of H_2O_2
295 generated by SOD because TrxR-1 has peroxidase activity, directly reducing H_2O_2[45]. Thus, ASX
296 protection against protein oxidation could explain why it also protected against the increase of
297 TrxR-1 activity because ASX spares this antioxidant enzyme. This protective effect of ASX against
298 changes in TrxR-1 activity has previously been reported in the aortic tissue from atherosclerotic
299 rabbits[16], but this is the first report on serum TrxR-1 activity.
300 PON1 is an enzyme almost exclusively located on serum HDL that is mainly responsible
301 for the breakdown of lipid peroxides before they can accumulate in LDL[7]. PON1 can also inhibit
302 cholesterol biosynthesis in arterial cells and it can stimulate HDL-mediated cholesterol efflux from

macrophages[46]. Despite the important effects of PON1, its enzymatic activity is sensitive to oxidative stress and is readily inactivated by oxidants[47]. Accordingly, PON1 activity was inhibited along with increased LDLox in serum of HC rabbits in the present study. All ASX doses prevented PON1 inhibition in our study. This protective effect could be explained by the absorption, metabolism and transport of xanthophylls, such as ASX, in the blood stream of Atlantic salmon[48] because these processes in salmon are similar to mammalian systems[48]. ASX is transferred to HDL from VLDL and LDL during the transport of these lipoproteins through the blood in salmon[38]. Thus, we suggest that ASX could be next to the attachment site of PON1 in HDL and exerting its antioxidant effects. Although PON1 activity was restored by ASX, this carotenoid did not prevent LDL oxidation in rabbits fed a cholesterol-enriched diet. These findings suggest that factors other than PON1 may influence LDL oxidation and that these factors could not be completely controlled by ASX. In fact, HDL-associated enzymes other than PON1, such as lecithin/cholesterol acyltransferase (LCAT) and platelet-activating factor acyl hydrolase (PAF-AH) were also implicated in the antioxidative properties of HDL[46]. Although the protective effect of ASX on PON1 has been described here for the first time, the effects of ASX on LCAT and PAF-AH still remain unknown.

All protective effects of ASX in the present study could be attributed to the high scavenger potential of ASX, which protects against free radicals generated by hypercholesterolemia, sparing antioxidant defences. ASX stabilises free radicals by adding them to its long double-bond chain rather than donating an atom or electron to the radical[13]. Consequently, it can resist chain reactions that occur when a fatty acid is oxidised, thus allowing it to scavenge or quench longer than antioxidants that cannot stop this chain reaction[13]. For these reasons, natural microalga or shrimp shell extracts containing ASX have been approved by the Food and Drug Administration (FDA) as nutraceuticals[14]. Furthermore, derivatives of ASX are being developed for pharmaceutical applications in human health where oxidative stress-mediated damage may be important, including cardiovascular diseases[14].

In recent years, a number of studies on ASX have demonstrated its antioxidant effects, such as against hepatocellular injury following ischemia/reperfusion[49] and oxidative stress, inflammation and apoptosis induced by high-glucose in kidney cells[50]. In addition, neuromodulatory effects of ASX have been described *in vitro*[51,52] and *in vivo*[53] as well as protective action against oxidative damage in diabetic rats[54] and UVA-induced oxidative damage[55]. Concerning cardiovascular diseases, ASX has been suggested as a potential treatment for oxidative stress and inflammation[14]. Although some reports have shown that ASX does not

protect against LDL oxidation[15] and aortic damage in rabbits[15,16], ASX prevented the oxidation of human LDL *in vitro*[56] and stabilised atherosclerotic plaques in rabbits[57].

In the present study, we demonstrated that ASX improves PON1 activity and oxidative status in HC rabbits but has no lipid-lowering effect. Thus, we propose that protection against protein oxidation and changes in PON1 and TrxR-1 activities are the likely mechanisms underlying the beneficial role of ASX in HC rabbits. Moreover, the absence of ASX effects on fatty acids could underlie the lack of lipid-lowering effect of this carotenoid.

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Legends

Fig. 1. Total cholesterol (A), triglycerides (B), HDL cholesterol (C) and LDL cholesterol (D) levels of rabbits fed with a cholesterol and/or ASX enriched diet. Data are expressed as means ± S.E.M (n = 6). *Different from control groups ($p < 0.05$).

Fig. 2. TBARS levels (A), protein carbonyl content (B), LDLox (C) and LDLoxAB (D) levels of rabbits fed with a cholesterol and/or ASX enriched diet. Data are expressed as means ± SEM (n = 6). *Different from control groups ($p < 0.05$). †Different from ASX 0-Chol group ($p < 0.05$). ‡ Different from ASX 0-control group ($p < 0.05$). MDA = malondialdehyde.

Fig. 3. PON1 (A), SOD (B) and TrxR1 (C) activities of rabbits fed with a cholesterol and/or ASX enriched diet. Data are expressed as means ± S.E.M (n = 6). *Different from control groups ($p < 0.05$). † Different from ASX 0-Chol group ($p < 0.05$).

Table 1 Fatty acid composition (% of total fatty acids) of serum from rabbits fed with a cholesterol and/or ASX enriched diet† (Mean values and standard error of the means for 6 animals per group)

Fatty acids	Control								Cholesterol							
	ASX0		ASX50		ASX100		ASX500		ASX0		ASX50		ASX100		ASX500	
	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
16 : 0	25.0	1.0	25.9	0.4	25.3	0.7	26.8	0.3	20.9*	0.3	21.8*	0.8	22.7*	1.2	23.3*	0.5
18 : 0	13.4	0.8	14.1	0.4	14.0	0.5	13.4	0.4	7.7*	0.4	7.6*	0.7	8.0*	0.7	8.3*	0.3
18 : 1*n*-9	19.4	1.8	17.2	0.9	17.2	0.9	19.0	0.1	30.2*	0.9	31.3*	0.6	29.4*	1.1	29.7*	0.4
18 : 2*n*-6	29.2	1.9	31.1	1.0	31.8	0.8	30.1	2.7	26.4	0.9	26.7	2.2	27.5	1.1	25.5	1.7
18 : 3*n*-3	4.1	1.0	4.0	0.3	3.7	0.5	4.0	0.3	3.3	0.2	3.3	0.2	3.9	0.4	3.6	0.4
20 : 4*n*-6	2.1	0.2	2.3	0.3	2.0	0.3	1.7	0.2	1.2*	0.1	1.5*	0.5	1.2*	0.1	1.2*	0.1
Σ SFA	43.4	1.9	45.6	0.8	45.1	1.7	45.3	1.2	33.7*	1.4	33.0*	2.5	34.2*	2.4	33.7*	1.7
Σ UFA	56.5	2.5	54.3	0.5	54.8	1.7	54.6	1.2	66.2*	1.5	66.9*	2.5	65.6*	2.4	66.1*	1.7
Σ MUFA	21.4	2.0	18.1	1.0	18.5	0.8	20.2	0.5	35.1*	0.7	35.4*	0.7	32.4*	1.5	33.8*	0.9
Σ PUFA	35.1	1.6	36.7	1.8	36.2	1.4	33.3	2.2	31.3	1.0	30.6	2.5	33.3	1.2	32.4	1.8
UFA/SFA ratio	1.4	0.1	1.3	0.0	1.3	0.1	1.3	0.1	2.0*	0.1	2.1*	0.2	2.0*	0.2	2.0*	0.1

ASX, astaxanthin; Σ SFA, sum of SFA; Σ UFA, sum of unsaturated fatty acids; Σ MUFA, sum of MUFA; Σ PUFA, sum of PUFA. * Different from control+ASX0 group ($p<0.05$). †Average content of C6 : 0, C8 : 0, C10 : 0, C12 : 0, C14 : 0, C14 : 1, C15 : 0, C15 : 1, 16 : 1, C17 : 0, C17 : 1, C18 : 3*n*-6, C20 : 0, C20 : 1*n*-9, C21 : 0 and C24 : 0, were lower than 0.5 % of total fatty acids and for this reason, are not shown in the table.

Figure 1

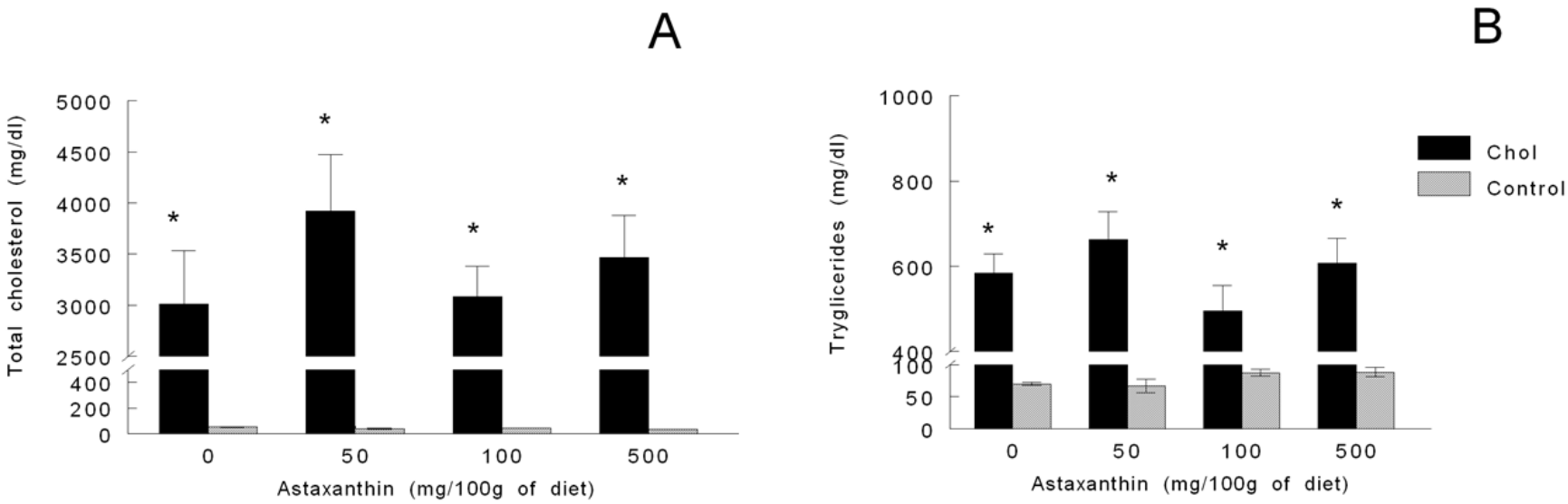

A
B
Total cholesterol (mg/dl)
5000
4500
4000
3500
3000
2500
400
200
0
0
50
100
500
Astaxanthin (mg/100g of diet)
Tryglicerides (mg/dl)
1000
800
600
400
100
50
0
Chol
Control

Figure 2

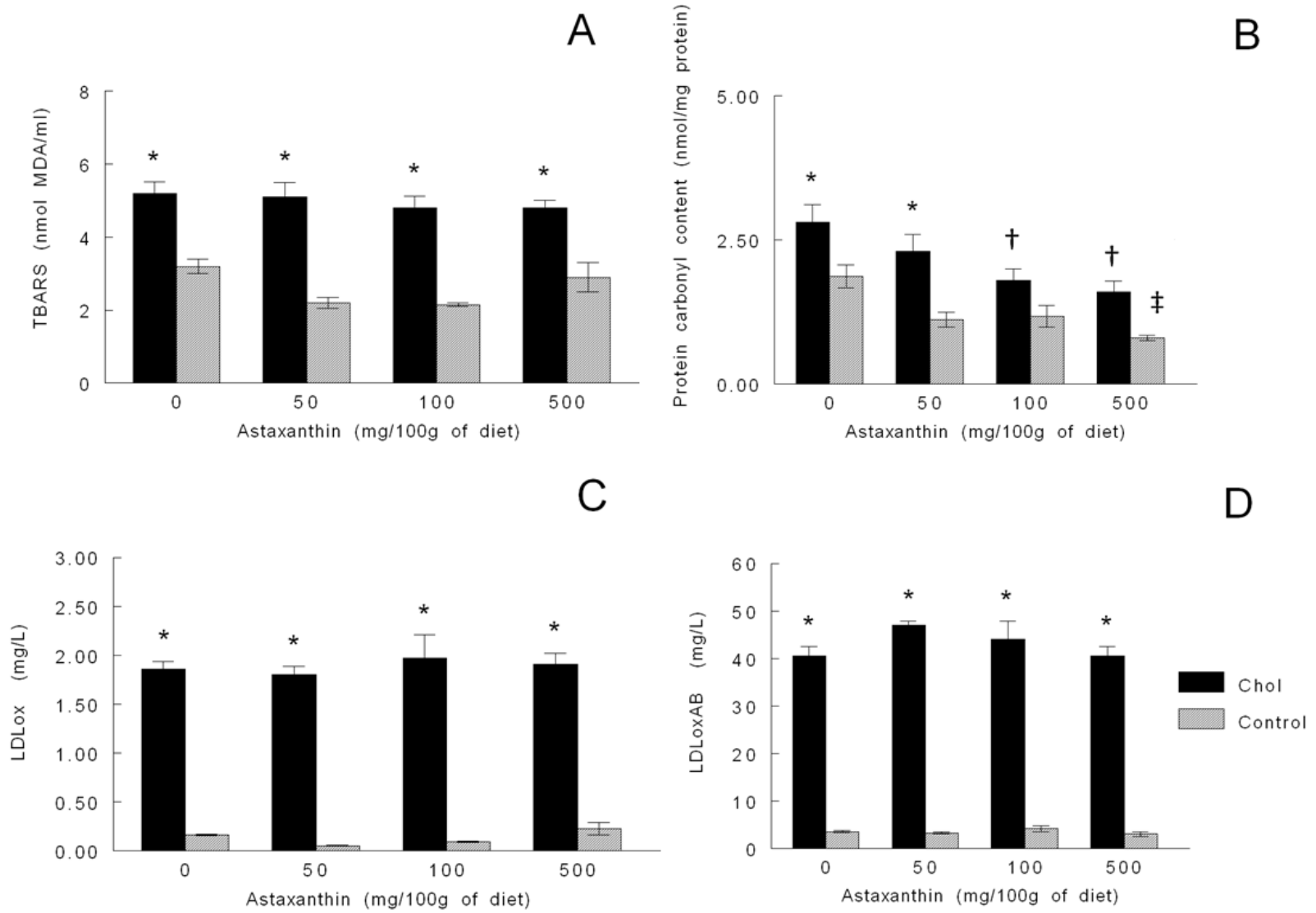
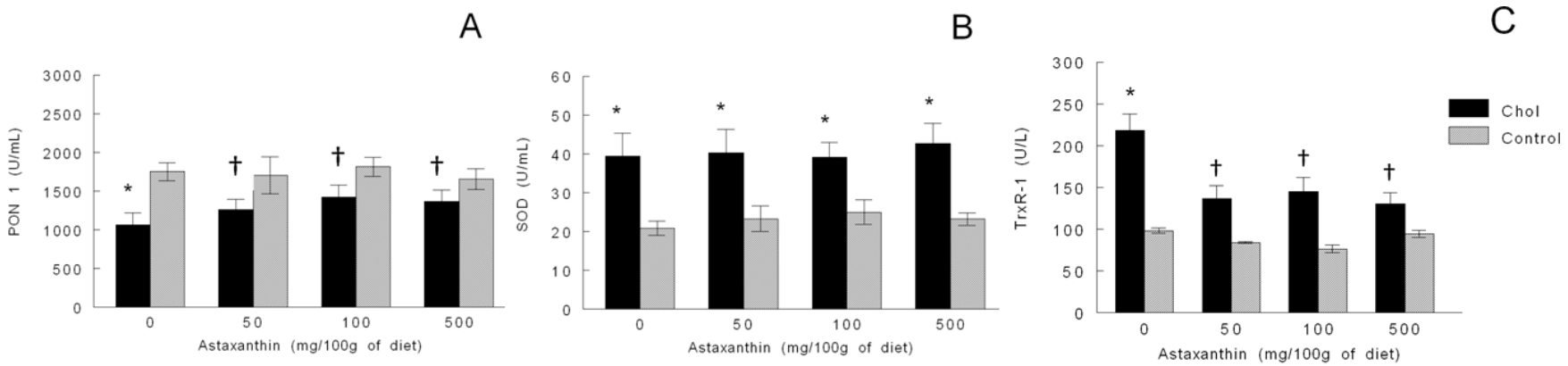

A
TBARS (nmol MDA/ml)
0
2
4
6
8
*
*
*
*
0
50
100
500
Astaxanthin (mg/100g of diet)
B
Protein carbonyl content (nmol/mg protein)
0.00
2.50
5.00
*
*
†
†
‡
0
50
100
500
Astaxanthin (mg/100g of diet)
C
LDLox (mg/L)
0.00
0.50
1.00
1.50
2.00
2.50
3.00
*
*
*
*
0
50
100
500
Astaxanthin (mg/100g of diet)
D
LDLoxAB (mg/L)
0
10
20
30
40
50
60
*
*
*
*
0
50
100
500
Astaxanthin (mg/100g of diet)
Chol
Control

Figure 3


A
B
C
PON 1 (U/mL)
SOD (U/mL)
TrxR-1 (U/L)
Astaxanthin (mg/100g of diet)
Chol
Control
*
†

PARTE III

DISCUSSÃO

O envolvimento do estresse oxidativo na etiologia da aterosclerose é conhecido uma vez que a liberação do ânion superóxido pelas células vasculares, no processo de formação de endoperóxidos cíclicos e prostaglandinas, pode ser responsável pelo início de processos oxidativos em algumas condições, tais como elevados níveis de colesterol na LDL (Lee e Prasad, 2003). Adicionalmente, a participação da lipoxigenase no processo de peroxidação lipídica na parede arterial, envolvendo a geração de hidroperóxidos lipídicos intermediários, está bem estabelecida (Steinberg, 1999). Os resultados do presente trabalho corroboram a participação da oxidação da LDL no processo aterogênico, uma vez que observamos níveis séricos elevados de LDLox concomitantemente com a formação de estrias gordurosas no tecido aórtico de coelhos hipercolesterolêmicos. A oxidação da LDL parece ocorrer em etapas distintas no interior da parede arterial, iniciando nos PUFA dos fosfolipídios da superfície da LDL e, a seguir, se propagando aos lipídios do núcleo hidrofóbico da lipoproteína, resultando em modificação oxidativa tanto dos PUFA como do colesterol e fosfolipídios. Após a propagação das reações de oxidação lipídica, iniciam-se os eventos de oxidação protéica, com modificação e degradação da Apo-B da LDL. (Batlouni, 1997).

No presente estudo, houve um aumento na oxidação lipídica no soro de coelhos e pacientes hipercolesterolêmicos, bem como no tecido aórtico de coelhos ateroscleróticos. Tal resultado indica que peroxidação lipídica é um evento importante durante a aterosclerose induzida por hipercolesterolemia, o que está de acordo com um estudo de Yang et al. (2008). Estes autores descrevem uma relação direta entre o índice aterogênico ([colesterol total – HDL] / colesterol total) e os níveis de malondialdeído, principal produto da reação de peroxidação lipídica, em pacientes em diferentes estágios de hiperlipidemia. Alguns estudos têm mostrado que

LDLs com alto conteúdo de MUFA são menos suscetíveis a peroxidação lipídica do que LDLs que contêm maior conteúdo de PUFA (Bonanome et al., 1992; Hargrove et al., 2000). Entretanto, no presente estudo os coelhos hipercolesterolêmicos apresentaram um aumento da peroxidação lipídica concomitante com um aumento nos níveis séricos de MUFA. Essa discrepância pode ser devido ao fato de que a esterificação do colesterol exógeno requer um aumento na síntese de MUFA, fazendo com que este seja facilmente incorporado às lipoproteínas (Ntambi, 1999). Dessa maneira, é possível que o efeito positivo dos MUFA nas LDLs tenha sido mascarado pelo aumento na incorporação do colesterol na LDL induzido pela síntese de MUFA. Essa hipótese é sustentada pelo aumento nos níveis de TBARS no soro de coelhos hipercolesterolêmicos mesmo com elevados níveis de MUFA nesses animais quando comparado ao grupo controle. Adicionalmente, foi observado um aumento na oxidação de proteínas no soro de coelhos e pacientes hipercolesterolêmicos no presente trabalho, o que sugere que a LDL encontra-se em sua forma altamente oxidada, com seus componentes lipídicos e protéicos modificados por processos de oxidação.

Os aumentos da peroxidação lipídica e da oxidação protéica no soro de coelhos e pacientes hipercolesterolêmicos foram acompanhados por uma elevação nos níveis séricos de LDLox, a qual induz a diferenciação de monócitos em macrófagos. Os macrófagos por sua vez, reconhecem e capturam a LDLox, originando as células espumosas, características das estrias gordurosas constituintes do ateroma (Batlouni, 1997). Em concordância, a elevação nos níveis de LDLox no soro de coelhos hipercolesterolêmicos foi acompanhada pela presença de estrias gordurosas no tecido aórtico. O acúmulo de LDLox na parede dos vasos estimula o recrutamento de leucócitos e plaquetas e a produção de anticorpos pelas células B (Angelique et al., 2005; Shoenfeld et al., 2004). De maneira semelhante, uma elevação nos níveis de LDLoxAB foi observada tanto em pacientes como em coelhos hipercolesterolêmicos no presente trabalho.

Embora os LDLoxAB sejam detectáveis em indivíduos saudáveis provavelmente como parte da reserva natural de anticorpos, uma elevação nos níveis de LDLoxAB, especificamente IgM, pode contribuir para manutenção de baixos níveis de LDLox no plasma (Fukumoto et al., 2000). Adicionalmente, LDLoxAB do tipo IgM bloqueia a captação de LDLox pelos macrófagos, prevenindo o acúmulo de LDLox e a formação de células espumosas (Shaw et al., 2001). Entretanto, a formação de células espumosas e estrias gordurosas no tecido aórtico de coelhos hipercolesterolêmicos foi observada neste estudo mesmo quando os níveis de LDLoxAB estavam elevados, o que sugere que os anticorpos produzidos não foram suficientes para conter a captação da LDLox pelos macrófagos e a formação da placa aterosclerótica. Adicionalmente, estudos recentes sugerem um efeito pró-aterogênico do LDLoxAB tipo IgG, pois os complexos desse anticorpo específico com a LDLox desencadeiam respostas inflamatórias ao se ligarem em receptores dos macrófagos (Shoenfeld et al., 2004; Kiener et al., 1995). Dessa maneira, os elevados níveis de LDLoxAB observados em coelhos hipercolesterolêmicos poderiam contribuir para a formação do ateroma. No presente trabalho observou-se também um aumento nos níveis de hs-CRP com o aumento dos níveis de LDL em pacientes. Além de induzir resposta imune, a LDLox desencadeia o processo inflamatório e em humanos um aumento crônico dos níveis de hs-CRP, um marcador de fase aguda da inflamação, é um fator de risco independente para doenças cardiovasculares (Chait et al., 2005). A elevação dos níveis de hs-CRP foi encontrada tanto em pacientes com níveis de LDL acima do valor considerado seguro (>160 mg/dl), quanto no soro de pacientes com níveis de LDL considerados clinicamente aceitáveis (130-160mg/dl). Esses resultados sugerem que os níveis séricos de hs-CRP são modificados no soro de humanos mesmo quando os níveis de LDL são considerados aceitáveis e que a hs-CRP circulante poderia ser um marcador de eventos coronarianos precoces. Essa hipótese é sustentada pela redução dos níveis

de hs-CRP e melhora no quadro de pacientes com doença cardiovascular estável por estatinas, independentemente dos níveis séricos de lipídios (Nissen et al., 2005).

Embora esteja bem estabelecida a importância da modificação oxidativa da LDL no processo aterosclerótico, outros lipídios séricos são suscetíveis à oxidação, como é o caso dos lipídios presentes na HDL (Bowry et al., 1992). A modificação oxidativa da HDL pode ocasionar efeitos que contribuem para o processo de aterogênese, como a redução da capacidade dessa lipoproteína em promover o efluxo de colesterol das células (Morel, 1994). A fim de se proteger do processo oxidativo e preservar suas funções, a HDL conta com a enzima PON1 associada a sua estrutura (Aviram et al., 1998). Assim, essa enzima protege a HDL, preservando suas funções anti-aterogênicas e, conseqüentemente, protege a LDL do processo oxidativo (Aviram et al., 1998). No presente estudo nós observamos uma redução na atividade da enzima PON1 no soro de coelhos hipercolesterolêmicos o que já foi observado em estudos anteriores onde a atividade da enzima foi inibida em indivíduos portadores de condições como a doença arterial coronariana (Karakaya et al., 1999), a hipercolesterolemia (Mackness et al., 1991b) e a hiperlipidemia (Paragh et al., 1998). Dessa maneira, nós podemos sugerir que a inibição da PON1 pode ter contribuído para a formação de estrias gordurosas no tecido aórtico de coelhos hipercolesterolêmicos no presente estudo. A redução na atividade da enzima PON1 ocorreu provavelmente devido a inibição da síntese hepática da PON1 induzida pela hiperlipidemia (Mackness et al, 1999). Adicionalmente, a inativação da PON1 pode ser o resultado da interação de lipídios oxidados da LDLox com o grupo SH livre no resíduo de cisteína 284 da PON 1 (Aviram et al., 1999). Apesar da redução na atividade da PON1 em coelhos hipercolesterolêmicos, nenhuma alteração na atividade da enzima PON1 foi observada em pacientes com níveis de LDL considerados de risco para ocorrência de doenças cardiovasculares. Tendo em vista que um aumento na concentração de PON em macrófagos é induzido a fim de

impedir a formação de placa aterosclerótica na parede arterial (Mackness et al., 1997), nós podemos sugerir que a ausência de alteração na atividade sérica da PON1 em pacientes hipercolesterolêmicos pode ser devido a um equilíbrio entre um aumento da PON1 como mecanismo de defesa contra a hipercolesterolemia e sua inativação por espécies oxidantes (Aviram, 2004). Assim, embora a PON1 seja apontada como indicador do risco independente para doenças cardiovasculares (Mackness et al., 2003), geralmente a atividade da enzima é avaliada no soro concomitantemente com a progressão na formação do ateroma. No presente estudo envolvendo humanos, a formação de ateroma não foi avaliada e por isso a importância da PON1 como fator de risco para doenças cardiovasculares não pode ser minimizada. Além disso, a associação entre a PON1 e os níveis séricos de LDL e LDLox foi observada em indivíduos com doença de Crohn, hipertensão e com pré-eclampsia (Bohen et al., 2009; Konukoglu et al., 2009; Uzun et al., 2005). Assim, mais estudos são necessários a fim de elucidar a relação entre os níveis de colesterol, estresse oxidativo e PON1 durante a HC, bem como para corroborar o papel da PON1 como indicador de eventos cardiovasculares subclínicos.

A produção EROs e o estresse oxidativo têm sido associados ao processo de aterogênese durante a hipercolesterolemia (Prasad e Kalra, 1993) e as enzimas antioxidantes SOD, CAT, GSHPx e TrxR-1 posuem a capacidade de proteger as células contra o dano oxidativo (Nordberg e Arnér, 2001). Além das enzimas antioxidantes, antioxidantes não enzimáticos como a GSH, que representa o principal composto contendo grupos tiólicos não protéicos (NPSH), podem também prevenir as injúrias induzidas por EROs (Nordberg e Arnér, 2001). No presente estudo nós observamos que a hipercolesterolemia altera o estatus antioxidante no soro e no tecido aórtico de coelhos. A atividade das enzimas SOD, CAT, TrxR-1 e os níveis de NPSH aumentaram no tecido aórtico de coelhos, bem como a atividade das enzimas SOD e TrxR-1 aumentaram no soro de coelhos e pacientes hipercolesterolêmicos. Essa elevação no estatus

antioxidante ocorreu provavelmente como uma resposta de defesa contra as EROs geradas pela hipercolesterolemia. Um aumento na expressão das SOD, CAT e TrxR-1 já foi descrito em macrófagos *in vitro* e em placas ateroscleróticas de humanos (Hägg et al., 2006). Em ambos os casos, o aumento da expressão das enzimas ocorreu provavelmente como resposta de defesa contra a LDLox (Hägg et al., 2006). Entretanto, o aumento na atividade das enzimas antioxidantes e nos níveis de NPSH não foi suficiente para prevenir a oxidação da LDL e o início do processo aterogênico no presente trabalho, uma vez que observamos elevados níveis séricos de LDLox e estrias gordurosas no tecido aórtico mesmo quando o estatus antioxidante estava elevado. Isso sugere, adicionalmente, que o estresse oxidativo induzido pela hipercolesterolemia excedeu a capacidade antioxidante endógena e induziu danos ao tecido aórtico. Adicionalmente, a formação de estrias gordurosas no tecido aórtico de coelhos observada em nosso estudo pode ser resultado da redução da atividade da enzima GSH-Px nesses animais, provavelmente devido à suscetibilidade dessa enzima a inibição por espécies oxidantes em placas ateroscleróticas (Lapena et al., 1998). A reduzida atividade da GSH-Px no tecido aórtico de coelhos ateroscleróticos também explicaria a elevação nos níveis de NPSH, uma vez que a GSH-Px inibida deixa de reduzir hidroperóxidos às custas de GSH.

Embora as alterações na atividade das enzimas antioxidantes SOD, CAT e GSH-Px durante hipercolesterolemia e a aterosclerose tenham sido amplamente descritas (Mantha et al., 1996; Erdinçler et al., 1997; Manjunatha e Srinivasan, 2007), alterações na atividade da enzima TrxR-1 no tecido aórtico e soro de coelhos hipercolesterolêmicos, bem como no soro de indivíduos com diferentes níveis de LDL foram demonstradas pela primeira vez no presente trabalho. A TrxR-1 pode reduzir hidroperóxidos diretamente (May et al., 2002) ou regenerar a proteína Trx-1, que servirá como equivalente redutor em múltiplos processos oxidativos (Wassmann et al., 2004). O sistema tioredoxina também pode regenerar proteínas inativadas por

condições oxidantes (Björnstedt et al., 1994). Assim, pode-se sugerir que o aumento na atividade da TrxR-1 sérica em coelhos e indivíduos com níveis crescentes de LDL poderia ser uma tentativa de contrabalancear a oxidação protéica observada em ambos os casos. Além disso, a remoção do H_2O_2 em excesso gerado pela SOD também poderia explicar o aumento da atividade da TrxR-1 observada no presente estudo. É interessante destacar que a elevação da atividade da TrxR-1 em humanos iniciou mesmo quando os níveis de LDL eram clinicamente aceitáveis, o que indica que essa enzima poderia ser um indicador precoce de alterações cardiovasculares. Entretanto, a falta de correlação entre os níveis de LDL e a atividade da TrxR-1 apontam a necessidade de elucidação do potencial dessa enzima como marcador de eventos cardiovasculares.

A oxidação da LDL é importante e, possivelmente, obrigatória no desenvolvimento das lesões ateroscleróticas (Batlouni, 1997). Assim, espera-se que a suplementação da dieta com compostos antioxidantes aumente a resistência da LDL contra processos oxidativos, prevenindo a incidência da aterosclerose. Antioxidantes provenientes da dieta, tais como as vitaminas C e E e carotenóides como licopeno, luteína e zeaxantina têm atraído interesse devido a alta capacidade desses compostos de remover EROs (Wilcox et al., 2008). Adicionalmente, compostos de selênio como o disseleneto de difenila são capazes de reduzir a hipercolesterolemia e estresse oxidativo em coelhos hipercolesterolêmicos devido às suas propriedades antioxidantes (de Bem et al., 2009).

No presente estudo o potencial antiaterogênico do carotenóide ASX foi avaliado no soro e tecido aórtico de coelhos. Embora todas as concentrações de ASX tenham prevenido o aumento na peroxidação lipídica causado por uma dieta hipercolesterolêmica no tecido aórtico, nenhuma concentração de ASX foi eficiente em prevenir a peroxidação lipídica no soro de coelhos hipercolesterolêmicos. O efeito protetor mais pronunciado da ASX contra a lipoperoxidação no

tecido aórtico em detrimento do soro pode ser explicado pela estrutura química deste carotenóide. A ASX circulante, devido à polaridade conferida pelos grupos hidroxila ligados a cadeia poliênica, localiza-se na superfície de quilomícrons e lipoproteínas, bem como pode estar ligada a albumina sérica (Aas et al., 2000). Assim, a localização da ASX não seria favorável para remover radicais livres no interior de lipoproteínas, local onde ocorrem as reações em cadeia de oxidação de lipídios (Noguchi, 2002). Contrariamente, nas células a ASX assume uma disposição espacial de modo que a cadeia poliênica remova radicais livres no interior hidrofóbico da membrana plasmática, enquanto os anéis polares removem radicais livres na superfície hidrofílica (figura 5) (Goto et al., 2001). Assim, no tecido aórtico a ASX removeria radicais livres eficientemente tanto no interior da membrana plasmática quanto na superfície, enquanto nas lipoproteínas o efeito antioxidante deste carotenóide seria mais pronunciado na superfície.

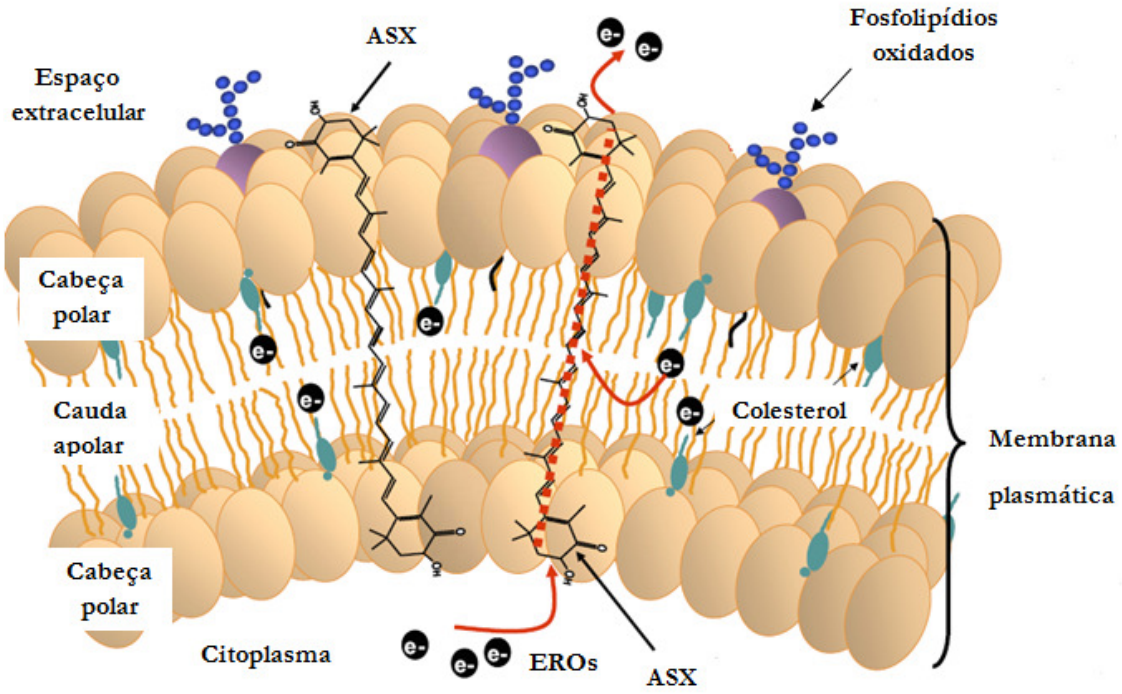


Figura 5: Orientação transmembrana da ASX. Adaptado de Paskow et al. (2008). EROs=espécies reativas de oxigênio; ASX=astaxantina.

De maneira semelhante, a ASX foi capaz de atenuar a elevação na atividade da SOD no tecido aórtico, mas não no soro de coelhos hipercolesterolêmicos. Uma vez que a ASX está em localização mais favorável para a remoção de EROs no tecido aórtico comparado ao soro, é plausível sugerir que a remoção de radicais livres pela ASX seja mais eficiente no tecido aórtico, poupando assim as defesas antioxidantes, como a enzima SOD.

Contrariamente ao efeito protetor da ASX no tecido aórtico em detrimento ao soro de coelhos hipercolesterolêmicos, observou-se no presente estudo que a todas as concentrações avaliadas de ASX atenuaram as alterações na atividade da TrxR-1 sérica causadas pela hipercolesterolemia. Entretanto, apenas a concentração de 50 mg% atenuou totalmente o efeito da hipercolesterolemia no tecido aórtico de coelhos enquanto as concentrações de 100 e 500mg% atenuaram parcialmente tais efeitos. Esse efeito protetor mais pronunciado no soro em comparação ao tecido aórtico pode ser um resultado direto do efeito protetor da ASX contra a oxidação protéica no soro. Uma vez que a elevação na atividade da TrxR-1 no soro de coelhos hipercolesterolêmicos parece ter ocorrido como uma resposta de defesa contra a oxidação protéica (Söderberg et al., 2000), a atenuação da oxidação de proteínas no soro pela ASX resultaria em um efeito atenuante também na atividade da TrxR-1. Apesar de a oxidação protéica causada pela hipercolesterolemia no tecido aórtico não ter sido avaliada no presente trabalho, a falta de proteção da ASX contra as alterações na atividade da TrxR-1 no tecido aórtico adicionalmente sugere que a produção de EROs se sobrepôs à capacidade antioxidante da ASX. Essa hipótese é sustentada pelo fato de que o efeito protetor da ASX contra alterações na atividade da SOD e peroxidação lipídica causadas pela hipercolesterolemia no tecido aórtico não resultou em proteção contra a formação de ateroma. Assim, os radicais livres excedentes exerceram seus efeitos na atividade da TrxR-1 e induziram a formação de placa ateromatosa.

Apesar da falta de efeito hipolipidêmico e antiaterogênico da ASX neste trabalho, estudos recentes destacam o potencial protetor desse carotenóide na prevenção de desordens associadas ao estresse oxidativo. Park et al. (2010) relataram o efeito protetor da ASX contra o estresse oxidativo e inflamação em indivíduos adultos saudáveis, bem como seu papel positivo na indução de resposta imune. Adicionalmente, a ASX preveniu a morte celular de neurônios *in vitro* induzida por peptídeo β-amilóide, o que indica um possível papel na prevenção e tratamento de doenças neurodegenerativas como a doença de Alzheimer (Chang et al., 2010). Um estudo de Cort et al. (2010) revelou a capacidade da ASX em reverter danos a retina causados por aumento na pressão intraocular em ratos.

CONCLUSÕES

Os resultados obtidos na presente Tese permitem concluir que:

1. Indivíduos com níveis séricos de LDL considerados de alto risco para ocorrência de doenças cardiovasculares (> 160 mg/dl) apresentam aumento na oxidação de lipídios e proteínas no soro, alterações na atividade de enzimas antioxidantes, bem como elevados níveis de LDLox, LDLoxAB e hs-CRP quando comparado a indivíduos com níveis de LDL considerados seguros (< 130 mg/dl). Tais resultados sugerem a ocorrência de processo oxidativo e inflamatório em indivíduos hipercolesterolêmicos.
2. A atividade da enzima TrxR-1 aumentou em indivíduos com níveis séricos de LDL considerados clinicamente aceitáveis (130-160 mg/dl) bem como em indivíduos hipercolesterolêmicos sugerindo que essa enzima poderia ser utilizada como um marcador de eventos cardiovasculares precoces. Adicionalmente, uma elevação na atividade da TrxR-1 foi observada no soro de coelhos hipercolesterolêmicos e no tecido aórtico de coelhos ateroscleróticos, indicando que essa enzima pode desempenhar um papel importante na hipercolesterolemia e aterosclerose.
3. Apesar da atividade da enzima PON1 estar inibida em coelhos hipercolesterolêmicos, sua atividade não foi afetada pela hipercolesterolemia em humanos, o que indica que estudos adicionais acerca da importância dessa enzima como preditor de eventos cardiovasculares devem ser conduzidos.
4. A adição de ASX na dieta atenuou o estresse oxidativo, mas não a hiperlipidemia e aterosclerose induzidas pela adição de colesterol na dieta, o que indica que outros fatores além do estresse oxidativo estão envolvidos no processo de aterogênese e que tais fatores não podem ser controlados pela ASX.

Considerando a participação do estresse oxidativo no processo de aterogênese, pode-se concluir que o uso de marcadores de estresse oxidativo como indicadores de eventos cardiovasculares deve ser considerado em um futuro próximo. Adicionalmente, mais estudos são necessários para elucidar completamente o papel de carotenóides como a ASX na prevenção e tratamento de doenças cardiovasculares.

PERSPECTIVAS

- Avaliar o potencial antiaterogênico de outros carotenóides visando uma possível ação na prevenção e tratamento de doenças cardiovasculares.
- Avaliar o potencial anitaterogênico de extratos de frutas nativas ricos em compostos antioxidantes.
- Padronizar metodologias mais específicas para avaliação de formação de placa ateromatosa, incluindo princípios de imunohistoquímica e marcadores de estabilidade do ateroma.
- Elucidar o papel da enzima TrxR-1 bem como da proteína Trx-1 durante a hipercolesterolemia e aterosclerose *in vitro* e *in vivo*.
- Avaliar o potencial de carotenóides na modulação da atividade da enzima PON 1.
- Elucidar o potencial da proteção conferida pela ASX sobre a atividade da TrxR-1 em patologias onde ocorre envolvimento do sistema tioredoxina.
- Realizar estudos de distribuição plasmática e tecidual de carotenóides específicos, elucidando o papel dos ácidos graxos nos processos de esterificação e transporte de carotenóides.

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ANEXOS

ANEXO I

Termo de consentimento livre e esclarecido assinado pelos participantes do estudo envolvendo seres humanos

TERMO DE CONSENTIMENTO LIVRE E ESCLARECIDO

Você está sendo convidado a participar de um estudo intitulado "POTENCIAL ANTIATEROGÊNICO DE CAROTENÓIDES IN VIVO E IN VITRO", que tem como objetivo geral avaliar os indicadores de estresse oxidativo em pacientes com diferentes níveis de colesterol e sua relação com o processo inflamatório. Este projeto é importante para verificar se diferentes níveis de colesterol afetam as defesas antioxidantes do organismo.

O estudo será desenvolvido no Núcleo Integrado de Desenvolvimento em Análises Laboratoriais (NIDAL) (Departamento de Tecnologia e Ciência dos Alimentos) da Universidade Federal de Santa Maria e no laboratório de Análises Clínicas Labimed na cidade de Santa Maria. Este estudo envolverá pacientes com diferentes níveis de colesterol da cidade de Santa Maria, Rio Grande do Sul. Os pacientes serão selecionados aleatoriamente no laboratório de Análises Clínicas Labimed antes de se submeterem a coleta de amostra. Após a realização dos exames laboratoriais, as amostras serão enquadradas nos grupos estudados. Eventualmente, quando a amostra pertencer a um grupo que já esteja completo, tal amostra não será utilizada e será descartada.

Os pesquisadores responsáveis pelo estudo são a Profa. Dra. Tatiana Emanuelli e a Msc. Paula Rossini Augusti, aluna do Programa de Pós Graduação em Bioquímica, da UFRGS. Em qualquer etapa do estudo você terá acesso aos pesquisadores responsáveis pelo estudo para esclarecimento de eventuais dúvidas

Este estudo obteve aprovação junto ao Comitê de Ética em Pesquisa da Universidade Federal de Santa Maria, com protocolo n° 23081.019182/2007-10

Procedimentos a serem realizados

Você deverá realizar apenas uma coleta, onde serão retirados cerca de 8 ml de sangue, sem qualquer custo, no laboratório de Análises Clínicas Labimed na cidade de Santa Maria. O sangue será coletado em tubos estéreis e individuais para cada paciente e posteriormente será armazenado por um período de no máximo 24 meses, para a realização de todas as análises previstas. O sangue coletado será armazenado no Núcleo Interado de Desenvolvimento em Análises Laboratoriais (NIDAL, Departamento de Tecnologia e Ciência dos Alimentos) da Universidade Federal de Santa Maria, sob responsabilidade da Professora Tatiana Emanuelli. Você receberá os resultados dos exames laboratoriais de colesterol total, colesterol LDL e HDL e triglicerídeos. Eventualmente, se você se enquadrar em um grupo que já esteja completo, sua amostra não será utilizada no estudo e será descartada.

Riscos individuais, possibilidade de exclusão e benefícios

A coleta de sangue é um procedimento de baixo risco e desconforto. No entanto, em alguns casos, pode ocorrer formação de hematoma local, que será tratado através da realização de massagem local com gel ou pomada contendo medicamento para promover a absorção do hematoma, sem nenhum custo.

Fica claro que você não é obrigado a participar do projeto. No caso de recusa você não terá nenhum prejuízo no seu atendimento rotineiro, nem represálias. A qualquer momento da pesquisa você é livre para retirar-se da mesma.

No caso de aceite, fica claro que não haverá benefício financeiro pela sua participação, nem prejuízos ou riscos a sua saúde.

Ao receber os resultados dos seus exames de colesterol total, colesterol LDL e HDL e triglicerídeos você será orientado a procurar atendimento médico, caso os valores encontrados apresentem alguma alteração.

Confidencialidade

Os dados obtidos com esta pesquisa se constituirão em publicações em revistas médico-científicas. Os seus dados serão analisados em conjunto com outros pacientes, e será garantida a confidencialidade dos dados que possam lhe identificar.

Utilização dos dados obtidos

O material coletado e os seus dados serão utilizados somente para esta pesquisa.

Telefones para contato com os pesquisadores

Prof. Dra. Tatiana Emanuelli – Departamento de Tecnologia e Ciência dos Alimentos – CCR
(55) 3220 8547
email: tatiemanuelli@smail.ufsm.br

Msc. Paula Rossini Augusti - Pós Graduação em Ciências Biológicas-Bioquímica – ICBS – UFRGS
Email: paula_augusti@terra.com.br
(55) 3220 8547, (55) 32254728, (55) 96070821

Acredito ter sido suficientemente informado a respeito das informações que li ou que foram lidas para mim, descrevendo o estudo "POTENCIAL ANTIATEROGÊNICO DE CAROTENÓIDES IN VIVO E IN VITRO". Eu discuti com a Profa. Dra. Tatiana Emanuelli sobre a minha decisão em participar nesse estudo. Ficaram claros para mim quais são os propósitos do estudo, os procedimentos a serem realizados, seus desconfortos e riscos, as garantias de confidencialidade e de esclarecimentos permanentes. Ficou claro também que minha participação é isenta de despesas e que tenho garantia do acesso a tratamento hospitalar quando necessário. Concordo voluntariamente em participar deste estudo e poderei retirar o meu consentimento a qualquer momento, antes ou durante o mesmo, sem penalidades ou prejuízo ou perda de qualquer benefício que eu possa ter adquirido, ou no meu atendimento neste Serviço.

Identificação do (a) paciente

Nome: _____

RG (número da identidade: _____

Assinatura: _____

Santa Maria, _____ de _____de 2007.

Declaro que obtive de forma apropriada e voluntária o Consentimento Livre e Esclarecido deste sujeito de pesquisa ou representante legal para a participação neste estudo.
Santa Maria, _____ de _____de 2007.

_____

Assinatura do responsável pelo estudo

ANEXO II

Normas para preparação de manuscritos para submissão ao periódico Clinical Biochemistry

Clinical Biochemistry

Official Journal of the Canadian Society of Clinical Chemists

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Guide for Authors

Introduction

Clinical Biochemistry publishes articles relating to the applications of molecular biology, biochemistry, chemistry and immunology to clinical investigation and to the diagnosis, therapy, and monitoring of human disease. Manuscripts are categorized as Analytical or Clinical Investigations and may be offered as Full Papers or as Short Communications. Critical Reviews are welcome, but contributors are encouraged to contact the Editor-in-Chief to avoid conflict with other forthcoming Reviews.

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Detailed Status Information

Manuscript #	BJN-2010-015476
Current Revision #	0
Submission Date	26th Jun 10
Current Stage	Under Review
Title	Dietary astaxanthin improves paraoxonase activity and oxidative status in hypercholesterolemic rabbits: a lipid-lowering independent effect
Running Title	Astaxanthin in hypercholesterolemia
Manuscript Type	Research Article
Special Section	N/A
Category	Metabolism and Metabolic Studies
Corresponding Author	Tatiana Emanuelli (tatiemanuelli@smail.ufsm.br) (Universidade Federal de Santa Maria)
Contributing Authors	Paula Augusti , Greicy Conterato , Sabrina Somacal , Rocheli Sobieski , Andréia Quatrin , Amanda Ruviaro , Luana Maurer , Marta Duarte
Sources of Funding	This work was supported by CNPq and FAPERGS. CNPq provided a research fellowship to T. Emanuelli, PhD degree fellowships to P.R.

	Augusti and G.M.M. Conterato, as well as a technical support fellowship. Scientific initiation fellowships from FAPERGS and CNPq are also acknowledged.
Abstract	Oxidative stress plays an important role in hypercholesterolemia and atherosclerosis. This study explored the effects of the antioxidant astaxanthin on the lipid profile, oxidative stress and paraoxonase activity in hypercholesterolemic rabbits. Male New Zealand white rabbits were fed a standard or a hypercholesterolemic diet (1 % cholesterol) alone or supplemented with 50, 100 or 500 mg % of astaxanthin for 60 days. Serum levels of total cholesterol, triglycerides, oxidised LDL and oxidised LDL antibodies were measured. Thiobarbituric acid reactive substances levels and carbonyl content in proteins were also measured as lipid and protein oxidation indicators. Paraoxonase and antioxidant enzymes activities as well as fatty acids composition were evaluated in serum. The hypercholesterolemic diet increased serum total cholesterol and triglycerides levels. This increase in serum lipids was accompanied by elevated oxidised LDL and oxidised LDL antibodies, as well as lipid and protein oxidation. In addition, hypercholesterolemia decreased SFA and increased unsaturated fatty acids levels in the serum. Astaxanthin (100 and 500 mg %) attenuated protein oxidation in hypercholesterolemic rabbits, whereas 500 mg % of astaxanthin reduced protein oxidation per se. In addition, the activities of antioxidant enzymes superoxide dismutase and thioredoxin reductase were enhanced, while paraoxonase activity was inhibited in hypercholesterolemic rabbits. Although all astaxanthin doses prevented changes in thioredoxin reductase and paraoxonase activities, the change in superoxide dismutase activity was not improved by astaxanthin. Our study suggests that astaxanthin could play a beneficial role in hypercholesterolemia by preventing protein oxidation as well as changes in thioredoxin reductase and paraoxonase activities.
Key Words	Thioredoxin reductase, Fatty acids, oxidised LDL, Thiobarbituric acid reactive substances
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Word Count	6000

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