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PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS BIOLÓGICAS: BIOQUÍMICA TOXICOLÓGICA

AVALIAÇÃO DE MARCADORES BIOQUÍMICOS, DE ESTRESSE OXIDATIVO E DO EFEITO ANTIOXIDANTE DA QUERCETINA NO HIPOTIREOIDISMO

TESE DE DOUTORADO

Adriana Santi

Santa Maria, RS, Brasil
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Adriana Santi

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Doutor em Bioquímica Toxicológica

Orientadora: Vania Lucia Loro
Co - orientador (a): Ivana Beatrice Mânica da Cruz

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elaborada por
Adriana Santi

como requisito parcial para a obtenção do grau de
Doutor em Bioquímica Toxicológica

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“As pessoas mais felizes não
têm as melhores coisas.
Elas sabem fazer o melhor das
oportunidades que aparecem
em seus caminhos.
A felicidade aparece para
aqueles que choram.
Para aqueles que se machucam.
Para aqueles que buscam
e tentam sempre.”

Clarice Lispector

RESUMO

Tese de Doutorado
Programa de Pós-Graduação em Ciências Biológicas: Bioquímica Toxicológica
Universidade Federal de Santa Maria

AVALIAÇÃO DE MARCADORES BIOQUÍMICOS, DE ESTRESSE OXIDATIVO E DO EFEITO ANTIOXIDANTE DA QUERCETINA NO HIPOTIREOIDISMO

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Local e Data da Defesa: Santa Maria, 28 de março de 2014.

. O hipotireoidismo é caracterizado por uma desordem decorrente da deficiência de hormônios tireoideanos, estando relacionado a disfunções no metabolismo lipídico e ao risco de desenvolvimento de doenças cardiovasculares. Entretanto, estas alterações no hipotioreodismo precisam ser melhor compreendidas. Assim, este trabalho teve como objetivo avaliar a associação de marcadores lipídicos, inflamatórios e de estresse oxidativo em pacientes com hipotireoidismo e o efeito antioxidante da quercetina nestes marcadores, utilizando como modelo experimental o hipotireoidismo induzido por metimazol em ratos. A metodologia e resultados são apresentados sob a forma de artigos. No artigo 1, foram avaliados biomarcadores de estresse oxidativo em 20 pacientes com hipotireoidismo subclínico (HSC) (49,12 ± 10,85 anos). Os níveis de substâncias reativas ao ácido tiobarbitúrico (TBARS), e as atividades das enzimas superóxido dismutase (SOD), catalase (CAT) e arilesterase (ARE) foram determinadas em pacientes com HSC e controles. Além disso, foram investigados os níveis de lipídeos plasmáticos: colesterol total (CT), triglicerídeos (TG) e as lipoproteínas de alta (HDL) e baixa densidade (LDL). Os níveis de lipoperoxidação determinado pela medida do TBARS e a atividade da enzima CAT estavam aumentados nos pacientes hipotireóideos, bem como os níveis plasmáticos de CT e colesterol LDL. A enzima ARE mostrou-se diminuída no grupo HSC. Foram evidenciadas correlações entre lipídeos plasmáticos e biomarcadores de estresse oxidativo e com o hormônio de estimulação da tireóide (TSH). O TSH foi correlacionado com TBARS, CAT e SOD. O segundo estudo (manuscrito 1) teve por objetivo investigar a associação entre biomarcadores inflamatórios e o hipotireoidismo clínico (HC). Foram determinados os níveis plasmáticos das citocinas: interleucina 1 (IL-1), interleucina 6 (IL-6), fator de necrose tumoral alfa (TNF- α), interferon gama (INF- ɣ) e os níveis de DNA circulante livre. Além disso, foram avaliados o perfil lipídico e marcadores pró-trombóticos (fibrinogênio e D-dímero). Os pacientes com HC apresentaram perfil pró-inflamatório, resultante dos níveis elevados das citocinas e do DNA livre. Os lipídeos e os marcadores pró-trombóticos também se apresentaram elevados. Associações significativas entre o perfil inflamatório e o perfil lipídico foram observadas nos pacientes hipotireóideos. No manuscrito 2 avaliou-se o efeito da quercetina sobre biomarcadores de estresse oxidativo em um modelo de hipotireoidismo induzido por metimazol (MMI) em ratos. O hipotireoidismo foi induzido pela administração de MMI na concentração de 20mg/100mL na água de beber, por um período de 30 dias.

Após este período, os animais receberam oralmente 10 ou 25 mg/kg de quercetina (QT) por um período de 8 semanas. Ratos machos wistar (n=60) foram divididos em seis grupos (grupo I, controle; grupo II, QT10; grupo III, QT25; grupo IV, hipotireóideo; grupo V, hipotireóideo + QT10; grupo VI, hipotireóideo + QT25). Os ratos hipotireóideos apresentaram níveis de TBARS hepático, renal e séricos aumentados, bem como os níveis de proteína carbonil (PCO) no fígado e os níveis de espécies reativas de oxigênio (ERO) no fígado e rins. A administração de quercetina (QT 10 e 25) diminuiu os níveis de TBARS em soro e rins, a PCO no fígado e a geração de ERO nos tecidos hepático e renal. Além disso, no grupo hipotireóideo foram observados altos níveis de TBARS no córtex cerebral e hipocampo. O tratamento com QT25 reduziu os níveis em ambos os tecidos. A administração de QT 25 aos ratos com hipotireoidismo diminuiu a atividade da SOD em fígado e sangue total e aumentou a atividade hepática da CAT. Os níveis de ácido ascórbico e a capacidade antioxidante total aumentaram no fígado e rins dos ratos após tratamento com QT10 e QT25. O conjunto dos resultados sugeriu associação entre estresse oxidativo e hipotireoidismo que pode ser potencialmente modulado por suplementação de antioxidantes como a quercetina. Estes achados são de grande importância no entendimento das disfunções bioquímicas e do status oxidativo no hipotireoidismo como também na busca de estratégias antioxidantes a serem utilizadas como coadjuvantes no tratamento desta disfunção.

Palavras – chave: hipotireoidismo; estresse oxidativo; lipídeos; inflamação; citocinas; metimazol; quercetina; antioxidante.

ABSTRACT

Thesis of Doctor's Degree
Programa de Pós-Graduação em Ciências Biológicas: Bioquímica Toxicológica
Universidade Federal de Santa Maria

EVALUATION OF LIPID, INFLAMMATORY AND OXIDATIVE STRESS MARKERS AND ANTIOXIDANT EFFECT OF QUERCETIN IN HYPOTHYROIDISM

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Data and Place of the defense: March, 28th, 2014, Santa Maria

Hypothyroidism is characterized by a disorder resulting from deficiency of thyroid hormones and is related to lipid metabolism dysfunction and cardiovascular diseases development risk. However, these changes in hypothyroidism need to be understood. Thus, this study aimed to evaluate the association between lipid, inflammatory and oxidative stress markers in patients with hypothyroidism and antioxidant effects of quercetin in these markers, using hypothyroidism experimental model induced by methimazole in rats. The methodology and results are presented in the form of articles. In article 1, were evaluated the oxidative stress biomarkers in 20 patients with subclinical hypothyroidism (SH) (49.12 ± 10.85 years). Thiobarbituric acid reactive substances (TBARS), superoxide dismutase (SOD), catalase (CAT) and arylesterase (ARE) were analyzed in SH patients and controls. In addition, were measured plasmatic lipids: total cholesterol (TC), triglycerides (TGs), high-density lipoprotein cholesterol (HDL-C) and low-density lipoprotein cholesterol (LDL-C). TBARS levels and CAT activity were higher in subclinical hypothyroidism patients, such as TC and LDL-C plasmatic levels. Arylesterase activity was lower in the SH group. Correlations were observed between plasmatic lipids and oxidative stress biomarkers and thyroid-stimulating hormone (TSH). TSH was correlated with TBARS, CAT, and SOD. The second study (manuscript 1) aimed to investigate the association between inflammatory biomarkers and overt hypothyroidism (OH). Plasmatic levels of cytokines were determinate: interleukin 1 (IL-1), interleukin 6 (IL-6), tumor necrosis factor alpha (TNF-α), interferon gamma (INF-ɣ) and the levels of cell free DNA (cf-DNA). Furthermore, we evaluated lipid profile and prothrombotic markers (fibrinogen and D-dimer). OH patients had pro-inflammatory profile, resulted from high levels of cytokines and cf-DNA. Lipids and prothrombotic markers also showed elevated. Significant associations between inflammatory status and lipid profile were observed in hypothyroid patients. Manuscript 2 evaluates the effect of quercetin on oxidative stress biomarkers in methimazole (MMI) - induced hypothyroid rats. Hypothyroidism was induced by administering MMI at 20 mg/100 ml in the drinking water, for 30 days. After this period, rats received orally 10 or 25 mg/kg of quercetin (QT) for 8 weeks. Sixty male wistar rats were randomly divided into six groups (group I, control; group II, QT10; group III, QT25; group IV, hypothyroid; group V, hypothyroid + QT10; group VI, hypothyroid + QT25). Hypothyroid rats showed hepatic, renal and serum TBARS levels increased, along with increased protein carbonyl (PCO) in liver and increased ROS levels in liver and kidney. Quercetin administration (QT10 and 25), was effective in decreasing TBARS levels in

serum and kidney, PCO in liver and ROS generation in liver and kidney tissues. Moreover, in hypothyroid group were observed high TBARS levels in cerebral cortex and hippocampus. QT25 treatment decreased the levels in both tissues. Administration of QT25 to hypothyroid rats resulted in decreased SOD activities in liver and whole blood and increased liver CAT activity. Ascorbic acid levels and total oxidative scavenging capacity (TOSC) were increased in liver and kidney rats after QT10 and QT25 treatment. These results suggest association between oxidative stress and hypothyroidism that may potentially modulated by antioxidant supplementation such as quercetin. These findings are of great importance in understanding the biochemical dysfunctions and oxidative status in hypothyroidism, as well as, in research of antioxidants strategies to be used as adjuncts in the treatment of this disorder.

Keywords: hypothyroidism; oxidative stress; lipids; inflammation; cytokines; methimazole; quercetin; antioxidant.

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LISTA DE ABREVIATURAS

ARE: arilesterase

CAT: catalase

CETP: proteína de transferência de colesterol esterificado

CK-MB: creatina quinase fração cardíaca

ER: espécies reativas

ERO: espécies reativas de oxigênio

GSH: glutationa reduzida

GSSG: glutationa oxidada

GPx: glutationa peroxidase

HC: hipotireoidismo clínico

Hct: homocisteína

HDL: lipoproteína de alta densidade

HSC: hipotireoidismo subclínico

HT: hormônios tireoideanos

H_2O_2: peróxido de hidrogênio

IL-1: interleucina 1

IL-6: interleucina 6

INF- ɣ: interferon gama

LDL: lipoproteína de baixa densidade

MDA: malondialdeído

MMI: metimazol

$O2^{\bullet -}$*:* ânion superóxido

PCO: proteína carbonil

PCR: proteína C-reativa

PON1: paraoxonase 1

RL: radicais livres

SOD: superóxido dismutase

SHNP: tióis não protéicos

TBARS: substâncias reativas ao ácido tiobarbitúrico

TG: triglicerídeos

TSH: hormônio tireoestimulador

T4: tiroxina

T3: triiodotironina

TNF- α: fator de necrose tumoral alfa

SUMÁRIO

1 INTRODUÇÃO

Nos seres humanos, a glândula tireóide consiste em dois lobos presos, nos dois lados da traquéia, por tecido conjuntivo. Os dois lobos são unidos por faixa de tecido tireóideo, ou istmo, situado logo abaixo da cartilagem cricóide (RHOADES & TANNER, 2005). É uma das maiores glândulas endócrinas, normalmente pesando de 15 a 20 gramas no adulto (GUYTON & HALL, 2011).

Os folículos tireoideanos representam a unidade funcional da glândula, sendo responsáveis pela síntese, armazenamento e liberação dos hormônios tireoideanos (HT): a tiroxina (T4) e a triiodotironina (T3) (McGEOWN, 2002) (Figura 1). Os hormônios T4 e T3 são derivados iodados do aminoácido tirosina sendo formadas pelo acoplamento dos anéis fenil de duas moléculas de tirosina iodada, por ligação éter. A estrutura resultante é a chamada iodotironina (RHOADES & TANNER, 2005). Em indivíduos saudáveis, o T4 total sérico apresenta concentração cerca de 60 vezes superior ao T3 total. O T3 é definido como hormônio “ativo”, sendo produzido pela biotransformação (por exemplo, deiodinação) do T4, por enzimas intracelulares dos tecidos periféricos (LUM et al., 1984).

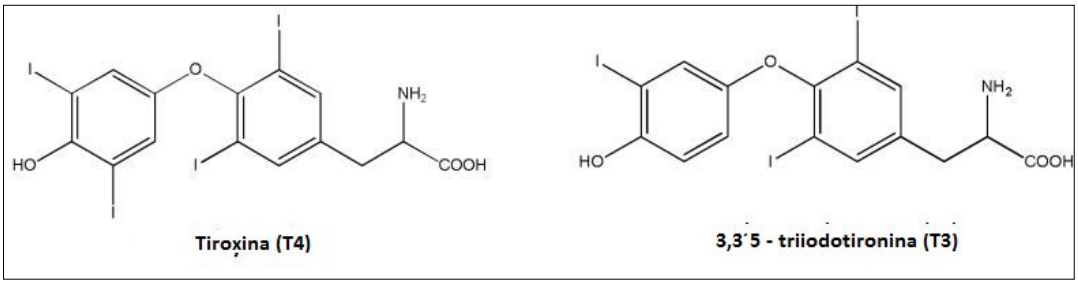

Figura 1 - Estrutura quimica dos hormônios tireoideanos (Adpatado de WANG; STAPLETON, 2010).

Os hormônios T3 e T4 regulam diversos processos fisiológicos, como a diferenciação, crescimento e metabolismo celular. Além disso, são fundamentais para o funcionamento normal dos tecidos, possuindo efeitos sobre o consumo de oxigênio e taxa metabólica. Alterações em seus níveis podem levar a anormalidades bioquímicas e clínicas, como o hipotireoidismo (OPPENHEIMER et al., 1987).

O hipotireoidismo clínico (HC) é uma disfunção caracterizada por valor sérico aumentado do hormônio tireoestimulador (TSH) e concentrações diminuídas de T3 e T4, na presença de sintomas clínicos manifestos (CESENAF et al., 2005). O hipotireoidismo subclínico (HSC) é definido por elevados níveis de TSH com níveis normais dos hormônios tireoideanos, T3 e T4 (KHANDELWAL & TANDON, 2012). Em estudo conduzido por SGARBI e colaboradores (2010), brasileiros descendentes de japoneses (idade >30 anos, n=1110) foram avaliados por um período de 7,5 anos, sendo observada a presença de HSC em 8,7% (n=99) da população avaliada.

Atualmente, alterações em vários parâmetros considerados fatores de risco para doenças cardiovasculares tem sido identificados em pacientes com hipotireoidismo. Estes fatores de risco incluem níveis séricos de homocisteína (Hct), de marcadores inflamatórios, disfunção endotelial (BIONDI; KLEIN, 2004) e dislipidemia (GAO et al., 2013).

Uma vez que os hormônios da tireóide agem de modo sistêmico, alterações endócrinas como o hipotireoidismo podem levar a disfunções e doenças metabólicas como é o caso da dislipidemia.

A dislipidemia é uma alteração metabólica comum em pacientes com doença tireoideana, e reflete o efeito dos hormônios tireoideanos sobre a síntese, absorção e metabolismo de lipídeos (ZHU; CHENG, 2010; PEPPA et al., 2011).Em estudo recente, a atividade da lipoproteína lipase foi reduzida em pacientes com hipotireoidismo, resultando em altos níveis de triglicerídeos. A proteína de transferência de colesterol esterificado (CETP) e a lipase hepática também se mostraram alteradas no hipotireoidismo, o que resultou em tamanhos menores de lipoproteínas de baixa densidade (LDL), em pacientes hipotireóideos com níveis de TSH >10 mIU/l (HERNÁNDEZ-MIJARES et al., 2013). As LDLs de tamanho menor podem ser facilmente concentradas nas paredes das artérias, são mais suscetíveis à oxidação e possuem menor afinidade por receptores de LDL, representando assim, fator de risco para o desenvolvimento de doenças arteriais coronarianas (DAC) via indução da aterosclerose (KOBA et al., 2008).

A aterosclerose é a principal causa de desenvolvimento de doenças cardiovasculares (LIBBY et al., 2002).É reconhecida como uma doença inflamatória crônica, possuindo a participação das imunidades inata e

adaptativa modulando sua progressão (HANSSON et al., 2002). O processo inflamatório é regulado pela presença de células imunes, como macrófagos e pela liberação de mediadores inflamatórios, como fatores de crescimento e citocinas. As citocinas são importantes reguladoras das imunidades inata e adaptativa e reconhecidas por atuarem em vários estágios da aterosclerose (TEDGUI; MALLAT, 2006). Muitas citocinas como as interleucinas (IL) 1, IL-6, IL-10, interferon gama (INF- ɣ) e fator de necrose tumoral alfa (TNF- α) são altamente expressas em áreas ateroscleróticas , possuindo propriedades pró e anti-aterogênicas (MEHRA et al., 2005; LIBBY et al., 2009).

A IL-6 está envolvida na diferenciação de linfócitos B, na ativação de linfócitos T e estimulação de proteínas de fase aguda (HEINRICHET al., 1990). O TNF-α é secretado na parede vascular por células musculares lisas endoteliais e por monócitos/macrófagos, sendo um potente indutor de inflamação local (ROSS, 1999). A IL-6 tem sido considerada um preditor independente do desenvolvimento de infarto do miocárdio e o TNF- α como indicador de risco para eventos coronários recorrentes, como morte cardiovascular após infarto do miocárdio (RIDKER et al., 2000).

Os níveis de DNA livre têm sido apontados como potencial marcador de processo inflamatório. Estudos recentes têm sugerido que o DNA livre circulante tem origem da necrose e apoptose celulares e uma pequena quantidade oriunda do DNA de linfócitos T (JAHR et al., 2001). Em estudo realizado por CHANG et al (2002), foram detectados níveis elevados de DNA livre em pacientes com infarto do miocárdio e sua associação com níveis elevados da enzima creatina quinase fração cardíaca (CK-MB), sugerindo-se sua utilização em conjunto com os marcadores cardíacos tradicionais.

A dislipidemia e o processo inflamatório no hipotireoidismo podem estar associados à geração de radicais livres (RL) e consequentemente ao desenvolvimento de estresse oxidativo. A inflamação e o estresse oxidativo podem contribuir para o inicio e progressão da aterosclerose (STOCKER; KEANEY, 2004). Pesquisas recentes tem demonstrado alterações no status oxidante/antioxidante no hipotireoidismo (TORUN et al., 2009; SANTI et al., 2010), apesar do estado hipometabólico. Desequilíbrios no status oxidante/antioxidante resultam de um aumento da geração de espécies reativas (ER) que por sua vez, estão envolvidos nos danos oxidativos à

estruturas celulares e moléculas, como lipídeos, proteínas e ácidos nucleicos (KEHRER et al., 1993).

O processo de peroxidação lipídica ocorre quando, por exemplo, radicais peroxil e hidroxil são adicionados a ácidos graxos insaturados ou uma cadeia carbônica de ácidos graxos sofre clivagem ao reagir com um elétron livre (RADWAŃSKA-WALA et al., 2008) gerando como produtos inúmeros compostos oxigenados, principalmente aldeídos como malondialdeído (MDA) e dienos conjugados. Este processo uma vez iniciado, é sustentado pela ação de radicais livres e pode levar a destruição oxidativa de membranas celulares (YANG et al., 2008).

A modificação de proteínas pelos RL podem levar a inativação e desnaturação de enzimas (STADTMAN; LEVINE, 2003). A carbonilação de proteínas é utilizada como marcador de oxidação proteica, podendo ser formada via radicais livres ou pelo ataque de aldeídos reativos como o MDA, formado durante a peroxidação lipídica (GRIMSRUD et al., 2008). Em estudo realizado por NANDA et al (2008), a peroxidação lipídica foi significativamente correlacionada com os níveis de proteína carbonil em pacientes com hipotireoidismo clínico.

Contudo, está bem estabelecida a função do sistema de defesa antioxidante enzimático no controle dos níveis de ER. O radical livre ânion superóxido ($O2^{\bullet -}$) produzido a partir da redução do oxigênio molecular, sofre dismutação pela enzima superóxido dismutase (SOD) gerando peróxido de hidrogênio (H_2O_2) que é então degradado à água pelas enzimas catalase (CAT) ou glutationa peroxidase (GPx) (HALLIWELL; GUTTERIDGE, 2001).

Além disso, as ER podem ser neutralizadas por antioxidantes não enzimáticos de baixo peso molecular, como a glutationa reduzida (GSH) e as vitaminas C e E (TAKANO et al., 2003). A GSH é a principal representante dos tióis não protéicos (SHNP), devido a sua abundância nas células (REED, 1990). Existem duas formas químicas desta molécula, a reduzida (GSH) e a oxidada (GSSG), sendo a razão entre as duas formas frequentemente usada na determinação do status redox celular (ISHII; YANAGAWA, 2007). Apresenta importante função na proteção contra o estresse oxidativo, através da remoção de muitas espécies reativas (WU et al., 2004).

A vitamina C (ácido ascórbico) é um nutriente essencial para a maioria dos tecidos vivos, sendo considerado um forte agente redutor (HA et al., 2009). Esta vitamina reage diretamente com os radicais superóxido e hidroxila, além do oxigênio singlete. Ademais, atua prevenindo a formação de hidroperóxidos lipídicos nas lipoproteínas plasmáticas, como por exemplo, na LDL, prevenindo assim a formação de placas ateroscleróticas (BENDICH ; LANGSETH, 1995).

O interesse em compostos fenólicos tem aumentando consideravelmente, devido as suas propriedades antioxidantes (sequestro de RL e quelante de metais) e seus possíveis efeitos benéficos para a saúde humana, como por exemplo, para o tratamento e prevenção do câncer e de doenças cardiovasculares (BRAVO, 1998). Dentre os flavonóides da dieta, a quercetina (3,5,7,3′,4′-pentahidroxiflavona) é o mais abundante, sendo encontrada em chás e vinho tinto e em várias frutas e vegetais, tais como cebolas, uvas, maçãs, morangos, cerejas e brócolis (BISCHOFF, 2008; HARNLY et al., 2006). Sua estrutura química está demonstrada na Figura 2. Flavonóides consistem em dois anéis benzenicos (A e B na Figura 2) ligados a um anel pirano (C na figura 2).

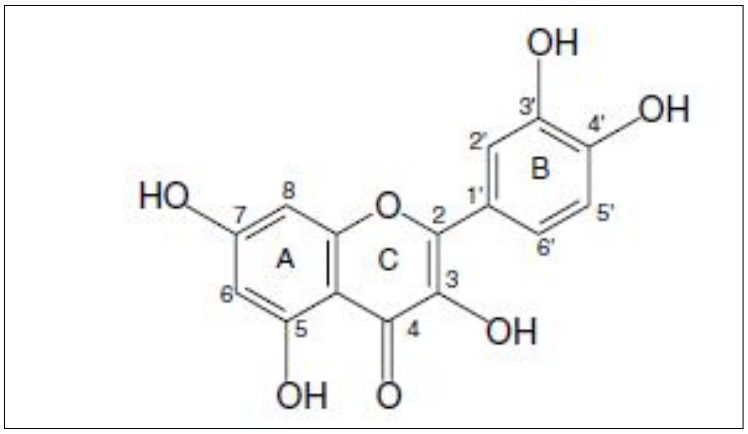

Figura 2 - Estrutura quimica da quercetina (Adpatado de HARWOOD et al., 2007).

Pesquisas sugerem que a quercetina, é um dos flavonóides que podem apresentar efeitos benéficos sobre o sistema cardiovascular (ARTS; HOLLMAN, 2005). Em estudos *in vitro,* a quercetina demonstrou ser um potente antioxidante, com atividade de sequestro de radicais peroxila (IOKU et al.,1995) e como inibidor da oxidação da LDL (DE WHALLEY et al., 1990). A

quercetina 3-*O*-β-d-glicosídeo (0,1%) mostrou-se um importante inibidor da peroxidação lipídica e diminuiu os níveis de lipídeos na aorta de coelhos submetidos à dieta hiperlipidêmica (2% de colesterol) (KAMADA et al., 2005).

Além disso, estudos tem demonstrado o efeito antioxidante da quercetina em patologias com envolvimento do estresse oxidativo, tais como o diabetes mellitus (MACIEL et al., 2013), isquemia-reperfusão (SHUTENKO et al., 1999) e a hipertensão (ROMERO et al., 2009). Entretanto, até o momento, o efeito antioxidante da quercetina no hipotireoidismo ainda não foi investigado.

Assim, devido à elevada prevalência desta disfunção endócrina e sua associação com o desenvolvimento de doenças cardiovasculares, torna-se importante investigar o envolvimento de marcadores lipídicos, inflamatórios e de estresse oxidativo nos pacientes hipotireóideos. Além disso, é de interesse cientifico e clínico a busca de estratégias antioxidantes, que minimizem os efeitos deletérios causados pelos radicais livres no hipotiredismo.

2 OBJETIVOS

2.1 Objetivo geral

Avaliar marcadores de estresse oxidativo, lipídicos e inflamatórios em pacientes com hipotireoidismo e o efeito da quercetina sobre marcadores de estresse oxidativo em modelo de hipotireoidismo induzido por metimazol em ratos.

2.2 Objetivos específicos

I. Avaliar marcadores de estresse oxidativo e o perfil lipídico em pacientes com hipotireoidismo subclínico;

II. Investigar a associação entre marcadores inflamatórios e o hipotireoidismo clínico;

III. Analisar o perfil lipídico e marcadores pró-trombóticos em pacientes com hipotireoidismo clínico;

IV. Determinar os efeitos da quercetina sobre marcadores oxidativos em ratos com hipotireoidismo induzido por metimazol;

V. Avaliar os efeitos da quercetina sobre os sistemas antioxidantes enzimático e não enzimático em ratos com hipotireoidismo induzido por metimazol.

3 DESENVOLVIMENTO

Os resultados que fazem parte desta tese estão apresentados sob a forma de artigo científico e manuscritos. Os itens Introdução, Materiais e Métodos, Resultados, Discussão e Referências encontram-se no próprio artigo e nos manuscritos. O artigo está disposto da mesma forma que foi publicado e os manuscritos conforme as normas das respectivas revistas científicas a que foram enviados.

3.1 ARTIGO 1:

ASSOCIATION OF LIPIDS WITH OXIDATIVE STRESS BIOMARKERS IN SUBCLINICAL HYPOTHYROIDISM

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Clinical Study

Association of Lipids with Oxidative Stress Biomarkers in Subclinical Hypothyroidism

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Objective. The aim of the present study was to evaluate the oxidative stress biomarkers in patients with subclinical hypothyroidism ($n = 20$) and health controls ($n = 20$). *Subjects and Methods*. Total cholesterol (TC), triglycerides (TGs), low-density lipoprotein-cholesterol (LDL-C), high-density lipoprotein cholesterol (HDL-C), thiobarbituric acid reactive substances (TBARSs), catalase (CAT), superoxide dismutase (SOD), and arylesterase (ARE) were analyzed. *Results*. TC, LDL-C, TBARS, and CAT were higher in subclinical hypothyroidism patients, whereas SOD did not change. Arylesterase activity was significantly lower in the SH group, compared with the control group. Correlation analyses revealed the association of lipids (TC and LDL-C) with both oxidative stress biomarkers and thyrotropin (TSH). Thyroid hormones were correlated only with triglyceride levels. In addition, TSH was significantly correlated with TBARS, CAT, and SOD. However, no significant correlations were observed after controlling TC levels. *Conclusions*. We found that SH patients are under increased oxidative stress manifested by reduced ARE activity and elevated lipoperoxidation and CAT activity. Secondary hypercholesterolemia to thyroid dysfunction and not hypothyroidism *per se* appears to be associated with oxidative stress in subclinical hypothyroidism.

1. Introduction

Subclinical hypothyroidism (SH), defined as an elevated serum thyroid stimulating hormone (TSH) level associated with serum thyroid hormone concentrations within the reference range, is found in 4–10% of individuals from Western populations [1, 2]. Patients with hypothyroidism have an increased risk of developing atherosclerosis, and the subclinical stage is also considered a risk factor for this disease [3, 4]. Some investigators have found this connection to be attributed to increased levels of total cholesterol (TC), low-density lipoprotein cholesterol (LDL-C), and apolipoprotein (apo) B [5, 6], whereas others did not observe any significant differences [7, 8].

Thyroid hormones are associated with the oxidative and antioxidative status of the organism. Depression of metabolism due to hypothyroidism has been reported to decrease oxidant production and thus protects tissues against oxidant damage [9, 10]. However, data on the oxidative status of hypothyroidism are limited and controversial [11–13].

Lipid peroxidation (LPO) is a free radical chain reaction, which is triggered by hydroxyl radical and leads to membrane break. It facilitates the alteration in the protein structure and function and promotes generation of free radicals (FRs) [14]. LPO is reported to be high in hyperlipidaemia, which is a consistent biochemical feature in hypothyroidism [15]. A study has shown that LPO in subclinical hypothyroid patients was similar to that in normal controls [16], while another study found increased LPO in hypothyroid patients [12].

The biological oxidative effects of free radicals on lipids, proteins, and DNA are controlled by a spectrum of antioxidants. Enzymatic protection against reactive oxygen species (ROS) and the breakdown products of peroxidized lipids and oxidized protein and DNA are provided by several enzyme systems such as superoxide dismutase (SOD) and catalase (CAT) [17]. SOD catalyzes the dismutation of the superoxide anion into hydrogen peroxide (H_2O_2), which is then deactivated to water (H_2O) by catalase or glutathione peroxidase (GPx) [18, 19].

High-density lipoproteins (HDLs) inhibit atherosclerosis development mainly by inducing reverse cholesterol transport [20]. However, other antiatherogenic effects of HDL have been reported, because of their apolipoprotein A-I (apo-AI) and paraoxonase 1 (PON1) content [21]. Arylesterase (AE), one of the enzymatic activities of paraoxonase-1, is known to play a protective role against peroxidation of LDL and other lipoproteins [22].

Given the high prevalence of SH in the general population, it is important to establish whether these alterations of thyroid function entail an oxidative stress and cardiovascular risk. Thus, the aim of this study was to assess the oxidative stress biomarkers and investigated their relation with lipid parameters in subjects with hypothyroidism.

2. Subjects and Methods

2.1. Subjects. Forty adult subjects from clinical laboratory LABIMED, Santa Maria, RS, Brazil were recruited for the present study. They were then classified into two groups-control group: 20 healthy subjects (47.20 ± 11.73 years) and the subclinical hypothyroidism (SH) group: 20 subjects newly diagnosed (49.12 ± 10.85 years). SH was defined as an elevated thyrotropin (TSH) (>4.5 mIU/L) and normal free thyroxine (FT4) level (8.7–22.6 nmol/L) [23]. Exclusion criteria were (1) lipid-lowering drugs, (2) antioxidant vitamin supplements, (3) acetylsalicylic acid, (4) antihistamines, (5) antihypertensive, (6) exposure to high-iodine condition, (7) smokers, (8) alcoholics, (9) pregnant, (10) hormone replacement therapy, (11) diabetes mellitus, and (12) acute, chronic, or malignant diseases. All subjects gave written informed consent to participate in the study. The protocol was approved by the Human Ethics Committee of the Federal University of Santa Maria (no. 23081.016996/2008).

2.2. Sample Collection. Blood samples were collected after 12 h overnight fasting by venous puncture into gray and red top Vacutainers (BD Diagnostics, Plymouth, UK) tubes. The samples were centrifuged for 15 min at 2500 ×g, and aliquots of serum were kept at −20°C for maximum of 4 weeks. An aliquot of whole blood was collected into sodium citrate (3.2%) and diluted 1 : 10 in saline solution for measurement of CAT and SOD activities.

2.3. Thyroid Profile. Thyroid profile was assessed by estimation of serums TSH, T3, and fT4 that were measured by chemiluminescent immunometric assay on IMMULITE 2000 (Siemens Healthcare Diagnostics, Los Angeles, USA). Detection limits for TSH was 0.004–14.000 mIU/L, FT4 were 3.9–77.2 pmol/L, and T3 was 0.29 nmol/L.

2.4. Lipid Profile. Serum total cholesterol (TC) and triglycerides (TG) concentrations were measured using standard enzymatic methods by use of Ortho-Clinical Diagnostics reagents on the fully automated analyzer (Vitros 950 dry chemistry system; Johnson & Johnson, Rochester, NY, USA). High-density lipoprotein cholesterol was measured in the supernatant plasma after the precipitation of apolipoprotein B-containing lipoproteins with dextran sulfate and magnesium chloride as previously described [24]. Low-density lipoprotein cholesterol (LDL-C) was estimated with the Friedewald equation [25].

2.5. Thiobarbituric Acid Reactive Substances Levels. Serum thiobarbituric acid reactive substances (TBARSs) were measured according to the modified method of Jentzsch et al. [26]. Serum was added to a reaction mixture containing 1% orthophosphoric acid, an alkaline solution of thiobarbituricacid-TBA, followed by heating for 45 min at 95°C. After cooling, samples and standards of malondialdehyde (0.03 mM) were read at 532 MDA/mL.

2.6. Catalase Activity. Whole blood catalase (CAT) activity was determined by the method of Aebi [27] by measuring the rate of decomposition of H_2O_2 at 240 nm. An aliquot of blood was homogenized in potassium phosphate buffer, pH 7.0. The spectrophotometric determination was initiated by the addition of sample into an aqueous solution of hydrogen peroxide 0.3 mol/L. The change in absorbance at 240 nm was measured for 2 min. CAT activity was calculated using the molar extinction coefficient (0.0436 $cm^2/\mu mol$), and results were expressed as U/g Hb.

2.7. Superoxide Dismutase Activity. Whole blood superoxide dismutase activity was measured as described by McCord and Fridovich [28]. In this method, SOD present in the sample competes with the detection system for superoxide anion. A unit of SOD is defined as the amount of enzyme that inhibits the rate of adrenalin oxidation by 50%. Adrenalin oxidation leads to the formation of the colored product, adrenochrome, which is detected spectrophotometrically. SOD activity is determined by measuring the rate of adrenochrome formation, observed at 480 nm, in a reaction medium containing glycine-NaOH (50 mM, pH 10.0) and adrenalin (1 mM). Basal measurements to calibrate the assay were performed in a reaction medium containing 1 mL of glycine-NaOH (50 mM, pH 10.0) and 17 μL of adrenalin (1 mM). This was used to determine the concentration in samples. The results were expressed as U/mg Hb.

2.8. Arylesterase Activity. Serum arylesterase activity was measured using phenylacetate (Sigma Co, London, UK) as the substrate. The phenol formed after the addition of a 40-fold diluted serum sample was measured spectrophotometrically at 270 nm following an established procedure [29]. Enzymatic activity was calculated from the molar absorptivity coefficient of the produced phenol, 1310 $M^{-1} cm^{-1}$. One unit of arylesterase activity was defined as 1 μmol phenol generated/min under the above conditions and expressed as U/L serum ([15], view record in scopus).

2.9. Hemoglobin Determination. Hemoglobin concentrations were measured in whole blood with a Pentra 120 analyzer (ABX, Montpellier, France). The results were expressed as g/dL.

2.10. Statistical Analysis. Data are presented as mean and standard deviation (SD). The nonparametric Mann-Whitney *U*-test was used to compare differences between groups. Spearman correlation was assessed to evaluate the correlations between the variables. Partial correlations were performed to control the associations between variables for total cholesterol levels. Statistical significance was assumed at $P < 0.05$. Data were analyzed using SPSS version 11.0 software (SPSS Inc., Chicago, IL, USA).

3. Results

There were no significant differences in age and body mass index (BMI) between groups. SH patients had significantly higher TSH, TC, LDL-C, and TC/HDL ratio with FT4 normal range than the control group (Table 1). In SH group TBARS and CAT were significantly higher than controls, while SOD did not change, as shown in Figure 1.

Arylesterase activity was significantly lower in the group with SH, compared with the control group (Figure 1). ARE did not show any correlation with thyroid hormones, lipids and oxidative stress biomarkers.

We observed a positive correlation between TC and TBARS ($r = 0.757$, $P < 0.0001$), TC and CAT ($r = 0.650$, $P < 0.0001$), LDL and TBARS ($r = 0.812$, $P < 0.0001$), LDL and CAT ($r = 0.644$, $P < 0.0001$), and LDL and SOD ($r = 0.540$, $P < 0.001$), as shown in Table 2.

The correlations between TSH, T3, and fT4 with oxidative stress biomarkers are shown in Table 3. TSH was significantly associated with TBARS and CAT ($r = 0.734$, $P = 0.000$; $r = 0.499$, $P = 0.004$, resp.). However, no significant correlation was observed after controlling for TC levels.

Table 1: Clinical and laboratory data of study participants.

	Control	Subclinical hypothyroidism
n	20	20
Age (years)	47.20 ± 11.73	49.12 ± 10.85
Male (%)	50	50
BMI (Kg/m^2)	21.30 ± 3.65	23.70 ± 3.20
TC (mmol/L)	4.28 ± 0.37	6.42 ± 0.84*
HDL (mmol/L)	1.51 ± 0.27	1.01 ± 0.19
LDL (mmol/L)	2.05 ± 0.43	4.61 ± 0.95*
TG (mmol/L)	1.72 ± 0.40	2.03 ± 0.69
TC/HDL	0.09 ± 0.03	0.16 ± 0.05*
TSH (mIU/L)	1.71 ± 0.78	11.62 ± 2.33*
T3 (nmol/L)	1.26 ± 0.23	1.15 ± 0.70
fT4 (pmol/L)	18.70 ± 5.54	19.09 ± 5.67

Data are expressed as mean ± SD. *$P < 0.001$. BMI: body mass index; TC: total cholesterol; HDL: high-density lipoprotein; LDL: low-density lipoprotein; TG: triglyceride; TSH: thyroid-stimulating hormone; T3: triiodothyronine; fT4: free thyroxine.

Table 2: Correlation analyses between oxidative stress biomarkers and lipid parameters in subclinical hypothyroidism and controls subjects.

	TBARS nmol MDA/mL	CAT U/g Hb	SOD U/mg Hb
TC (mmol/L)	0.757**	0.650**	0.209
HDL (mmol/L)	−0.302	−0.268	−0.258
LDL (mmol/L)	0.812**	0.644**	0.540*
TG (mmol/L)	0.113	0.433	0.333

*$P < 0.001$; **$P < 0.0001$. TC: total cholesterol; HDL: high-density lipoprotein; LDL: low-density lipoprotein; TG: triglyceride; TBARS: thiobarbituric acid reactive substances; CAT: catalase; SOD: superoxide dismutase.

4. Discussion

In the present study we have demonstrated that patients with subclinical hypothyroidism had altered lipid profiles, reduced ARE activity, increased lipid peroxidation, and induction of enzymatic defense when compared with control subjects. Hypercholesterolemia is a common feature in hypothyroidism since thyroid hormones upregulate LDL-receptor expression [30]. In a substantial number of studies, TC and/or LDL-C seem to be elevated in SH compared with controls [31–33]. In this respect, our results showed that subjects with SH had significantly higher levels of TC, LDL-C, TG, and TC/HDL-C ratio thus displaying a more atherogenic lipid profile when compared with healthy individuals.

The level of lipid profiles is influenced by many factors. The present research has shown that thyroid hormones change the lipid profiles. Thyroid hormones may stimulate hydroxymethylglutaryl coenzyme A (HMG CoA), the key enzyme of cholesterol biosynthesis, and induce an increased synthesis of cholesterol. Additionally, the LDL-C receptor gene contains a thyroid hormone responsive element (TRE) that could allow triiodothyronine (T3) to modulate the gene expression of the LDL-C receptor resulting in an increase of LDL-C receptor synthesis. Thyroid hormones and their function are low in target tissue in SH, and researchers conjectured that SH influences lipid profiles by the above-mentioned mechanism [34, 35]. We report here a positive correlation between TSH and total cholesterol and LDL fraction as well as thyroid hormones (T3 and FT4) showing correlation with triglyceride levels. TSH was also associated with deleterious changes in serum lipids, particularly HDL-C, LDL-C, and the ratio of LDL-C to HDL-C as suggested by recent investigations [36–38].

Thyroid dysfunctions increase LPO reactions and ROS as documented by recent studies [39, 40]. LPO is an autocatalytic mechanism leading to oxidative destruction of cellular membranes [41]. Such destruction can lead to cell death and to the production of toxic and reactive aldehyde metabolites called free radicals, where malondialdehyde (MDA) is the most important. It is known that ROS would lead to oxidative damage of biological macro10molecules, including lipids, proteins, and DNA [10, 42]. We observed increased concentrations of TBARS in the circulation of SH patients. Moreover, TBARS was correlated with TSH, TC, and LDL cholesterol. However, after controlling TC levels, the association between TSH and TBARS was not significant suggesting

TABLE 3: Correlations of serums TSH, T3, and fT4 with oxidative stress biomarkers in the whole population before and after controlling for total cholesterol (TC) levels.

Biomarkers	TSH				T3				fT4			
	Before		After		Before		After		Before		After	
	r	P	r	P	r	P	r	P	r	P	r	P
TBARS, nmol MDA/mL	0.734	0.000	0.149	0.497	−0.137	0.522	0.034	0.875	−0.008	0.969	0.217	0.319
CAT, U/g Hb	0.499	0.004	0.036	0.870	−0.278	0.124	−0.153	0.485	−0.269	0.137	−0.121	0.580
SOD, U/mg Hb	0.330	0.065	0.061	0.781	−0.106	0.566	0.084	0.702	0.014	0.938	0.169	0.440

$P < 0.05$ was considered statistically significant. TSH: thyroid-stimulating hormone; T3: triiodothyronine; fT4: free thyroxine; TBARS: thiobarbituric acid reactive substances; CAT: catalase; SOD: superoxide dismutase.

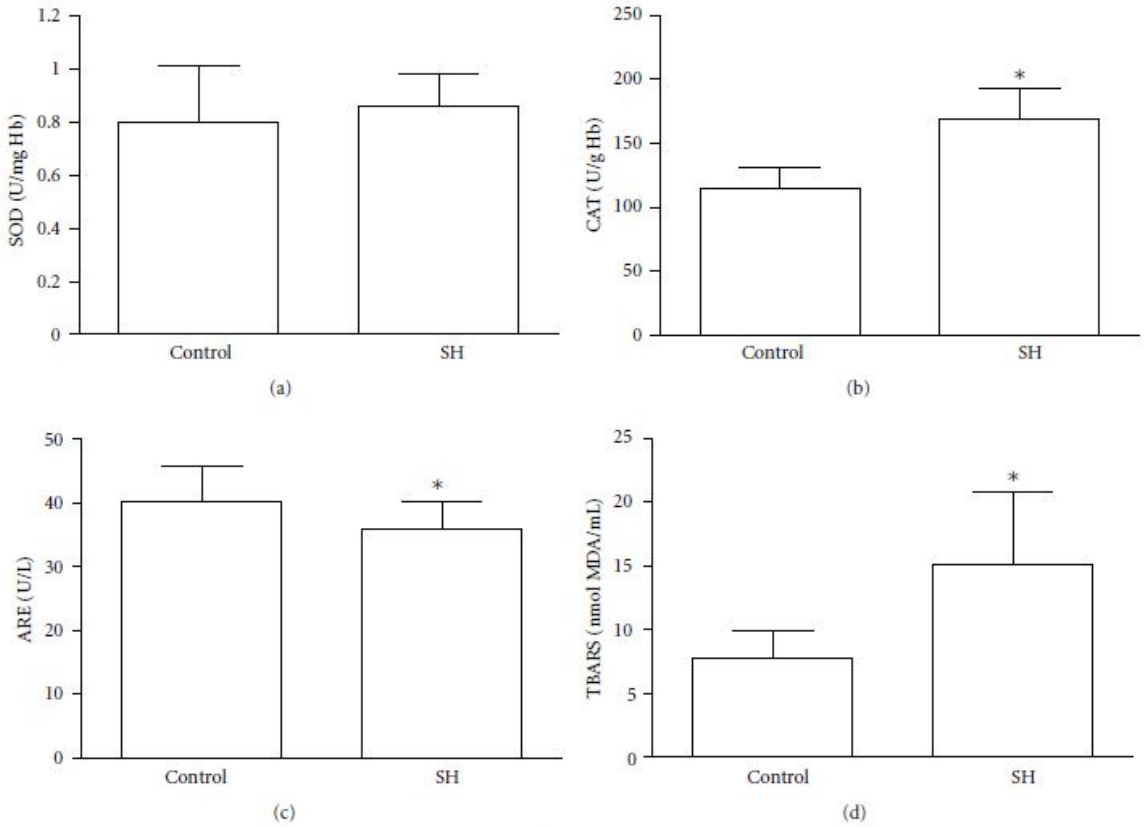

FIGURE 1: The values of SOD (a), CAT (b), ARE (c), and TBARS (d) in control and subclinical hypothyroidism (SH) groups. $*P < 0.05$.

that increased LPO could be attributed to lipid levels in hypothyroid status. Similar results were found by Nanda et al. (2008), where a significant correlation between TSH and MDA was lost after nullifying the effects of each of the coronary lipid risk factors among hypothyroid subjects [15].

Free radical-scavenging enzymes such as SOD and CAT are the first line of cellular defense against oxidative injury, decomposing O_2^- and H_2O_2 before interacting to form a more reactive hydroxyl radical (OH). These enzymes protect the red cells against O_2- and H_2O_2-mediated lipid peroxidation [19]. We have observed an increased activity of CAT in the SH group. In addition, our study shows an association between lipid parameters (CT and LDL) and CAT or SOD activities. Recently, Duarte et al. (2010) demonstrated that CAT was significantly higher in subjects with hypercholesterolemia [43]. On the other hand, some studies have reported no changes in CAT activity in hypothyroid patients [15, 44]. We also observed that the associations between TSH and TBARS, CAT, and SOD were lost when lipids (cholesterol) were annulled. Therefore, oxidative stress is likely to be potentially related to the secondary hypercholesterolemia to thyroid dysfunction and not directly to thyroid hormone levels in subclinical hypothyroidism. This result is consistent with our previous study showing that hypercholesterolemia has a stronger influence on the development of oxidative stress in overt hypothyroid (OH) patients [45].

It is supposed that LDL particles can be protected from free radical-induced oxidation by an HDL-linked enzyme, paraoxonase 1 (PON1). PON1 is found in tissues such as liver, kidney, intestine, and also serum [46]. It may possess antiatherogenic and anti-inflammatory properties, resulting from its ability to destroy modified phospholipids and to prevent cumulation of oxidized lipids in lipoproteins [47]. We found lower PON1 arylesterase activity in SH patients than controls, suggesting oxidative stress. Similar results were found by other researchers [48, 49]. In addition, epidemiological evidence demonstrates that low PON1 activity is associated with increased risk of cardiovascular events [50] and is an independent risk factor for cardiovascular disease [51].

In conclusion, our study shows an increase in the oxidative stress biomarkers in the circulation of patients with subclinical hypothyroidism. Oxidative stress biomarkers seem to be associated with secondary hypercholesterolemia to hypothyroidism, whereas hypothyroidism *per se* does not cause oxidative stress in SH patients. On the other hand, high-plasma lipids can be considered as an oxidation substrate for the oxidative stress [52]. Thus, we suggest monitoring of oxidant/antioxidant status and lipid levels in SH patients, because we have found associations between serum TSH levels and serum lipids levels, showing thyroid dysfunction influence on lipid metabolism and consequently on oxidant/antioxidant status in these patients. However, further studies are necessary to evaluate a larger series of patients, with a longer duration of subclinical hypothyroidism.

Conflict of Interests

There is no conflict of interests that could be perceived as prejudicing the impartiality of the research reported.

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3.2 MANUSCRITO 1:

OVERT HYPOTHYROIDISM IS ASSOCIATED WITH BLOOD INFLAMMATORY BIOMARKERS DEPENDENT OF LIPID PROFILE

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OVERT HYPOTHYROIDISM IS ASSOCIATED WITH BLOOD INFLAMMATORY BIOMARKERS DEPENDENT OF LIPID PROFILE

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Abstract

Objectives: To investigate the association between inflammatory biomarkers and overt hypothyroidism (OH).

Design and methods: We measured inflammatory cytokines, interleukin-1 (IL-1), interleukin-6 (IL-6), tumor necrosis factor-α (TNF- α) and interferon- ɣ (IFN-ɣ) as well as cell-free DNA (cf-DNA) levels in 40 OH patients and 40 healthy controls. Total cholesterol, high and low density lipoprotein subfractions, triglyceride, fibrinogen, and D-dimer were recorded.

Results: Increased inflammatory profile was evidenced through significant elevations in the concentrations of all cytokines and cf-DNA levels in the OH group. Lipids and prothrombotic markers were also increased in hypothyroid subjects. A significant association between the inflammatory cytokines and lipid profile was observed. A multivariate analysis showed that this result was independent of the sex, age and BMI status of the subjects.

Conclusions: Hypothyroidism is associated with proinflammatory state. Lipid abnormalities have a stronger influence on inflammation, increasing cardiovascular risk and atherosclerosis development in hypothyroidism.

Keywords: hypothyroidism; lipids; cytokines; inflammation

1. Introduction

Overt hypothyroidism (OH) is characterized by high thyrotropin (TSH) blood concentration, low triiodothyronine and thyroxine levels, and alterations of plasma lipid concentration, which could also be involved in the progress of atherosclerosis [1].Hypercholesterolemia is very common in OH patients, mainly due to higher low-density lipoprotein cholesterol (LDL-chol) levels. In addition, OH presents association with other atherosclerosis risk factors such as diastolic hypertension, coagulopathy and impaired endothelial function. As described in the review performed by Ichiki (2010) who summarized the basic and clinical studies on the role of thyroid hormone in atherogenesis, emerging risk factors have been associated with atherosclerosis and OH as a high C-reactive protein levels [2].

Atherosclerosis is currently regarded as a chronic inflammatory disease of the vascular wall [3, 4] in which cells of both innate and acquired immune systems are implicated. The low-grade chronic inflammation is maintained by the chronic activation of autoimmune reactions against self-proteins modified by oxidative stress that sustains endothelial dysfunction and plaque development [5]. In these terms, a potential OH association with inflammatory cytokines could be expected.

Thyroid hormones influence specific immune responsiveness as well as several aspects of innate and adaptive immunity. However, the relationship between thyroid hormones and immune cells is complex and needs to be clarified with additional investigations. T3 and T4 are able to modulate immune

responses through both genomic and nongenomic mechanisms and at physiological concentrations [6].

The acute phase response to inflammation is characterized by the combination of hepatocyte-derived plasma proteins induced by the inflammatory cytokines tumor necrosis factor-α (TNF- α) and interleukin-1 (IL-1) as well as those induced by interleukin-6 (IL-6) [7]. A key regulator of the inflammatory response is IL-6, which stimulates the synthesis of acute phase proteins including C-reactive protein (CRP) and fibrinogen (elevated levels which increase plasma viscosity and erythrocyte sedimentation rate), as well as the release of white blood cells from bone marrow into circulating blood [4,8]. Monocytes, fibroblasts, and lymphocytes cells produce IL-6 [9]. Moreover, IL-1 has been identified as a chemical mediator released from monocytes/macrophages and exhibits important biologic activity in inflammatory and immunologic responses [10].

TNF-α is another important cytokine produced by macrophages and T-cells in response to bacteria and virus infections. It is a powerful modulator of the immune response, mediating the induction of adhesion molecules and other cytokines [7]. This molecule is responsible for producing changes in the hypothalamic-pituitary thyroid axis by directly acting on each level. It is also an important modulator of the immunologic reactions produced by interferon- ɣ (IFN- ɣ) of HLA class II molecules in human thyroid follicular cells [11]. Despite the relevance of these molecules in metabolic dysfunctions associated to atherogenesis studies of alternating cytokine levels and OH are still incipient.

Another emerging inflammatory biomarker is the cell-free DNA (cf-DNA) that has been associated with outcome in several conditions as ischemic heart

failure, stroke, mesenteric ischemia, preeclampsia, and reports concerning the outcomes after cardiac arrest that found association of circulating DNA quantities at admission with mortality [12]. High cf-DNA levels were also described in patients with different cancer diagnosis as colon, lung, breast, stomach, and esophagus. The sources of cell-free DNA in plasma are primarily necrosis and apoptosis. In a healthy person, it is believed that cf-DNA enters circulation via apoptosis of lymphocytes and other nucleated cells [13].

Despite the potential influence of OH on blood inflammatory biomarkers there are few studies investigating this potential association. Therefore, we performed here a case-control study that evaluated the association between inflammatory biomarkers and OH. Lipid, prothrombotic and other biochemical markers related with body functions were also evaluated in the sample studied here.

2. Methods

2.1 Study design

A case-control study was performed with eighty subjects enrolled prospectively from clinical laboratory LABIMED, located in Santa Maria-RS, Brazil. Subjects were divided into two groups as follows: control group, 40 healthy subjects (male =18; female = 22) and the recent OH group, 40 patients (male= 19; female = 21) without previous pharmacological treatment. OH was defined as thyroid-stimulating hormone (TSH) higher than 10 mIU/L and low free thyroxine (fT4) and triiodothyronine (T3) levels [14]. Subjects with previous diseases and dysfunctions that could influence the results were excluded. The exclusion criteria were as follows: subjects taking lipid-lowering drugs,

antioxidant vitamin supplements, acetylsalicylic acid, antihistamines, antihypertensive, and exposure to high iodine condition, smokers, alcoholics, pregnant women, women on hormone replacement therapy, diabetics and subjects with acute, chronic or malignant diseases. The protocol was approved by the Human Ethics Committee of the Federal University of Santa Maria (number 87/2010). All subjects gave written informed consent to participate in the study.

2.2 Biochemical determinations

Blood samples were collected after 12 h overnight fasting by venous puncture into blue, gray and red top Vacutainers® (BD Diagnostics, Plymouth, UK) tubes. The samples were centrifuged for 15 min at 2500 x *g*, and aliquots of serum were kept at -20ºC for maximum of 4 weeks. Serum TSH, T3 and fT4 concentrations were measured by chemiluminescent immunometric assay on IMMULITE 2000® (Siemens Healthcare Diagnostics, Los Angeles, USA). Detection limits for TSH was 0.004 - 14.000 mIU/L, FT4 was 3.9 - 77.2 pmol/L and T3 was 0.29 nmol/L.

The biochemical markers were spectrophotometry determined using Hitachi U-2800A® equipment (Hitachi High – Technologies Corporation, Japan). High-density lipoprotein cholesterol was measured in the supernatant plasma after the precipitation of apolipoprotein B-containing lipoproteins with dextran sulfate and magnesium chloride as previously described [15]. Low-density lipoprotein cholesterol (LDL-C) was estimated with the Friedewald equation [16].

The cytokines IL-1, IL-6, TNF- α and IFN-ɣ were analyzed using ELISA capture, according to the manufacturer's instructions (Biomyx Technology, San Diego, CA). The D-dimer levels were measured by immunoturbidimetric method on Cobas INTEGRA 400® (Roche Diagnostics, Basel, Switzerland). Fibrinogen levels were measured using coagulation analyzer Sysmex® CA-1500 (Siemens Healthcare Diagnostics, Los Angeles, USA).

The cf-DNA was quantified using PicoGreen fluorescent assay done according to protocol supplied by the manufacturer (Quant-iT™ Picogreen® dsDNA kit, Invitrogen, USA). Picogreen dye was diluted 1:200 with TE buffer (10mM Tris-HCl, 1mM EDTA, pH 7.5) and incubated with plasma DNA in the dark at room temperature for 5 min. To minimize photo bleaching effects, time for fluorescence measurement was kept constant for all samples. PicoGreen with DNA was recorded at 528 nm using an excitation wavelength of 485 nm. All the fluorescence measurements were recorded on a SpectraMax M2/M2e Multi-mode Plate Reader, Molecular Devices Corporation, Sunnyvale, CA, USA.

2.3 Statistical analysis

Data are presented as mean and standard deviation (SD). Statistical differences between groups were evaluated by Student t test. Multivariate analysis using logistic regression (*Backward wald*) were performed to evaluate if sex, age and BMI variables could to present some influence in the significant results obtained from univariate analysis. To test the potential influence of hormonal status, lipid profile and prothrombotic variables on inflammatory cytokine and cf-DNA levels an additional Pearson correlation test was

performed considering just OH subjects. Statistical significance was assumed at $p<0.05$. Data were statistically analyzed using SPSS software (version 19.0).

3. Results

Baseline characteristics of the study (Table 1) showed that, as expected the OH group presented higher TSH and lower T3 and fT4 levels as well as higher levels of autoimmune thyroid markers, anti-thyroperoxidase antibodies (anti-TPOAbs) and anti-thyroglobulin antibodies (anti- TgAbs). The OH subjects also presented higher levels of cholesterol total, LDL-chol and triglycerides, fibrinogen and D-dimer and lower levels of HDL-chol than control subjects despite the age and BMI to be similar between groups.

Table 1 here

Hereafter proinflammatory cytokines as well as cf-DNA levels were compared between groups. As can see in Figure 1, the inflammatory cytokines levels were significantly higher in OH when compared to control subjects. The cf - DNA levels were also higher in OH than healthy control subjects. The association between cytokines and cf- DNA with OH was independent of sex, age and BMI variables.

Figure 1 here

Potential correlation between inflammatory biomarkers and thyroid hormonal status, lipids and prothrombotic in OH patients was also evaluated (Table 2). In general hormonal status was not associated with cytokine and cf-DNA levels in OH subjects. On the other hand, lipid profile of OH subjects presented important correlations with inflammatory biomarkers studied here. Positive association was observed between total cholesterol and IL-1, IL-6 and

IFN- ɣ cytokines. LDL-cholesterol also presented a positive correlation with IL-6 and INF-ɣ and triglycerides with IL-1 and TNF-α. Negative correlation between HDL-cholesterol and TNF-α cytokine was also observed. Prothrombotic variables did not present correlation with inflammatory cytokines. Cf-DNA levels were not correlated with any variables investigated here.

Table 2 here

Considering that lipid profile are highly associated with atherosclerosis and subsequent cardiovascular risk, an additional analysis was performed when the OH patients were categorized in respect of lipid profile cut-off points: cholesterol total (> 240 mg/dL), LDL-chol (> 130 mg/dL), triglycerides (> 150 mg/dL) and HDL-chol (< 50 mg/dl) and the cytokines levels that presented significant correlation were compared. As can see in Figure 2, the OH with cholesterol > 240 mg/dL presented significant higher IL-6 and IFN-ɣ levels than subjects with lower cholesterol levels. The OH subjects with lower HDL-chol levels also presented higher significant TNF-α levels. Again, these associations were independent of sex, age and BMI variables.

Figure 2 here

4. Discussion

The present study was performed to evaluate the potential association between OH and blood inflammatory biomarkers (cytokines and cf-DNA) as well as the influence of hormonal status and cardiovascular risk factors know to be associated with OH: lipid profile and prothrombotic variables.

We found higher levels of total cholesterol, LDL cholesterol and triglycerides and lower levels of HDL cholesterol in the OH group. These

findings are in agreement with results of other recent investigations, which have showed dyslipidemia associated with hypothyroid status [17-19]. The association between lipid profile alterations and OH observed in our study is well established in previous studies and explain, in part, the potential association between hypothyroidism and cardiovascular diseases [20, 21]. A causal mechanism to explain the association between lipid profile alteration and OH is related to the fact that thyroid hormones upregulate the LDL-receptor expression. Thus, the low levels of T3 and T4 found in hypothyroidism promote a reduction in catabolism of lipoproteins leading to hypercholesterolemia [22, 23]. Hypothyroidism has also been associated with alterations in prothrombotic variables. Data reported in the literature have shown higher levels of D-dimer [24] and fibrinogen [25] in patients with overt hypothyroidism. Our results corroborate this association since OH subjects presented higher prothrombotic variables, suggesting a potential hypercoagulable state, which may augment the already existing risk for atherosclerotic complications.

From the confirmation of the association between OH and lipid and prothrombotic alterations, the levels of five inflammatory biomarkers, four cytokines (IL-1, IL-6, TNF- α and IFN - ɣ), and cf-DNA levels were performed between case-control groups. The results showed higher levels of these biomarkers in OH subjects indicating the occurrence of a low-grade inflammatory state that is in consonance with atherosclerosis development.

It is widely recognized that OH is associated with increased risk of atherosclerosis. Hyperlipidemia is one of the major risk factors leading to early

atherosclerotic vascular diseases. Atherosclerosis has been considered a chronic inflammatory disease, involving both the innate and adaptive immune systems, which modulate the initiation and progression of the lesions, and potentially devastate thrombotic complications [26, 27]. Cytokines such as IL-1 and TNF-α are proximal components of inflammatory cascades of systemic mediators activating the endothelium which leads to an endothelial dysfunction and moreover alters the balance within lymphocytic subpopulations containing distinct arsenals of secretory mediators such as interferons, interleukins and chemokines [28]. Other cytokines, such as TNF-α or IFN-γ, are central mediators of inflammatory reactions [29]. TNF-α along with interferon-γ and IL-1 stimulate IL-6 production by smooth muscle cells. IL-6 gene transcripts are expressed in human atheromatous lesions, and IL-6 is the main hepatic stimulus for CRP production [30].

To evaluate if the association between inflammatory biomarkers and OH is a primary event triggered by hormonal status or is a secondary event triggered by lipid and prothrombotic status, a correlation analysis between these variables was carried out considering just OH patients. Results showed clearly that higher cytokine levels present direct correlation with dyslipidemia suggesting that the inflammatory status of OH patients is modulated by lipid profile.

Inflammation is associated with alterations in lipid metabolism that may be mediated by cytokines. The inflammatory profile in subjects with OH is higher in those with total cholesterol levels over 240 mg/dl than in those with lower total cholesterol levels, as evidenced by the higher IL-6 and INF- ɣ levels between the former group of patients. In addition, TNF – α was higher in the OH

subjects with HDL-chol lower 50 mg/dl. According to these data, we can confirm an association between the degree of hyperlipidemia and inflammatory status in hypothyroid subjects. In respect to the association between lipid metabolism and inflammatory response several experimental studies have demonstrated that the cytokines can affect hepatic enzymes responsible for the synthesis and catabolism of lipids. The administration of TNF – α and IL-1 to rats results in an acute stimulation of hepatic fatty acid synthesis [31]. TNF or IL-1 administration to Syrian hamsters increased serum cholesterol levels, and decreased HDL cholesterol levels because cytokines increased hepatic HMG CoA reductase mRNA levels [32]. According to these data, we can confirm an association between the degree of hyperlipidemia and inflammatory status in hypothyroid subjects, suggesting the role of inflammatory cytokines in lipid homeostasis.

Furthermore, we can suggest that inflammatory condition observed in OH group did not involve protrombotic and thyroid autoimmunity markers because neither of the parameters displayed significant associations with inflammatory markers (IL-1, IL-6, TNF-α and INF-γ), although most patients of the OH group showed autoimmune basis for hypothyroidism. Hashimoto thyroiditis (HT) is the most common etiology of hypothyroidism and is characterized by chronic lymphocyte infiltration of thyroid glands [33]. Its prevalence is linked to genetic predisposition and is also influenced by environmental factors, among which iodine plays a pivotal role [34]. Therefore, we can suggest that inflammatory condition observed in the OH patients is lipid profile dependent.

In this study, the concentrations of cf-DNA were significantly elevated in the OH subjects compared with the healthy controls, suggesting chronic low-grade proinflammatory state and increased death cellular rate in

hypothyroidism. Necrosis, apoptosis, and possibly “active secretion” are thought to be the mechanisms by which cell-free nucleic acids enter the circulation [35]. Cui et al (2013) demonstrated that Cf-DNA levels are higher in patients with acute coronary syndrome than in patients with stable angina and healthy control, indicating that cf-DNA may be a valuable marker for diagnosing and predicting the severity of coronary artery lesions [36]. Another study suggests that the measurement of cell-free DNA may complement troponin and CK-MB in the diagnosis of myocardial infarction [37]. In very old individuals, plasma cf-DNA level was positively associated with the plasma levels of CRP and IL-1ra and inversely associated with the HDL cholesterol level presenting a novel biomarker candidate for systematic inflammation related to aging [38]. However, we did not observe correlations between the cf-DNA levels and the hormonal, lipid and prothrombotic status. These results indicate that the release of DNA from cells in hypothyroidism is likely to be a result of primary inflammation associated with high lipid levels.

In conclusion, our data suggest that higher lipid concentrations are associated with inflammatory status observed in hypothyroidism. Moreover, higher cf-DNA levels and prothrombotic markers could be additional risk factors for atherosclerotic cardiovascular disease in such subjects. However, further studies in a larger sample are required to elucidate the mechanisms of atherosclerosis development in hypothyroidism and provide new strategies for lipid and inflammation management in this disease.

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Conflict of Interest

The authors declare that there are no conflicts of interest.

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Table 1. Clinical and laboratory data of study participants

	Control	OH patients
Age (years)	51.3 ± 10.4	47.6 ± 8.5
BMI (Kg/m^2)	24.1 ± 3.5	26.7 ± 3.76
TSH (mIU/L)	1.61 ± 1.07	12.26 ± 2.79 ***
T3 (nmol/L)	1.64 ± 0.49	0.52 ± 0.13 ***
fT4 (pmol/L)	85.26 ± 28.38	19.8 ± 0.83 ***
Anti-TPOAbs (IU/ml)	7.81 ± 6.02	375.3 ± 83.19 ***
Anti- TgAbs (IU/ml)	7.93 ± 7.60	72.74 ± 14.65 ***
Total cholesterol (mmol/L)	4.12 ± 0.69	7.05 ± 0.95 **
HDL cholesterol (mmol/L)	1.69 ± 0.37	1.23 ± 0.26 **
LDL cholesterol (mmol/L)	1.98 ± 0.63	5.07 ± 0.95 ***
Triglycerides (mmol/L)	1.17 ± 0.32	1.64 ± 0.56 **
Fibrinogen (mg/dL)	197.9 ± 44.16	388.3 ± 28.9 ***
D-dimer (ng/mL)	76.9 ± 34.7	169.1 ± 22.24 ***

Data are expressed as mean ± SD. BMI, body mass index; TSH, thyroid-stimulating hormone; T3, triiodothyronine; fT4: free thyroxine; Anti-TPO Abs, anti-thyroperoxidase antibodies; Anti- Tg Abs, anti-thyroglobulin antibodies. Significance value = ** $p < 0.01$; *** $p < 0.001$ estimated by Student t test.

Table 2. Correlations between total inflammatory cytokines and cf-DNA and thyroid hormones, lipid profile and prothrombotic variables in OH patients (n=40)

Variables	IL-1 (pg/mL)		IL-6 (pg/mL)		TNF-α (pg/mL)		IFN-γ (ìg/ml)		cf-DNA (ng/mL)	
	r	p	r	p	r	p	r	p	r	p
TSH (mIU/L)	0,09	0,586	0,20	0,213	0,23	0,150	0,07	0,660	0,03	0,874
T3 (nmol/L)	0,32	0,056	0,22	0,181	-0,03	0,872	0,27	0,089	-0,03	0,887
fT4 (pmol/L)	0,24	0,132	0,12	0,472	0,29	0,066	0,03	0,861	0,04	0,850
Anti-TPOAbs (IU/ml)	0,17	0,306	0,07	0,681	0,25	0,127	0,15	0,366	0,24	0,194
Anti- TgAbs (IU/ml)	0,05	0,744	0,11	0,510	-0,13	0,434	-0,04	0,827	-0,19	0,318
Total cholesterol (mmol/L)	0,33	0,036	0,46	0,003	0,04	0,822	0,47	0,002	0,18	0,341
HDL cholesterol (mmol/L)	0,00	0,984	-0,06	0,726	-0,45	0,003	-0,09	0,591	0,07	0,698
LDL cholesterol (mmol/L)	0,24	0,137	0,41	0,009	0,05	0,745	0,44	0,005	0,12	0,518
‘Tryglicerides (mmol/L)	0,35	0,027	0,25	0,117	0,40	0,010	0,23	0,159	0,15	0,408
Fibrinogen (mg/dL)	0,27	0,093	0,17	0,308	-0,15	0,349	0,11	0,499	0,04	0,824
D-dimer (ng/mL)	0,20	0,209	-0,27	0,091	-0,02	0,901	-0,32	0,055	-0,02	0,934

IL-1, Interleukin-1; IL-6, interleukin-6; TNF- α, tumor necrosis factor-α; IFN-ɣ, interferon- ɣ; cf-DNA, cell-free DNA; TSH, thyroid-stimulating hormone; T3, triiodothyronine; fT4, free thyroxine; Anti-TPO Abs, anti-thyroperoxidase antibodies; Anti-Tg Abs, anti-thyroglobulin antibodies. $p < 0.05$ according to Pearson correlation test.

Figure legends

Fig. 1: Inflammatory cytokines and cf- DNA levels in controls and OH patients. (A) IL-1, (B) IL-6 ,(C) TNF-α, (D) IFN-ɣ and (E) Cf - DNA . IL-1, Interleukin-1; IL-6, interleukin-6; TNF- α, tumor necrosis factor-α; IFN-ɣ, interferon- ɣ; cf-DNA, cell-free DNA. Significance value = ** p <0.01; *** p <0.001 estimated by Student t test.

Fig. 2: Inflammatory cytokines levels according to lipid profile cut-off points in OH patients. (A) IL-6 (pg/mL), (B) IFN-ɣ and (C) TNF-α. Cut-off values for elevated total cholesterol (TC) were >240 mg/dL and for decreased HDL cholesterol were <50mg/dL. IL-6, interleukin-6; IFN-ɣ, interferon - ɣ; TNF- α, tumor necrosis factor-α. Significance value = * p <0.05, ** p <0.01 estimated by Student t test.

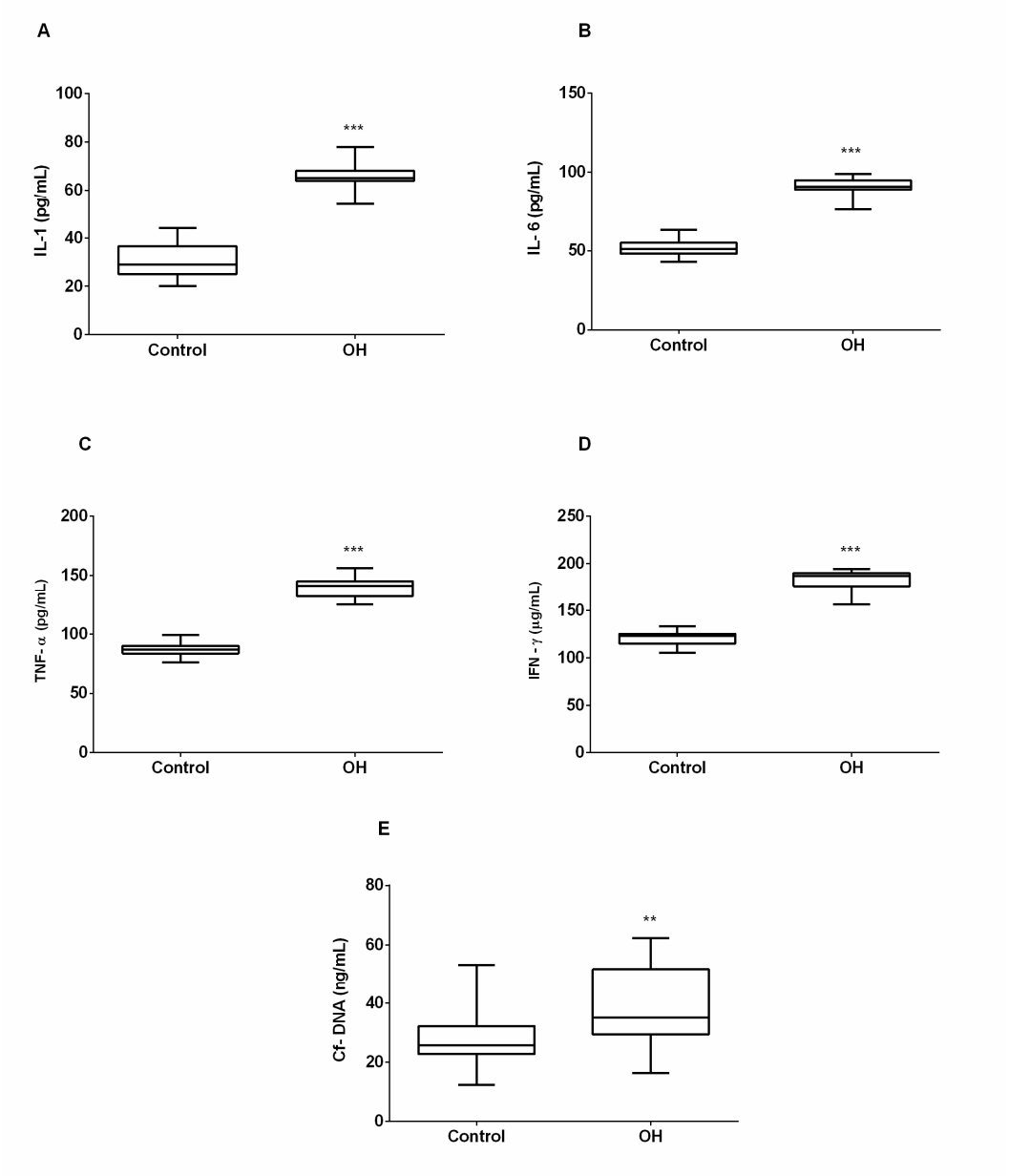
A
IL-1 (pg/mL)
100
80
60
40
20
0
***
Control
OH
B
IL- 6 (pg/mL)
150
100
50
0
***
Control
OH
C
TNF- α (pg/mL)
200
150
100
50
0
***
Control
OH
D
IFN - γ (μg/mL)
250
200
150
100
50
0
***
Control
OH
E
Cf- DNA (ng/mL)
80
60
40
20
0
**
Control
OH

Fig. 1

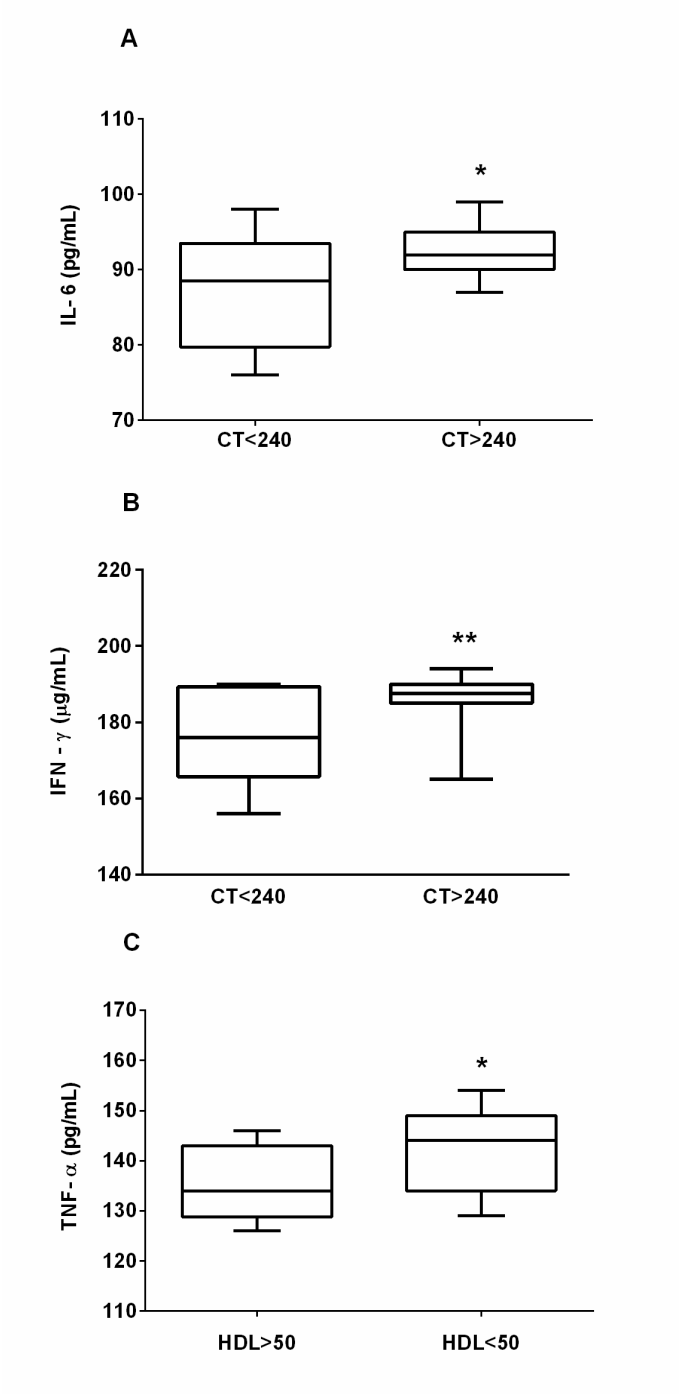
A
110
100
90
80
70
IL- 6 (pg/mL)
*
CT<240
CT>240
B
220
200
180
160
140
IFN - γ (μg/mL)
**
CT<240
CT>240
C
170
160
150
140
130
120
110
TNF- α (pg/mL)
*
HDL>50
HDL<50

Fig. 2

3.3 MANUSCRITO 2:

EFECTS OF QUERCETIN ON OXIDATIVE STRESS BIOMARKERS IN METHIMAZOLE – INDUCED HYPOTHYROID RATS

Adriana Santi, Jucimara Baldissareli, Camila R. Murussi, Glaecir R. M. Dias, Charlene C. de Menezes, Daniela Zanini, Fátima H. Abdalla, Gustavo R. Thomé, Caroline C. Martins, Maria Rosa C. Schetinger, Vania L. Loro.

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EFFECTS OF QUERCETIN ON OXIDATIVE STRESS BIOMARKERS IN METHIMAZOLE – INDUCED HYPOTHYROID RATS

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Running title: Effects of quercetin on oxidative stress in hypothyroid rats.

Figures: 4

Tables: 4

References: 49

Abstract

The objective of the present study was to evaluate the effect of quercetin on oxidative stress biomarkers in methimazole (MMI) - induced hypothyroidism male rats. Hypothyroidism was induced by administering MMI at 20 mg/100 ml in the drinking water, for 1 month. After achieved hypothyroidism, rats received orally 10 or 25 mg/kg of quercetin (QT) for 8 weeks. Sixty male wistar rats were randomly divided into six groups (group I, control; group II, QT10; group III, QT25; group IV, hypothyroid; group V, hypothyroid + QT10; group VI, hypothyroid + QT25). Liver, kidney and serum TBARS levels significantly increased in hypothyroid rats when compared to controls, along with increased protein carbonyl (PCO) in liver and increased ROS levels in liver and kidney tissues. QT10 and QT25 were effective in decreasing TBARS levels in serum and kidney, PCO levels in liver and ROS generation in liver and kidney. MMI - induced hypothyroidism also increased TBARS levels in cerebral cortex and hippocampus that in turn were decreased in rats treated with QT25. Moreover, the administration of QT25 to hypothyroid rats resulted in decreased SOD activities in liver and whole blood and increased liver CAT activity. Liver and kidney ascorbic acid levels were restored with quercetin supplementation at both concentrations. QT10 and QT25 also significantly increased total oxidative scavenging capacity in liver and kidney tissues from hypothyroid rats. These findings suggest that MMI - induced hypothyroidism increases oxidative stress parameters and quercetin administration could exert beneficial effects against redox imbalance in hypothyroid status.

Keywords: quercetin; hypothyroidism; methimazole; oxidative stress; antioxidant.
Number of words: 249

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1. Introduction

A great number of evidence suggests the role of THs in the generation of reactive oxygen species (ROS) due to their effect on the general metabolism of body (1). Hypothyroidism has been associated with a submetabolic state and lowered energy and oxygen metabolism (2). However, many clinical and experimental studies have shown the association between hypothyroidism and imbalance redox (3-5). Likewise, in our earlier reports, we demonstrated that overt and subclinical hypothyroidism modulates several oxidative stress and antioxidant defense parameters in humans (6, 7). It was described that, despite being a hypometabolic state, increased lipid peroxide levels can occur in hypothyroidism due to slower clearance rate (8).

In order to protect tissues from these damaging effects, the organism possesses enzymatic and non enzymatic antioxidant systems (9). Free radical-scavenging enzymes such as superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx) are the first line of cellular defense against oxidative injury, decomposing $O2^{\circ -}$ and H_2O_2 before interacting to form the more reactive hydroxyl radical (°OH) (10). Small intracellular antioxidants such as vitamin E and C, β-carotene, ubiquinone, lipoic acid, urate, and glutathione are categorized under non enzymatic pathways (11).

Currently, antioxidant therapies have been employed such as possible strategies to maintain the redox homeostasis by directly quenching excessive ROS or protecting endogenous antioxidative enzyme activities against oxidative stress (12). Quercetin (3,5,7,3',4'-pentahydroxyflavone) is one of the several naturally-occurring dietary flavonol compounds, and is found in various vegetables, fruits, and

red wine (13,14).The compound has been extensively studied for its various pharmacological properties including anti-inflammatory (15), vasodilator (16), anti-mutagenic (17) and antioxidant (18). So far, the impact of quercetin on oxidative/antioxidative status in hypothyroidism has not been systematically investigated. Therefore, the present study aimed to investigate the effect of quercetin on oxidative stress biomarkers in methimazole (MMI) - induced hypothyroid rats.

2. Materials and methods

2.1 *Chemicals*

≥99% methimazole, 95% quercetin, 5,5 '-dithio-bis-2-nitrobenzoic acid (DTNB), thiobarbituric acid (TBA), trichloroacetic acid (TCA), malondialdehyde (MDA) and Coomassie brilliant blue G were obtained from Sigma Chemical Co (St. Louis, MO, USA). All other reagents used in the experiments were of analytical grade and of the highest purity

2.2 Animals

Experiments were carried out in 50-70 day-old adult male Wistar strain rats (*Rattus norvegicus*) supplied by the Central Animal House of the Federal University of Santa Maria (Santa Maria, Brazil). Animals were maintained at 25 °C ± 2 °C under standard conditions in the animal room. All animal procedures were approved by the Ethics Committee for Animal Care from the Federal University of Santa Maria (protocol under number: 083/2012).

2.3 *Experimental induction of hypothyroidism*

Initially, rats were divided into two groups: control (n=40), given water and hypothyroid (n=40) given 20% methimazole (MMI) in drinking water for 30 days (19). After this period, animals were euthanized and blood samples were collected by cardiac puncture for measurement of thyroid hormones (see section 2.12) that in turn confirmed hypothyroidism in the animals (n=5) (data not shown).

2.4 Treatment with quercetin

After rats achieved hypothyroidism or control period, they were divided into six groups (n=10 rats per group) as follows:

- Group I: Control group in which animals have never received any treatment (euthyroid);
- Group II: QT10 group in which animals received quercetin (10mg/kg/day) for 8 weeks orally by gavage;
- Group III: QT25 group in which animals received quercetin (25mg/kg/day) for 8 weeks orally by gavage;
- Group IV: Hypothyroid group in which animals received 20% MMI in drinking water for 8 weeks;
- Group V: Hypothyroid plus QT10 group in which animals received 20% MMI in drinking water and quercetin (10mg/kg/day) concurrently for 8 weeks;
- Group VI: Hypothyroid plus QT25 group in which animals received 20% MMI in drinking water and quercetin (25mg/kg/day) concurrently for 8 weeks;

Euthyroid group received only drinking water and animals of IV, V and VI groups had achieved hypothyroidism and thereafter continue receiving 20% MMI in drinking water for 8 weeks. Treatment with quercetin 10 and 25 mg/kg body weight

were administered by gavage in groups II, III, V and VI, between 10 and 12 a.m. once a day for 8 weeks, at a volume not exceeding 0.1 mL/100g rat weight. Ethanol 25% was used as the vehicle solution for treatment with quercetin. A control/ethanol group (n=10) was used to verify if vehicle solution alter the parameters studied. However, no changes were observed (data not shown). Euthyroid (group I) and hypothyroid (group IV) rats received drinking water by gavage at same volume of antioxidant.

2.5 Sample preparation

Twenty-four hours after the treatment, the animals were previously anesthetized with halothane 3% associated with tramadol hydrochloride (8 mg/kg subcutaneously) and submitted to euthanasia. The samples of liver and kidney were quickly removed, placed on ice and homogenized within 10 min in cold 50 mM Tris HCl pH 7.4 (1/10, w/v) and centrifuged at 2,400 x g for 10 min, except for protein carbonyl content determination in which the homogenate was used without centrifugation. The low-speed supernatants (S1) were separated and used for the assays. The brain was separated into cerebral cortex, hypothalamus, hippocampus, cerebellum, and striatum. The samples were placed in a solution of 10 mM Tris–HCl, pH 7.4 and centrifuged at 2,400 x g for 10 min, to yield the S1 fraction.

2.6 Reactive oxygen species (ROS) and total oxyradical scavenging capacity (TOSC) levels

ROS were determined in the fresh supernatant fraction of liver and kidney by the method of Viarengo et al (20). Tissue samples were prepared through homogenization (1:5 –w/v) in a buffer containing Tris-HCl (100 mM, pH 7.75), EDTA

(2 mM) and $MgCl_2$ (5mM). The homogenates were centrifuged at 10,000 x g for 20 min at 4°C. ROS was expressed as area of ROS per mg of protein. In the same supernatant, TOSC was determined using the method described by Amado et al (21). TOSC was expressed as the relative area of remaining ROS per milligram of protein, an index which is inversely proportional to total oxyradical scavenging capacity.

2.7 *Determination of lipid peroxidation*

Lipid peroxidation in liver, kidney and brain structures was estimated colorimetrically by measuring thiobarbituric acid reactive substances (TBARS) using the method described previously by Ohkawa et al (22). In short, the reaction mixture contained 200 µL of samples of S1 from liver, kidney and brain structures or standard (MDA-malondialdehyde 0.03 mM), 200 µL of 8.1% sodium dodecylsulfate (SDS), 750 µL of acetic acid solution (2.5 M HCl, pH 3.5) and 750 µL of 0.8% TBA. The mixtures were heated at 95ºC for 60 min. TBARS tissue levels were expressed as nmol MDA/mg protein.

Serum TBARS levels were measured according to the modified method of Jentzsch et al (23). Serum was added to a reaction mixture containing 1% ortho-phosphoric acid, an alkaline solution of thiobarbituric acid-TBA, followed by heating for 45 min at 95ºC. After cooling, samples and standards of malondialdehyde (0.03 mM) were read at 532 nm against the blank of the standard curve. Results were expressed as nmol MDA/mL.

2.8 Protein carbonyl (PCO) level determination

The protein carbonyl determination was carried out as described by Reznick

and Packer (24). Aliquots of 1,000 µl of the diluted homogenates were incubated in three tubes. In two tubes, it was added 200 µl of 10 mM 2,4-dinitrophenylhydrazine in 2.0 M HCl. The other tube contained only 200 µl of 2.0 M HCl solution (blank). Tubes were incubated for 60 min at room temperature in dark and were shaken with a Vortex mixer every 15 min. After that, 0.5 ml of denaturizing buffer (sodium phosphate buffer, pH 6.8, containing 3 % sodium dodecyl sulfate (SDS), 1.5 ml of ethanol, and 1.5 ml of hexane were added. The mixture was shaken with a vortex mixer for 40 s and centrifuged at 2,400×g for 15 min. The pellet obtained was separated and washed two times with 1 ml of ethanol: ethyl acetate (1:1, v/v). Absorbance was measured at 370 nm. PCO levels were expressed as nanomoles of carbonyl per milligram of protein.

2.9 Superoxide dismutase (SOD) and catalase (CAT) activities

With the purpose of performing the SOD assay (25) liver, kidney and whole blood were adequately diluted with Tris-HCl pH 7.4 at a proportion of 1:40 (w/v) and 1:60(w/v) respectively. Briefly, epinephrine undergoes auto-oxidation at pH 10.2 to produce adrenochrome, a colored product that was detected at 480 nm. The addition of samples (10, 25, 50 µL) containing SOD inhibited the auto-oxidation of epinephrine. The rate of inhibition was monitored during 180 s. The amount of enzyme required to produce 50% inhibition was defined as 1 unit of enzyme activity.

For the CAT assay, whole blood, liver and kidney were homogenized in 50mM potassium phosphate buffer, pH 7.5, at a proportion of 1:9 (w/v) and 1:5 (w/v), respectively. The homogenate was centrifuged at 2000 g for 10 min to yield a supernatant that was used for the enzyme assay. CAT activity was measured by the method of Nelson and Kiesow (26). The reaction mixture contained 50 mM

potassium phosphate buffer (pH 7), 10mM H_2O_2 and 20 µL of the supernatant. The rate of H_2O_2 reaction was monitored at 240 nm for 2 min at room temperature. The enzymatic activity was expressed in units mg/protein (One unit of the enzyme is considered as the amount of CAT which decomposes 1 µmol of H_2O_2 per min at pH 7, 00 at 25 °C.)

2.10 Ascorbic acid (Vitamin C) and non-protein thiol group (NPSH) content

Hepatic and renal ascorbic acid (AA) levels were determined by the method of Roe (27).Proteins of liver and kidney were precipitated in a cold 10% trichloroacetic acid (TCA) solution at a proportion of 1:1 (v/v) and submitted to centrifugation again. This supernatant was then used for analysis. A 300 µL aliquot of sample in a final volume of 575 µL of solution was incubated for 3 h at 37° C then 500 µL H_2SO_4 65% (v/v) was added to the medium. The reaction product was determined using a color reagent containing 4.5 mg/mL dinitrophenyl hydrazine (DNPH) and CuSO4 (0.075 mg/ mL). Ascorbic acid levels are expressed as µg ascorbic acid/g tissue.

NPSH was measured spectrophotometrically with Ellman's reagent (28). An aliquot of 100 µL for liver and 200 µL for kidney in a final volume of 900 µL of solution was used for the reaction. The reaction product was measured at 412 nm after the addition of 10 mM 5-5-dithio-bis (2-nitrobenzoic acid) (DTNB) (0.05 mL). A standard curve using cysteine was added to calculate the content of thiol groups in samples, and was expressed as µmol SH/g tissue.

2.11 Biochemical analysis

Plasmatic aspartate aminotransferase (AST), alanine aminotransferase (ALT),

total cholesterol (TC) and glucose were determined using commercial kits (Labtest, Diagnóstica S.A., Minas Gerais, Brazil).

2.12 Thyroid hormones determination

Plasma levels of total thyroxine (tT4) and total triiodothyronine (tT3) were measured by microparticle enzyme immunoassay (MEIA) using AxSYM® system (Abbott Laboratories, Abbott Park, Illinois, USA), according to suppliers' instructions.

2.13 Protein determination

Protein was measured by the method of Bradford (29) using bovine serum albumin as standard.

2.14 Statistical analysis

Data were analyzed statistically by two-way ANOVA followed by the Tukey's multiple tests. Results are expressed as the mean ± S.E.M. Differences were considered significant when the probability was $p < 0.05$.

3. Results

3.1 Body weight, plasma tT3 and tT4 levels and biochemical parameters

MMI exposure caused a significant reduction in the body weight of animals when compared to the control group, which was partially protected with quercetin administration. Moreover, treatment with methimazole was effective in establishing a hypothyroid state, decreasing tT3 and tT4 levels in animals (groups IV, V and VI). Plasmatic biochemical parameters (AST, ALT, TC and glucose) did not change

among the experimental groups studied (Table 1).

3.3 ROS and TOSC levels

The concentrations of liver and kidney ROS and TOSC of MMI-induced hypothyroid rats are shown in Fig. 1 and 2, respectively. A significant increase in ROS levels was observed in liver and kidney tissues of MMI-induced hypothyroid rats when compared to those of the control group. QT10 and QT25 protected against the increase of ROS levels observed in hypothyroidism (Fig. 1). With respect to TOSC levels, liver and kidney showed significantly increased relative area, signifying a loss of total ROS scavenging capacity in hypothyroid animals when compared to the control group. Quercetin showed significantly decreased relative area in liver (QT10 and QT25) and kidney (QT25), indicating an augmentation of total ROS scavenging capacity in hypothyroid rats (Fig. 2).

3.4 *TBARS and protein carbonyl (PCO) levels*

There was a significant increase in liver TBARS in Group IV rats when compared to euthyroid group. Administration of quercetin did not reduce hepatic lipid peroxidation in comparison to hypothyroid rats. In addition, MMI-induced hypothyroid state resulted in significant increases in kidney and serum TBARS levels with regard to control rats and it were modified by QT10 and QT25 with respect to hypothyroid group (Table 2).

Protein carbonyl levels in liver were increased in hypothyroid group when compared to controls, while treatment with QT10 and QT25 decreased the levels. No

alteration in kidney protein carbonyl content was observed in hypothyroidism (Table 2).

Table 3 shows TBARS levels in cerebral structures of rats. As can be observed, levels in cerebral cortex and hippocampus were significantly increased in the hypothyroid group when compared to the control group. The administration of QT25 to experimental hypothyroid rats significantly reinstates the elevated TBARS levels in both tissues to near normalcy.

3.5 Activities of antioxidant enzymes

The effect of quercetin on the activities of enzymatic antioxidants SOD and CAT in liver, kidney and whole blood are presented in Table 4. A marked increase in hepatic, renal and whole blood SOD activities were observed in the MMI treated groups when compared to their control groups. The administration of QT25 (group VI) decreased hepatic and whole blood SOD activities in hypothyroid animals. Moreover, MMI treatment resulted in a significant decrease in the liver CAT activity when compared to the control group. The administration of QT25 to hypothyroid rats resulted in a marked increase in the liver CAT activity when compared to Group IV rats.

3.6 Levels of non enzymatic antioxidants

MMI treatment resulted in a significant decrease in liver and kidney AA levels in comparison to Group I rats. However, quercetin was able to prevent these reductions in the hypothyroid/QT10 and hypothyroid/QT25 groups when compared to hypothyroid rats (Fig. 3). Hepatic and renal NPSH contents were lower in hypothyroid group when compared to control rats. Treatment with quercetin no was effective in

reversing these decreases (Fig.4).

Discussion

In this investigation, the effect of quercetin on serum, whole blood and tissues oxidative stress biomarkers were reported in MMI – induced hypothyroidism in male rats. Our present results clearly show that the quercetin prevented oxidative damage for reduced ROS generation, lipid and protein oxidation, increased ROS scavenging capacity and AA levels and influenced antioxidant enzymes. Some authors have reported that the administration of exogenous antioxidant can be associated with traditional TH therapy (30) or a preferable therapeutic agent against hypothyroid-induced oxidative stress (31).It is known that quercetin may exhibit antioxidant properties due to its chemical structure, particularly the presence and location of the hydroxyl (-OH) substitutions (32).

Thyroid hormones are implicated in the regulation of oxidative metabolism, and thereby play an important role in ROS generation (33). Lipid peroxidation (LPx) may produce injury by compromising the integrity of membranes and by covalent binding of reactive intermediates to important biological molecules such as reduced glutathione (GSH) (34). In the present study, TBARS levels increased in serum, liver and kidney of hypothyroid animals (Table 2). Moreover, an increase in kidney and liver levels of ROS was found in MMI – treated rats (Fig.1). Increased LPx and free radical formation in rats with hyporthyroidism have led to the suggestion that free radicals may play a role in the tissue injury in this disease. However, quercetin treatment was effective in decreasing the TBARS levels in serum and kidney and ROS generation in liver and kidney of V and VI groups. Similar results were found by Jena et al (31) that showed co-administration of vitamin E and curcumin to

hypothyroid rats resulted in amelioration of LPx level in kidney cortex. In addition, in hypothyroid animals supplemented with 0.5% taurine plasma MDA levels were restored to those seen in control animals (35). Treatment with quercetin (10mg/Kg) for 5 weeks reduced plasma malondialdehyde levels in hypertensive rats (36). Thus, results of the present work suggest that ROS generation and lipid peroxidation may be attenuated by quercetin supplementation in hypothyroid dysfunction.

It has been reported that thyroid hormones T3 and T4 are essential for appropriate brain development and function (37). Brain is extremely susceptible to oxidative damage since it may present high levels of ROS due to its high aerobic metabolism and blood perfusion, besides having relatively poor enzymatic antioxidant defenses (38). We reported here increased TBARS levels in cerebral cortex and hippocampus of hypothyroid rats. Quercetin was effective in decreasing lipid peroxidation in both brain structures only at the highest concentration (25mg/kg) (Table 3). Likewise, Cano-Europa et al (39) observed that hypothyroidism increases ROS production and induces selective oxidative stress in hippocampus of adult rats. In addition, Jena et al (40) showed that curcumin-supplementation to hypothyroid rats reduced LPx levels in cerebellum and cerebral cortex. Thus, these decreases in TBARS levels could be good indicators of lowered lipid peroxidation in brain with quercetin treatment in hypothyroidism. Quercetin has been shown to have a beneficial role in neuroprotection through a strong antioxidant activity (41, 42).

It is clear from the present findings that hypothyroidism is associated with prooxidative status, as evidenced by the elevated lipid peroxidation and ROS generation. Also, a concurrent increase in liver PCO content implies that liver cells are under oxidative stress. It can be supposed that increased lipid peroxidation and ROS production in liver could be contributing for increased protein damage in this

tissue. Protein carbonylation is the most widely used marker for protein oxidation formed by oxidation either via free radicals or via the attack of reactive aldehydes such as MDA formed during lipid peroxidation (43). Curcumin, a yellow polyphenol, was reported to decrease protein carbonylation in hypothyroid rat renal cortex (31). In our hypothyroid animals, quercetin at both concentrations employed was adequate to protect the liver cells against oxidative injury by reducing PCO levels (Table 2). These results suggest that the quercetin by scavenging properties may prevent hepatic oxidative damage in hypothyroidism.

Alterations in the antioxidant capacity may play an important role in influencing tissue susceptibility to oxidative processes (44). It is observed that hypothyroidism induced by MMI treatment resulted in the augmentation of the SOD activities in whole blood, liver and kidney, which is accompanied with a decrease in liver catalase activity (Table 4). These results indicate that hypothyroidism induces activation in SOD activity possible due the excess free radical formation which leads to increased lipid and protein damage. On the contrary, in hypothyroid liver, the reduction of the enzyme CAT should potentiate the effect of the increased free radical production, resulting in increased tissue sensitivity to oxidant injury mediated mainly by H_2O_2 overproduction. Whole blood and liver SOD activities reduced and liver CAT was restored near control values following QT25 to hypothyroid rats. In addition, we describe here an increase in ROS scavenging capacity (decreased TOSC area; Fig. 2) in liver and kidney tissues after quercetin treatment. Thus, these results could be related to the fact that quercetin showed antioxidative properties influencing antioxidant defense systems of the tissues in hypothyroid rats, and so far contributing for improved antioxidant status of the organism.

To explore effects of quercetin on non-enzymatic antioxidants, we have

investigated AA and NPSH levels in hypothyroid rats. Ascorbic acid functions physiologically as a water-soluble antioxidant by virtue of its high reducing power (45). We have found decreased AA levels in liver and kidney tissues from hypothyroid rats that in turn were restored by QT10 and QT25 (Fig.3). Moreover, we have found that NPSH content in hypothyroid animals also decreased in liver and kidney tissues. However, the levels were not modified by quercetin administration (Fig 4). On the contrary, Konukoglu et al. (46) found that the levels of the antioxidant plasma protein thiol decreased in hypothyroid patients and were normalized with thyroxine therapy. In another study, vitamin E supplementation significantly increased liver and kidney GSH levels in propylthiouracil treated animals (47).

Hypothyroidism increased not only ROS generation but also serum and tissues lipid peroxidation and liver protein oxidation. Membrane lipids and lipids in circulating lipoproteins such as low-density lipoprotein (LDL) can interact with ROS resulting in lipid peroxidation. The similar form a peptide chain or specific amino acids can be cleaved by oxidants. By reducing the radical initiators, ascorbic acid can prevent lipid and protein oxidation and radical propagation (48). Moreover, GSH plays an important role in the detoxification of hydroperoxides generated by the respiratory chain. Lipid peroxidation is an important regulator of intra-mitochondrial GSH (49).

Thus, we suggest that insufficiency of the antioxidant defense system may be due to increased utilization as an antioxidant defense against increased ROS in hypothyroidism. In fact, QT10 and QT25 administration were effective in restoring ascorbic acid levels and increasing scavenging capacity thus protecting liver and kidney cells against oxidative stress.

In conclusion, our results indicated that MMI-induced hypothyroidism

increased oxidative stress biomarkers. Quercetin treatment for eight weeks caused a recovery of changed parameters. At doses of 10 and 25 mg/kg this flavonoid was effective in decreasing ROS generation, lipid and protein oxidative damage. Furthermore, quercetin at the highest dose showed ameliorated scavenging capacity and influenced enzymatic and non-enzymatic antioxidants in hypothyroid animals. These findings are of great importance, given the increased use of quercetin as dietary supplement and therapeutic agent, its effects on hypothyroid dysfunction should be considered, included a possible beneficial effects on cell redox status. Nevertheless, further studies are needed to investigate the precise mechanism(s) of action of quercetin on oxidative stress biomarkers in rat under hypothyroidism.

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Conflict of Interest

The authors declare that there are no conflicts of interest.

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Table 1: Effects of quercetin on final body weight, tT3 and tT4 levels and biochemical parameters of MMI-induced hypothyroid rats

Parameters	Groups					
	I	II	III	IV	V	VI
Final body weight (g)	334.30 ± 17.60	341.50 ± 9.32$^{\#}$	370.80 ± 13.18$^{\#}$	252.90 ± 8.34*	264.30 ± 12.67*	314.60 ± 17.63$^{\#}$
tT3 (ng/ml)	0.45 ± 0.03	0.42 ± 0.03	0.54 ± 0.03	0.27 ± 0.04*	0.29 ± 0.02*	0.28 ± 0.03*
tT4 (µg/ml)	2.26 ± 0.06	2.74 ± 0.31	2.92 ± 0.18	0.12 ± 0.06*	0.47 ± 0.07*	0.75 ± 0.16*
AST (U/L)	79.73 ± 2.68	86.67 ± 5.20	67.50 ± 5.53	66.08 ± 3.54	78.27 ± 2.36	80.25 ± 4.34
ALT (U/L)	52.25 ± 2.95	47.00 ± 2.04	56.00 ± 3.21	57.23 ± 2.7	44.30 ± 4.92	47.18 ± 3.90
Total cholesterol (mg/dL)	90.90 ± 6.38	89.05 ± 5.55	98.40 ± 3.41	103.9 ± 5.72	98.05 ± 4.67	92.65 ± 5.22
Glucose (mg/dL)	106.10 ± 4.45	107.70 ± 7.85	104.80 ± 2.83	109.30 ± 3.69	110.70 ± 9.72	95.02 ± 3.52

Group I (euthyroid), Group II (QT 10 mg/kg), Group III (QT 25 mg/kg), Group IV (hypothyroid) and Group V (hypothyroid with QT 10 mg/kg) and Group VI (hypothyroid with QT 25 mg/kg). Data are expressed as means ± S.E.M of 10 animals. (*) Denoted $p<0.05$ as compared with the control group. ($^{\#}$) Denoted $P<0.05$ as compared with the hypothyroid group (two-way ANOVA/Tukey´s multiple range test).

Table 2: Effect of quercetin on TBARS and PCO levels in different tissues and serum of MMI-induced hypothyroid rats

Parameters	Groups					
	I	II	III	IV	V	VI
Liver TBARS	2.96 ± 0.82	3.03 ± 0.89$^{\#}$	3.38 ± 0.83$^{\#}$	4.70 ± 0.65*	4.03 ± 0.41	3.96± 0.40
Kidney TBARS	3.10 ± 0.81	3.02 ± 0.89	3.22± 1.07	4.24 ± 0.25*	3.018 ± 0.75$^{\#}$	2.43± 0.62$^{\#}$
Serum TBARS	39.98 ± 5.77	33.55±7.44$^{\#}$	40.61 ± 8.26$^{\#}$	51.91 ± 4.91*	39.53.± 9.63$^{\#}$	40.73± 5.92$^{\#}$
Liver PCO	2.48 ± 0.39	3.20± 0.30	1.79 ± 0.21$^{\#}$	4.45 ± 0.29*	2.84 ± 0.25$^{\#}$	2.74± 0.18$^{\#}$
Kidney PCO	5.43 ± 0.24	5.20 ± 0.66	4.80 ± 0.53	6.017 ± 0.23	5.02 ± 0.63	3.79 ± 0.47

'Group I (euthyroid), Group II (QT 10 mg/kg), Group III (QT 25 mg/kg), Group IV (hypothyroid) and Group V (hypothyroid with QT 10 mg/kg) and Group VI (hypothyroid with QT 25 mg/kg). Data are expressed as means ± S.E.M of 10 animals. TBARS, thiobarbituric acid reactive substances; TBARS levels are expressed as nmol MDA/mg protein in tissues and as nmol MDA/mL in serum. PCO, protein carbonyl; PCO levels are expressed as nmol carbonyl/mg protein.(*) Denoted *P*<0.05 as compared with the control group. ($^{\#}$) Denoted *P*<0.05 as compared with the hypothyroid group (two-way ANOVA/Tukey´s multiple range test)

Table 3: Effect of quercetin on TBARS levels in brain structures of MMI-induced hypothyroid rats

Brain Structure	Group					
	I	II	III	IV	V	VI
Cerebral cortex	1.98 ± 0.24	1.38 ± 0.17 $^{\#}$	2.00 ± 0.55$^{\#}$	3.78 ± 0.63*	2.49 ± 0.32	1.38 ± 0.24$^{\#}$
Hypothalamus	1.59 ± 0.26	2.47 ± 0.30	1.28 ± 0.12$^{\#}$	3.32 ± 0.63	2.62 ± 0.37	1.64 ± 0.51
Hippocampus	1.44 ± 0.42	1.51 ± 0.23	1.41 ± 0.15$^{\#}$	2.88 ± 0.31*	1.89 ± 0.39	1.63 ± 0.13$^{\#}$
Cerebellum	1.48 ± 0.15	1.73 ± 0.45	1.35 ± 0.48	2.14 ± 0.21	0.93 ± 0.14	1.35 ± 0.48
Striatum	1.04 ± 0.12	1.15 ± 0.15	0.74 ± 0.09	2.35 ± 0.53	1.92 ± 0.52	1.67 ± 0.57

Group I (euthyroid), Group II (QT 10 mg/kg), Group III (QT 25 mg/kg), Group IV (hypothyroid) and Group V (hypothyroid with QT 10 mg/kg) and Group VI (hypothyroid with QT 25 mg/kg). Data are expressed as means ± S.E.M of 10 animals. TBARS, thiobarbituric acid reactive substances; TBARS levels are expressed as nmol MDA/mg protein.(*) Denoted $P<0.05$ as compared with the control group. ($^{\#}$) Denoted $P<0.05$ as compared with the hypothyroid group (two- way ANOVA/Tukey´s multiple range test)

Table 4. Effect of quercetin on antioxidant enzymes in liver, kidney and whole blood of MMI-induced hypothyroid rats

Enzymes	Groups					
	I	II	III	IV	V	VI
Liver SOD	9.41 ± 0.70	10.71 ± 0.54	9.96± 1.01	12.51 ± 0.69*	11.15± 0.53	8.70 ± 0.72#
Kidney SOD	6.90 ± 0.63	7.32 ± 0.92#	8.63 ± 0.68#	12.02 ± 0.80*	11.57 ± 0.62*	11.24 ± 0.77*
Whole blood SOD	6.55 ±0.67	5.03 ± 0.35#	6.21 ± 0.27#	9.52 ± 0.65*	9.07 ± 0.56*	6.13 ± 0.61#
Liver CAT	4.20 ± 0.37	4.37 ± 0.37#	5.22 ± 0.57#	1.63± 0.13*	3.34 ± 0.27	3.91 ± 0.40#
Kidney CAT	1.30 ± 0.15	1.20 ± 0.17	1.11 ± 0.15	1.54 ± 0.09	1.16 ± 0.03	1.28 ± 0.36
Whole blood CAT	1.20 ± 0.14	1.38 ± 0.20	1.46 ± 0.17	1.00 ± 0.17	1.10 ± 0.17	0.83 ± 0.06

Group I (euthyroid), Group II (QT 10 mg/kg), Group III (QT 25 mg/kg), Group IV (hypothyroid) and Group V (hypothyroid with QT 10 mg/kg) and Group VI (hypothyroid with QT 25 mg/kg). Data are expressed as means ± S.E.M of 10 animals. SOD, superoxide dismutase; SOD activities are expressed as U SOD/mg protein; CAT, catalase; CAT activities are expressed as U/mg protein. (*) Denoted $P<0.05$ as compared with the control group. (#) Denoted $P<0.05$ as compared with the hypothyroid group. (Two - way ANOVA/Tukey´s multiple range test)

Figure legends

Fig. 1: Effect of quercetin on ROS levels in (A) liver and (B) kidney of MMI-induced hypothyroid rats. Group I (euthyroid), Group II (QT 10 mg/kg), Group III (QT 25 mg/kg), Group IV (hypothyroid) and Group V (hypothyroid with QT 10 mg/kg) and Group VI (hypothyroid with QT 25 mg/kg). Data are expressed as means ± S.E.M of 10 animals. ROS, reactive oxygen species; ROS are expressed as area of ROS / mg protein. (*) Denoted $P<0.05$ as compared with the control group. ($^{\#}$) Denoted $P<0.05$ as compared with the hypothyroid group (two-way ANOVA/Tukey´s multiple range test)

Fig. 2: Effect of quercetin on TOSC levels in (A) liver and (B) kidney of MMI-induced hypothyroid rats. Group I (euthyroid), Group II (QT 10 mg/kg), Group III (QT 25 mg/kg), Group IV (hypothyroid) and Group V (hypothyroid with QT 10 mg/kg) and Group VI (hypothyroid with QT 25 mg/kg). Data are expressed as means ± S.E.M of 10 animals. TOSC, total oxyradical scavenging capacity; TOSC are expressed as relative area of ROS /mg protein. (*) Denoted $P<0.05$ as compared with the control group. ($^{\#}$) Denoted $P<0.05$ as compared with the hypothyroid group (two-way ANOVA/Tukey´s multiple range test)

Fig. 3: Effect of quercetin on AA levels in (A) liver and (B) kidney of MMI-induced hypothyroid rats. Group I (euthyroid), Group II (QT 10 mg/kg), Group III (QT 25 mg/kg), Group IV (hypothyroid) and Group V (hypothyroid with QT 10 mg/kg) and Group VI (hypothyroid with QT 25 mg/kg). Data are expressed as means ± S.E.M of 10 animals. AA, ascorbic acid; AA levels are expressed as µg ascorbic acid/g tissue. (*) Denoted $P<0.05$ as compared with the control group.

(#) Denoted *P*<0.05 as compared with the hypothyroid group (two-way ANOVA/Tukey´s multiple range test).

Fig. 4: Effect of quercetin on NPSH levels in (A) liver and (B) kidney of MMI-induced hypothyroid rats. Group I (euthyroid), Group II (QT 10 mg/kg), Group III (QT 25 mg/kg), Group IV (hypothyroid) and Group V (hypothyroid with QT 10 mg/kg) and Group VI (hypothyroid with QT 25 mg/kg). Data are expressed as means ± S.E.M of 10 animals. NPSH, non-protein thiol group; NPSH are expressed as µmol SH/g tissue. (*) Denoted *P*<0.05 as compared with the control group. (#) Denoted *P*<0.05 as compared with the hypothyroid group (two-way ANOVA/Tukey´s multiple range test)

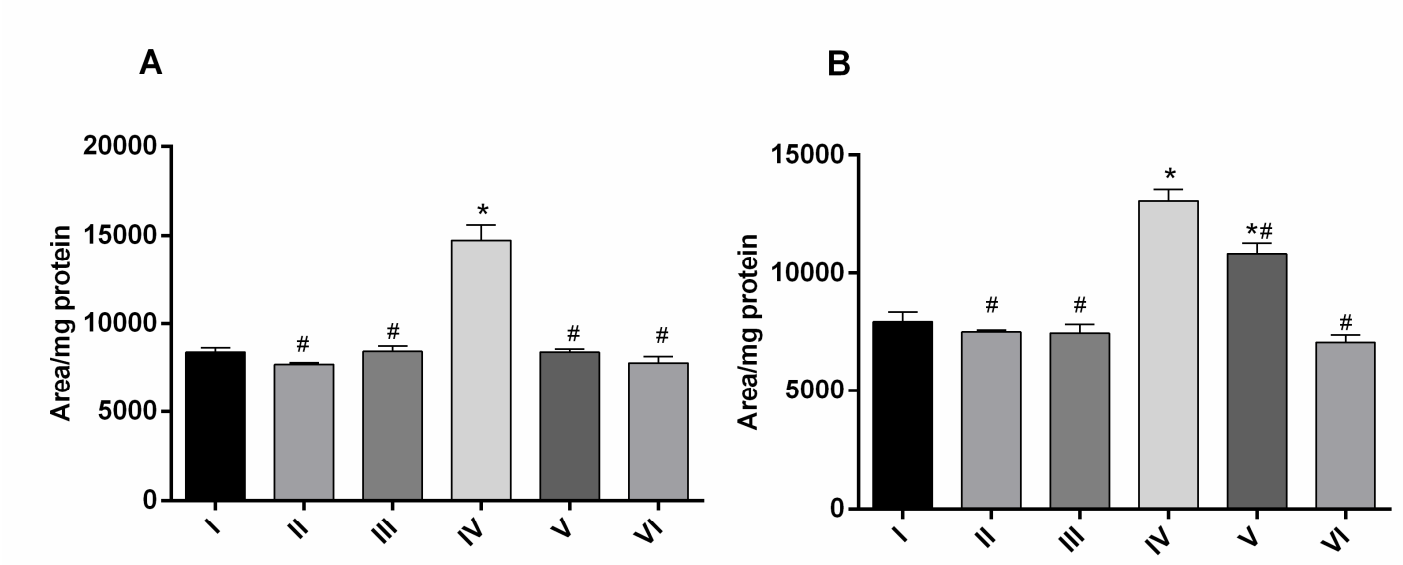
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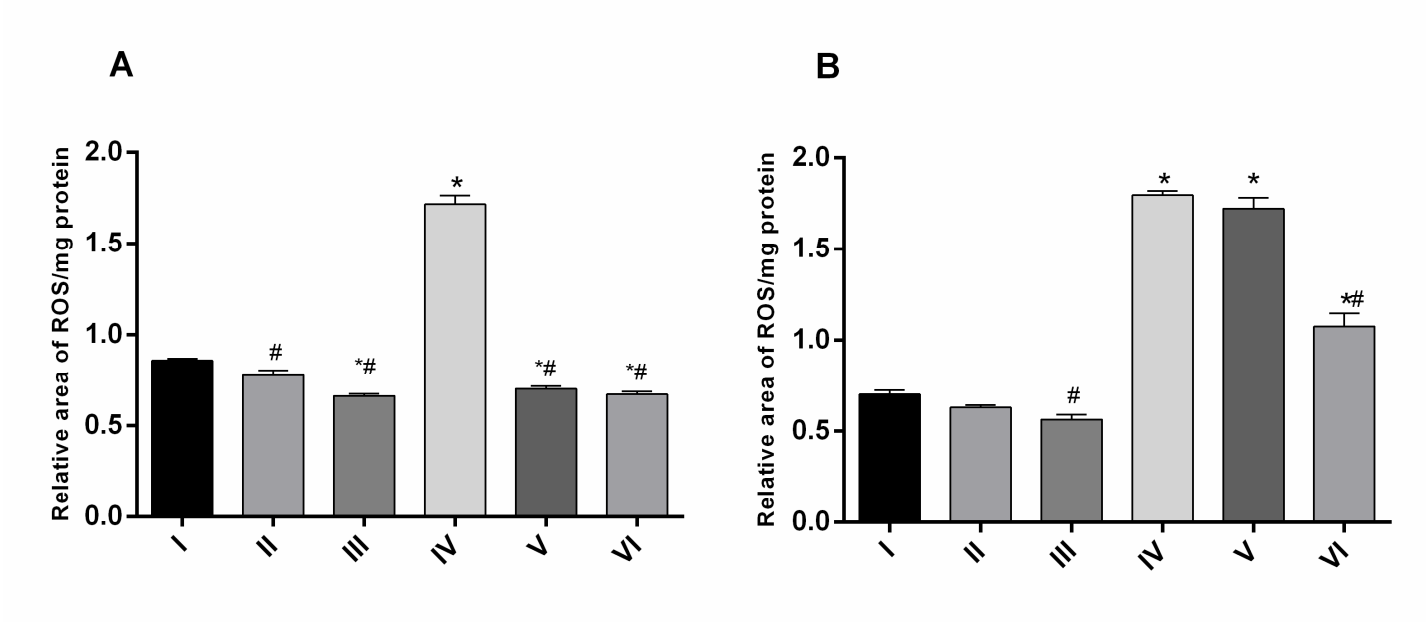
Fig. 1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VI

Fig. 2

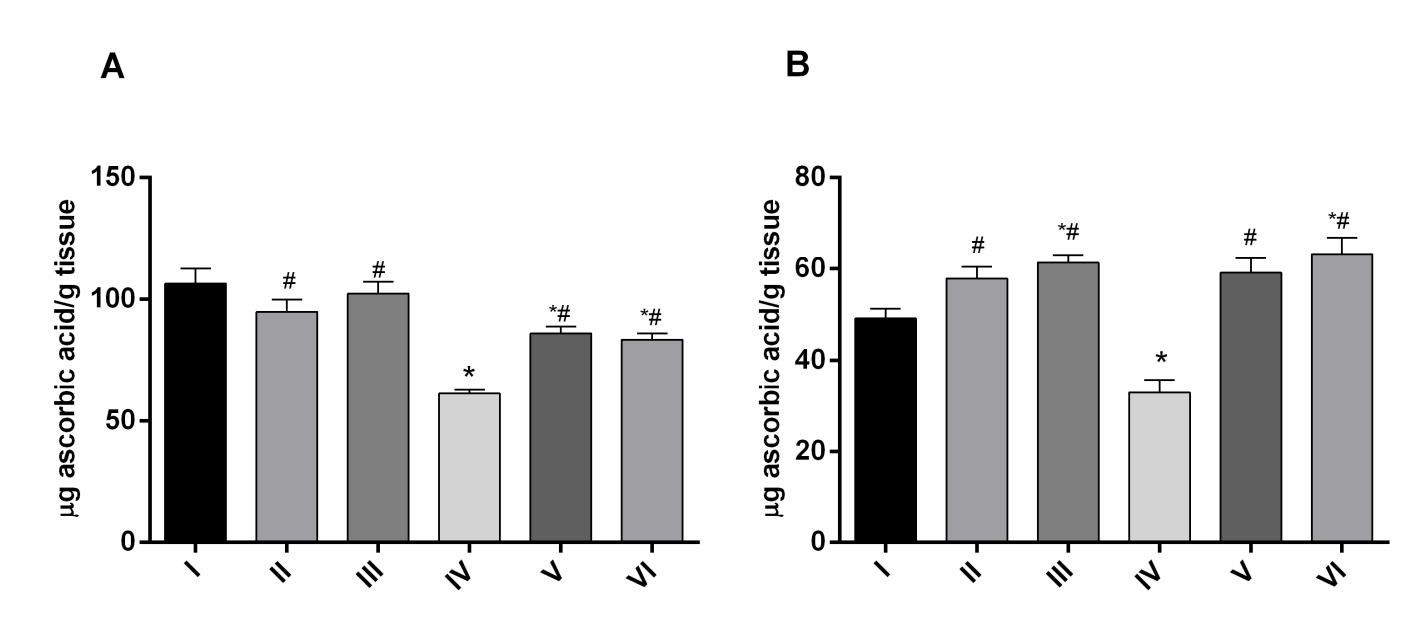
A
µg ascorbic acid/g tissue
150
100
50
0
#
#
*
*#
*#
I
II
III
IV
V
VI
B
µg ascorbic acid/g tissue
80
60
40
20
0
#
*#
*
#
*#
I
II
III
IV
V
VI

Fig. 3

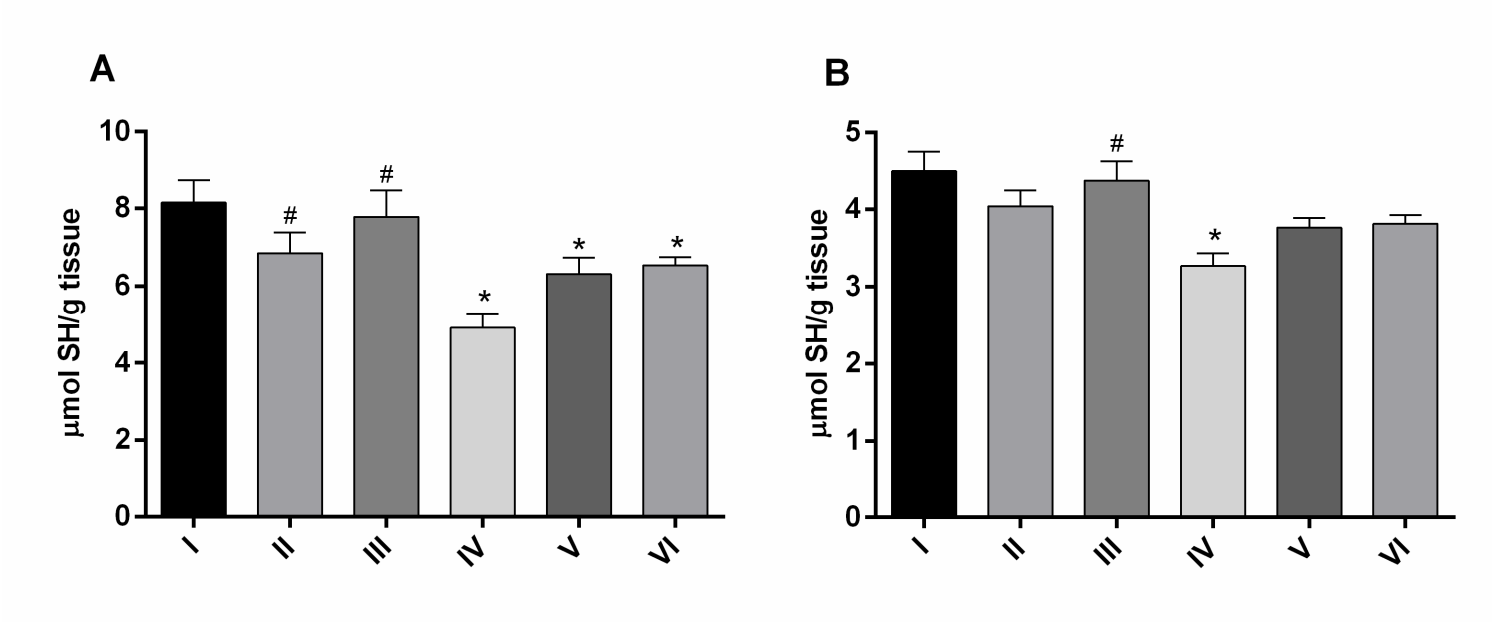
A
µmol SH/g tissue
10
8
6
4
2
0
#
#
*
*
*
I
II
III
IV
V
VI
B
µmol SH/g tissue
5
4
3
2
1
0
#
*
I
II
III
IV
V
VI

Fig. 4

4 DISCUSSÃO

No artigo 1, avaliou-se a associação entre biomarcadores de estresse oxidativo e o perfil lipídico em pacientes com hipotireoidismo subclínico (HSC). Níveis elevados de lipídeos plasmáticos colesterol total, colesterol LDL e triglicerídeos, bem como níveis diminuídos de colesterol HDL foram observados no grupo HSC quando comparados com o grupo controle, demonstrando um importante fator de risco para o desenvolvimento de doenças cardiovasculares. No hipotireoidismo a hiperlipidemia é um achado frequente, sendo observado em estudos conduzidos com hipotireoidismo nas formas clínica (NANDA et al., 2007; KUTLUTURK et al., 2013) e subclínica (WALSH et al., 2005; NAKAJIMA et al., 2013).

Os níveis de peroxidação lipídica (TBARS) mostraram-se aumentados no grupo HSC. Está bem estabelecido que os hormônios tireoideanos interferem nos níveis de colesterol, metabolismo hepático e na síntese de colesterol (DORY; ROHEIM, 1981). Como o metabolismo lipídico é dependente dos HT, o hipotireoidismo associado à hiperlipidemia contribui para a geração de RL e para o estresse oxidativo. Um dos mecanismos propostos é que a hiperlipidemia fornece um pool de substratos que facilita a taxa de peroxidação lipídica (SUBUDHI et al., 2009).

Muitos estudos têm associado o hipotireoidismo subclínico ao estresse oxidativo. Em estudo recente realizado por REDDY et al (2013) foram observados níveis plasmáticos elevados de malondialdeído (MDA), bem como indução da enzima antioxidante glutationa peroxidase (GPX) em eritrócitos de pacientes com HSC. HARIBABU et al (2013) evidenciou estresse oxidativo através de níveis elevados de MDA e proteína carbonil nos pacientes hipotireóideos estudados. Na presente investigação, os níveis de TBARS apresentaram correlações positivas com colesterol total, colesterol LDL e com os níveis de TSH. Entretanto, após efetuarmos um controle pelos níveis de colesterol total a associação entre TBARS e TSH deixou de ser significativa. Portanto, nossos resultados sugerem a influência do perfil lipídico no dano oxidativo aos lipídeos no hipotireoidismo.

Além disso, foi observado um aumento na atividade da CAT, enquanto

que a enzima SOD apresentou tendência de aumento, porém não significativo no grupo HSC. A indução da CAT reflete possivelmente o excesso da geração de peróxido de hidrogênio, radical detoxificado por esta enzima. De forma similar ao TBARS, parâmetros lipídicos mostraram associações significativas com as atividades das enzimas antioxidantes avaliadas neste estudo. Os níveis de TSH foram correlacionados com a atividade da CAT e esta correlação foi perdida após o controle pelos níveis de colesterol. Assim, os resultados aqui descritos mostraram a influência dos níveis de colesterol total e LDL sob a indução do sistema antioxidante no hipotireoidismo.

A paraoxonase 1 (PON1) é uma enzima encontrada na circulação associada com lipoproteínas de alta densidade (HDL), desempenhando assim, importante papel no sistema antioxidante do organismo (CAMPS et al., 2009). Em nosso estudo, os pacientes com hipotireoidismo subclinico apresentaram atividade da arilesterase (ARE) diminuída, quando comparados com os indivíduos controles. De maneira similar, a indução do hipotireoidismo, resultou em um aumento do estresse oxidativo e reduziu a atividade da PON1 em ratos (SARANDOL et al., 2005). FERRÉ e colaboradores (2013) observaram significantes associações entre a atividade sérica da PON1 com altos níveis de TG e baixos níveis de HDL- C em crianças obesas, sugerindo assim a relação desta enzima com risco aumentado para doenças cardiovasculares. Embora não observamos associações significativas entre a atividade da ARE sérica com lipídeos plasmáticos, hormônios tireoideanos e estresse oxidativo nos pacientes com HSC, a redução da ARE sugere uma diminuição nas defesas antioxidantes frente ao status pró-oxidativo associado ao hipotireoidismo.

No manuscrito 1 foi avaliada a associação entre biomarcadores inflamatórios e o hipotireoidismo clínico (HC). Além disso, verificamos o perfil lipídico e marcadores pro-trombóticos dos pacientes.

Os resultados demonstraram altos níveis das citocinas pró-inflamatórias, IL-1, IL-6, TNF- α e IFN- ɣ em pacientes com hipotireoidismo clínico quando comparados com o grupo controle. A aterosclerose é considerada uma doença inflamatória crônica e muitas citocinas participam do seu desenvolvimento (KIRI et al., 2003). Inicialmente, os macrófagos são ativados nas placas ateroscleróticas, seguido por um aumento da expressão de moléculas da classe II do complexo principal de histocompatibilidade. Este processo torna os

macrófagos importantes células apresentadoras de antígenos para desenvolvimento de uma resposta imune específica. A LDLox é o antígeno ativador que promove uma resposta TH1, seguida pela produção de citocinas como IFN- ɣ e TNF – α (STEMME et al., 1995; ANGERIO, 2009).

Estudos têm sugerido que a função do INF- ɣ na modulação da resposta inflamatória está associada à aterosclerose, uma vez que pacientes com angina estável e instável, bem como, com miocardite apresentam níveis plasmáticos elevados desta citocina (FERNANDES et al., 2004). A principal função desta citocina é ativar monócitos/macrófagos, com um consequente aumento da apoptose, da expressão de moléculas de adesão endoteliais e da síntese de outras citocinas pró-inflamatórias como a IL-1 e a IL-6 (CORRÊA et al, 2011).

A IL-1 é secretada na parede das artérias principalmente por monócitos e macrófagos e também por células endoteliais e células musculares lisas (BEVILACQUA et al., 1984). Sua expressão esteve aumentada em artérias coronárias humanas afetadas pela aterosclerose (GALEA et al., 1996). CHAMBERLAIN et al (2009) demonstraram associação entre IL-1 com inflamação arterial, estresse oxidativo e aumento da pressão arterial .

Neste contexto, já está bem estabelecida a relação entre hipotireoidismo e o desenvolvimento de aterosclerose e de consequentes doenças cardiovasculares relacionadas. Entretanto, o papel das citocinas no HC ainda permanece sem ser completamente elucidado. Para isso, neste estudo foram realizadas correlações a fim de avaliar se a associação entre os biomarcadores inflamatórios e o HC é um evento associado ao status hormonal ou um evento secundário associado aos status lipídico e pró-trombóticos dos pacientes. Os resultados apontaram claramente que os elevados níveis de citocinas possuem correlação direta com a dislipidemia apresentada pelos pacientes avaliados.

Os pacientes hipotireóideos aqui investigados apresentaram elevados níveis de lipídeos plasmáticos, colesterol total, colesterol LDL e triglicerídeos e níveis diminuídos de colesterol HDL, sugerindo elevado risco cardiovascular. Dentre os principais fatores de risco para o desenvolvimento da aterosclerose destaca-se a hipercolesterolemia. Sabe-se que os hormônios tireoideanos possuem influência sob o metabolismo e deposição do colesterol (NESS; LOPEZ, 1995) e isto pode contribuir para a associação entre hipotireoidismo e

desordens lipídicas. Estudos envolvendo pacientes com HC confirmam esta associação (TORUN et al., 2009; BAMASHMOOS et al., 2013). De maneira similar, em estudo anterior realizado pelo grupo de pesquisa que participa desta investigação, foi mostrado um aumento de marcadores de estresse oxidativo em indivíduos com HC e a forte influência da hipercolesterolemia no desenvolvimento desta condição (SANTI et al., 2010).

Os níveis de IL-6 e INF-γ foram mais elevados em pacientes com níveis de colesterol acima de 240 mg/dL. Da mesma maneira, os níveis de TNF- α foram superiores em pacientes com níveis de colesterol HDL < 50mg/dL (Manuscrito 1/Figura 2). Estes resultados reforçam a associação anteriormente citada, destacando a influência do status inflamatório no metabolismo lipídico ou vice-versa.

Além do status pró-aterogênico evidenciado pelos altos níveis de lipídeos, os pacientes apresentaram níveis elevados de marcadores pró-trombóticos, fibrinogênio e D-dímero, que contribuem para a elevada prevalência de aterosclerose no hipotireoidismo. Estudos epidemiológicos têm demonstrado que os níveis de fibrinogênio podem predizer o risco futuro para infarto do miocárdio e AVC (WILHELMSEN et al., 1984; SCARABIN et al., 1998). Níveis elevados de D - dímero foram observados em pacientes com IAM e também foram utilizados para predizer eventos cardiovasculares futuros em indivíduos saudáveis (LOWE et al., 2004). De forma semelhante aos resultados aqui descritos, GURSOY et al (2006) observou níveis elevados de fibrinogênio em pacientes com HC enquanto que CHADAREVIAN et al (2001) observou altos níveis de D-dímero nestes pacientes.

Ademais, foi aqui examinado outro marcador inflamatório nos pacientes com HC, os níveis plasmáticos de DNA livre. O hipotireoidismo aumentou os níveis de DNA livre (37.76 ± 13.80 ng/mL) quando comparados com os indivíduos controles (27.25 ± 7.55 ng/mL). Entretanto, este marcador não foi correlacionado com os hormônios tireoideanos, perfil lipídico e marcadores pró-trombóticos. Com isso, sugere-se que a liberação de DNA das células possivelmente esteja ocorrendo secundariamente ao processo inflamatório desencadeado pela dislipidemia em pacientes com HC. Estudos recentes apontam que os níveis de DNA livre podem ser utilizados como marcadores de diagnóstico e de predição de lesões ateroscleróticas (CUI et al., 2013) e

também como coadjuvante complementando as dosagens de troponina e CK-MB no diagnóstico de IAM (CHANG et al., 2003). Assim, também é sugerido o DNA livre como um biomarcador candidato na detecção de processo inflamatório e na predição de risco para doenças cardiovasculares associadas ao hipotireoidismo.

No manuscrito 2 foram investigados os efeitos da quercetina sobre biomarcadores de estresse oxidativo no hipotireoidismo induzido por metimazol em ratos machos. Os resultados deste trabalho demonstraram que o grupo de ratos tratados com metimazol (MMI) teve um aumento da geração de espécies reativas de oxigênio (ERO) em conjunto com um aumento da oxidação de lipídeos e de proteínas. As defesas antioxidantes enzimáticas e não enzimáticas também foram modificadas pela indução do hipotireoidismo nos animais bem como, a capacidade antioxidante total. Assim, podemos concluir que o hipotireoidismo induzido por metimazol causou um desequilíbrio no status redox dos ratos.

O uso de antioxidantes exógenos tem sido proposto para reduzir o estresse oxidativo e danos associados a esta condição, devido as suas capacidades de sequestrar radicais livres e espécies reativas, que na maioria das vezes se encontram elevados nestas situações (DIPLOCK et al., 1998). A quercetina é considerada um potente sequestrador de ERO, juntamente com outros membros da família dos flavonoides. Sua ação antioxidante é atribuída a presença de dois farmacófaros antioxidantes dentro da molécula, que possuem configuração ótima para a eliminação de RLs (BOOTS et al., 2008). O estudo também demonstrou aqui que a administração de quercetina por um período de 08 semanas, foi efetiva em diminuir a geração de ERO no fígado e rim de ratos hipotireóideos. Além disso, foi obervada diminuição nos níveis de peroxidação lipídica em rim, soro, córtex cerebral e hipocampo, bem como, os níveis de proteína carbonil no fígado. Assim, estes resultados sugerem que a quercetina atua protegendo contra a geração de ERO e consequentemente contra danos oxidativos a lipídeos e a proteínas no hipotireoidismo.

O *status* antioxidante celular determina a suscetibilidade aos danos oxidativos, sendo frequentemente alterado em reposta ao estresse oxidativo. Enzimas antioxidantes como a SOD e a CAT e antioxidantes como as vitaminas C e E, protegem as células da peroxidação lipídica (AFANAS'EV et

al., 1989). O presente estudo demostrou um desequilíbrio nas atividades das enzimas SOD e CAT em sangue total e tecidos de ratos com hipotireoidismo. A suplementação com quercetina (QT25) mostrou-se eficaz no restabelecimento dos mecanismos de defesa enzimáticos principalmente no tecido hepático. Assim, estes resultados indicam para o efeito antioxidante da quercetina, melhorando o status antioxidante principalmente na maior concentração utilizada neste experimento.

Em estudo realizado por ARTS et al (2004) a administração de quercetina aumentou em seis vezes a capacidade antioxidante total do plasma quando comparada com o antioxidante de referência, trolox, um antioxidante hidrossolúvel derivado da vitamina E. De forma similar, nós observamos que a quercetina foi capaz de aumentar a capacidade antioxidante total nos tecidos hepáticos e renal de ratos com hipotireoidismo, representado pela diminuição da área relativa de ERO (Manuscrito 2;Fig.2). Ademais, o efeito benéfico da quercetina foi observado pelo aumento dos níveis de ácido ascórbico em fígado e rim de ratos expostos ao MMI. Simultaneamente, a estes achados os marcadores pró-oxidativos diminuíram nos tecidos de ratos hipotireóideos tratados com quercetina, contribuindo também, para uma melhora nas defesas antioxidantes.

Por fim, os resultados destes estudos demonstraram que a disfunção no metabolismo lipídico, secundária a disfunção tireoideana está associada ao estresse oxidativo, como foi evidenciado nos pacientes com hipotireoidismo subclínico. Além disso, sugere-se o papel dos lipídeos no desenvolvimento de processo inflamatório e no perfil pró-aterogênico comumente observado nesta disfunção endócrina. A administração do antioxidante quercetina mostrou-se benéfica, reduzindo o estresse oxidativo em ratos hipotireóideos.

Considerando-se a alta prevalência de hipotireoidismo na população em geral e as disfunções secundárias frequentemente associadas tais como as desordens lipídicas, os resultados deste trabalho são de grande importância na elucidação do status redox celular e bioquímico dos pacientes. Além disso, sugere-se a utilização de estratégias antioxidantes, como o uso da quercetina como potencial coadjuvante no tratamento clássico do hipotireoidismo.

5 CONCLUSÃO

- A partir dos resultados obtidos, pode-se concluir que o hipotireoidismo subclínico está associado ao estresse oxidativo e alterações nos parâmetros lipídicos. Além disso, nos pacientes com hipotireoidismo clinico, o processo inflamatório está associado às alterações do perfil lipídico. Assim, a hipercolesterolemia secundária à disfunção endócrina possui forte influência no status pró – oxidativo e inflamatório dos pacientes com hipotireoidismo.

- Além destas alterações, ressalta-se o aumento de marcadores pró-trombóticos, D- dímero e fibrinogênio que constituem também importante fator de risco para doenças cardiovasculares.

- Ademais, o hipotireoidismo induzido por metimazol gerou um desequilíbrio no status redox dos ratos; e a quercetina atuou protegendo contra os danos oxidativos gerados por esse desequilíbrio em diferentes tecidos e sangue, via aumento dos mecanismos de defesa do organismo.

- Dessa forma, futuros estudos devem ser desenvolvidos com a finalidade de avaliar o efeito antioxidante da quercetina em pacientes com a disfunção endócrina.

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