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INFLUÊNCIA DE ESTRÓGENOS E PROGESTINAS SOBRE A ATIVIDADE DA SUPERÓXIDO DISMUTASE E O ESTATUS OXIDATIVO EM MULHERES.

TESE DE DOUTORADO

TAÍS CRISTINA UNFER

Santa Maria, RS, Brasil
2013

INFLUÊNCIA DE ESTRÓGENOS E PROGESTINAS SOBRE A ATIVIDADE DA SUPERÓXIDO DISMUTASE E O ESTATUS OXIDATIVO EM MULHERES.

por

Taís Cristina Unfer

Tese apresentada ao Curso de Doutorado do Programa de Pós-Graduação em Farmacologia, Área de Concentração Farmacologia, da Universidade Federal de Santa Maria (UFSM, RS), como requisito parcial para a obtenção do grau de **Doutora em Farmacologia**.

Orientador: Profa. Dra. Tatiana Emanuelli

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elaborada por
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como requisito parcial para a obtenção do grau de
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“... Pesquiso para constatar,
constatando, intervenho, intervindo,
educo e me educo. Pesquiso para
conhecer o que ainda não conheço e
comunicar ou anunciar a
novidade...”
(Paulo Freire)

RESUMO

Tese de Doutorado
Programa de Pós-Graduação em Farmacologia
Universidade Federal de Santa Maria

INFLUÊNCIA DE ESTRÓGENOS E PROGESTINAS SOBRE A ATIVIDADE DA SUPERÓXIDO DISMUTASE E O ESTATUS OXIDATIVO EM MULHERES.

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Local e Data da Defesa: Santa Maria, 20 de maio de 2013

O déficit de estrogênio, que acompanha a menopausa pode ser relacionado às alterações metabólicas e ao aumento do estresse oxidativo, observados na fase não reprodutiva feminina. A terapia de reposição hormonal é utilizada para atenuar os sintomas da menopausa. Ela é prescrita como reposição de estrogênio ou uma combinação de estrogênio com progestina. A superóxido dismutase (SOD) é uma enzima chave no controle dos níveis de espécies reativas de oxigênio e, moduladores da SOD podem ser úteis como agentes terapêuticos em desordens associadas ao estresse oxidativo. Os objetivos deste estudo foram avaliar: i) os efeitos, *in vitro*, de estrógenos e progesterona, naturais e sintéticos, sobre a atividade da SOD presente em sangue humano, e ii) o efeito da terapia hormonal com estrogênio ou estrogênio mais progestinas sobre os marcadores de estresse oxidativo no sangue de mulheres na pós-menopausa e a relação entre esses marcadores e os níveis séricos de estradiol e progesterona. O efeito, *in vitro*, de hormônios esteroides (acetato de 17β-estradiol (E_2), progesterona, 3-benzoato de 17β-estradiol e 17-acetato de medroxiprogesterona) foi avaliado na enzima purificada a partir de eritrócitos humanos (CuZnSOD) (Sigma), e em amostras de eritrócitos (CuZnSOD citosólica) e de plasma rico em plaquetas (PRP) (CuZnSOD, citosólica e extracelular, e MnSOD, mitocondrial), obtidas a partir de homens e mulheres saudáveis. Os hormônios, em concentrações baixas (fisiológica), causaram uma estimulação, dose dependente, da atividade da CuZnSOD eritrocitária, embora, este efeito tenha sido suprimido em concentrações mais elevadas. Ademais, a combinação de um estrogênio com uma progestina apresentou um efeito sinérgico sobre a atividade da CuZnSOD eritrocitária. No PRP a atividade da MnSOD não foi afetada por hormônios, enquanto que a atividade da CuZnSOD foi modulada apenas pelos esteroides naturais. Quatro grupos de mulheres foram selecionados para avaliar marcadores sanguíneos de estresse oxidativo: mulheres na pré-menopausa (n = 24), mulheres na pós-menopausa sem terapia hormonal (TH) (n = 31), mulheres na pós-pausa utilizando TH, composta apenas de estrogênio (ET) (n = 12), ou de uma combinação de estrogênio mais progestinas (EPT) (n = 16). Os níveis de proteína carbonilada, peroxidação lipídica e da atividade de catalase e glutationa peroxidase (GPx) não diferiram entre os grupos. No entanto, as atividades das isoformas da SOD (CuZn e MnSOD) e o poder antioxidante total do plasma (FRAP) foram significativamente maiores em mulheres na pós-menopausa sob EPT em comparação com mulheres na pós-menopausa sem TH, enquanto que a ET aumentou apenas a atividade da CuZnSOD em mulheres na pós-menopausa. A duração da TH e os níveis séricos de E_2 foram positivamente correlacionados com a atividade da CuZnSOD e com o poder antioxidante total do plasma (FRAP), enquanto que os níveis de progesterona foram positivamente correlacionados com a atividade da CuZnSOD e negativamente correlacionados com os níveis de proteína carbonilada. O poder antioxidante total do plasma foi positivamente correlacionada com a atividade da CuZnSOD e da GPx. O presente estudo demonstrou, pela primeira vez, que os hormônios esteroides, naturais e sintéticos, têm um efeito direto e bifásico na atividade da CuZnSOD eritrocitária humana *in vitro*. Também foi observado que a terapia de reposição hormonal aumenta a capacidade antioxidante de mulheres na pós-menopausa devido a um aumento das defesas antioxidantes enzimáticas (SODs) e que este efeito é ainda mais pronunciado com o uso de terapia hormonal combinada (estrogênio e progestinas).

Palavras-chave: Terapia de reposição hormonal, menopausa, enzimas antioxidantes, atividade antioxidante total, CuZnSOD, MnSOD.

ABSTRACT

PhD Thesis
Graduate Program on Pharmacology
Federal University of Santa Maria, RS, Brazil

INFLUENCE OF ESTROGENS AND PROGESTINS ON THE SUPEROXIDE DISMUTASE ACTIVITY AND THE OXIDATIVE STATUS IN WOMEN.

AUTHOR: TAIS CRISTINA UNFER
ADVISOR: TATIANA EMANUELLI
Date and Place of the defense: May 20th, 2013, Santa Maria

The deficit of estrogen that accompanies menopause may be involved in the metabolic changes and increased oxidative stress during non-reproductive female life. Hormone replacement therapy (HRT) has been used to attenuate the menopausal symptoms. It is prescribed either as the replacement of estrogen alone or the combination of estrogen with progestins. Superoxide dismutase (SOD) is a key enzyme in the control of reactive oxygen species levels and SOD modulators may have potential use as therapeutic agents in oxidative stress-associated disorders. The objectives of this study were to evaluate: i) the effects of natural and synthetic estrogens and progestins on the activity of SOD from human blood *in vitro*; and ii) the effect of the hormone therapy with estrogen or estrogen plus progestin on the markers of oxidative stress in the blood of postmenopausal women and the relationship among these markers and the serum levels of estradiol and progesterone. The *in vitro* effect of steroid hormones (17 β-estradiol 17-acetate, progesterone, β-estradiol 3-benzoate and medroxyprogesterone 17-acetate) was evaluated in the enzyme purified from human erythrocytes (CuZnSOD) (Sigma) and in samples of erythrocytes (cytosolic CuZnSOD) and platelets-rich plasma (PRP) (MnSOD and cytosolic and extracellular CuZnSOD) obtained from healthy men and women. Hormones caused a dose-dependent stimulation of erythrocyte CuZnSOD activity at low concentrations (physiological), but this effect was abolished at higher concentrations. The combination of an estrogen with a progestin had a synergic effect on the erythrocyte CuZnSOD activity. In the PRP the activity of MnSOD was not affected by hormones, whereas the CuZnSOD activity was modulated only by the natural, but not by the synthetic hormone derivatives. Four groups of women were selected to evaluate blood markers of oxidative stress: premenopausal women (n=24), postmenopausal women without hormone therapy (HT) (n=31), postmenopausal women with estrogen-only HT (ET) (n=12) and estrogen plus progestin HT (EPT) (n=16). The levels of protein carbonyl, lipid peroxidation and the activity of catalase and glutathione peroxidase did not differ among groups. However, the activities of SOD isoforms (CuZn and MnSOD) and total plasma antioxidant power (FRAP) were significantly higher in postmenopausal women under EPT compared with postmenopausal women without HT, whereas ET increased only the activity of CuZnSOD in postmenopausal women. The duration of HT and serum E_2 levels were positively correlated with the activity of CuZnSOD and the total antioxidant power of plasma (FRAP levels), whereas progesterone levels were positively correlated with the activity of CuZnSOD and negatively correlated with protein carbonyl levels. The total antioxidant power of plasma was positively correlated to the CuZnSOD activity and to the GPx activity. The present study demonstrated for the first time, that the natural and synthetic steroid hormones have a direct biphasic effect on CuZnSOD activity of human erythrocytes *in vitro*. We also observed that the hormone replacement therapy increase the antioxidant status of postmenopausal women due to an increase of the enzymatic antioxidant defenses and this effect is more remarkable with the combined hormone therapy (estrogen plus progestin).

Keywords: Hormone replacement therapy, menopause, antioxidant enzymes, total antioxidant activity, CuZnSOD, MnSOD.

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MANUSCRITO

LISTA DE ABREVIATURAS

CAT	Catalase
CuZnSOD	Cobre e zinco superóxido dismutase
BenzE_2	3-benzoato de 17β-estradiol
EROs	Espécies reativas de oxigênio
E_2	17-β estradiol
FRAP	Habilidade do plasma em reduzir o íon férrico (poder antioxidante total)
FSH	Hormônio folículo estimulante
GPx	Glutationa peroxidase
GSH	Glutationa reduzida
HRT	Hormone Replacement Therapy
LPO	Peroxidação lipídica
MPA	17-acetato de medroxiprogesterona
MnSOD	Manganês superóxido dismutase
PRP	Plasma rico em plaquetas
SOD	Superóxido dismutase
TBARS	Substâncias reativas ao ácido tiobarbitúrico
TRE	Terapia de reposição de estrogênios
TRH	Terapia de reposição hormonal

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SUMÁRIO

APRESENTAÇÃO

Esta Tese de Doutorado é composta pela apresentação de um artigo científico original, publicado no periódico *Free Radical Research* (2013) e um manuscrito científico submetido ao periódico *Basic & Clinical Pharmacology & Toxicology*.

O trabalho está organizado da seguinte forma: primeiramente é apresentada a **INTRODUÇÃO** e os **OBJETIVOS** que guiam a Tese de doutorado. A seguir é apresentado o primeiro **ARTIGO ORIGINAL**, já publicado na revista *Free Radical Research* com fator de impacto é de 2,878. Em seguida, tem-se a apresentação do manuscrito de um segundo manuscrito, cujos resultados também compõem esta Tese de Doutorado, e que foi enviado para apreciação pelo periódico *Basic & Clinical Pharmacology & Toxicology*, cujo fator de impacto é de 2,179.

As seções, Material e Métodos e Resultados, encontram-se descritas no artigo e no manuscrito. Em seguida apresentam-se os itens **DISCUSSÃO DOS RESULTADOS** e **CONCLUSÕES**, no qual são explanados interpretações e comentários gerais sobre os resultados obtidos neste estudo.

As **REFERÊNCIAS BIBLIOGRÁFICAS** referem-se somente às citações que aparecem nos itens **INTRODUÇÃO** e **DISCUSSÃO DOS RESULTADOS** deste trabalho.

1 INTRODUÇÃO

Há condições fisiológicas que somente as mulheres vivenciam e que podem ter impactos negativos na saúde delas. Algumas destas condições, como a gravidez, o parto e a menopausa, não são doenças, mas processos fisiológicos que podem apresentar riscos à saúde e exigem atenção (OMS, 2011). O climatério é definido pela Organização Mundial da Saúde (OMS) como uma fase biológica, que compreende a transição entre o período reprodutivo e o não reprodutivo da vida da mulher (OMS, 1996). A menopausa é um marco dessa fase, correspondendo ao último ciclo menstrual devido à perda da função folicular ovariana e, é reconhecida após doze meses de amenorréa (Figura 1). Torna-se difícil determinar a idade de ocorrência da menopausa, devido à variabilidade individual e populacional. Contudo, estima-se que naturalmente ela ocorra em média aos 51 anos de idade (GREENDALE et al., 1999; HALE & BURGER, 2009).

Durante a vida reprodutiva feminina, os níveis de hormônios hipofisário e esteroides oscilam a cada ciclo menstrual (Brasil, 2008) (Figura 2). Ao passo que, na menopausa, as concentrações plasmáticas de hormônios esteroides caem para níveis indetectáveis ou próximos daqueles observados no início da fase folicular do ciclo menstrual normal (Hale & Burger, 2009). Sendo assim, as principais características do envelhecimento reprodutivo, observadas na bioquímica clínica incluem: reduções dos níveis séricos de 17β-estradiol (E_2) (Figura 3A), comumente referido como estrogênio ou estrógeno, e dos níveis de progesterona (Figura 3B), além do aumento dos níveis séricos do hormônio folículo estimulante (FSH) (HALE & BURGER, 2009). O FSH é um dos hormônios secretados pela hipófise anterior que estimula o crescimento folicular e a síntese de E_2, o qual depois de produzido, exerce um *feedback* negativo sobre a liberação do hormônio hipofisário. Considerando que na menopausa não se tem mais a atividade folicular, e consequentemente a produção de estrogênios, os níveis de FSH tendem a estar constantemente aumentados (HALE & BURGER, 2009) e assim, representam melhores indicadores da fase não reprodutiva (Figura 1) (SOULES et al., 2001), uma vez que, a variabilidade individual pode tornar os níveis de esteroides plasmáticos distintos mesmo após a menopausa (HALE & BURGER, 2009).

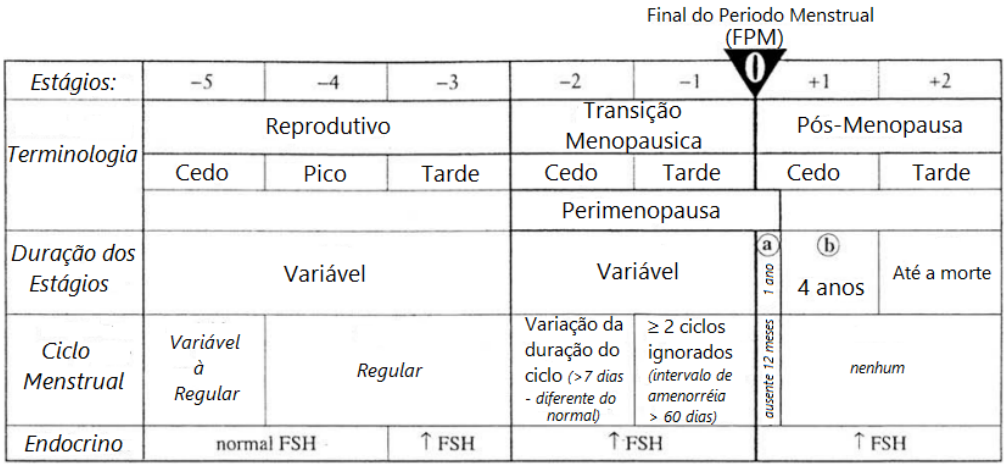

Figura 1- Estágios/nomenclatura do envelhecimento normal reprodutivo segundo os critérios internacionais estabelecidos (adaptado de The Stages of Reproductive Aging - STRAW) (SOULES et al., 2001).*

Os estrogênios incluem vários grupos de compostos que diferem na estrutura química e propriedades em geral, mas tem em comum sua função biológica: estimulação do desenvolvimento e a manutenção das características sexuais femininas. Esses hormônios esteroidais são produzidos e secretados principalmente pelo folículo ovariano, e os receptores para estrógenos são abundantes em todo o corpo (HALE & BURGER, 2009), resultando em ações genômicas e não genômicas (KUMAR et al., 2010). Devido às funções regulatórias dos estrogênios em vários órgãos e sistemas, o déficit desses hormônios provoca as reações fisiopatológicas responsáveis pelos sintomas característicos da menopausa, como as ondas de calor, as disfunções urogenitais, a atrofia e secura vaginal, a perda da libido e a depressão; bem como o aumento no risco de desenvolvimento de doenças crônicas como as cardiopatias e a osteoporose (GREENDALE et al., 1999; BRUCE et al., 2009). Estudos sugerem, ainda, que a diminuição dos níveis circulantes de estrogênios poderia ser a responsável pelo aumento do estresse oxidativo em mulheres após a menopausa (TREVISAN et al., 2001; KE et al., 2003; BEDNAREK-TUPIKOWASKA et al., 2004; STEVENSON et al., 2009).

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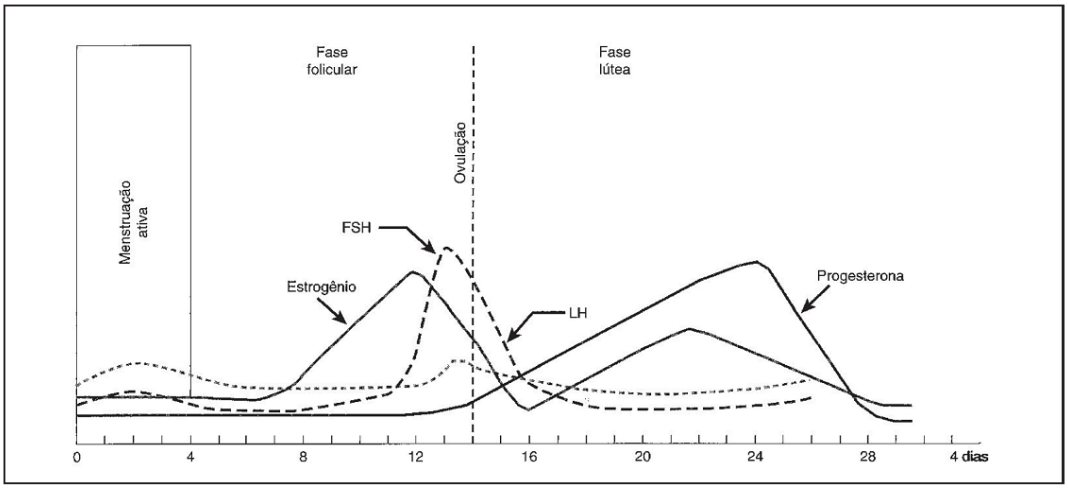

Figura 2 - Representação esquemática do ciclo reprodutivo feminino de 28 dias (adaptado de OTOMO-CORGEL & STEINBERG, 2002).*

A progestina natural em humanos é a progesterona (Figura 3B), um hormônio esteroide que, juntamente com os estrogênios, regula os órgãos acessórios durante o ciclo menstrual e é particularmente importante na manutenção da gravidez. Em mulheres não grávidas a progesterona é secretada principalmente pelo corpo lúteo, uma massa glandular no ovário, formada por um folículo ovariano após a liberação de seu óvulo (Figura 2). Durante a gravidez a placenta é a principal fonte deste hormônio (HALE & BURGER, 2009). Nas células, o efeito das progestinas ocorre através de alterações na transcrição de genes (AFRICANDER et al., 2011) e seus efeitos fisiológicos podem ser influenciados pela expressão de diferentes isoformas de receptores esteroidais (TURGEON et al., 2004). Além disso, a presença de receptores para esteroides na membrana plasmática sinaliza a possibilidade de mecanismos de ação rápidos e não genômicos (ALZAMORA et al., 2000; BOONYARATANAKORNKIT et al., 2004; NARAYANAN et al., 2008). Progestinas são utilizadas em formulações contraceptivas, na terapia de reposição hormonal (TRH), em tratamentos ginecológicos e terapias contra o câncer (GREENDALE et al., 1999; AFRICANDER et al., 2011). A progesterona natural e as progestinas sintéticas interagem não apenas com receptores para progesterona, mas também com outros receptores para hormônios esteroides, incluindo assim, efeitos androgênicos e anti-androgênicos (SITRUK-WARE, 2004).

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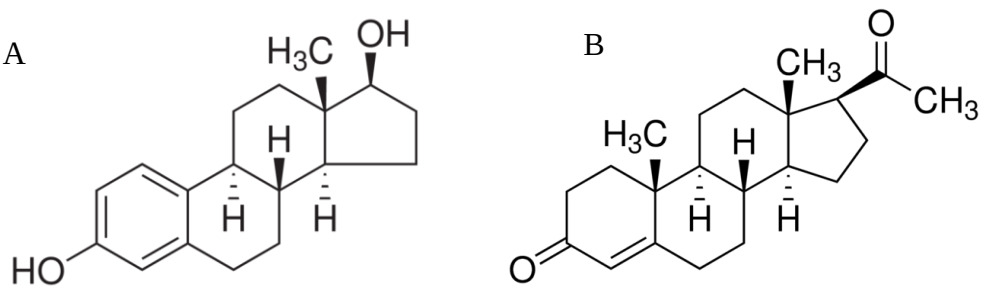

Figura 3 - Estrutura química do 17-β estradiol (A) e da progesterona (B) (Sigma Aldrich – Catálogo de Produtos).

1.1 Terapia de Reposição Hormonal (TRH)

A TRH consiste na administração exógena de hormônios sexuais femininos, naturais e sintéticos, sendo efetiva para compensar os efeitos da queda na produção de estrogênios após a menopausa. As terapias hormonais são uma intervenção complexa, para manter ou restaurar o estado fisiológico, que podem provocar efeitos positivos (sobre o remodelamento ósseo, calorões e sexualidade), e alguns efeitos prejudiciais à saúde, e ainda controversos, como a hiperplasia e o desenvolvimento de câncer endometrial, câncer de mamas e cardiopatias (WHI, 2002; WHI 2007; SKOUBY & JESPERSEN, 2009; STEVENSON et al., 2009; SCHUMACHER et al., 2007; WHITE et al., 2010). A reposição hormonal pode ser prescrita como terapia de reposição de estrógenos (TRE), ou uma combinação de estrógenos com uma ou mais progestinas, genericamente chamadas de TRH. Comumente, as doses de progestinas, prescritas em associação com os estrogênios visam à prevenção da hiperplasia e do câncer endometrial (GREENDALE, et al., 1999; KUMAR et al., 2010).

A menopausa é considerada o fim da proteção natural contra doenças relacionadas ao envelhecimento, como a osteoporose, doenças coronarianas, diabetes, Alzheimer e Parkinson (GREENDALE, et al., 1999; STEVENSON et al., 2009; AQUINO et al., 2012) e vem sendo reconhecida como um fator de risco para o estresse oxidativo (CRIST et al., 2009; SÁNCHEZ-RODRÍGUEZ et al., 2012). Com base em estudos de observação populacional, mulheres na pós-menopausa, que utilizam TRH, apresentam uma redução de 30-50% nas causas de mortalidade em relação as que não usam (GREENDALE et al., 1999). De fato, tem sido demonstrado que a terapia com estrogênios ou estrogênios mais progestinas previne a osteoporose, reduz o índice de fraturas (CAULEY et al., 2003) e protege a função endotelial e

o sistema cardiovascular (SUBBIAH, 1998; BUREAU et al., 2002). Contudo, os estudos ainda são contraditórios em relação ao mecanismo de proteção exercida pela TRH contra o estresse oxidativo em mulheres menopausadas (GURDOL et al., 1997; ÖZDEN et al., 2001; BUREAU et al., 2002; BEDNAREK-TUPIKOWASKA et al., 2004; NAZIROGLU et al., 2004; SIGNORELLI et al., 2005; BEDNAREK-TUPIKOWASKA et al., 2006; DELIBASE et al., 2006; UNFER et al., 2006).

O estresse oxidativo é definido como o desequilíbrio entre fatores oxidantes e antioxidantes, a favor dos oxidantes, prejudicando a integridade celular (SIES, 1985; 2000). Espécies reativas de oxigênio (EROs) são continuamente formadas e degradadas por organismos aeróbicos. A sua formação em concentrações fisiológicas é requerida para a função celular normal. No entanto, quantidades excessivas levam ao aumento do estresse oxidativo (NORDBERG & ARNÉR, 2001). As EROs incluem um grande número de moléculas quimicamente reativas e derivadas do oxigênio, como exemplo o ânion radical superóxido ($O_2^{\bullet-}$), o peróxido de hidrogênio (H_2O_2) e o radical hidroxila ($^{\bullet}OH$). (Figura 4)

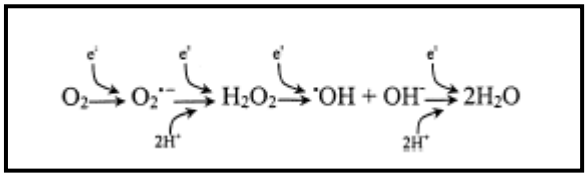

Figura 4 - Formação das espécies reativas de oxigênio, a partir do oxigênio molecular, com sucessivas transferências de elétrons (adaptado de NORDBERG & ARNÉR, 2001).

A superprodução de EROs pode provocar alterações em várias biomoléculas como proteínas (STADTMAN & LEVINE, 2000), lipídios e lipoproteínas (YLÄ-HERTTUALA, 1999), e ácido desoxirribonucleico (DNA) (MARNETT, 2000). Propõe-se que o estresse oxidativo esteja envolvido no processo de envelhecimento, tanto por induzir danos no DNA mitocondrial (FINKEL & HOLBROOK, 2000), quanto por outros mecanismos como a redução na atividade antioxidante celular natural (KASAPOGLU & ÖZDEN, 2001; ÍNAL et al., 2001; FUJIMOTO et al., 2010). HARMAN (1956) foi o primeiro a propor o papel vital dos radicais livres no envelhecimento e demonstrar que existia uma diminuição nos sistemas de defesa antioxidantes dependente da idade. Estudos também sugerem o envolvimento de EROs, no desenvolvimento de doenças como aterosclerose (DARLEY-USMAR et al., 1997;

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FUJIMOTO et al., 2010), câncer, diabetes (FINKEL & HOLBROOK, 2000) e na osteoporose (BASU et al., 2001; SANDUKJI et al., 2011). Além disso, o desequilíbrio entre fatores pró e antioxidantes também tem sido associado a mudanças fisiológicas subsequentes à menopausa, sendo observados nesta fase maiores danos oxidativos e alterações nas atividades das enzimas antioxidantes naturais (GURDOL et al., 1997; BEDNAREK-TUPIKOWASKA et al., 2001; ÖZDEN et al., 2001; BEDNAREK-TUPIKOWASKA et al., 2004; SIGNORELLI et al., 2005; UNFER et al., 2006).

As defesas antioxidantes incluem fatores não enzimáticos, intrínsecos e extrínsecos, e a atividade natural enzimática. As enzimas antioxidantes protegem as células aeróbicas e demais estruturas corporais de injúrias oxidativas causadas por EROs, geradas durante o metabolismo. Dentre elas, algumas das mais estudadas são a superóxido dismutase (SOD), a catalase (CAT) e a glutationa peroxidase (GPx) (Figura 5).

1.2 Superóxido Dismutase (SOD)

A atividade da SOD representa a primeira linha de defesa contra o estresse oxidativo, pois detoxificam o $O_2^{\bullet -}$, em um processo de dismutação, produzindo o H_2O_2, que pode ser reduzido por ação das enzimas CAT e GPx. A atividade superóxido dismutase está representada em humanos sob diferentes isoformas nos compartimentos celulares: CuZnSOD citoplasmática (SOD1) e extracelular (SOD3) e MnSOD (SOD2), exclusivamente mitocondrial (Figura 5) (NORDBERG & ARNÉR, 2001). Estudos demonstraram uma diminuição nas atividades da SOD (ÍNAL et al., 2001) e da GPx, e um aumento na peroxidação lipídica (KASAPOGLU & ÖZDEN, 2001) em decorrência do envelhecimento humano. Além disso, condições patológicas, como a obesidade e o diabetes, também estão associadas a disfunções na dismutação do ânion radical superóxido (JOHNSON & GIULIVI, 2005). De fato, tem sido proposto que o estresse oxidativo seja o mais importante mecanismo responsável pelas alterações associadas à idade no que se refere, a atividade da SOD e ao aumento da apoptose celular em ratos idosos (KOKOSKA *et al.*, 2001). Além do mais, existem evidências, *in vitro* e *in vivo*, de que hormônios esteroides podem regular a expressão e a função da SOD (SUGINO et al., 2002; STREHLOW et al., 2003; FENG & ZHANG, 2005; MOORTHY et al., 2005; UNFER et al., 2006; PAJOVIĆ & SAIČIĆ, 2008).

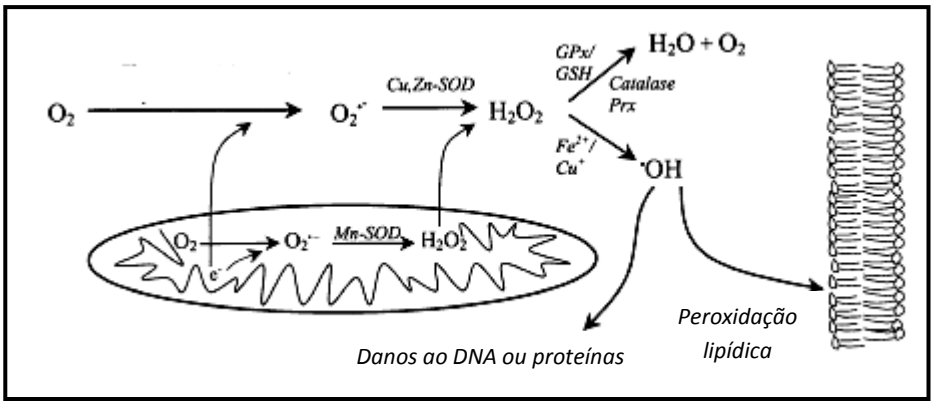

Figura 5 - Esquema simplificado não estequiométrico dos sistemas oxidante e antioxidante nas células (adaptado de NORDBERG & ARNÉR, 2001).

1.3 A SOD e os hormônios esteroides

CHAO et al. (1994), constataram que o tratamento, *in vitro*, de macrófagos de ratos com concentrações fisiológicas de E_2, progesterona e testosterona promoveu alterações significativas na produção de $O_2^{\bullet-}$ e H_2O_2. De fato, o E_2 contém um grupo fenol (Figura 2A) que pode ser efetivo na remoção de $^{\bullet}OH$, resultando em produtos hidroxilados (HALIWELL & GROOTVELD, 1987). Além do mais, tem sido demonstrado que o estrogênio diminui a produção de EROs e o estresse oxidativo *in vitro* e *in vivo* (HUBER et al., 1989; SUBBIAH et al., 1993; AYERS et al., 1998; THIBODEAU et al., 2002; BEDNAREK-TUPIKOWASKA et al., 2004; BEDNAREK-TUPIKOWASKA et al., 2006; AL-GUBORY et al., 2008; KUMAR et al., 2010). Contudo, existem relatos de desenvolvimento de carcinomas, estimulado pelo estrogênio, e esse efeito genotóxico poderia estar relacionado à geração de radicais livres durante sua metabolização até catecolestrogênios e, subsequentemente, até quinonas (LIEHR, 1990; TONIOLO et al., 1995; CAO et al., 1998; KATTAN et al., 2008; KUMAR et al., 2010). Os efeitos estrogênicos, ora identificados como pró-oxidante, ora como antioxidante devem-se ao tipo de célula estudada, já que os tipos de receptores presentes em uma ou outra célula variam largamente. Além do mais, o contexto da resposta hormonal também deve ser considerado, uma vez que as ações via receptor de estrogênios podem ser genômicas ou não genômicas (KUMAR et al. 2010). De fato, os hormônios esteroides parecem exercer efeitos distintos na expressão e atividade das isoformas da SOD dependendo do tecido avaliado.

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O E_2 aumentou a expressão e a atividade da CuZnSOD extracelular e da MnSOD em cultura de células da musculatura lisa vascular (STREHLOW et al., 2003). O E_2 também reverteu a redução da atividade da MnSOD cerebral (FENG & ZHANG, 2005) e a expressão da CuZnSOD extracelular e MnSOD em tecido aórtico de ratas (STREHLOW et al., 2003) após a ooforectomia. O tratamento com progesterona aumentou a expressão e a atividade da CuZnSOD e da MnSOD em culturas de células endometriais humanas (SUGINO et al., 2002), mas diminuiu a expressão e a atividade da CuZnSOD extracelular e MnSOD em cultura de células vasculares (WASSMANN et al., 2005). Por outro lado, o E_2 sozinho não apresentou efeito na atividade da SOD em cultura de células endometriais humanas (SUGINO et al., 2002), mas aumentou a expressão da CuZnSOD extracelular e MnSOD em cultura de células vasculares (STREHLOW et al., 2003; WASSMANN et al., 2005). Entretanto, os estudos encontrados divergem quanto ao efeito da combinação de estrogênio com progestina, no que se refere à redução do estresse oxidativo avaliada em diferentes tecidos (SUGINO et al., 2002; STREHLOW et al., 2003; WASSMANN et al., 2005).

Segundo MOORTHY et al. (2005), a atividade da SOD em ratas, diminui significativamente com a idade em vários tecidos, sendo esse efeito mais pronunciado nos tecidos hepático e cardíaco. Após a terapia hormonal, a atividade da SOD aumentou no grupo de animais idosos tratados com a terapia combinada de E_2 mais progesterona (MOORTHY et al., 2005). Em cultura de células endometriais humanas, o estradiol potencializou os efeitos da progesterona sob a expressão da SOD (SUGINO et al., 2002), enquanto que em cultura de células vasculares lisas, também humanas, a progesterona bloqueou o efeito estimulatório do estradiol (WASSMANN et al., 2005). Tais fatos indicam que o E_2 e a progesterona, podem modular a atividade da SOD em tecidos que apresentem receptores celulares para estes hormônios. Eles também demonstraram que os efeitos do E_2 são seletivos para a SOD, sem mudanças na expressão da GPx e CAT. No entanto, os estudos que investigaram a influência dos hormônios esteroides nas atividades das isoformas da SOD em culturas celulares ou *in vivo*, têm focado principalmente as alterações na expressão enzimática.

Avaliando mulheres na pré e na pós-menopausa sem TRH, BEDNAREK-TUPIKOWSKA et al. (2001), detectaram que existe uma correlação negativa entre o E_2 endógeno e a peroxidação lipídica (LPO) no soro e, uma associação positiva entre a concentração desse hormônio e a atividade da GPx em eritrócitos. Esses autores também sugerem que o E_2 exerce ação antioxidante, não apenas por sua estrutura química (fenólica), mas provavelmente devido a sua influência na atividade de enzimas antioxidantes

(BEDNAREK-TUPIKOWASKA et al., 2006). No entanto, o papel das progestinas como uma parte essencial da TRH na defesa antioxidante ainda permanece obscuro. Progestinas administradas sozinhas ou em combinação com estrógenos diminuem os níveis de peróxido na membrana de plaquetas (TRANQUILLI et al., 1995) e aumentam a capacidade antioxidante total no soro (BEDNAREK-TUPIKOWSKA et al., 2004), em mulheres pós-menopáusicas. Contudo, as progestinas, em associação terapêutica com estrógenos não amplificaram o efeito antioxidante deste último sobre a oxidação lipídica no soro de mulheres na pós-menopausa (BEDNAREK-TUPIKOWSKA et al., 2004). Estudos observaram uma diminuição nos níveis, plasmático e eritrocitário, de substâncias reativas ao ácido tiobarbitúrico (TBARS) e, um aumento nos níveis de glutationa reduzida (GSH), e da atividade, eritrocitária e plasmática, da GPx (ÖZDEN et al., 2001; NAZIROGLU et al., 2004) e da CAT (NAZIROGLU et al., 2004) em mulheres na pós menopausa com terapia hormonal combinada. Contudo, BUREAU et al. (2002) não encontraram diferença na atividade eritrocitária da GPx e da SOD entre mulheres na pós-menopausa com e sem TRH. Sendo assim, os estudos avaliando a influência da TRH na atividade das enzimas antioxidantes em mulheres na menopausa ainda limitados, principalmente no que se refere à metodologia, população estudada, o tempo e a composição hormonal da terapia.

Em um estudo anterior, nós demonstramos que a TRH impede a diminuição da atividade sanguínea da SOD total em mulheres na pós-menopausa (UNFER et al., 2006). Além disso, observamos uma tendência de maior atividade da SOD no sangue total de mulheres na pós-menopausa que fazem uso de TRH combinada de E_2 mais progesterona, quando comparado com o grupo de mulheres utilizando apenas estrogênio na terapia hormonal (UNFER et al., 2006). No entanto, as isoformas da SOD não foram avaliadas e também não foi possível avaliar as terapias hormonais separadamente, terapia somente com estrógenos ou a terapia combinada de estrógenos com progestinas, uma vez que, o pequeno número de pacientes prejudicou a divisão do grupo com TRH.

Enfim, os hormônios, principalmente a progesterona, parecem regular de forma diferenciada, a atividade das isoformas da SOD e em vários tecidos, o que representa particular importância em situações em que existem flutuações nos perfis hormonais, como na menopausa e nas TRH. Além do mais, não foram encontrados estudos avaliando o efeito de estrogênios, progestinas e seus derivados sintéticos (utilizados em fórmulas farmacêuticas de TRH) sobre a atividade das isoformas da SOD presentes no sangue humano. Tampouco, foram encontrados estudos avaliando os efeitos de diferentes TRH (estrogênios e estrogênio

mais progesterona), sobre a atividade antioxidante das isoformas sanguíneas da SOD em mulheres na pós-menopausa.

2 OBJETIVOS

2.1 Objetivo geral

O presente trabalho tem como objetivo geral investigar a influência de estrogênios e progestinas sobre a atividade da superóxido dismutase (SOD) e o estatus oxidativo de mulheres.

2.2 Objetivos específicos

- Avaliar, *in vitro,* os efeitos de estrógenos e progestinas naturais e sintéticos sobre a atividade da CuZnSOD purificada de eritrócitos humanos e das isoformas da SOD em amostras de eritrócitos (CuZnSOD citoplasmática) e de plasma rico em plaquetas (MnSOD e CuZnSOD citoplasmática e extracelular).

- Avaliar os efeitos da terapia de reposição hormonal com estrógenos ou com estrógenos mais progestinas sobre a atividade das isoformas sanguíneas da SOD (CuZn e MnSOD), da catalase e da glutationa peroxidase, e o perfil dos indicadores do estresse oxidativo em mulheres após a menopausa.

- Relacionar os níveis séricos dos hormônios esteroides naturais, estradiol e progesterona com os parâmetros de estresse oxidativo avaliados em mulheres.

3 ARTIGO ORIGINAL

Non-genomic, direct modulatory effect of 17 b-estradiol, progesterone and their synthetic derivatives on the activity of human erythrocyte CuZn superoxide dismutases

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ORIGINAL ARTICLE

Non-genomic, direct modulatory effect of 17β-estradiol, progesterone and their synthetic derivatives on the activity of human erythrocyte CuZn superoxide dismutase

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Abstract

This study aimed to evaluate whether natural or synthetic steroid hormones could directly modulate the activity of the different superoxide dismutase (SOD) isoforms found in human blood fractions without changing enzyme expression. Enzyme samples of human erythrocytes, the human platelet-rich plasma fraction (PRP) or isolated CuZnSOD, which was purified from human erythrocytes were pre-incubated with natural steroids (17β-estradiol 17-acetate and progesterone) and their synthetic derivatives (β-estradiol 3-benzoate and medroxyprogesterone 17-acetate). Then, CuZn and MnSOD activities were measured using the xanthine/xanthine oxidase/nitroblue tetrazolium method. Hormones had no effect on MnSOD activity from the PRP, but we show for the first time that natural and synthetic steroid hormones have a direct, bell-shaped effect on the activity of CuZnSOD from both male and female human erythrocytes. Low (physiological) hormone concentrations caused a dose-dependent increase in enzyme activity, which disappeared at higher hormone concentrations. In addition, the combination of synthetic and natural estrogens and progestins had a synergistic stimulatory effect on the activity of CuZnSOD from human erythrocytes. The molecular interaction between CuZnSOD and steroid hormones was preliminarily studied. Natural hormones did not change the electrophoretic mobility of SOD under denaturing conditions, but they did increase the absorption spectra of SOD in the 230–290 nm range. These data suggest that hormone-mediated modulation of CuZnSOD is related to subtle changes in protein conformation, possibly related to Trp and Phe residues. We propose that this effect may account for the physiological regulation of enzyme activity during conditions where steroid hormones undergo alterations as the ovulatory cycle.

Keywords: Mn superoxide dismutase, hormone replacement therapy, β-estradiol 3-benzoate, medroxyprogesterone

Introduction

Superoxide dismutases (SOD; EC 1.15.1.1.) play a key role in protecting cells from oxidative stress because they eliminate superoxide anion radicals ($O_2^{\cdot -}$) that are produced under physiological conditions or in oxidative stress-related diseases [1,2]. CuZnSODs are present in the cell cytoplasm (SOD1) and extracellular milieu (SOD3), whereas MnSOD is located in the mitochondrial matrix (SOD2) and is the first-line defense against superoxide radicals produced by the respiratory chain [2,3].

Hormone replacement therapy (HRT) is a complex intervention that is used to attenuate menopausal symptoms. HRT consists of either the replacement of estrogen alone or the combination of estrogen with progestins [4]. There is some evidence that sex steroid hormones regulate the expression and function of SODs. Estradiol increased the expression and activity of extracellular CuZnSOD and MnSOD in vascular smooth muscle cell cultures [5]. This effect was apparently due to the activation of the estradiol receptor and was selective for SOD, with no change in the expression of glutathione peroxidase or catalase. Estradiol also antagonized the ovariectomy-induced reduction of MnSOD activity in the brain [6] and the reduction of extracellular CuZnSOD and MnSOD expression in the aorta of rats [5].

Regarding the combined use of progesterone in HRT, we found higher blood SOD activity in postmenopausal women receiving HRT compared to those not receiving HRT [7]. Interestingly, a trend of higher SOD activity was observed in women receiving combined estradiol plus progesterone therapy compared to those who received only estrogen [7]. Similarly, the administration of estradiol and progesterone to female rats prevented the age-induced decrease in cytosolic CuZnSOD activity in various tissues [8].

However, steroid hormones seem to differentially affect SOD expression and activity depending on the tissue type.

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Progesterone treatment increased the expression and activity of CuZn and MnSOD in human endometrial cell cultures [9] but decreased the expression and activity of extracellular CuZnSOD and MnSOD in vascular smooth muscle cell cultures [10]. Likewise, estradiol alone had no effect on SOD activity in human endometrial cell cultures [9] but increased the expression of extracellular CuZnSOD and MnSOD in vascular smooth muscle cell cultures [5,10]. Estradiol augmented the effects of progesterone on SOD expression in human endometrial cell cultures [9], whereas progesterone blocked the effects of estradiol on SOD expression in human vascular smooth muscle cell cultures [10].

Studies investigating the influence of steroid hormones on SOD isoforms in cell culture or *in vivo* have mainly focused on changes in enzyme expression. However, the effect of estrogens, progestins or their synthetic derivatives in human blood SOD isoforms has not been studied. Since SOD enzymes from different tissues seem to be differentially affected by steroids, this study aimed to evaluate whether natural or synthetic steroid hormones could directly affect the activity of different SOD isoforms found in human blood fractions.

Materials and methods

Human subjects

This research protocol was approved by the local ethics committee of the Federal University of Santa Maria (Brazil) (CAAE n° 0120.0.243.000-06). Four healthy women (age range, 20–28 years) and three men (age range 20–30 years) were included in the study as blood donors, after giving informed consent. In order to obtain blood samples with low levels of steroid hormones, all the female subjects were in the follicular phase of the ovulatory cycle. The subjects did not receive any medication prior to blood collection.

Blood sampling and sample preparation

Peripheral blood was collected with citrate-phosphate-dextrose-adenine anticoagulant and was immediately centrifuged at 1500 × *g* for 12 min to obtain the erythrocyte and platelet-rich plasma (PRP) samples, which were stored at − 20°C for up to one month prior to analysis. Erythrocyte samples were lysed and used exclusively as a source of CuZnSOD (SOD1 or cytoplasmic SOD), whereas PRP was used as a source of both MnSOD (SOD2 or mitochondrial SOD) and CuZnSODs (cytoplasmic SOD1, and extracellular SOD3).

The purified CuZnSOD (SOD1) enzyme, which was isolated from human erythrocytes, was obtained from Sigma (St. Louis, MO, USA; catalog code S9636) and diluted in purified water before use.

Blood analysis

The serum levels of 17β-estradiol and progesterone were determined in all blood samples by immunoassay with Immulite® Estradiol and Immulite® 2000 Progesterone kits (Diagnostic Products Corporation, Los Angeles, CA, USA). The limits of quantification for the E2 and progesterone assays were 20.0 pg/mL (73 pM) and 0.2 ng/mL (0.6 nM), respectively.

Hematocrit and hemoglobin values, which ranged from 38.8%–39.4% and 12.4–13.3 g/dL, respectively, were determined using a Beckman/Coulter T890 hematology analyzer with diluent solution diaton-3 (Daigon, Belo Horizonte, MG, Brazil). Protein levels in the PRP, which ranged from 3.17–7.85 g/dL, were determined according to Lowry et al. [11], using bovine serum albumin (Sigma, St. Louis, MO, USA) as a standard.

Superoxide dismutase activity assay

To assess the time course of hormone effects on SOD activity, purified CuZnSOD (18 U/mL) was pre-incubated in the presence of vehicle (control) or hormones (2 nM) at 37°C for up to 24 h before assessing the enzyme activity. Hormone stock solutions (200 μM) were prepared in ultrapurified water using ethanol (0.038%) and Tween-80 (1%). Hormone working solutions were prepared from the stock solution by dilution with ultrapurified water. A vehicle solution containing only ethanol and Tween 80 was prepared in a similar way. The vehicle was incubated with the samples following the same procedure used for the hormones and was used as a control to account for any effects of these solubilizing agents alone.

In the following enzyme assays, the samples (erythrocytes, PRP or the purified CuZnSOD) were pre-incubated for 24 h at 37°C in the presence of vehicle (control) or steroid hormones (0.3–10^5 nM). Four hormones were evaluated: the natural steroids 17β-estradiol 17-acetate (E2) (Riedel-de-Häen, Steinhein, Germany) and progesterone and their synthetic derivatives, β-estradiol 3-benzoate (E2Benz) and medroxyprogesterone 17-acetate (MPA) (Sigma, St. Louis, MO, USA). The quantification of E2 and progesterone levels in the incubation assay (assessed by immunoassay, as described in the blood analysis section) revealed no loss of hormones during the pre-incubation period.

After pre-incubation, the SOD activity was measured by an indirect assay based on the competitive reaction between SOD and nitroblue tetrazolium (NBT) chloride (Sigma, St. Louis, MO, USA) [12]. The rate of increasing absorbance at 560 nm indicates the reduction of NBT to blue formazan by superoxides, which are generated by the xanthine/xanthine oxidase system. To assess the total SOD (CuZn plus Mn SOD) activity, the enzyme that was previously pre-incubated with the vehicle or hormone was added to a buffered medium containing diethylene triamine pentaacetic acid (1 mM), xanthine (0.1 mM), catalase (1 U/mL), and NBT (56 μM). The enzymatic reaction was initiated by adding xanthine oxidase. This assay medium contained the vehicle or the same hormone concentration used in the pre-incubation assay. MnSOD activity was quantified in the same assay medium but in the presence of 5 mM KCN, which inhibits CuZnSOD activity. CuZnSOD activity was determined as the

difference between total SOD activity and MnSOD activity. One unit of activity was defined as the amount of protein necessary to achieve half-maximal inhibition of the NBT reaction, and the activity was expressed as units per milligram of protein or units per milligram of Hb in the case of erythrocytes.

To determine whether steroid hormones could directly affect the NBT reaction, which could bias the SOD assay, additional controls containing the same hormone concentrations used in the NBT reaction assay, but without the SOD enzyme sample, were used. These controls revealed that neither the hormones nor the vehicle affected the complex assay, as they had no direct effect on the reduction of NBT to blue formazan (data not shown). To provide additional evidence that steroid hormones could not directly inhibit xanthine oxidase or scavenge superoxide anions, which would decrease the superoxide flux generated by the xanthine oxidase system, we used a single assay, as described by Cos et al. [13]. The reaction assay contained vehicle or hormones (0.3–10^5 nM), xanthine (0.3 mM), hydroxylamine (0.2 mM), EDTA (0.1 mM), xanthine oxidase (6 mU/mL) and phosphate buffer solution (80 mM, pH 7.5). After 30 min of incubation, HCl was added to stop the reaction and superoxide production was measured using the nitrite method, whereas uric acid was measured by the increase in absorbance at 290 nm. Steroid hormones (0.3–10^5 nM) did not decrease the production of superoxide or uric acid catalyzed by xanthine oxidase compared to vehicle-containing controls (data not shown).

Because steroid hormones are especially important for the physiology of women, all experiments were initially conducted using female samples compared to the purified enzyme. Then, to determine whether SOD from men had the same behavior we evaluated the effect of E2 and progesterone on the activity of CuZnSOD from male erythrocytes and PRP.

Electrophoretic mobility of CuZnSOD incubated with E2 and progesterone

Purified CuZnSOD (180 U/mL) was pre-incubated with vehicle, 3 or 18 nM E2, or 10 or 60 nM progesterone at 37°C for 24 h. The concentration of CuZnSOD that was used in this assay was 10-fold higher than the concentration that was used in the enzyme activity assays, because we could not detect any electrophoretic band at lower protein concentration. After pre-incubation, samples were solubilized in an electrophoresis sample buffer (62.5 mM Tris–HCl, pH 6.8, 1% (w/v) sodium-dodecyl-sulfate, 10% (v/v) glycerol), fractionated by SDS–PAGE in 15% polyacrylamide gels (2-hour run at constant 85 V) and electro-blotted onto nitrocellulose membranes. Protein loading and electro-blotting efficiency were verified using Ponceau S staining, and the membrane was blocked in Tween-Tris buffered saline (TTBS: 100 mM Tris–HCl, pH 7.5, containing 0.9% NaCl and 0.1% Tween-20) containing 5% albumin. Membranes were incubated overnight at 4°C with a CuZnSOD antibody (Millipore-Chemicon) at a 1:1000 dilution, in TTBS. The membranes were then washed with TTBS, incubated in an anti-rabbit IgG peroxidase-linked secondary antibody for 1 h (1:5000 dilution range), and washed again, and the immunoreactivity was then detected by enhanced chemiluminescence, using a West Pico kit from Pierce (Rockford, USA). Blots were developed so that they were in the linear range used for densitometry.

Absorbance spectra of CuZnSOD incubated with E2 and progesterone

Measurements were performed using a SpectraMax M5 multi-mode microplate reader in the UV/visible absorbance mode (Molecular Devices, LLC, Sunnyvale, USA). The light source was a Xenon flash lamp, and the grating bandwidth was < 4 nm. Purified CuZnSOD (18 U/mL) was pre-incubated with vehicle, E2, progesterone or a combination of E2 and progesterone at 37°C for 24 h. For control purposes, solutions containing only the hormones were also pre-incubated at 37°C for 24 h. The absorption spectra were then collected from 230 to 430 nm, at 10-nm steps, using disposable, semi-micro UV-cuvettes, with a 1 cm optical path (Plastibrand). All measurements were performed at room temperature against blanks that contained only the vehicle that was used to prepare the enzyme or hormone solutions.

Statistical analyses and EC_{50} calculation

Data were analyzed by one-way analysis of variance (ANOVA), followed by Duncan's test when appropriate. The results were expressed as means ± standard error (S.E.M.) of three independent experiments. For the activity of SOD from blood samples, each experiment was conducted using a sample from a different donor. In each experiment, all concentrations of a given hormone were evaluated in parallel using the same blood sample. The effective concentration of hormone required to stimulate 50% of enzyme activity (EC_{50}) was determined by non-linear regression analyses using GraphPad Prism software (version 5.04). The EC_{50} values were compared for statistical significance using confidence intervals.

Results

To determine the effect of steroid hormones on the different isoforms of the antioxidant enzyme SOD in human blood, we used erythrocyte samples as the source of cytoplasmic CuZnSOD, PRP as the source of both MnSOD and CuZnSODs (cytoplasmic and extracellular) and commercial purified CuZnSOD from human erythrocytes (Sigma®) as an isolated enzyme. The background physiological levels of E2 found in the female and male blood samples used in the present study were < 73 pM, whereas the background levels of progesterone ranged from 0.6 to 2.9 nM for the female samples and from 0.6 to 0.8 nM for

the male samples. The concentration of steroid hormones added to the incubation assay, which was used to assess their effect on SOD, ranged from 0.3 to 10^5 nM. Thus, the background physiological levels of E2 did not contribute to the hormone concentration in the incubation assay, while the background levels of progesterone only significantly contribute at the lowest hormone concentrations that were evaluated.

With the concentrations evaluated, the steroid hormones had no effect on the reduction of NBT to blue formazan in the absence of SOD enzyme preparation (data not shown). This finding indicates that the evaluated steroid hormones did not act as formazan oxidants in our assay conditions. In addition, hormones did not affect the superoxide flux generated by the xanthine-xanthine oxidase system, which is responsible for the reduction of NBT to blue formazan. Thus, hormones did not act as inhibitors of xanthine oxidase or scavengers of superoxide anions. Therefore, the observed effects could be attributed to the specific action of steroid hormones on the SOD enzyme preparations.

To assess the time course of hormone effects on SOD activity, samples of purified CuZnSOD were pre-incubated *in vitro* with E2, progesterone, E2Benz or MPA for up to 24 h before assessing the enzyme activity (Figure 1). All of the hormones that were tested significantly and immediately increased the enzyme activity after they were added to the assay (1 min of pre-incubation), and pre-incubation with the hormones for up to 24 h did not cause any further change in the enzyme activity. Therefore, because of logistics, we adopted a 24 h pre-incubation condition for all of the other experiments performed in this study.

Effect of the natural steroid hormones, 17β-estradiol and progesterone on SOD activity

The concentration-response curves for the effect of E2 on CuZnSOD activity in female erythrocytes (SOD1, Figure 2A), female PRP (SOD1 and SOD3, Figure 2B) and the purified enzyme (SOD1, Figure 2C) were convex and bell shaped. Erythrocyte CuZnSOD activity increased by up to 8-fold when incubated with E2 (2–3 nM). However, this increase disappeared at E2 concentrations higher than 3 nM, and CuZnSOD activity returned to the control value when incubated with 10^2 nM E2 (Figure 2A). Similar behavior was observed for CuZnSOD activity in the PRP (Figure 2B). Enzyme activity was increased by up to 24-fold in response to 10 nM E2, but this increase disappeared at E2 concentrations higher

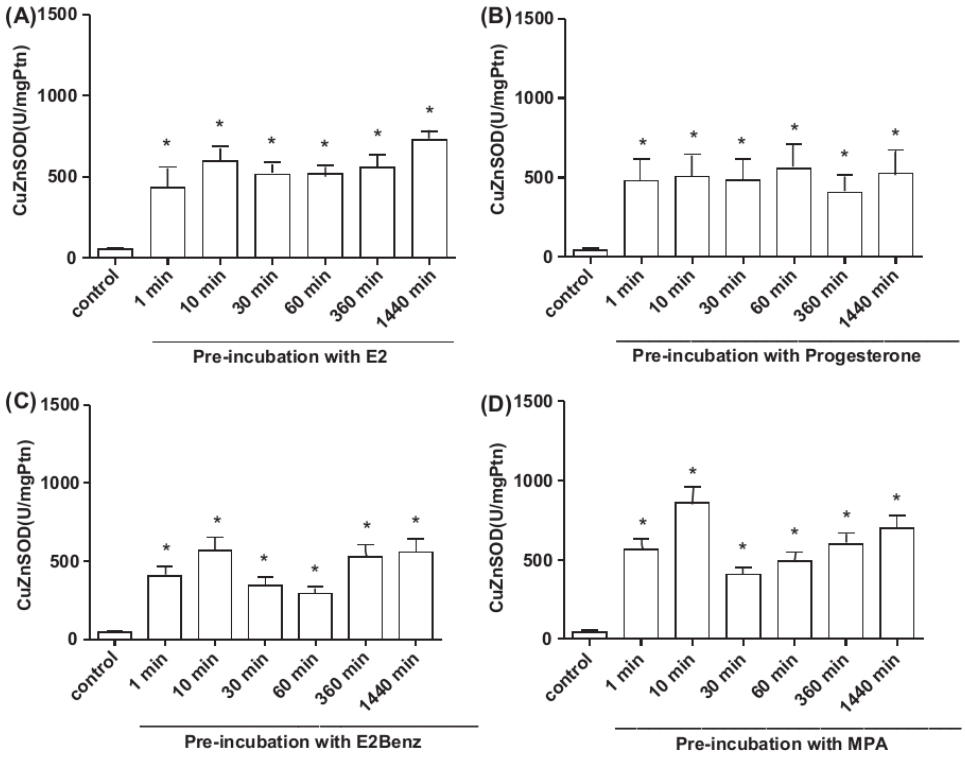

Figure 1. Time-course of 17β-estradiol (E2) (A), progesterone (B), β-estradiol 3-benzoate (E2Benz) (C) and medroxyprogesterone 17-acetate (MPA) (D) effects on the activity of purified CuZnSOD that was obtained from human erythrocytes. Enzyme samples were pre-incubated with vehicle (control) or hormones (2 nM) for different durations. The results are mean ± SEM (n = 3). *Different from control ($p < 0.05$).

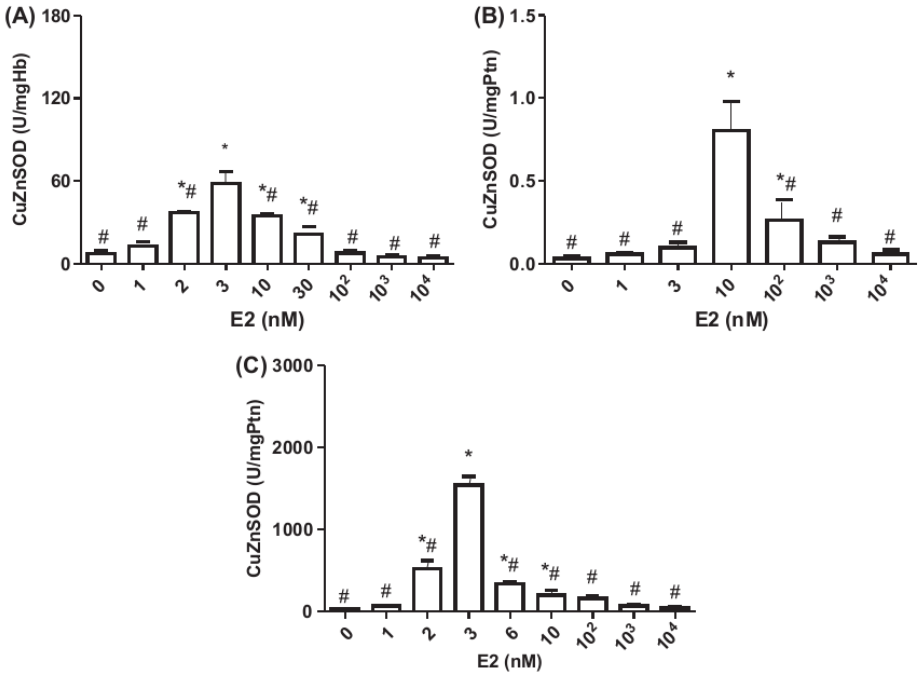

Figure 2. 17β-Estradiol (E2), a natural steroid hormone, exhibited a bell-shaped dose-dependent effect on CuZnSOD activity in female erythrocytes (EC_{50} for stimulation = 1.9 nM; confidence interval, 1.6–2.2 nM) (A), in female platelet-rich plasma samples (EC_{50} for stimulation = 6.2 nM; confidence interval, 4.6–8.3 nM) (B) and in the purified enzyme that was obtained from human erythrocytes (EC_{50} for stimulation = 2.2 nM; confidence interval, 2.1–2.3 nM) (C). Enzyme samples were pre-incubated for 24 hours with vehicle (control) or increasing concentrations of E2. The results are mean ± SEM (n = 3). *Different from control ($p < 0.05$). #Different from the hormone concentration that yielded the highest enzyme activity.

than 10 nM. CuZnSOD activity returned to the control value when incubated with 10^3 nM E2 (Figure 2B). To confirm whether the observed effects were due to a direct interaction between E2 and CuZnSOD, we evaluated the effect of E2 on purified CuZnSOD that was obtained from human erythrocytes (Sigma®) (Figure 2C). Purified CuZnSOD exhibited a similar behavior to that of non-purified CuZnSOD obtained from erythrocytes. CuZnSOD activity increased by up to 51-fold when incubated with E2 (2–3 nM). However, this increase disappeared at E2 concentrations above 3 nM, and CuZnSOD activity returned to the control value when incubated with 10^2 nM E2 (Figure 2C). The EC_{50} values for the stimulation by E2 were similar for both the purified and non-purified CuZnSOD from human erythrocytes (2.2 and 1.9 nM, respectively), but the EC_{50} values were slightly higher for the activity of CuZnSOD obtained from PRP (6.2 nM) (Figure 2). E2 had no effect on the activity of MnSOD in female PRP (data not shown).

SOD activity is inhibited at high concentrations of H_2O_2 [12]. Thus, we used CAT (1 U/mL) in our incubation assay to prevent the inhibition of SOD by excess of H_2O_2. However, the decrease in SOD activity observed at the highest hormone concentrations could be due to enzyme inhibition by H_2O_2, which could have exceeded the removal capacity of CAT. To test this hypothesis, we evaluated the effect of E2 (10^2 and 10^3 nM) on the activity of purified CuZnSOD obtained from human erythrocytes (Sigma®) in the presence of increasing CAT concentrations (1 to 20 U/mL). SOD activity did not change with increasing CAT concentrations (data not shown), indicating that no excess H_2O_2 was formed in our assay conditions.

As was observed for E2, the concentration-response curves for the effect of progesterone on CuZnSOD activity in female erythrocytes were convex and bell shaped (Figure 3A). When erythrocyte SOD was incubated with progesterone (0.3–3 nM) the enzyme activity increased by up to 19-fold. However, this increase disappeared when the enzyme was incubated with progesterone concentrations above 3 nM, and CuZnSOD activity returned to the control value when incubated with 10^2 nM progesterone. Progesterone also increased the activity of CuZnSOD in the female PRP; however, we did not find any decrease in enzyme stimulation even with 10^4 nM progesterone (Figure 3B). CuZnSOD purified from human erythrocytes (Sigma®) displayed a similar behavior to that of the non-purified enzyme, where enzyme activity increased by up to 20-fold in response to 10^2 nM progesterone (Figure 3C). Although the enzyme activity decreased at progesterone concentrations above 10^2 nM, it was still significantly higher than the control value even at a concentration of 10^4 nM. The EC_{50} value for enzyme stimulation by progesterone was lower for non-purified CuZnSOD (1.0 nM) compared to purified CuZnSOD (5.0 nM) and CuZnSOD

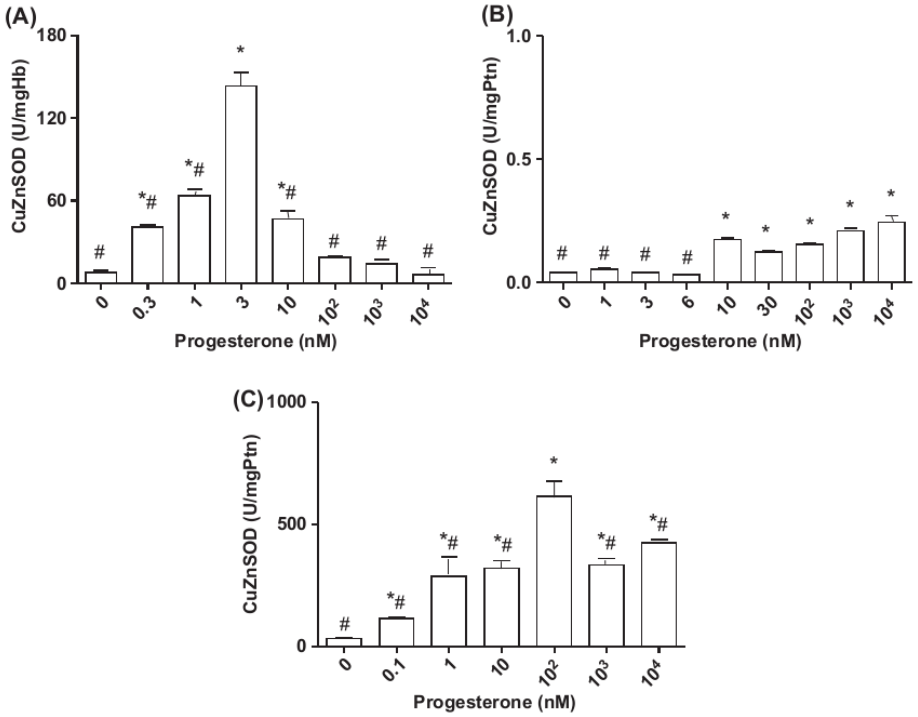

Figure 3. Progesterone, a natural steroid hormone, exhibited a bell-shaped dose-dependent effect on CuZnSOD activity in female erythrocytes (EC_{50} for stimulation = 1.0 nM; confidence interval, 0.8–1.4 nM) (A), in female platelet-rich plasma samples (EC_{50} for stimulation = 41.7 nM; confidence interval, 16.2–107.1 nM) (B) and in the purified enzyme that was obtained from human erythrocytes (EC_{50} for stimulation = 5.0 nM; confidence interval, 2.4–10.5 nM) (C). Enzyme samples were pre-incubated for 24 hours with vehicle (control) or increasing concentrations of progesterone. The results are mean ± SEM (n = 3). *Different from control ($p < 0.05$). #Different from the hormone concentration that yielded the highest enzyme activity.

from PRP (41.7 nM) (Figure 3). In contrast, progesterone had no effect on the activity of MnSOD in female PRP (data not shown).

To verify whether the effects of hormones on the activity of SOD from male donors were similar to those observed for female donors we evaluated the effect of E2 and progesterone in erythrocytes and PRP obtained from male donors (Figure 4). Similar to the female enzyme preparations, the concentration-response curves for the effect of E2 on CuZnSOD activity in male erythrocytes (SOD1, Figure 4A) and PRP (SOD1 and SOD3, Figure 4B) were convex and bell shaped. Erythrocyte CuZnSOD activity was increased by up to 7-fold when incubated with E2 (2–3 nM). However, this increase disappeared at E2 concentrations above 3 nM, and CuZnSOD activity returned to the control value when incubated with 10^2 nM E2 (Figure 4A). Similar behavior was observed for CuZnSOD activity in the PRP (Figure 4B). CuZnSOD activity was increased by up to 4-fold (10 nM E2). However, this increase disappeared when the enzyme was incubated with E2 at concentrations above 10 nM, and CuZnSOD activity returned to the control value when incubated with 10^2 nM E2 (Figure 4B). The EC_{50} values following enzyme stimulation by E2 were similar for both male and female erythrocytes (2.2 and 1.9 nM, respectively) and PRP (4.0 and 6.2 nM, respectively) (Figures 2 and 4).

As observed for E2, the concentration-response curves for the effect of progesterone on CuZnSOD activity in male erythrocytes were convex and bell shaped (Figure 4C). When male erythrocyte SOD was incubated with progesterone (1–3 nM), the enzyme activity was increased by up to 6-fold. However, this increase disappeared when the enzyme was incubated with progesterone at concentrations above 3 nM, and CuZnSOD activity returned to the control value when incubated with 10 nM progesterone. Progesterone (10 nM) also increased the activity of CuZnSOD in the PRP; however, we did not find any decrease in enzyme stimulation, even following treatment with 10^4 nM progesterone (Figure 4D). The EC_{50} values for progesterone-mediated stimulation of the enzyme were similar for both male and female erythrocytes (0.9 and 1.0 nM, respectively). However, the enzyme from male PRP had a significantly lower EC_{50} for progesterone stimulation compared to the female PRP (3.7 and 41.7 nM, respectively) (Figures 3 and 4).

We observed only minor differences between the stimulatory effects of natural hormones on SOD activity from male and female donors. For this reason and because the hormone replacement therapy with synthetic compounds is used only in women, the following assays were conducted only in the samples from female donors and in the purified CuZnSOD.

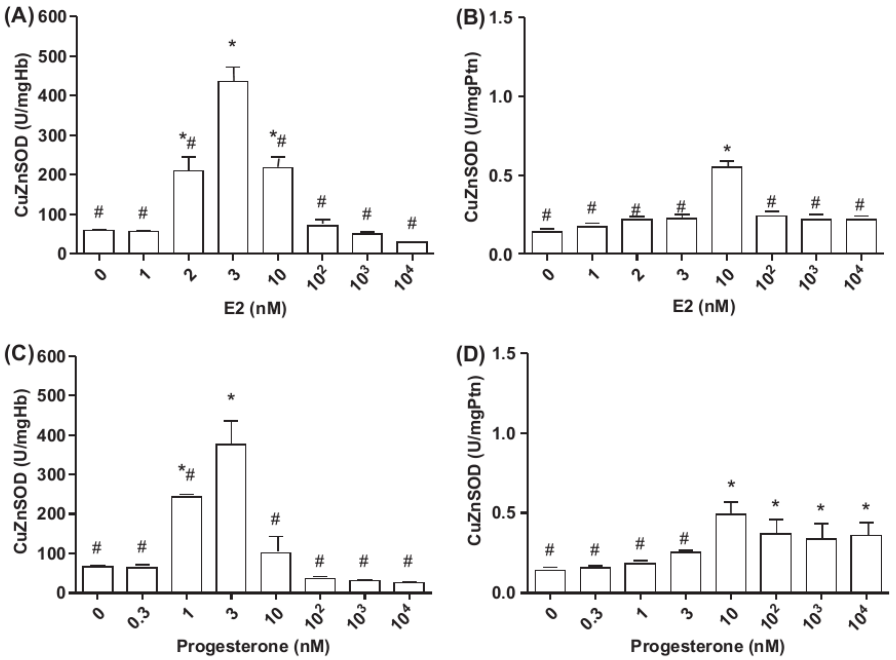

Figure 4. Steroid hormones exhibited a bell-shaped dose-dependent effect on CuZnSOD activity in samples from male donors. 17β-Estradiol (E2) effect on erythrocytes (EC_{50} for stimulation = 2.2 nM; confidence interval, 2.0–2.3 nM) (A) and on platelet-rich plasma samples (EC_{50} for stimulation = 4.0 nM; confidence interval, 3.0–5.4 nM) (B). Progesterone effect on erythrocytes (EC_{50} for stimulation = 0.9 nM; confidence interval, 0.4–1.9 nM) (C) and on platelet-rich plasma samples (EC_{50} for stimulation = 3.7 nM; confidence interval, 2.4–5.5 nM) (D). Enzyme samples were pre-incubated for 24 hours with vehicle (control) or increasing concentrations of hormones. The results are mean ± SEM (n = 3). *Different from control ($p < 0.05$). #Different from the hormone concentration that yielded the highest enzyme activity.

Effect of the synthetic steroid hormones, β-estradiol 3-benzoate and medroxyprogesterone 17-acetate, on SOD activity

Because synthetic steroid hormones are especially important for women receiving HRT, we evaluated the effect of β-estradiol 3-benzoate (E2Benz) and medroxyprogesterone 17-acetate (MPA) on enzymes obtained from female donors and purified enzyme. Figures 5 and 6 show the concentration-response curves for the effects of E2Benz and MPA on the activity of CuZnSOD from female erythrocytes and the purified enzyme. Similar to the natural steroids, the concentration-response curves for these synthetic derivatives were convex and bell shaped in both the non-purified and purified CuZnSOD from human erythrocytes.

The activity of the non-purified erythrocyte CuZn-SOD from female donors was increased by up to 7-fold

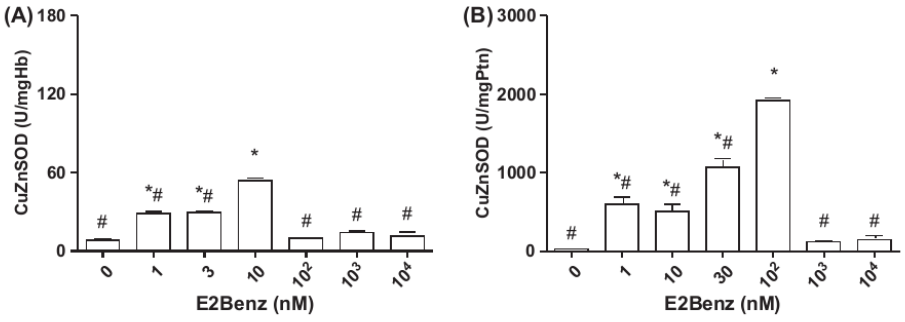

Figure 5. β-Estradiol 3-benzoate (E2Benz), a synthetic estradiol derivative, exhibited a bell-shaped dose-dependent effect on CuZnSOD activity in female erythrocytes (EC_{50} for stimulation = 1.9 nM; confidence interval, 1.2–3.1 nM) (A) and in the purified enzyme that was obtained from human erythrocytes (EC_{50} for stimulation = 16.4 nM; confidence interval, 7.6–35.5 nM) (B). Enzyme samples were pre-incubated for 24 hours with vehicle (control) or increasing concentrations of E2Benz. The results are mean ± SEM (n = 3). *Different from control ($p < 0.05$). #Different from the hormone concentration that yielded the highest enzyme activity.

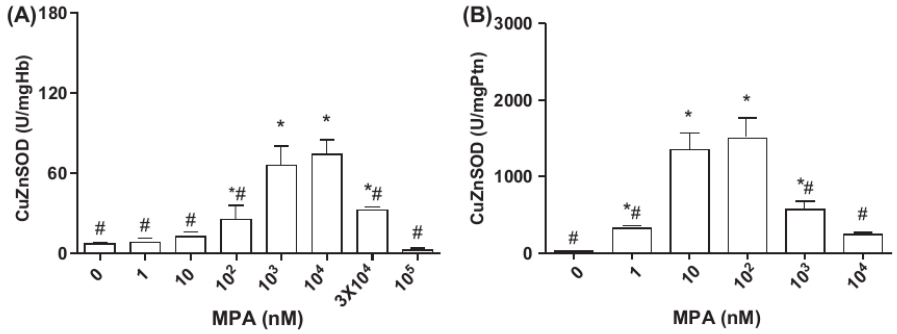

Figure 6. Medroxyprogesterone 17-acetate (MPA), a synthetic progesterone derivative, exhibited a bell-shaped dose-dependent effect on CuZnSOD activity in female erythrocyte (EC_{50} for stimulation = 230.8 nM; confidence interval, 167.0–319.0 nM) (A) and in the purified enzyme that was obtained from human erythrocytes (EC_{50} for stimulation = 2.4 nM; confidence interval, 2.2–2.6 nM) (B). Enzyme samples were pre-incubated for 24 hours with vehicle (control) or increasing concentrations of MPA. The results are mean ± SEM (n = 3). *Different from control ($p < 0.05$). #Different from the hormone concentration that yielded the highest enzyme activity.

when incubated with E2Benz (1–10 nM), whereas the activity of the purified enzyme was increased by up to 64-fold (1–10^2 nM of E2Benz) (Figure 5). However, this increase disappeared when the E2Benz concentration was increased; the non-purified enzyme returned to the control value when incubated with 10^2 nM E2Benz, and the purified enzyme returned to the control value when incubated with 10^3 nM E2Benz (Figure 5). The EC_{50} value for E2Benz stimulation was lower in the non-purified erythrocyte samples (1.9 nM) compared to purified CuZnSOD (16.4 nM) (Figure 5). E2Benz had no effect on the activity of CuZnSODs or MnSOD in the female PRP (data not shown).

The non-purified erythrocyte CuZnSOD activity from female donors was increased by up to 10-fold in the presence of MPA (10^2–10^4 nM), whereas the purified enzyme activity was increased by up to 50-fold (1–10^2 nM of MPA) (Figure 6). However, this increase disappeared when the MPA concentration was increased; the non-purified enzyme returned to the control value when incubated with 10^5 nM MPA and the purified enzyme returned to the control value when incubated with 10^4 nM MPA (Figure 6). The EC_{50} value for MPA stimulation was higher in the non-purified erythrocyte samples (230.8 nM) compared to purified CuZnSOD (2.4 nM) (Figure 6). MPA had no effect on the activity of CuZnSODs and MnSOD in the female PRP (data not shown).

Effect of combined steroid hormones on SOD activity

Because HRT usually involves a combination of steroidal hormones (an estrogen plus a progestin compound), we also assessed the effect of combined steroid hormones on the SOD activity. In these experiments, we used enzyme taken from female donors and purified enzyme.

As observed for E2, the concentration-response curves for the effect of the combination of E2 plus MPA on CuZnSOD activity were convex and bell shaped in all of the enzyme preparations evaluated (Figure 7). The activity of non-purified erythrocyte CuZnSOD from female donors was increased by up to 4-fold at 1 nM E2 plus MPA (0.5 nM for each hormone), whereas the purified enzyme activity increased by up to 82-fold in response to 1 nM E2 plus MPA. Thus, the combination of E2 plus MPA reduced the hormone concentration required to stimulate CuZnSOD from human erythrocytes. In response to incubation with the combined hormones, maximal stimulation occurred at concentrations far below the EC_{50} values for the individual hormones to stimulate the non-purified (1.9 nM for E2 and 230.8 nM for MPA) and purified enzyme (2.2 nM for E2 and 2.4 nM for MPA). These results indicate that E2 and MPA have a synergistic, bell-shaped effect on the activity of both the non-purified and purified CuZnSOD from human erythrocytes.

The combination of E2 plus MPA increased the activity of CuZnSODs in the female PRP by up to 6-fold (10 nM E2 plus MPA) (Figure 7B). The stimulatory potency observed for the combined hormones was similar to that observed for the stimulation using E2 alone (EC_{50} = 6.2 nM). Thus, considering that MPA alone had no effect on CuZnSOD from the PRP, these results indicate that there is no synergistic effect of these hormones on this enzyme preparation. The combination of E2 plus MPA had no effect on the activity of MnSOD in the female PRP (data not shown).

As observed for the isolated hormones, the combination of E2Benz plus progesterone had no effect on the activity of MnSOD from the female PRP (data not shown), but it displayed a convex, bell-shaped effect on the activity of CuZnSOD from erythrocytes (Figure 8). The activity of non-purified erythrocyte CuZnSOD from female donors increased by up to 8-fold in response to 1 nM E2Benz plus progesterone (0.5 nM for each hormone), whereas purified CuZnSOD activity increased by up to 86-fold (at 0.3 nM E2Benz plus progesterone). Thus, the combination of E2Benz plus progesterone reduced the hormone concentration required to stimulate CuZnSOD from human erythrocytes. In response to incubation with the combined hormones, maximal stimulation occurred at concentrations far below the EC_{50} values required for the

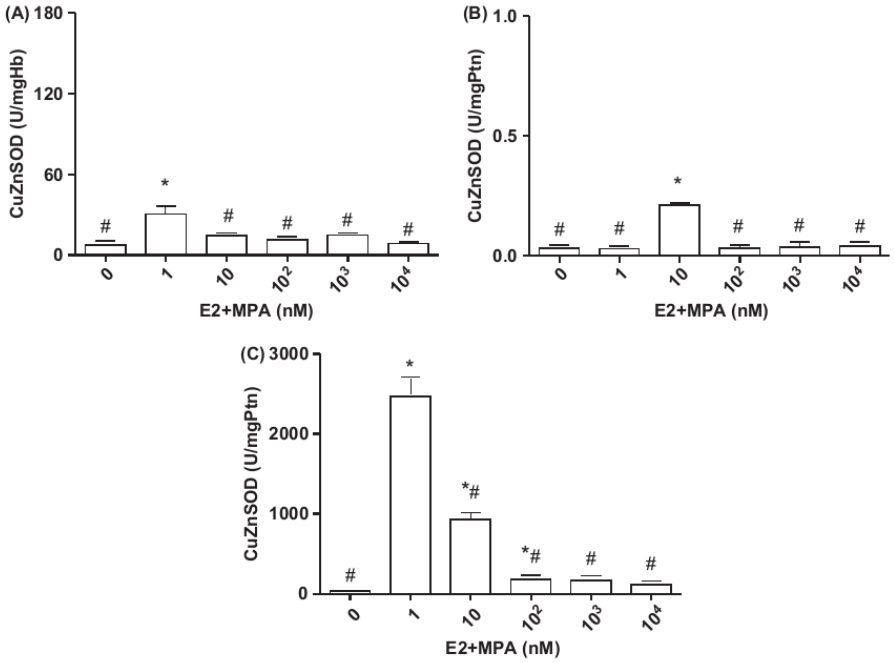

Figure 7. The combination of steroid hormones 17β-estradiol (E2) plus medroxyprogesterone 17-acetate (MPA) exhibited a bell-shaped dose-dependent effect on CuZnSOD activity in female erythrocytes (A), female platelet-rich plasma samples (B) and in the purified enzyme that was obtained from human erythrocytes (C). Enzyme samples were pre-incubated for 24 hours with vehicle (control) or increasing concentrations of hormones. The hormones were combined at equimolar concentrations and the values shown in the figure are the final concentration, i.e. the sum of the individual concentrations of each hormone. The results are mean ± SEM (n = 3). *Different from control ($p < 0.05$). #Different from the hormone concentration that yielded the highest enzyme activity.

individual hormones to stimulate the non-purified (1.9 nM for E2Benz and 1.0 nM for progesterone) and purified enzyme (16.4 nM for E2Benz and 5.0 nM for progesterone). These results indicate that E2Benz and progesterone have a synergistic, bell-shaped effect on both non-purified and purified CuZnSOD from human erythrocytes.

Under physiological conditions, both E2 and progesterone are found in the bloodstream; thus, we investigated the combination of these natural steroid hormones on the activity of purified CuZnSOD obtained from erythrocytes (Figure 9). The combination of E2 plus progesterone increased the activity of purified CuZnSOD. However, as was observed for progesterone alone, enzyme activity decreased at progesterone concentrations above 10^2 nM but it was still significantly higher than the control value even at a concentration of 10^4 nM. Purified CuZnSOD

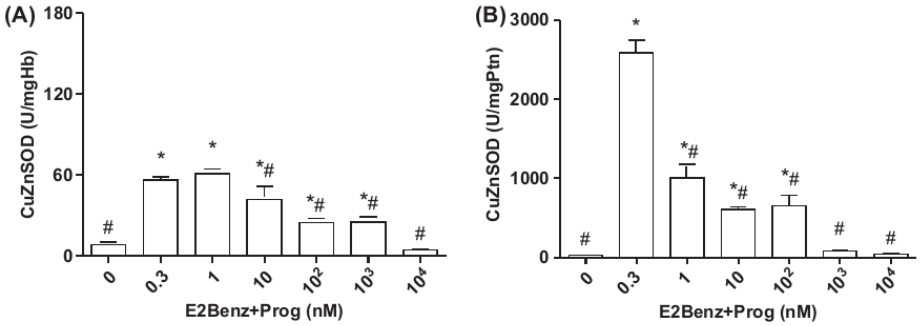

Figure 8. The combination of steroid hormones β-estradiol 3-benzoate (E2Benz) plus progesterone exhibited a bell-shaped dose-dependent effect on CuZnSOD activity in female erythrocytes (A) and in the purified enzyme that was obtained from human erythrocytes (B). Enzyme samples were pre-incubated for 24 hours with vehicle (control) or increasing concentrations of hormones. The hormones were combined at equimolar concentrations, and the values shown in the figure are the final concentration, i.e., the sum of the individual concentrations of each hormone. The results are mean ± SEM (n = 3). *Different from control ($p < 0.05$). #Different from the hormone concentration that yielded the highest enzyme activity.

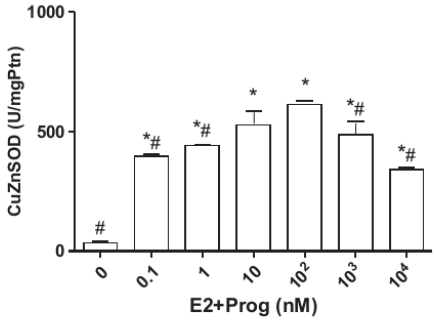

Figure 9. The combination of steroid hormones 17β-estradiol (E2) plus progesterone exhibited a dose-dependent effect on purified enzyme that was obtained from human erythrocytes. Enzyme samples were pre-incubated for 24 hours with vehicle (control) or increasing concentrations of hormones. The hormones were combined at equimolar concentrations, and the values shown in the figure are the final concentration, i.e., the sum of the individual concentrations of each hormone. The results are mean± SEM (n = 2). *Different from control ($p < 0.05$). #Different from the hormone concentration that yielded the highest enzyme activity.

activity was increased by up to 17-fold (10^2 nM E2 plus progesterone). The combination of E2 plus progesterone reduced the hormone concentration required to stimulate purified CuZnSOD from human erythrocytes. The EC_{50} for the stimulation of the purified enzyme by the combined hormones was 0.13 nM (i.e., the sum of the individual concentrations of each hormone; confidence interval, 0.04–0.39), whereas the EC_{50} values for the isolated hormones were 2.2 nM and 5.0 nM for E2 and progesterone, respectively. These results indicate that E2 and progesterone have a synergistic, bell-shaped effect on purified CuZnSOD from human erythrocytes.

Preliminary study on the molecular interaction between CuZnSOD and 17β-estradiol and progesterone

To investigate the molecular mechanism that is responsible for the modulation of CuZnSOD activity by steroid hormones, we evaluated the electrophoretic mobility and absorbance spectra of CuZnSOD incubated with E2 and progesterone.

Electrophoresis can be used to identify molecular changes in protein structure, which are indicated by band shifts in the electrophoretic profile of the protein. Here, we used denaturing SDS-PAGE, which induces a loss of native protein conformation by disrupting weak interactions, such as hydrogen bonding, salt bridges and Van de Waals forces. This method allows for the identification of covalent modifications that cause dramatic changes in the molecular weight of a protein. The electrophoretic mobility of purified CuZnSOD incubated with estrogen (3 and 18 nM) or progesterone (10 and 60 nM) was assessed using western blotting (Figure 10). The area represented in the figure corresponds to a16 KDa band observed in the electroblotted gels. We observed no difference in the mobility of the enzyme in the presence or absence of either hormone, regardless of the concentration (e.g., lanes 1 and 3 vs. lanes 2 and 4, Figrue 10A and B). The CuZnSOD bands in both the hormone and vehicle-treated groups did not appear as one homogenous band, which was expected according to the technical specifications from the datasheet provided by the antibody manufacturer. For this reason, we also ran a mixture (1:1) of the samples from lane 1 (CuZnSOD plus hormone at the lowest concentration) and lane 2 (CuZnSOD plus the respective vehicle) in lane 5 to detect any band shift among the samples in the same lane. An additional mixture of samples from lanes 3 and 4 is also depicted in lane 6. We did not observe any changes in electrophoretic mobility in any group, indicating that the hormones did not cause structural modifications to CuZnSOD that were capable of changing its electrophoretic mobility.

The absorption spectrum of purified CuZnSOD increased significantly in the presence of 3 nM E2 (Figure 11A) or 10 nM progesterone (Figure 11B), especially in the 230–290 nm range compared to the spectrum of SOD alone. Simultaneous incubation of SOD with E2 plus progesterone (final concentration 0.1 nM) caused a greater change in the absorption spectrum of CuZnSOD compared to the spectra changes observed when the enzyme was incubated with each hormone separately (Figure 11C). The steroid hormones alone had almost no absorbance in this wavelength range at the low concentrations used (0.1–10 nM; Figure 11A–C). However, at 10^3 nM both E2 and progesterone had a remarkably high absorbance in the 230–250 nm range. When the purified enzyme was incubated with 10^3 nM E2 or progesterone, there was a significant increase in absorbance in the 230–240 nm range compared to the absorbance that would be expected from the sum of the individual absorbances of these molecules (Figure 11D–E). This behavior is similar to that observed at low hormone concentrations. In contrast, from 250 nm onward, there was a decrease in the absorbance compared to the value that would be expected from the sum of the individual absorbances of these molecules (Figure 11D–E).

Discussion

We demonstrated for the first time that steroid hormones directly modulate the activity of cytosolic CuZnSOD obtained from human erythrocytes *in vitro*. Both natural and synthetic estrogens and progestins displayed a bell-shaped effect on the activity of enzyme taken from females; low hormone concentrations caused a dose-dependent increase in enzyme activity that disappeared at higher hormone concentrations. In addition, the combination of two natural hormones (E2 plus progesterone) or a synthetic derivative plus a natural hormone (E2 plus MPA and E2Benz plus progesterone) had a synergistic effect on the activity of CuZnSOD from female erythrocytes. Moreover, the effect of natural steroids on the activity of the enzyme from male erythrocytes was virtually equal to that observed for the female enzyme.

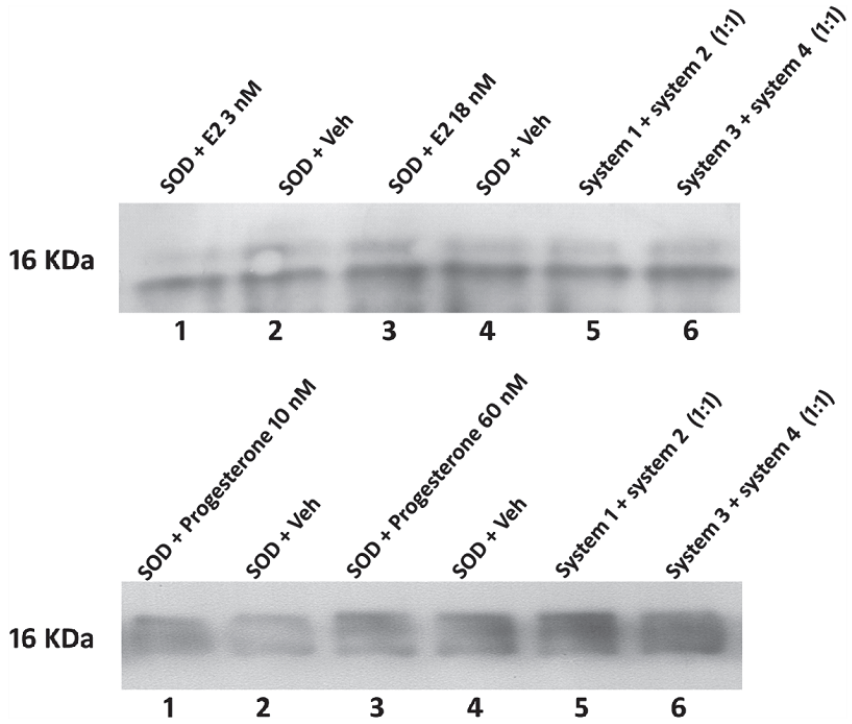

Figure 10. Representative immunoblot of purified CuZnSOD incubated with different concentrations of 17β-estradiol (E_2) and progesterone for 24 h and subjected to SDS–PAGE in 15% polyacrylamide gel. (A) Purified CuZnSOD obtained from human erythrocytes (180 U/mL) was incubated in the presence of 3 nM E_2 (**lane 1**) vs. vehicle (**lane 2**) and 18 nM E_2 (**lane 3**) vs vehicle (**lane 4**). **Lane 5** is a 1:1 mixture of the incubation systems present in lanes 1 and 2, whereas **lane 6** is a 1:1 mixture of the incubation systems presented in lanes 3 and 4. (B) Purified CuZnSOD obtained from human erythrocytes (180 U/mL) was incubated in the presence of 10 nM progesterone (**lane 1**) vs. vehicle (**lane 2**) and 60 nM progesterone (**lane 3**) vs vehicle (**lane 4**). **Lane 5** is a 1:1 mixture of the incubation systems presented in lanes 1 and 2, whereas **lane 6** is a 1:1 mixture of the incubation systems present in lanes 3 and 4. The area represented in the figure corresponds to the 16 KDa band of the electroblotted gels.

Blood levels of E2 in women vary with the ovulatory cycle, with lower levels during the follicular phase (<0.6 nM) and higher levels during the luteal phase and the periovulatory period (0.1–1.5 nM) [14]. Similarly, progesterone levels in women are also lower during the follicular phase (<3.6 nM) and higher during the luteal phase (3–68 nM) [14]. Importantly, an increase in CuZnSOD activity was observed at E2 and progesterone concentrations within the physiological range for premenopausal women. However, the E2 levels found in men (approximately 0.1 nM) [15] are lower than those required for SOD stimulation. Although male progesterone levels (<3 nM) [16] are within the range required for CuZnSOD stimulation, its contribution to the modulation of enzyme activity is uncertain because male progesterone levels do not undergo gross fluctuation in physiological conditions. These findings suggest that steroid hormones may be physiological modulators of CuZnSOD activity in human erythrocytes, especially in women.

Previous data from *in vivo* studies support the potential modulation of SOD activity by steroid hormones. For example, we found lower blood SOD activity in postmenopausal women compared to premenopausal women and higher blood SOD activity was observed in postmenopausal women receiving HRT compared to those not receiving HRT [7]. In addition, *in vivo* studies suggest that steroid hormones regulate CuZnSOD activity from other tissues [8,9,17,18]. However, previous evidence indicates that the steroidal regulation of CuZnSOD activity might be attributed to the modulation of enzyme expression [9]. This hypothesis was further supported by cell culture assays [9], which also demonstrated steroidal modulation of the expression of MnSOD and extracellular CuZnSOD [5,10]. However, in the present study the effect of steroids on SOD activity cannot be related to changes in enzyme expression because the purified CuZnSOD showed a similar behavior to the non-purified enzyme. In addition, when erythrocytes emerge from the bone marrow, they lose their nucleus, ribosomes and mitochondria, and therefore, are incapable of protein synthesis [19]. Moreover, the stimulatory effect of E2 occurred even with a very short pre-incubation period (1 min). Previous studies on the modulation of SOD activity by steroid hormones did not investigate a direct effect of these hormones on enzyme activity. Thus, we cannot rule out the possibility that the modulation of cytosolic CuZnSOD by steroid hormones

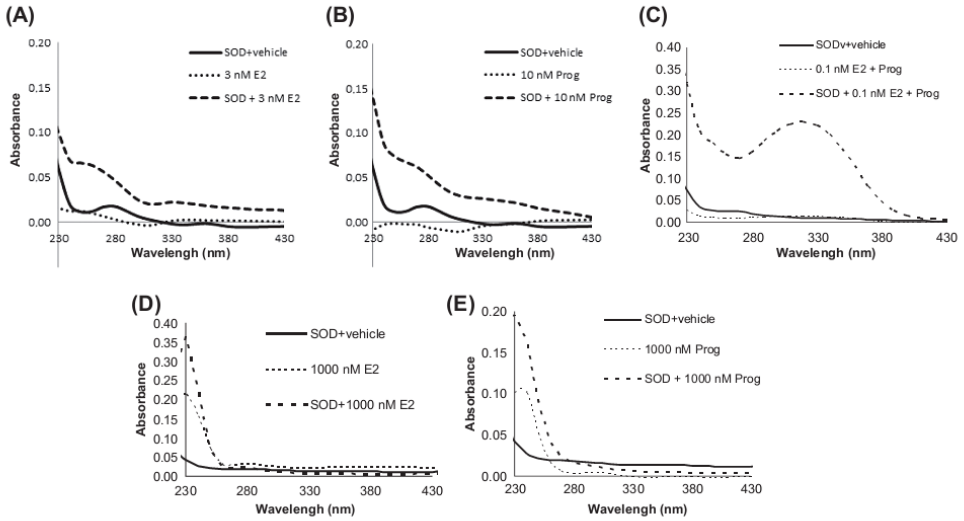

Figure 11. Changes in the absorption spectra of purified CuZnSOD obtained from human erythrocytes in the presence of natural steroids. The absorption spectra of CuZnSOD (SOD) were evaluated in the presence of vehicle, 3 nM 17β-estradiol (E2) (A), 10 nM progesterone (Prog) (B), an equimolar combination of E2 plus Prog, at final concentration of 0.1 nM (C), 10^3 nM E2 (D) or 10^3 nM Prog (E). Enzyme samples were pre-incubated for 24 hours with hormones.

in vivo results from both changes in enzyme expression and a non-genomic direct modulation of enzyme activity. Moreover, we propose that the non-genomic direct modulation of enzyme activity by steroids is particularly important for erythrocytes because they do not synthesize proteins. Erythrocytes have a life span of approximately 120 days. Therefore, only direct modulation of enzyme activity would be able to cause rapid changes in erythrocyte CuZnSOD activity.

A recent study revealed that the mitochondrial intermembrane space CuZnSOD (IMS SOD) from rat liver is activated by E2, and this activation is dependent on mitochondrial cytochrome P450 [20]. The authors provided evidence that substrates of mitochondrial P450, including E2, are effective sources of superoxide anions (via P450), which could mediate the activation of IMS SOD through oxidation of critical thiol groups on the enzyme [20]. However, this P450-dependent mechanism could not be responsible for the enzyme stimulation demonstrated in this study because stimulation was observed with the purified enzyme.

E2 contains a phenol ring that can scavenge hydroxyl radicals [21]. It also decreases the production of reactive oxygen species *in vitro* [22]. However, this effect was only observed at high, non-physiological hormone concentrations [22]. Thus, this antioxidant effect could not be involved in SOD modulation because we observed modulation at physiological E2 concentrations. Furthermore, the steroid effect was not due to an artifact in our enzyme assay conditions because the steroid hormones did not act as inhibitors of xanthine oxidase or scavengers of superoxide anion; therefore, they did not alter the superoxide fluxes in our assay conditions. In addition, steroid hormones also did not act as formazan oxidants *per se* in our assay conditions. Thus, the observed effects could be attributed to specific, non-genomic, modulation of enzyme activity by steroids. Huang et al. [23] demonstrated *in vitro* that 2-methoxyestradiol, an E2 derivative that cannot bind to the estrogen receptor, binds to and inhibits the activity of CuZn and MnSOD at high concentrations (IC_{50} ~20 μM). Although the steroid hormones evaluated in the present study did not inhibit SOD activity compared to the control, we propose that their binding to CuZnSOD could be involved in the specific modulation of enzyme activity.

To provide preliminary data on the molecular interaction between CuZnSOD and steroid hormones we evaluated the electrophoretic migration and the absorption spectra of enzyme incubated with E2 and progesterone. The eletrophoretic mobility of proteins is shifted only after dramatic changes in their molecular weight. Our results indicate that E2 and progesterone did not induce such changes in an *in vitro* system containing these hormones and the purified protein. For example, these results exclude the possibility of protein dimerization. However, the molecular mass and size of both E2 and progesterone are very unlikely to induce a significant shift in CuZnSOD electrophoretic mobility even if the formation of a covalent complex between the protein and one or a few molecules of the hormone occurred during incubation. These findings suggest that the regulation of CuZnSOD by hormones is related to subtle changes in protein conformation due to either covalent binding or weak molecular interactions. Supporting this proposal, the absorption spectra of purified CuZnSOD were increased in the presence of E2 and progesterone compared to the enzyme alone, indicating that they do interact. Interestingly, only low hormone concentrations

increased enzyme absorption in the 250–300 nm range, whereas this effect disappeared in the presence of high hormone concentrations. This behavior is compatible with the bell-shaped effect of hormones on enzyme activity. In contrast, the combination of E2 and progesterone, which had a synergic effect on enzyme activity, caused a greater change in the enzyme absorbance compared to the effect of each hormone separately.

The absorbance spectrum of human CuZnSOD changes in the region between 250 and 300 nm, when the number and type of essential metals in the enzyme are changed [24]. This change was ascribed to the dominance of phenylalanine (Phe) (250–265 nm) and tryptophan (Trp) absorption (275–290 nm). Human CuZnSOD contains four Phe residues and one Trp residue, which are thought to form a ring surrounding the active site metals [24]. Trp UV absorbance is an indicator of solvent penetration in the enzyme structure and the movement of a Cu-binding histidine (His) residue (46 or 120) [24]. Phe absorbance is sensitive to changes in the active site of the enzyme, and it was shown to increase when Cu is added to the apoenzyme [24]. Thus, our spectra data suggest that Trp and Phe residues could be involved in the non-covalent binding of the steroid hormones to the enzyme. In fact, a hydrogen bridge between an E2 3-OH group and a His or Trp residue is critical for the binding of E2 to various proteins, including nuclear estrogen receptors [25] and protein disulfide isomerase [26]. Hormone binding to these proteins is very fast, which is consistent with the pre-incubation time required for the stimulation of SOD by steroid hormones (less than 1 min). Nevertheless, the changes in the spectra of SOD could also result from a change in the conformation of Phe and Trp residues in the protein structure due to the binding of steroids to other amino acid residues. Thus, the specific domains of CuZnSOD involved in the interaction with steroids and the conformational changes induced by hormone binding must be further explored in detailed structural studies.

As observed for the natural steroids, synthetic steroid hormones also modulated the activity of CuZnSOD in erythrocytes. E2Benz and MPA are constituents of contraceptive pills commonly used by premenopausal women, whereas post-menopausal women use these compounds for HRT [27]. Because both contraceptive pills and HRT usually contain a combination of an estrogen plus a progestin, it is noteworthy that these hormones had a synergistic effect on the modulation of erythrocyte CuZnSOD activity.

Unlike the activity of erythrocyte CuZnSOD, steroid hormones did not directly modulate the activity of MnSOD (from human platelets found in PRP). Mature, wild-type, human MnSOD is a mixed α-helix and β-sheet structure composed of four identical subunits, each containing one atom of manganese. In comparison, CuZnSOD is composed of two identical subunits, each of which binds one Cu and one Zn ion and displays the β-barrel fold [2]. The structural differences between these SOD isoforms may be responsible for their different susceptibility to steroidal modulation.

In addition to MnSOD, PRP also contains cytosolic and extracellular CuZnSOD isoforms. In fact, extracellular SOD accounts for most of the SOD activity in plasma [28]. Extracellular SOD is a secretory, tetrameric, glycoprotein that is found in interstitial spaces and extracellular fluids. Its amino acid sequence is homologous to cytosolic CuZnSOD, apart from the N- and C-terminal extensions. CuZnSOD activity in the PRP of female donors was modulated by E2 and progesterone but not by E2Benz and MPA, which suggests that extracellular SOD has different sensitivity to steroid modulation compared to cytosolic CuZnSOD. The CuZnSOD activity in the PRP of male donors was also modulated by E2 and progesterone, but it was more sensitive to progesterone stimulation than the enzyme from female donors. This difference may be related to the background physiological levels of progesterone found in female samples, which were slightly higher than those found in male samples, and may have masked the hormone effect at the lowest concentrations.

Conclusion

In this present study, we demonstrated for the first time that natural and synthetic steroid hormones have a non-genomic direct bell-shaped effect on the activity of CuZnSOD from human erythrocytes, which is independent of changes in enzyme expression. We propose that this effect may account for the physiological regulation of enzyme activity during conditions where steroid hormones undergo variations, including the ovulatory cycle, menopause, and HRT. In addition, these results highlight the importance of monitoring serum hormone levels during HRT because of its relationship to SOD activity.

Declaration of interest

The authors report no declarations of interest. The authors alone are responsible for the content and writing of the paper.

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References

[1] McCord JM, Fridovich I. Superoxide dismutase: an enzymic function for erythrocuprein (hemocuprein). J Biol Chem 1969;244:6049–6055.
[2] Perry JJP, Shin DS, Getzoff ED, Tainer JA. The structural biochemistry of the superoxide dismutases. Biochim Biophys Acta 2010;1804:245–262.
[3] Halliwell B, Gutteridge JMC. The antioxidants of human extracellular fluids. Arch Biochem Biophys 1990;280:1–8.
[4] Hulley S, Grady D. Postmenopausal hormone treatment. JAMA 2009;301:2493–2495.

[5] Strehlow K, Rotter S, Wassmann S, Adam O, Grohé C, Laufs K, et al. Modulation of antioxidant enzyme expression and function by estrogen. Circul Res 2003;93:170–177.
[6] Feng Z, Zhang J. Long-term melatonin or 17h-estradiol supplementation alleviates oxidative stress in ovariectomized adult rats. Free Radic Biol Med 2005;39:195–204.
[7] Unfer TC, Conterato GMM, da Silva JCN, Duarte MMMF, Emanuelli T. Influence of hormone replacement therapy on blood antioxidant enzymes in menopausal women. Clin Chim Acta 2006;369:73–77.
[8] Moorthy K, Sharma D, Basir SF, Baquer NZ. Administration of estradiol and progesterone modulate the activities of antioxidant enzyme and aminotransferases in naturally menopausal rats. Exp Gerontol 2005;40:295–302.
[9] Sugino N, Karube-Harada A, Kashida S, Takiguchi S, Kato H. Differential regulation of copper-zinc superoxide dismutase and manganese superoxide dismutase by progesterone withdrawal in human endometrial stromal cells. Mol Human Reprod 2002;8:68–74.
[10] Wassmann K, Wassmann S, Nickenig G. Progesterone antagonizes the Vasoprotective effect of estrogen on antioxidant enzyme expression and function. Circul Res 2005;97: 1046–1054.
[11] Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the folin phenol reagent. J Biol Chem 1951;193:265–275.
[12] Spitz DR, Oberley LW. An assay for superoxide dismutase activity in mammalian tissue homogenates. Anal Biochem 1989;179:8–18.
[13] Cos P, Ying L, Calomme M, Hu JP, Cimanga K, Poel BV, et al. Structure-activity relationship and classification of flavonoids as inhibitorsof xanthine oxidase and superoxide scavengers. J Nat Prod 1998;61:71–76.
[14] Vankrieken L, Van der Horst FAL, Castracane VD. An international multicenter reference range study of normal menstrual cycles using IMMULITE® reproductive hormone assays. Clin Chem 1999;45:A92.
[15] Tchernof A, Labrie F, Langer AB, Prud'homme D, Bouchar C, Tremblay A, et al. Relationships between endogenous steroid hormone, sex hormone-binding globulin and lipoprotein levels in men: contribution of visceral obesity, insulin levels and other metabolic variables. Atherosclerosis 1997;133:235–244.
[16] Evans SM, Foltin RW. Exogenous Progesterone Attenuates the Subjective Effects of Smoked Cocaine in Women, but not in Men. Neuropsychopharmacology 2006;31:659–674.
[17] Kumar P, Kale RK, Baquer NZ. Estradiol enhances membrane linked ATPases, antioxidant enzymes and membrane fluidity and reduces lipid peroxidation and lipofuscin concentration in aged rat liver. J Aging Res 2011;2011:article ID 580245, 8 p.
[18] Kumar P, Kale RK, McLean P, Baquer NZ. Protective and anti-aging effects of 17β estradiol on altered age related neuronal parameters in female rat brain. Neurosci Lett 2011; 502:56–60.
[19] Nikolić-Kokić A, Blagojević D, Spasić MB. Complexity of free radical metabolism in human erythrocytes. J Med Biochem 2010;29:189–195.
[20] Iñarrea P, Casanova A, Alava MA, Iturralde M, Cadenas E. Melatonin and steroid hormones activate intermembrane Cu,Zn-superoxide dismutase by means of mitochondrial cytochrome P450. Free Radic Biol Med 2011;150:1575–1581.
[21] Kumar S, Lata K, Mukhopadhyay S, Mukherjee TK. Role of estrogen receptors in pro-oxidative and anti-oxidative actions of estrogens: a perspective. Biochim Biophys Acta 2010; 1800:1127–1135.
[22] Thibodeau PA, Kachadourian R, Lemay R, Bisson M, Day BJ, Paquette B. In vitro pro- and antioxidant properties of estrogens. J Ster Biochem Mol Biol 2002;81:227–236.
[23] Huang P, Feng L, Oldham EA, Keating MJ, Plunkett W. Superoxide dismutase as a target for the selective killing of cancer cells. Nature 2000;407:390–395.
[24] Mailer K, Addetia R, Livesey DL. UV spectrocopic studies of human erythrocyte superoxide dismustase. J Inorg Biochem 1989;37:151–161.
[25] Weatherman RV, Fletterick RJ, Scanlan TS. Nuclear-receptor ligands and ligand-binding domains. Annu Rev Biochem 1999;68:559–581.
[26] Fu XM, Wang P, Ting Zhu B. Characterization of the estradiol-binding site structure of human protein disulfide isomerase (PDI). PLoS ONE 2011;6:e27185.
[27] Sitruk-Ware R. Pharmacological profile of progestins. Maturitas 2004;47:277–283.
[28] Adachi T, Wang XL. Association of extracellular-superoxide dismutase phenotype with the endothelial constitutive nitric oxide synthase polymorphism. FEBS Lett 1998;433: 166–168.

4 MANUSCRITO

Estrogen plus progestin hormone therapy is associated with increased blood superoxide dismutase activity and plasma total antioxidant capacity in postmenopausal women

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Estrogen plus progestin hormone therapy is associated with increased blood superoxide dismutase activity and plasma total antioxidant capacity in postmenopausal women

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Abstract: This cross-sectional study was aimed to evaluate the behavior of blood antioxidant enzymes (superoxide dismutase-SOD, catalase and glutathione peroxidase), plasma total antioxidant capacity and oxidative damage (lipid oxidation and protein carbonyl levels) and their relationship with the serum levels of steroid hormones in postmenopausal women with estrogen alone or estrogen plus progestin therapy. Blood was collected from premenopausal women (n=24), postmenopausal women without hormone therapy (n=31), postmenopausal women with estrogen therapy (n=12) and estrogen plus progestin therapy (n=16). The CuZn and MnSOD activities and the plasma total antioxidant power were significantly higher in the postmenopausal women under estrogen plus progestin therapy than in the postmenopausal women without hormone therapy. The estrogen therapy increased only CuZnSOD activity compared to the postmenopausal women without hormone therapy. However, no differences were observed in the levels of lipid or protein oxidation or in the non-enzymatic plasma antioxidants (uric acid and albumin) among the groups. The duration of hormone therapy and serum estrogen levels were positively correlated to the blood CuZnSOD activity and to plasma total antioxidant power, whereas the serum progesterone levels were positively correlated to the CuZnSOD activity and negatively correlated to carbonyl groups. Interestingly, the total antioxidant power of plasma was positively correlated to the CuZnSOD and glutathione peroxidase activities. We conclude that hormone therapy with estrogens plus progestins increases blood MnSOD and CuZnSOD activity in postmenopausal women, leading to an increased plasma total antioxidant capacity. This finding may be relevant to the prevention of oxidative stress-related disorders in postmenopausal women.

Keywords: Hormone therapy, estrogen, progestin, menopause, superoxide dismutase, total antioxidant capacity.

Introduction

Menopause is associated with a decrease in circulating estrogen and progesterone levels, and increase in follicle stimulating hormone levels [1]. This natural loss of estrogen increases the risk of cardiovascular diseases, osteoporosis, depression, and neurodegenerative diseases [2-4]. Interestingly, menopause was also found to be a risk factor for oxidative stress [5-7] and antioxidants were suggested to have a beneficial role against menopause symptoms and its associated disorders [8].

Hormone replacement therapy is a complex and controversial intervention that is used to attenuate the menopause-associated disorders [3,9-11]. It is prescribed either as the replacement of estrogen alone or the combination of estrogen with progestins [3,9,12]. Some studies suggested that hormone therapy protects postmenopausal women against oxidative stress [13-19].

Besides acting as a sexual hormone, estradiol contains a phenol group that was effective to remove $OH^{\bullet}$ *in vitro* [20,21] and it has been shown that estrogen reduces oxidative stress *in vitro* [22-26] and *in vivo*[14,16,27]. Progestins alone or combined with estrogen decreased the level of peroxide in platelet membranes [28]. However, progestins in association to the estrogen therapy did not amplify the antioxidant effect the latter one in postmenopausal women [14,16]. Thus, the role of progestins in the antioxidant effects of hormone replacement therapy remains controversial.

There is some evidence that the expression and function the antioxidant enzymes superoxide dismutases (SODs) can be regulated by estrogen and progesterone [17,29-31]. Most of these studies were performed on cell cultures or *in vivo* and focused on changes in enzyme expression. The expression and activities of the different SODs isoforms (CuZn and MnSOD) seem to be differentially affected by estrogens and/or progestins, depending on the tissue evaluated [29,32,33]. Moreover, we recently demonstrated that *in vitro* estrogens and

progestins have a non-genomic direct and synergic stimulatory effect on human erythrocyte CuZnSOD activity [31].

We previously demonstrated that hormone replacement therapy prevents the decrease on blood total SOD activity that occurs in postmenopausal women [17]. However, it is not known which SOD isoforms are affected and whether this effect was due to estrogens or progestins because in that study the reduced number of subjects did not allow us to divide them into groups with different types of hormone therapy. Thus, previous investigations on the effect of hormone replacement therapy on human SOD activity did not evaluate the behavior of different enzyme isoforms found in blood (CuZn or Mn SOD) nor did they evaluate whether the presence of progestins contributes to the effect of the hormone replacement therapy [14,16,17] .

The present study reported the behavior of blood antioxidant enzymes, plasma total antioxidant capacity and markers of oxidative damage (lipid oxidation and protein carbonyl levels) in postmenopausal women with estrogen alone or estrogen plus progestin therapy. In addition, we also evaluated the relationship between the oxidative status and the serum levels of steroid hormone.

Material and methods

Human subjects. The research protocol was approved by the local ethics committee of Federal University of Santa Maria (Brazil) (CAAE n° 0120.0.243.000-06) and all the women gave their informed consent prior to the inclusion in the study. Subjects were recruited among women patients of Rheumatology Ambulatory from Santa Maria University Hospital and from private clinics. Subjects were given a short questionnaire to obtain information about race, age, menopause state, smoking, alcohol consumption, physical exercise, hormonal therapy, and diagnosed diseases. Women with case history of alcoholism, smoking, diabetes,

renal, hepatic or thyroid chronic disease, as well as those using anti-inflammatory drugs and vitamins were excluded from the study. Subjects were also questioned about the frequency and amount of intake of antioxidant-rich foodstuffs, like fruits and vegetables. Blood systolic and diastolic pressure, weight and Eighty-three women were included in this case-control study and divided into 4 groups: premenopausal women (n = 24), postmenopausal women without hormone therapy (n =31), postmenopausal women with estrogen therapy (ET) (n =12) and postmenopausal women with estrogen plus progestin therapy (EPT) (n =16). Premenopausal women were those with regular menses, whereas postmenopausal women were those >12 months of amenorrhea in according with international criteria [34]. No hormone therapy was prescribed only in terms of this work; all the women included in the postmenopausal group with hormonal therapy were taking hormones for at least three months prior to the inclusion in the study and collection of blood samples.

Sample collection. Blood samples were taken from the cubical vein after overnight fasting. Whole EDTA-blood samples were used to determine the hematocrit, the hemoglobin and total blood protein content. An aliquot was centrifuged and the plasma obtained was used to determine the ferric-reducing ability of plasma. A non-heparinized blood sample was centrifuged and serum was immediately used for aspartate and alanine aminotransferases, creatinine, albumin, uric acid, total cholesterol, low-density lipoprotein-cholesterol (LDL), high-density lipoprotein cholesterol (HDL), triglycerides, follicle-stimulating hormone, progesterone, 17β-estradiol, thiobarbituric acid reactive substances and protein carbonyl groups analysis. An aliquot of heparinized whole blood was stored at -20°C for no more than 1 week before analyses of antioxidant enzymes activity. This storage time was settled to allow for similar conditions for samples that were collected at different times.

Biochemical and hematological parameters. Hematocrit and hemoglobin were determined automatically by Sysmex XE 5000 (Symex Corporation). Aspartate and alanine aminotransferases, creatinine, albumin, uric acid, total cholesterol, low-density lipoprotein cholesterol (LDL), high-density lipoprotein cholesterol (HDL), triglycerides and blood total protein were determined by routine kits (Roche Diagnostics, Mannheim, Germany).

Hormone assays. Serum 17β-estradiol, progesterone and follicle-stimulating hormone levels were determined by immunoassay with Immulite® Estradiol, Immulite® 2000 Progesterone and Immulite® 2000 follicle-stimulating hormone kits (Diagnostic Products Corporation, Los Angeles, CA, USA). The limits of quantification for the 17β-estradiol, progesterone and follicle-stimulating hormone assays were 20 pg/mL, 0.2 ng/mL, and 0.10 mIU/mL, respectively.

Activity of blood antioxidant enzymes. The superoxide dismutase (SOD) activity was measured by an indirect assay based on the competitive reaction between SOD and nitroblue tetrazolium chloride (Sigma, St. Louis, MO, USA) [35]. The rate of increase in the absorbance at 560 nm indicates the reduction of nitroblue tetrazolium chloride to blue formazan by superoxide, which are generated by the xanthine/xanthine oxidase system. The enzymatic reaction was initiated by adding xanthine oxidase. MnSOD activity was quantified in the same assay medium but in the presence of 5 mM KCN, which inhibits CuZn-SOD activity. CuZnSOD activity was determined as the difference between total SOD activity and MnSOD activity. One unit of activity was defined as the amount of protein necessary to achieve half-maximal inhibition of the nitroblue tetrazolium chloride reaction, and the activity was expressed as units per milligram of hemoglobin.

The glutathione peroxidase (GPx) activity was determined using glutathione reductase and nicotinamide adenine dinucleotide phosphate-oxidase (NADPH). The method is based on the oxidation of NADPH, which is indicated by the decrease in absorbance at 340 nm [36] and the activity was expressed as μmol of NADPH per minute per gram of hemoglobin. Catalase (CAT) activity was measured spectrophotometrically [37] using hydrogen peroxide as substrate and the activity was expressed as K per gram of hemoglobin.

Levels of blood parameters of oxidative stress and plasma total antioxidant power. Lipid oxidation, measured as thiobarbituric acid reactive substances levels, was assessed after the addition of 7.2 mM of butylated hydroxytoluene to prevent further oxidation. The reaction was performed as previously described [38], the products were extracted with n-butanol and quantified at 535 nm using a standard curve of 1,1,3,3-tetraethoxypropane. Protein oxidation was assessed as plasma protein carbonyl content based on the reaction of the carbonyl groups with 2,4-dinitrophenylhydrazine to form 2,4 dinitrophenylhydrazone [39]. Samples were read at 370 nm and the carbonyl content was calculated using the molar absorption coefficient for aliphatic hydrazones (22,000 M^{-1} cm^{-1}).

To evaluate the ‘‘total antioxidant power’’ we measured the ability of plasma to withstand the oxidative effects of reactive species purposefully generated in the reaction mixture according to Benzie and Strain [40]. This method measures the ferric-reducing ability of plasma as the increase in absorption at 593 nm when a ferric-tripyridyltriazine (Fe III-TPTZ) complex is reduced to the ferrous (Fe II) form.

Statistical analysis. Data were analyzed by one-way analysis of variance (ANOVA), for variables that followed the ANOVA assumptions. The results of each group were post hoc compared to the group of postmenopausal women without hormone therapy using the

Dunnett's test. Variables that did not follow the ANOVA assumptions were analyzed by Kruskal-Wallis followed by Mann-Whitney's comparison test. The association among biochemical parameters, activity of antioxidant enzymes, and the characteristics of the study groups were assessed using Pearson's correlation for variables that had a normal distribution and by Spearman's rank order correlation for variables that did not exhibit a normal distribution. Results are expressed as means ± standard error (S.E.M.). Data were analyzed using the Statistica 6.0 software system (Statsoft Inc., 2001).

Results

The characteristics of the studied groups are shown in Table 1. The premenopausal group and the postmenopausal group with estrogen plus progestin therapy had lower mean age than the postmenopausal group without hormone therapy, but no age differences were observed between this group and the postmenopausal women with estrogen therapy. The state in menopause and the composition of the hormone replacement therapy (estrogen alone or estrogen plus progestin) were used to classify the patients. No significant differences were observed in the duration of menopause of the postmenopausal groups under hormone therapy compared to that without hormone therapy. Likewise, no significant difference was observed in the duration of hormone therapy between women with estrogen and estrogen plus progestin therapy. The body mass index, the systolic and diastolic blood pressure and the regularity of physical exercise of the premenopausal group and of the postmenopausal groups under hormone therapy did not differ from that of the postmenopausal women without hormone therapy (Table 1). Similarly, the frequency and amount of dietary intake of antioxidant-rich foodstuffs did not differ among the groups (data not shown).

As expected, serum estradiol levels were significantly higher in the premenopausal women and in the postmenopausal women with estrogen or estrogen plus progestins therapy

compared to the postmenopausal women without hormone therapy (Table 2). The serum progesterone levels were significantly higher while the serum follicular stimulating hormone levels were significantly lower in the premenopausal women than in the postmenopausal women without hormone therapy (Table 2). However, no differences were observed in the progesterone and follicular stimulating hormone levels between the postmenopausal women without and with hormone therapy (Table 2).

Aspartate aminotransferase, alanine aminotransferase, creatinine, uric acid, albumin, hemoglobin and hematocrit, which are indicators of hepatic, renal and blood disturbances, were not significantly different among postmenopausal women without hormone therapy and the other groups (Table 3). Total blood proteins (Table 3) were significantly higher in the postmenopausal group with estrogen plus progestin therapy than in the postmenopausal women without hormone therapy. We also evaluated serum lipids as indicators of dyslipidemia. The serum total cholesterol, HDL, LDL and triglycerides were not significantly different among postmenopausal women without hormone therapy and the other groups (Table 3).

The blood activity of antioxidant enzymes of the studied groups are shown in Table 4. One-way ANOVA revealed that the postmenopausal groups under estrogen or estrogen plus progestin therapy showed significantly increased CuZnSOD activities when compared to the postmenopausal women without hormone therapy. The premenopausal women had a tendency of higher CuZnSOD activity compared to the postmenopausal women without hormone therapy ($p<0.1$). Furthermore, MnSOD activity was significantly higher in the postmenopausal women under estrogen plus progestins therapy than in the postmenopausal women without hormone therapy. The mean age of the postmenopausal group with estrogen plus progestin therapy was significantly lower than the postmenopausal women without hormone therapy (Table 1). Because the activity of SOD has been reported to decrease with

the increase of age [30,41], we also evaluated the differences of SODs activity among groups by analysis of covariance, using age as a covariate. This statistical analysis revealed the same result at $p < 0.05$. Catalase and glutathione peroxidase activities were not significantly different among postmenopausal women without hormone therapy and the other groups. Because catalase and glutathione peroxidase could be important to remove the hydrogen peroxide that is generated during the dismutation of superoxide anion radical by SOD, we have also assessed the SOD/(catalase + glutathione peroxidase) ratio to verify a possible imbalance among these activities. The SOD/(catalase + glutathione peroxidase) ratio was not significantly different among postmenopausal women without hormone therapy and the other groups (Table 4).

The blood indicators of oxidative stress are shown in Table 5. The serum thiobarbituric acid reactive substances and protein carbonyl groups levels were not significantly different among postmenopausal women without hormone therapy and the other groups. However, the plasma total antioxidant power, evaluated by ferric-reducing ability of plasma, was significantly higher in the postmenopausal group with estrogen plus progestin therapy than in the postmenopausal women without hormone therapy.

The duration of hormone therapy and the serum estradiol levels were positively correlated to the blood CuZnSOD activity (Fig. 1A and 1C) and to the total antioxidant power of plasma (ferric-reducing ability of plasma values; Fig. 1B and 1D). In addition, the serum progesterone levels were positively correlated to the blood CuZnSOD activity (Fig. 1E) and negatively correlated to the serum levels of protein carbonyl groups (Fig. 1F). However, no significant correlation was found between progesterone levels and total antioxidant power of plasma values or between the levels of protein carbonyl groups and the duration of hormone therapy or estradiol levels (data not shown). In addition, no significant correlation was found among thiobarbituric acid reactive substances levels, glutathione peroxidase, catalase or

MnSOD activity and the duration of hormone therapy, estradiol or progesterone levels (data not shown). The age or the duration of menopause also had no significant correlation with any blood antioxidant enzyme activity or oxidative stress parameter. Interestingly, the total antioxidant power of plasma was positively correlated to the CuZnSOD activity (Fig. 1G) and to the glutathione peroxidase activity (Fig. 1H).

Discussion

We evaluated the effect of different types of hormone therapy on the activity of blood SOD isoforms (CuZn and MnSOD) and on other indicators of the oxidative status in postmenopausal women. In this context, we observed that blood CuZnSOD activity, was increased in postmenopausal women under both types of hormone replacement therapy (estrogen alone and hormone therapy with estrogen plus progestin) compared to the postmenopausal women without hormone therapy. Moreover, blood CuZnSOD activity was positively correlated to the duration of hormone therapy and to the blood levels of estrogen and progesterone. We recently demonstrated *in vitro* that both estrogen and progesterone can directly stimulate CuZnSOD activity from human erythrocytes at concentrations within the physiological range, and they had a synergic effect when combined. In addition, the human gene promoters of CuZnSOD and MnSOD have progesterone receptors, which indicate that progesterone can regulate the expression of these antioxidant enzymes [42,43]. Indeed, progestins stimulated the expression of MnSOD mRNA in T47D human breast cancer cells [44] and in endometrial cells culture [32]. However, progesterone antagonized the overexpression of extracellular CuZnSOD and MnSOD induced by estrogen in cultured vascular smooth muscle cells [33]. Furthermore, in the present study only the combined hormone therapy with estrogen plus progestin increased blood MnSOD activity in postmenopausal women. This effect is probably related to the increased expression of

MnSOD caused by steroid hormones, because no direct stimulatory effect by steroid hormones on blood MnSOD activity was observed *in vitro* [31]. In fact, in endometrial cells culture estrogen potentiated the increase of MnSOD expression caused by progesterone, but had no effect *per se* [32].

Azevedo *et al.* [45] found no effect of hormone replacement therapy on SOD and glutathione peroxidase activities, but they found increased catalase activity in peritoneal macrophages from ovariectomized rats treated with estradiol plus progesterone [45]. In the present study, we did not find influence of hormone therapy on blood catalase or glutathione peroxidase activities in postmenopausal women. Likewise, the activities of glutathione peroxidase and catalase did not differ between ovulating and non-ovulating women [17,46], and the expression of catalase or glutathione peroxidase was not altered by progesterone in cultured vascular smooth muscle cells [33]. In fact, the effect of estrogens on SOD expression in human endometrial stromal cells [29] and follicular fluid [47] was apparently due to the activation of the estradiol receptor and was selective for SOD, with no change in the expression of glutathione peroxidase or catalase. Moreover, catalase and the GSH-dependent erythrocyte antioxidant defense were not affected by ovarian hormone disturbances in regularly menstruating women that result in anovulation with markedly lower plasma estradiol concentrations [46].

Despite being an antioxidant enzyme, an increase of SOD activity may occasionally induce oxidative stress due to the accumulation of peroxide. This oxidative stress occurs when the increase of SOD activity is not accompanied by increased activity of hydrogen peroxide-removing enzymes [48-50] and is characterized by an increased SOD/(catalase + glutathione peroxidase) ratio [48,49]. However, in the present study the postmenopausal women under hormone therapy had no imbalance in the SOD/(catalase + glutathione peroxidase) activities and no increase in the lipid (thiobarbituric acid reactive substances levels) or protein (protein

carbonyl groups) oxidation compared to the postmenopausal women without hormone therapy. These findings suggest that the increased SOD activity may be beneficial to this group.

The total antioxidant capacity measured by the ferric-reducing ability of plasma allows an assessment of the performance of the entire antioxidant system which is very useful [40]. Along with the increased CuZnSOD and MnSOD activities, we also observed an increase on the total antioxidant capacity in postmenopausal women under estrogen plus progestin therapy, but not in women under therapy with estrogen alone. This increased antioxidant capacity was not accompanied by changes in the albumin or uric acid levels, which are major non-enzymatic antioxidant components of the plasma. In addition, the total antioxidant capacity of plasma was positively correlated to CuZnSOD and glutathione peroxidase activities. These results indicate that the improvement of antioxidant capacity triggered by the estrogen plus progestin therapy is related to the changes in the activity of these antioxidant enzymes, rather than to the direct free radical scavenging antioxidant effect of estradiol [20,23]. Interestingly, the plasma total antioxidant capacity was positively correlated to the duration of hormone therapy and to serum estradiol levels. Accordingly, a previous prospective studies showed that the estrogen therapy increased the total blood antioxidant status of postmenopausal women but there was no additional increase with the association of progestins in the hormones therapy [14,51]. However, the duration of hormone therapy in these studies was much lower (up to 4 months) than in the present study.

Despite the increased plasma total antioxidant capacity of postmenopausal women under estrogen plus progestin therapy no changes were observed in the lipid or protein oxidation in this group. This result is in agreement with previous studies that showed no differences on plasma lipid oxidation in postmenopausal women with or without hormone replacement therapy [52] or between ovulating and non-ovulating women [46]. Accordingly,

no correlation was found between the erythrocyte or plasma thiobarbituric acid reactive substances levels and the duration of hormone replacement therapy [13]. On the other hand, some studies showed increased protein carbonyl levels [53] and lipid oxidation in the plasma from postmenopausal women, which were decreased by the combined hormone therapy [13-15]. Although, we did not find differences on thiobarbituric acid reactive substances and protein carbonyl levels between premenopausal and postmenopausal women, we found a negative correlation between progesterone levels and protein carbonyl levels.

Our results indicate that the combined therapy with estrogen and progestin was associated with an increase in the total antioxidant capacity of plasma in postmenopausal women and this increase was associated to the increased activity of blood CuZnSOD and MnSOD isoforms. The therapy with estrogen alone increased the activity of CuZnSOD, but not MnSOD activity or the total antioxidant capacity of plasma. These results suggest that the antioxidant properties of the hormone replacement therapy are due to an increase of the enzymatic antioxidant defenses and are more remarkable with the association of estrogens and progestins. To the best of our knowledge, this is the first work demonstrating the effect of different types of hormone replacement therapy on the SOD isoforms in human blood. These findings may play an important role in the development of menopause-related symptoms and diseases.

The case-control design is a limitation of the present study because it provide less evidence for causal inference than randomized trials. However, there is some ethical constraints in clinical randomized trials, because the hormone replacement therapy may be unrecommended for some patients. Although the total number of subjects enrolled in this study was large, the small number of subjects in the groups under hormone replacement therapy is another limitation. It was particularly difficult to find voluntaries under hormone therapy, because ultimately there was a dispute on the benefits and risks of this treatment [54;

55]. Nevertheless, the results obtained were corroborated by the various blood markers evaluated and are in agreement with our previous study where we demonstrated the direct stimulatory effects of natural and synthetic steroid hormones on human CuZnSOD activity *in vitro* [31].

We conclude that hormone therapy with estrogens plus progestins increases blood MnSOD and CuZnSOD activity in postmenopausal women, leading to an increased plasma total antioxidant capacity compared to the postmenopausal women that are not under hormone therapy. Thus, it is likely that hormone replacement therapy, in addition to reducing the menopause-related symptoms, may also play a role to improve the antioxidant status.

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References

1 Hale GE, Zhao X, Hughes CL, Burger HG, Robertson DM, Fraser IS. Endocrine features of menstrual cycles in middle and late reproductive age and the menopausal transition classified according to the Staging of Reproductive Aging Workshop (STRAW) staging system. J Clin Endocrinol Metab 2007;92:3060-7.

2 Sowers JR. Diabetes mellitus and cardiovascular disease in women. Arch Intern Med 1998; 58:617-21.

3 Greendale GA, Lee NP, Arriola ER. The menopause. The Lancet 1999;353:571-80.

4 Aquino EM, Barreto SM, Bensenor IM, Carvalho MS, Chor D, Duncan BB, Lotufo PA, Mill JG, Molina MDEL C, Mota EL, Passos VM, Schmidt MI, Szklo M. brazilian longitudinal study of adult health (ELSA-Brasil): Objectives and Design. Am J Epidemiol 2012;175:315-24.

5 Trevisan M, Browne R, Ram M, Muti P, Freudenheim J, Carosella AM, Armstrong D. Correlates of markers of oxidative status in the general population. Am. J. Epidemiol 2001;154(4):348-56.

6 Signorelli SS, Neri S, Sciacchitano S, Pino LD, Costa MP, Marchese G, Celotta G, Cassibba N, Pennisi G, Caschetto S. Behavior of some indicators of oxidative stress in postmenopausal and fertile women. Maturitas 2006;53:77-82.

7 Sánchez-Rodríguez MA, Zacarías-Flores M, Arronte-Rosales A, Correa-Muñoz E, Mendoza-Núñez VM. Menopause as risk factor for oxidative stress. Menopause 2012;19:361-7.

8 Miquel J, Ramírez-Boscá A, Ramírez-Bosca JV, Alperi JD. Menopause: a review on the role of oxygen stress and favorable effects of dietary antioxidants. Arch Gerontol Geriatr 2006;42:289-306.

9 WHI-Writing Group For The Women's Health Initiative Investigators (WGWHIII). Risk and benefits of estrogen plus progesterone in healthy postmenopausal women. JAMA 2002;288:321-33.

10 Rossouw JE, Prentice RL, Manson JE, Wu L, Barad D, Barnabei VM, Ko M, Lacroix AZ, Margolis KL, Stefanick ML. Postmenopausal hormone therapy and risk of cardiovascular disease by age and years since menopause. JAMA 2007;297:1465-77.

11 Hulley S, Grady D, Bush T, Furberg C, Herrington D, Riggs B, Vittinghoff E. Heart and Estrogen/progestin Replacement Study (HERS) Research Group. Randomized

trial of estrogen plus progestin for secondary prevention of coronary heart disease in postmenopausal women. JAMA 1998;280:605-13.

12 Skouby SO, Jespersen J. Progestins in HRT: sufferance or desire? Maturitas 2009;20:371-5.

13 Özden S, Dildar K, Kadir YH, Gülizar K. The effects of hormone replacement therapy on lipid peroxidation and antioxidant status. Maturitas 2001;38:165-70.

14 Bednarek-Tupikowsa G, Bohdanowicz-Pawlak A, Bidzinska B, Milewicz A, Atonowicz-Juchniewicz J, Andrzejak R. Serum lipid peroxides and total antioxidant status in postmenopausal women on hormone replacement therapy. Gynecol Endocrinol, 2004;19:57-63.

15 Naziroğlu M, Simşek M, Simşek H, Aydilek N, Ozcan Z, Atilgan R. The effects of hormone replacement therapy combined with vitamins C and E on antioxidant levels and lipid profiles in postmenopausal women with Type 2 diabetes. Clin Chim Acta 2004;344:63-71.

16 Bednarek-Tupikowska G, Tworowska U, Jedrychowska I, Radomska B, Tupikowski K, Bidzinska-Speichert B, Milewicz A. Effects of oestradiol and oestroprogestin on erythrocyte antioxidative enzyme system activity in postmenopausal women. Clin Endocrinol (Oxf) 2006;64:463-8.

17 Unfer TC, Conterato GM, Da Silva JC, Duarte MM, Emanuelli T. Influence of hormone replacement therapy on blood antioxidant enzymes in menopausal women. Clin Chim Acta. 2006;369:73-7.

18 Gökkusu C, Tata G, Ademoğlu E, Tamer S. The benefits of hormone replacement therapy on plasma and platelet antioxidant status and fatty acid composition in healthy postmenopausal women. Platelets 2010;21:439-44.

19 Gökkuşu, C.; Özbek, Z.; Tata, G. Hormone replacement therapy: relation to homocysteine and prooxidant-antioxidant status in healthy postmenopausal women. Arch Gynecol Obstet 2012;285:733-9.

20 Haliwell B, Grootveld M. The measurement of free radical reactions in humans. Some thoughts for future experimentation**.** FEBS Letters 1987;213:9-16.

21 Subbiah MT. Mechanisms of cardioprotection by estrogens. Proc Soc Exp Biol Med 1998;217:23-9.

22 Huber LA, Scheffler E, Poll T, Ziegler R, Dresel HA. 17β-estradiol inhibits LDL oxidation and cholesteryl macrophages. Free Radic Res 1989;8:167-73.

23 Subbiah MT, Kessel B, Agrawal M, Rajan R, Abplanalp W, Rymaszewski Z. Antioxidant potential of specific estrogens on lipid peroxidation. J Clin Endocrinol Metab 1993;77:1095-7.

24 Ayres S, Abplanalp W, Liu J, Subbiah MT. Mechanism involved in the protective effect of estradiol-17β on lipid peroxidation and DNA damage. Am J Physiol 1998; 274:1002-8.

25 Thibodeau PA, Kachadourian R, Lemay R, Bisson M, Day BJ, Paquette B. *In vitro* pro- and antioxidant properties of estrogens. J Steroid Biochem Mol Biol 2002;81: 227-36.

26 Wójcik M, Kosior-Korzecka U, Bobowiec R. Cytoprotective action of 17β-oestradiol against iron-induced hepatic oxidative stress *in vitro*. Bull Vet Inst Pulawy 2010;54:259-63.

27 Al-Gubory KH, Bolifraud P, Garrel C. Regulation of key antioxidant enzymatic systems in the sheep endometrium by ovarian steroids. Endocrinology 2008;149: 4428-34.

28 Tranquilli AL, Mazzanti L, Cugini AM, Cester N, Garzetti GG, Romanini C. Transdermal estradiol and medroxyprogesterone acetate in hormone replacement therapy are both antioxidants. Gynecol Endocrinol 1995;9:137-41.

29 Strehlow K, Rotter S, Wassmann S, Adam O, Grohé C, Laufs K, Böhm M, Nickenig G. Modulation of antioxidant enzyme expression and function by estrogen. Circ Res 2003;93:170-7.

30 Moorthy K, Sharma D, Basir SF, Baquer NZ. Administration of estradiol and progesterone modulate the activities of antioxidant enzyme and aminotransferases in naturally menopausal rats. Exp Gerontol 2005;40:295-302.

31 Unfer TC, Maurer LH, Kemerich DM, Figueiredo C, Duarte MMF, Emanuelli T. Non-genomic, direct modulatory effect of 17 b -estradiol, progesterone and their synthetic derivatives on the activity of human erythrocyte CuZn superoxide dismutase. Free Radic Res 2013;47:219-32.

32 Sugino N, Karube-Harada A, Kashida S, Takiguchi S, Kato H. Differential regulation of copper-zinc superoxide dismutase and manganese superoxide dismutase by progesterone withdrawal in human endometrial stromal cells. Mol Hum Reprod 2002;8:68-74.

33 Wassmann K, Wassmann S, Nickenig G. Progesterone antagonizes the Vasoprotective effect of estrogen on antioxidant enzyme expression and function. Circ Res 2005;97:1046-54.

34 Soules MR, Sherman S, Parrot E. Stages of Reproductive Aging Workshop (STRAW). J Womens Health, 2001;10:843-48.

35 Spitz DR, Oberley LW. An assay for superoxide dismutase activity in mammalian tissue homogenates. Anal Biochem 1989;179:8-18 .

36 Paglia DE, Valentine WN. Studies on the quantitative and qualitative characterization of erythrocyte glutathione peroxidase. J Lab Clin Med 1967;70:158-69.

37 Aebi H. Catalase *in vitro*. Methods Enzymol 1984;105:121-6

38 Ohkawa H, Ohishi N, Yagi K. Assay for lipid hidroperoxides in animal tissue by thiobarbituric acid reaction. Anal Biochem 1979;95:351-8.

39 Levine RL, Garland D, Oliver CN, Amici A, Climent I, Lenz AG, Ahn BW, Shaltiel S, Stadtman ER. Determination of carbonyl content in oxidatively modified proteins. Methods Enzymol 1990;186:464-78.

40 Benzie IFF, Strain JJ. The ferric reducing ability of plasma (FRAP) as a measure of "antioxidant power": The FRAP assay. Anal Biochem 1996;239:70-6.

41 Ceballos-Picot, Trivier J M, N icole A, Sinet PM, Thevenin M. Age-correlated modifications of copper -zinc superoxide dismutase and glutathione-r elated enzyme activities in human erythrocytes. Clin Chem. 1992;38:66-70.

42 Kim HT, Kim HY, Nam JW, Lee HJ, Rho HM, Jung G. Study of 5'-flanking region of human Cu/Zn superoxide dismutase. Biochem Biophys Res Commun 1994;201:1526-33.

43 Wan XS, Devalaraja MN, St Clair DK. Molecular structure and organization of the human manganese superoxide dismutase gene. DNA Cell Biol 1994;13:1127-36.

44 Holley AK, Kiningham KK, Spitz DR, Edwards DP, Jenkins JT, Moore MR. Progestin stimulation of manganese superoxide dismutase and invasive properties in T47D human breast cancer cells. J Steroid Biochem Mol Biology 2009;117:23-30.

45 Azevedo RB, Lacava ZGM, Miyasaka CK, Chaves SB, Curi R. Regulation of antioxidant enzyme activities in male and female rat macrophages by sex steroids. Braz J Med Biol Res 2001;34: 683-7.

46 Lutosławska G, Tkaczyk J, Panczenko-Kresowska B, Hübner-Woźniak E, Skierska E, Gajewski AK. Plasma TBARS, blood GSH concentrations, and erythrocyte antioxidant enzyme activities in regularly menstruating women with ovulatory and anovulatory menstrual cycles. Clin Chim Acta 2003;331:159-63.

47 Borrás C, Gambini J, Gómez-Cabrera C, Sastre J, Pallardó F, Mann G, Viña J. 17β-oestradiol up-regulates longevity-related, antioxidant enzyme expression via the ERK1 and ERK2 [MAPK]/NFκ B cascade. Aging Cell 2005;4:113-8.

48 Pinho RA, Andrades ME, Oliveira MR, Pirola AC, Zago MS, Silveira PCL, Dal-Pizzol F, Moreira JCF: Imbalance in SOD/CAT activities in rat skeletal muscles submitted to treadmill training exercise. Cell Biol Int 2006;30:848-53.

49 Bambini-Junior V, Rodrigues L, Behr GA, Moreira JCF, Riesgo R, Gottfried C: Animal model of autism induced by prenatal exposure to valproate: behavioral changes and liver parameters. Brain Res 2011;1408:8-16.

50 Augusti PR, Ruviaro AR, Quatrin A, Somacal S, Conterato GMM, Vicentini JT, Duarte MMF, Emanuelli T. Imbalance in superoxide dismutase/thioredoxin reductase activities in hypercholesterolemic subjects: relationship with low density lipoprotein oxidation. Lipids Health Dis 2012;11:79.

51 Delibasi T, Kockar C, Celik A, Kockar O. Antioxidant effects of hormone replacement therapy in postmenopausal women. Swiss Med Wkly 2006;136:510-4.

52 Bureau I, Anderson RA, Arnaud J, Raysiguier Y, Favier AE, Roussel AM. Trace mineral status in postmenopausal women: impact of hormonal replacement therapy. J Trace Elem Med Biol 2002;16:9-13.

53 Polac I, Borowiecka M, Wilamowska A, Nowak P. Oxidative stress measured by carbonyl groups level in postmenopausal women after oral and transdermal hormone therapy. J Obstet Gynaecol 2012; 38:1177-81.

54 Writing Group for the Women's Health Initiative Investigators (WGWHIII). Risk and benefits of estrogen plus progesterone in healthy postmenopausal women. JAMA 2002;288:321-33.

55 Writing Group for the Women's Health Initiative Investigators (WGWHIII). Postmenopausal hormonal therapy and risk of cardiovascular disease by age and years since menopause. JAMA 2007;297(13):1465-77.

Table 1 – Characteristics of the studied groups (means ± S.E.M.)

	Postmenopausal women without HT (n = 31)	Postmenopausal women with ET (n = 12)	Postmenopausal women with EPT (n = 16)	Premenopausal women (n = 24)
Age (years)	60.5 ± 1.2 (47.0-75.0)	57.2 ± 1.6 (45.0-65.0)	55.7 ± 0.7* (51.0-60.0)	44.7 ± 0.8* (36.0-50.0)
Duration of menopause (months)	144.2± 17.3 (12.0-408.0)	146.7± 21.5 (27.0-264.0)	93.1 ± 15.6 (6.0-240.0)	0
Duration of HT (months)	0	93.1 ± 23.5 (3.0-240.0)	57.6 ± 12.4 (3.0-148.0)	0
BMI	26.7 ± 0.8 (18.0-36.7)	25.3 ± 1.1 (21.9-32.9)	24.9 ± 0.7 (19.4-28.9)	25.3 ± 0.8 (19.2-33.8)
Systolic blood pressure (mmHg)	127.1 ± 2.3 (100.0-150.0)	125.0 ± 2.3 (120.0-140.0)	125.0 ± 2.3 (100.0-180.0)	122.5 ± 2.2 (100.0-140.0)
Diastolic blood pressure (mmHg)	81.6 ± 1.9 (50.0-100.0)	80.0 ± 0.0 (80.0)	81.2 ± 2.6 (60.0-100.0)	80.0 ± 1.6 (60.0-90.0)

*Different from the postmenopausal women without HT (ANOVA followed by Dunnett´s test; $p < 0.05$). No significant differences were observed in the BMI among postmenopausal women without HT and the other groups or in the duration of menopause among postmenopausal women without HT and the other postmenopausal groups (ANOVA; $p > 0.05$). No significant differences were observed in HT between the two groups on hormone therapy (Mann-Whitney´s test, $p > 0.05$). Physical exercise level: 0 – no exercise; 1 – light (twice/week); 2 - moderate (3-4 times/week); 3 - intense (5-7 times/week) HT: hormone therapy; ET: estrogen therapy; EPT: estrogen plus progestin therapy; BMI: body mass index.

Table 2 – Serum hormone levels of the studied groups (means ± S.E.M.)

	Postmenopausal women without HT (n = 31)	Postmenopausal women with ET (n = 12)	Postmenopausal women with EPT (n = 16)	Premenopausal women (n = 24)
E_2 (pg/mL)	46.6 ± 11.9 (7.0-220.0)	80.9 ± 15.3* (7.0-220.0)	95.3 ± 30.8* (7.0-525.9)	75.6 ± 12.7* (7.0-181.1)
Progesterone (ng/mL)	0.7 ± 0.1 (0.05-2.8)	0.4 ± 0.1 (0.2-1.3)	0.6 ± 0.2 (0.2-2.8)	4.2 ±1.5* (0.2-28.2)
FSH (mIU/mL)	65.4 ± 5.8 (7.9-122.1)	53.6 ± 10.5 (15.4-152.0)	41.2 ± 7.2 (5.5-108.9)	15.2 ± 4.4* (0.2-70.0)

*Different from the postmenopausal women without HT (ANOVA followed by Dunnett´s test; $p < 0.05$). HT: hormone therapy; ET: estrogen therapy; EPT: estrogen plus progestin therapy; BMI: body mass index; E_2: 17β-estradiol; FSH: follicle-stimulating hormone.

Table 3 – Biochemical parameters of the studied groups (means ± S.E.M.)

	Postmenopausal women without HT (n = 31)	Postmenopausal women with ET (n = 12)	Postmenopausal women with EPT (n = 16)	Premenopausal women (n = 24)
AST (IU/L)	25.0 ± 3.8 (11.0-120.0)	23.2 ± 1.4 (17.0-36.0)	21.7 ± 1.1 (16.0-28.0)	21.0 ± 2.6 (11.0-72.0)
ALT (IU/L)	26.5 ± 4.4 (9.0-115.0)	22.3 ± 1.4 (12.0-32.0)	24.0 ± 2.9 (11.0-55.0)	26.4 ± 4.0 (9.0-104.0)
Creatinine (mg/mL)	0.64 ± 0.02 (0.40-0.90)	0.67 ± 0.02 (0.60-0.80)	0.62 ± 0.02 (0.50-0.80)	0.68 ± 0.02 (0.40-0.90)
Uric acid (mg/dL)	4.2 ± 0.2 (2.3-6.1)	4.4 ± 0.2 (3.3-6.7)	4.5 ± 0.2 (3.2-6.2)	4.3 ± 0.1 (2.4-6.1)
Albumin (g/dL)	4.3 ± 0.1 (3.3-5.0)	4.6 ± 0.01 (4.5-4.6)	4.3 ± 0.05 (3.7-4.6)	4.3 ± 0.05 (3.8-4.8)
Total blood proteins (ng/mL)	60.5 ± 2.0 (2.0-39.4)	60.7 ± 2.3 (42.0-70.7)	66.7 ± 1.3* (56.0-76.9)	62.6 ± 1.7 (37.9-79.5)
Hemoglobin (g/dL)	13.2 ± 0.2 (10.8-14.6)	13.1 ± 0.3 (11.2-14.9)	13.4 ± 0.2 (12.4-15.6)	12.9 ± 0.2 (11.0-14.9)
Hct (%)	40.3 ± 0.5 (33.8-45.2)	39.1 ± 0.9 (34.1-45.0)	41.5 ± 0.6 (38.0-47.1)	39.1 ± 0.6 (34.2-45.2)
Total cholesterol (mg/dL)	226.5 ± 7.8 (148.0-311.0)	209.2 ± 8.6 (134.0-250.0)	211.2 ± 7.1 (175.0-277.0)	201.2 ± 7.8 (139.0-286.0)
HDL (mg/dL)	60.0 ± 3.1 (36.0-95.0)	63.9 ± 4.9 (50.0-104.0)	56.6 ± 2.6 (36.0-75.0)	52.0 ± 2.5 (32.0-80.0)
LDL (mg/dL)	145.9 ± 7.4 (89.0-227.0)	135.5 ± 7.4 (101.6-193.0)	128.1 ± 6.4 (81.0-174.4)	122.5 ± 7.5 (59.0-194.2)
TG (mg/dL)	136.2 ± 9.3 (62.0-236.0)	137.3 ± 16.9 (62.0-270.0)	130.7 ± 21.0 (78.0-433.0)	132.3 ± 12.3 (59.0-283.0)

No significant differences were observed among postmenopausal women without HT and the other groups. HT: hormone therapy; ET: estrogen therapy; EPT: estrogen plus progestin therapy; HDL: high-density lipoprotein; LDL: low-density lipoprotein; TG: triglycerides; AST: aspartate aminotransferase; ALT: alanine aminotransferase; Hct: hematocrit.

Table 4 – Blood activity of antioxidant enzymes in the studied groups (means ± S.E.M.)

	Postmenopausal women without HT (n = 31)	Postmenopausal women with ET (n = 12)	Postmenopausal women with EPT (n = 16)	Premenopausal women (n = 24)
CuZnSOD (U/mg 52)	32.1 ± 3.2 (9.4-73.0)	47.1 ± 5.3* (19.7-80.7)	51.3 ± 7.2* (21.8-119.1)	44.9 ± 6.7 (10.3-142.0)
MnSOD (U/mg Hb)	0.54 ± 0.05 (0.17-1.40)	0.63 ± 0.09 (0.24-1.20)	0.92 ± 0.10* (0.22-2.00)	0.52 ± 0.06 (0.21-1.30)
CAT (K/gHb)	0.52 ± 0.06 (0.03-1.20)	0.58 ± 0.09 (0.09-1.40)	0.80 ± 0.13 (0.20-2.10)	0.80 ± 0.10 (0.16-2.20)
GPx (µmol NADPH/min/g Hb)	28.5 ± 2.7 (10.2-89.1)	24.5 ± 4.4 (8.1-57.4)	38.6 ± 6.7 (12.8-103.3)	34.2 ± 4.9 (9.1-101.5)
SOD/(CAT+GPx)	1.5 ± 0.2 (0.2-4.7)	3.1 ± 0.7 (0.4-7.5)	1.7 ± 0.3 (0.3-4.7)	2.2 ± 0.6 (0.2-15.3)

No significant differences were observed in the CAT and GPx activity or in the SOD/(CAT+GPx) ratio among postmenopausal women without HT and the other groups.
*Different from the postmenopausal women without HT (ANOVA followed by Dunnett´s test; $p < 0.05$). HT: hormone therapy; ET: estrogen therapy; EPT: estrogen plus progestin therapy; SOD: superoxide dismutase; CAT: catalase; GPx: glutathione peroxidase; NADPH, nicotinamine adenine dinucleotide phosphate reduced; SOD/(CAT+GPx) ratio.

Table 5 – Blood indicators of oxidative stress in the studied groups (means ± S.E.M.)

	Postmenopausal women without HT	Postmenopausal women with ET	Postmenopausal women with EPT	Premenopausal women
TBARS (μmol MDA/L plasma)	19.8 ± 1.1 (13.2-44.0) (n = 30)	18.8 ± 1.7 (11.6-33.7) (n = 12)	21.8 ± 2.1 (7.7-29.9) (n = 16)	19.0 ± 1.0 (11.1-31.2) (n = 24)
Protein carbonyl groups (nmol/mg protein)	1.50 ± 0.21 (0.28-4.10) (n=18)	1.60 ± 0.22 (0.49-2.60) (n=10)	1.90 ± 0.23 (0.50-3.30) (n=13)	1.70 ± 0.16 (0.36-2.60) (n=16)
FRAP (mmol Fe^{2+}-TPTZ/L plasma)	1.09 ± 0.06 (0.71-1.40) (n=13)	1.22 ± 0.06 (0.99-1.40) (n=7)	1.43 ± 0.09* (1.10-2.00) (n=12)	1.21 ± 0.04 (0.96-1.40) (n=13)

No significant differences were observed in the TBARS or protein carbonyl groups levels among postmenopausal women without hormone therapy and the other groups.

*Different from the postmenopausal women without HT (ANOVA followed by Dunnett´s test; $p < 0.05$). HT: hormone therapy; ET: estrogen therapy; EPT: estrogen plus progestin therapy; FRAP: ferric-reducing ability of plasma; Fe^{2+}-TPTZ: ferrous-tripyridyltriazine complex; TBARS, thiobarbituric acid reactive substances; MDA: malondialdehyde.

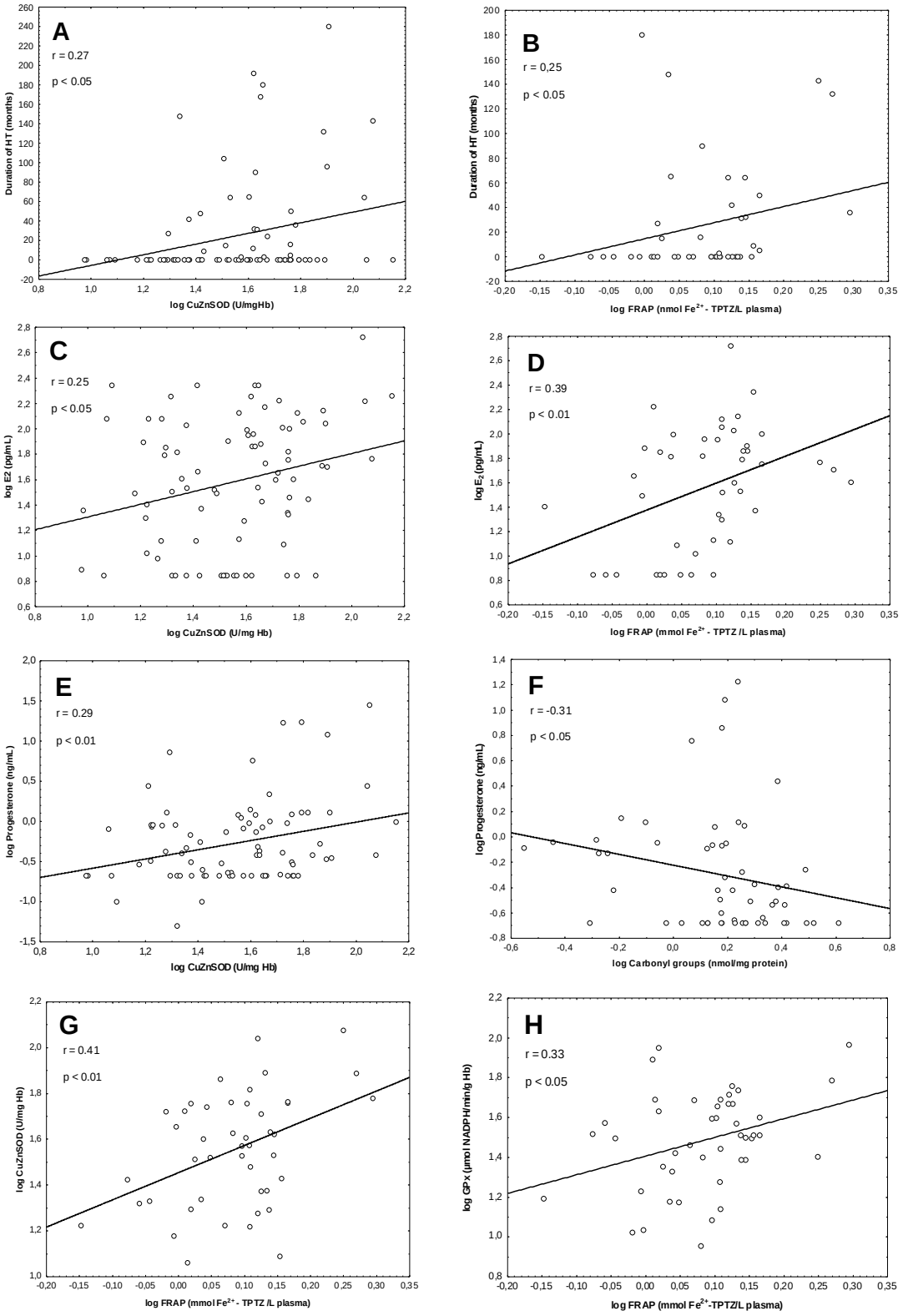


Figure 1 – Significant correlations among the duration of hormone therapy, serum E_2 and progesterone levels and indicators of oxidative status. HT: hormone theraphy; E_2: 17β-estradiol; FRAP: ferric-reducing ability of plasma; Fe^{2+}-TPTZ: ferrous-tripyridyltriazine complex; Gpx: glutathione peroxidase; SOD: superoxide dismutase.

5 DISCUSSÃO DOS RESULTADOS

Os hormônios esteroides exercem diversas funções no organismo, do desenvolvimento à manutenção de características sexuais, distribuição de gordura corporal, coagulabilidade sanguínea, etc.. Nas mulheres, os níveis circulantes de estrógenos e progesterona variam muito em cada ciclo menstrual normal e, na menopausa, fase que determina o fim da função folicular ovariana, observam-se sinais e sintomas atribuídos ao déficit de hormônios esteroides. Além do mais, a menopausa tem sido considerada o fim da proteção natural contra doenças relacionadas ao envelhecimento e foi recentemente indicada como um fator de risco para o estresse oxidativo. A reposição dos hormônios esteroides, através da TRH, é um dos principais tratamentos utilizados para prevenir doenças e diminuir os sinais e sintomas após a menopausa. Existem evidências *in vitro* e *in vivo*, especialmente em animais, de que os hormônios E_2 e progesterona, afetam a atividade de enzimas antioxidantes, em especial a SOD, e a formação de EROs. Contudo, os resultados divergem em relação ao aumento ou diminuição da expressão e atividade da SOD, dependendo do tecido estudado. E não foram encontrados estudos avaliando os efeitos de hormônios esteroides, naturais e sintéticos sobre a atividade das isoformas da SOD presentes no sangue humano (CuZn e MnSOD).

Os resultados apresentados nesta tese iniciam por um estudo, *in vitro*, onde avaliamos o efeito de estrógenos e progestinas, naturais e sintéticos, sobre a atividade da CuZnSOD purificada humana e das isoformas da SOD (CuZnSOD e MnSOD), presentes em frações de sangue humano, eritrócitos e plasma rico em plaquetas (PRP) (artigo 1). Nós demonstramos, pela primeira vez, que os estrógenos e as progestinas, naturais e sintéticos, exercem um efeito bifásico sobre a atividade da SOD citoplasmática (CuZnSOD) *in vitro;* ou seja, em baixas concentrações (fisiológicas), os hormônios aumentaram a atividade da CuZnSOD de forma dose-dependente, sendo esse efeito anulado em presença de concentrações mais altas, quando comparado com o controle. Além do mais, a combinação de dois hormônios naturais (E_2 mais progesterona) ou, a combinação de um hormônio natural com um derivado sintético (E_2 mais acetato de medroxiprogesterona - MPA ou benzoato de estradiol - E_2Benz mais progesterona), evidenciaram um efeito sinérgico sobre a atividade da CuZnSOD eritrocitária, purificada ou não. Ainda que os estrógenos e as progestinas tenham maior impacto sobre o organismo feminino, esses hormônios naturais, também são encontrados em homens. Com base nisso,

nós também avaliamos, *in vitro*, o efeito dos esteroides naturais sobre a atividade da CuZnSOD obtida de eritrócitos masculinos e os resultados obtidos foram virtualmente iguais aos observados em eritrócitos femininos.

Nas mulheres, durante a fase folicular, os níveis de E_2 são baixos (<0,6 nM) e aumentam durante a fase lútea e o período pré-ovulatório do ciclo (0,1-1,5 nM). Da mesma forma, os níveis de progesterona em mulheres não grávidas, também são menores durante a fase folicular (<0,36 nM) e se elevam na fase lútea (3-68 nM) (VANKRIEKEN et al., 1999). Por conseguinte, salientamos que o aumento da atividade da CuZnSOD eritrocitária, observado em nosso estudo, *in vitro*, ocorreu em presença de concentrações fisiológicas de E_2 e progesterona. Níveis estes, encontrados tanto em mulheres na pré-menopausa quanto em mulheres na pós-menopausa, utilizando terapias hormonais. No entanto, os níveis de E_2 encontrados em homens (aproximadamente 0,1 nM) (TCHERNOF et al., 1997) são menores que as concentrações requeridas para estimular a SOD. Além do mais, embora, os níveis masculinos de progesterona (< 3 nM) (EVANS & FOLTIN, 2006) estejam dentro da faixa de concentração requerida para estimulação da CuZnSOD, a sua contribuição para a modulação da atividade enzimática é incerta, pois os níveis de progesterona em homens não variam substancialmente em condições fisiológicas. De qualquer forma, nossos resultados sugerem que os hormônios esteroides podem ser moduladores fisiológicos da atividade antioxidante da CuZnSOD em eritrócitos humanos, especialmente em mulheres.

Estudos sugerem a modulação da atividade da SOD por hormônios esteroides e tecidos humanos (SUGINO et al., 2002) ou animais (MOORTHY et al., 2005; KUMAR et al., 2011). Todavia, todos estes estudos atribuíram a regulação esteroidal da atividade da CuZnSOD e/ou MnSOD apenas à modulação da expressão enzimática (SUGINO et al., 2002; STREHLOW et al., 2003; WASSMANN et al., 2005) em culturas celulares. Em nosso estudo *in vitro*, os efeitos dos esteroides sobre a atividade da CuZnSOD não podem ser relacionados a mudanças na expressão enzimática, uma vez que, a enzima purificada demonstrou um comportamento similar a enzima não purificada em presença das diferentes concentrações hormonais. Além disso, sabe-se que quando os eritrócitos saem da medula óssea, eles perdem seus núcleos, ribossomos e mitocôndrias e, portanto, são incapazes de realizar síntese proteica (EVANS & FOLTIN, 2006). Adicionalmente, nós observamos o efeito estimulatório do E_2 sobre a atividade enzimática da CuZnSOD após um período muito curto de pré-incubação (1 minuto), o qual seria incompatível com o processo de síntese de novas proteínas. Na verdade, os estudos anteriores a respeito da modulação da SOD por hormônios esteroides, não

investigaram o efeito direto, não genômico, destes hormônios sobre a atividade enzimática. Ainda assim, nós não podemos excluir definitivamente a possibilidade de que, *in vivo*, a modulação da atividade da CuZnSOD por hormônios esteroides resulte de ambos mecanismos, genômico e não genômico (ou direto). Nós também propomos que o efeito modulador direto, não genômico, é particularmente importante para a SOD em eritrócitos, pois estas células não sintetizam proteínas. Os eritrócitos têm uma meia vida de aproximadamente 120 dias, portanto, apenas um efeito modulador direto na atividade enzimática poderia ser importante para promover uma mudança rápida na atividade da CuZnSOD eritrocitária.

Recentemente, um estudo revelou que no espaço intermembrana mitocondrial de hepatócitos de ratos, uma SOD dependente de CuZn é ativada por E_2 e, esta ativação é dependente de citocromo P450 (IÑARREA et al., 2011). Esses autores evidenciaram que substratos do complexo enzimático P450 mitocondrial, incluindo o E_2, são fontes efetivas de ânions radicais superóxido e podem mediar a ativação da CuZnSOD presente no espaço intermembrana via oxidação de grupos tióis da enzima (IÑARREA et al., 2011). Contudo, esse mecanismo dependente de P450 não pode ser o responsável pela estimulação enzimática observada em nosso estudo, pois o efeito estimulatório também foi encontrado em avaliações com a enzima purificada.

O E_2 contém um anel fenólico capaz de remover radicais hidroxila (KUMAR et al., 2010) e diminuir a produção de EROs *in vitro* (THIBODEAU et al., 2002). Entretanto, esses efeitos foram observados somente em presença de concentrações muito altas de E_2 (THIBODEAU et al., 2002), e não podem estar envolvidos na modulação da atividade da SOD, uma vez que nós observamos o efeito estimulatório apenas em concentrações hormonais semelhantes aquelas encontradas fisiologicamente. Além disso, como utilizamos um método indireto de avaliação da atividade da SOD, que é o mais utilizado na literatura, avaliamos a possibilidade de que os efeitos observados tivessem sido produzidos por artefatos metodológicos. Os resultados indicaram que os resultados obtidos em nosso estudo de fato podem ser atribuídos a uma modulação específica direta (não genômica) da atividade da CuZnSOD eritrocitária humana por hormônios esteroides. Nós propomos que os hormônios esteroides possivelmente se liguem na estrutura enzima, assim como já foi demonstrado para o 2-metoxiestradiol, um derivado do E_2, que não se liga a receptores de estrogênios, e que ligou e inibiu a atividade da CuZn e da MnSOD em altas concentrações (IC_{50} ~20 µM) (HUANG et al., 2000).

A fim de investigar uma possível interação molecular entre a CuZnSOD e os hormônios esteroides, nó avaliamos a migração eletroforética e o espectro de absorção da enzima incubada com E_2 e/ou progesterona. Nossos resultados indicaram que o E_2 e a progesterona não induzem mudanças drásticas no peso molecular da CuZnSOD, como dimerização proteica, pois a mobilidade eletroforética da proteína não foi modificada após incubação com os hormônios. Ademais, devido ao seu baixo peso molecular, a ligação dos hormônios esteroides a CuZnSOD, por si só, não modificaria a mobilidade eletroforética da proteína. Avaliamos também o efeito dos hormônios sobre o espectro de absorção UV/visível da enzima para identificar alguma interação mais sutil que pudesse estar ocorrendo entre estas moléculas. Observamos então, que o espectro de absorção da CuZnSOD purificada aumentou em presença de E_2 e de progesterona, quando comparado com o espectro da enzima isolada, indicando que os hormônios e a enzima de fato interagem de alguma forma. O aumento na absorção da enzima na faixa de 250-300 nm ocorreu apenas em presença de baixas concentrações hormonais, o que é compatível com o efeito bifásico dos hormônios sobre a atividade da enzima. Além disso, a combinação de E_2 mais progesterona, que exerceu um efeito sinérgico na atividade enzimática, provocou também uma maior alteração no espectro de absorção da enzima quando comparado com o efeito de cada hormônio separadamente.

Estudos anteriores demonstraram que alterações no espectro de absorbância da CuZnSOD humana nas região entre 250 e 300 nm, podem ser atribuídas a modificações em resíduos dos aminoácidos fenilalanina (Phe) (250 - 265 nm) e triptofano (Trp) (275-290 nm) (MAILER et al., 1989). A CuZnSOD humana contém quatro resíduos de Phe e um resíduo de Trp, que formam um anel em torno do sítio ativo contendo os metais (MAILER et al., 1989). A absorbância ultravioleta (UV) do Trp é um indicador de penetração do solvente na estrutura da enzima e do movimento de um resíduo de histidina (His) responsável pela ligação do cobre (posição 46 ou 120) (MAILER et al., 1989). Já a absorbância da Phe é sensível a alterações no sítio ativo da enzima, e ela aumenta quando o Cu é adicionado à apoenzima (MAILER et al., 1989). Desta forma, os resultados do espectro de absorção da SOD sugerem que resíduos de Trp e de Phe podem estar envolvidos na ligação não covalente dos hormônios esteroides a estrutura da CuZnSOD. Foi demonstrado que a formação de uma ponte de hidrogênio entre o grupo 3-OH do E_2 e um resíduo de His ou de Trp é crítica para a ligação do E_2 a várias proteínas, incluindo os receptores de estrogênio nuclear (WEATHERMAN et al., 1999) e a proteína dissulfito isomerase (FU et al., 2011). A ligação de hormônios a proteínas é muito rápida, o que está de acordo com a pré-incubação requerida em nosso estudo para a

estimulação da SOD por hormônios esteroides (menos de 1 min). Entretanto, as alterações no espectro de absorção da SOD podem também resultar de uma alteração na conformação dos resíduos de Phe e Trp na estrutura proteica, devido à ligação de esteroides em outros resíduos de aminoácidos. Assim, os domínios específicos da CuZnSOD envolvidos na interação com os esteroides e as mudanças conformacionais induzidas por essa ligação ainda devem ser explorados em estudos estruturais mais detalhados, oferecendo novas perspectivas de trabalho.

Assim como observado para os esteroides naturais, os esteroides sintéticos também modularam a atividade da CuZnSOD em eritrócitos. O E_2Benz e a MPA são constituintes de pílulas anticoncepcionais utilizadas por mulheres não menopausadas, e também reposições hormonais indicadas após a menopausa (SITRUK-WARE, 2004). Tanto os contraceptivos quanto as terapias hormonais geralmente contêm uma combinação de um estrógeno, mais uma progestina, sendo importante salientar que nós observamos o efeito sinérgico dos hormônios sobre a modulação da atividade da CuZnSOD eritrocitária.

Ao contrário do observado na atividade da CuZnSOD eritrocitária, os hormônios esteroides, naturais e sintéticos, não modularam diretamente a atividade da MnSOD presente em plaquetas humanas. A MnSOD nativa humana possui uma estrutura mista com α-hélice e folha β, constituída por quatro subunidades idênticas, cada uma contendo um átomo de manganês. Em contrapartida, a CuZnSOD é constituída por duas subunidades idênticas, cada uma ligada a um íon Cu e um íon Zn, apresentando estrutura β-barril (PERRY et al., 2010). As diferenças estruturais entre estas isoformas da SOD podem ser responsáveis pela sua diferença na sensibilidade à modulação pelos hormônios esteroides.

Além da MnSOD, o PRP contém também as isoformas, CuZnSOD citosólica e extracelular. A CuZnSOD extracelular é a responsável pela maior parte da atividade da SOD do plasma (ADACHI & WANG, 1998). A SOD extracelular é uma glicoproteína tetramérica, que se encontra nos espaços intersticiais e nos fluidos extracelulares e a sua sequência de aminoácidos é homóloga a encontrada na CuZnSOD citosólica. Em nosso estudo, a atividade da CuZnSOD presente no PRP de mulheres foi modulada por E_2 e por progesterona, mas não por seus derivados sintéticos, E_2Benz e MPA, o que sugere que a isoforma extracelular tem uma sensibilidade diferente à modulação hormonal quando comparada com a CuZnSOD citosólica. A atividade da CuZnSOD do PRP de homens também foi modulada apenas por E_2 e progesterona, sendo mais sensível à estimulação pela progesterona do que a enzima obtida de mulheres. Esta diferença pode estar relacionada aos níveis fisiológicos de progesterona um

pouco mais elevados em mulheres do que em homens, que podem assim ter mascarado o efeito de baixas concentrações do hormônio.

Os hormônios esteroides têm funções amplas no organismo humano, com especial diferenciação em mulheres, que apresentam flutuações hormonais na fase reprodutiva e déficit significativo na fase não reprodutiva, que pode ser revertido com o uso de TRH. Alguns estudos demonstram que a diminuição nos níveis de E_2, ocorrida na menopausa, está associada a um aumento do risco de doenças e do stress oxidativo em mulheres (GURDOL et al., 1997; BEDNAREK-TUPIKOWASKA et al., 2001; ÖZDEN et al., 2001; BEDNAREK-TUPIKOWASKA et al., 2004 ; SIGNORELLI et al., 2005; BRUCE et al., 2009; POLAC et al., 2012; SÁNCHEZ-RODRÍGUEZ et al., 2012). Nós conseguimos, *in vitro*, demonstrar que os estrógenos e as progestinas estimulam diretamente a atividade da CuZnSOD, incluindo o efeito sinérgico de particular importância no contexto da TRH após a menopausa. Sendo assim, também avaliamos em um estudo transversal o efeito *ex vivo*, de diferentes TRH, sobre a atividade das isoformas sanguíneas da SOD e outros parâmetros de estresse oxidativo em mulheres após a menopausa.

Em média, as mulheres avaliadas em nosso estudo estavam no estágio +2 do envelhecimento normal reprodutivo, segundo os critérios internacionais (STRAW) (SOULES et al., 2001). Além do mais, os níveis séricos de E_2 e progesterona eram significativamente menores, enquanto os níveis de FSH eram maiores, em mulheres na pós-menopausa em comparação com as mulheres não menopausadas.

A reposição hormonal ainda é a terapêutica mais indicada para reverter os efeitos do hipoestrogenismo menopáusico pode ser prescrita como terapia contendo somente estrogênios (TE) ou em uma combinação de estrogênios com progestinas (TEP) (GREENDALE et al., 1999; KUMAR et al., 2010). Da mesma forma que POLAC *et al.* (2012), nós observamos que o uso da terapia hormonal após a menopausa restaura significativamente os níveis séricos de E_2. A progesterona tem um metabolismo rápido, o que resulta em uma meia-vida biológica curta (SCHUMACHER et al., 2007) e, nós não observamos diferença nos níveis séricos de progesterona entre os grupos estudados.

Estudos demonstram que a atividade da SOD apresenta-se alterada durante os ciclos hormonais (KASAPOVIC et al., 2001; LUTOSLAWSKA et al., 2003) e após gonadectomias (KASAPOVIC et al., 2001). Em um estudo anterior, nós demonstramos que a atividade sanguínea da SOD total diminui em mulheres após a menopausa em comparação com mulheres não menopausadas (UNFER et al., 2006). No presente estudo, a fim de minimizar

picos muito altos de E_2 e progesterona nas mulheres pré-menopáusicas em comparação com as mulheres menopausadas, a coleta das amostras de sangue de mulheres em condições ovulatórias normais, foi realizada no início do período folicular do ciclo menstrual normal, quando os níveis de esteroides se comparam aos observados após a menopausa (HALE & BURGER, 2009). Sendo assim, nós observamos apenas uma tendência de menor atividade SOD após a menopausa em relação às mulheres ainda em idade reprodutiva.

Tem sido sugerido que a TRH, além de aliviar ou eliminar os sintomas, previne doenças (BRUCE et al., 2009) e parece reduzir o estresse oxidativo na menopausa (ÖZDEN et al., 2001; BEDNAREK-TUPIKOWSKA et al., 2004; NAZIROGLU et al., 2004; BEDNAREK-TUPIKOWSKA et al., 2006, DELIBASI et al., 2006; PAJOVIĆ E SAIČIĆ, 2008; GÖKKUSU et al., 2012; POLAC et al., 2012). Nós observamos que mulheres sob ambas as terapias hormonais (TE e TEP) apresentam uma maior atividade da isoforma CuZnSOD sanguínea em relação às mulheres na menopausa que não utilizam TRH. Além disso, semelhante ao observado por GÖKKUSU *et al.* (2012), a atividade da CuZnSOD foi positivamente correlacionada com o tempo de terapia hormonal e com os níveis sanguíneos de E_2 e progesterona. De fato, em nosso estudo *in vitro*, observamos que o estrogênio e a progesterona, em concentrações fisiológicas, estimulam diretamente a atividade da CuZnSOD eritrocitária humana, e possuem ainda um efeito sinérgico. Sendo assim, demonstramos pela primeira vez que os hormônios esteroides, estrogênios e progestinas, modulam a atividade da CuZnSOD eritrocitária humana *in vitro* e *ex vivo*.

Os genes promotores das enzimas CuZn e MnSOD humanas possuem receptores de progesterona, o que indica que a progesterona pode também regular a expressão destas enzimas antioxidantes (KIM et al. 1994; WAN et al. 1994; WASSMAN et al, 2005; HULLEY et al. 2009). Adicionalmente, as progestinas estimulam a expressão de MnSOD RNAm em culturas de células de câncer de mama (T47D) (HULLEY et al., 2009) e em células endometriais humanas (SUGINO et al., 2002). Contudo, a progesterona antagonizou uma super expressão de CuZnSOD extracelular e de MnSOD, induzidas pelo estrogênio em cultura de células lisas da musculatura vascular (WASSMANN et al., 2005). Além disso, no presente estudo, apenas a terapia hormonal combinada (TEP), aumentou a atividade da MnSOD em mulheres na menopausa. Esse resultado pode estar relacionado ao efeito dos hormônios esteroides sobre a expressão da MnSOD, uma vez que, *in vitro* não encontramos efeito estimulatório direto dos hormônios sobre a atividade da MnSOD presente no PRP. Em cultura de células endometriais humanas, SUGINO et al. (2002), observaram que o estrogênio

potencializou o aumento na expressão da MnSOD, causada pela progesterona, mas não teve efeito *per se*.

AZEVEDO et al. (2001) não encontraram efeitos da terapia hormonal na atividade das enzimas SOD e GPx presentes em macrófagos obtidos de ratas ooforectomizadas. Embora, esses mesmos autores tenham observado aumento na atividade da CAT em macrófagos de ratas anovulatórias tratadas com E_2 mais progesterona (AZEVEDO et al., 2001), nós não encontramos influências das TRH nas atividades sanguíneas da CAT ou GPx em mulheres na pós-menopausa. Do mesmo modo, outros estudo observaram que as atividades da GPx e da CAT não diferiram entre mulheres com ou sem capacidade ovulatória (LUTOSLAWSKE et al., 2003; Unfer et al., 2006), e que a expressão destas enzimas não sofreu alteração na presença de progesterona em culturas de células da musculatura lisa vascular (WASSMANN et al., 2005). De fato, os efeitos dos estrogênios na expressão da SOD humana em células endometriais (STREHLOW et al., 2003) e em fluído folicular (BORRÁS et al., 2005) se deve, aparentemente, a ativação de receptores de estradiol e, foi seletiva para SOD, sem alterar a expressão da GPx ou da CAT. Além do mais, a atividade da CAT e as defesas antioxidantes dependentes de GSH em eritrócitos não sofreram alterações em mulheres com distúrbios menstruais, que resultam em anovulação com concentrações plasmáticas de E_2 significativamente mais baixas (LUTOSLAWSKA et al., 2003).

Apesar de ser uma enzima antioxidante, o aumento da atividade da SOD pode ocasionalmente induzir ao estresse oxidativo devido ao acúmulo de peróxidos. Esse estresse oxidativo ocorre quando o aumento na atividade da SOD não é acompanhado pelo aumento da atividade das enzimas removedoras de H_2O_2 (PINHO et al., 2006; BAMBINI-JUNIOR et al., 2011; AUGUSTI et al., 2012) e é caracterizado por um aumento da razão SOD/(CAT+GPx) (PINHO et al., 2006; BAMBINI-JUNIOR et al., 2011). Contudo, no presente estudo, não observamos desequilíbrio na relação SOD/(CAT+GPx), nem aumento dos níveis de oxidação lipídica (TBARS) ou proteica (proteína carbonil) nas mulheres pós-menopáusicas tratadas com TRHs em relação aquelas sem TRH. Sendo assim, nossos resultados sugerem que o aumento na atividade da SOD é benéfico em mulheres sob terapia hormonal após a menopausa.

A capacidade antioxidante total sérica, determinada pela capacidade do plasma em reduzir o íon férrico (FRAP), permite uma avaliação completa do desempenho antioxidante de diferentes moléculas (BENZIE & STRAIN, 1996). Juntamente com o aumento da atividade das isoformas sanguíneas da SOD, nós também observamos um aumento na capacidade

antioxidante total em mulheres na pós-menopausa utilizando terapia hormonal combinada (TEP), mas não em mulheres tratadas apenas com estrogênios (TE). Esse aumento na capacidade antioxidante total não foi acompanhado por alterações plasmáticas nos níveis de albumina ou ácido úrico, que são os principais antioxidantes não enzimáticos do plasma (DELIBASI et al., 2006). Ademais, a capacidade antioxidante total plasmática foi positivamente correlacionada com as atividades da CuZnSOD e da GPx. Esses resultados indicam que a melhoria na capacidade antioxidante, desencadeada pela TEP, está muito mais relacionada às alterações na atividade destas enzimas antioxidantes, do que a um efeito antioxidante direto do E_2 como removedor de radicais livres (HALIWELL & GROOTVELD, 1987; SUBBIAH, 1998). A capacidade antioxidante total do plasma foi positivamente correlacionada com a duração da terapia hormonal e com os níveis séricos de E_2. Da mesma forma, estudos prospectivos demonstram que a capacidade antioxidante total é significativamente menor no sangue de mulheres na pós-menopausa e que aumenta com o uso de terapias hormonais compostas somente de estrogênios, embora sem efeito adicional da associação de progestinas a terapia (BEDNAREK-TUPIKOWSKA et al., 2004; DELIBASE et al., 2006). Contudo, a duração da terapia hormonal nesses estudos foi muito menor (até 4 meses) do que a avaliada por nós.

A oxidação de proteínas e os produtos de peroxidação lipídica são marcadores de estresse oxidativo em amostras clínicas (FINKEL E HOLBROOK, 2000; DALLE-DONE et al., 2003; POLAC et al., 2012). Apesar de termos observado que a TEP promoveu um aumento na atividade antioxidante total do plasma em mulheres após a menopausa, nós não encontramos nenhuma alteração nos parâmetros avaliando a oxidação de lipídios e de proteínas neste grupo. Esse resultado está em acordo com estudos prévios que também não encontraram diferenças nos níveis plasmáticos de oxidação lipídica em mulheres na pós-menopausa com ou sem TRH (BUREAU et al., 2002; UNFER et al. 2006), ou entre mulheres com e sem condições ovulatórias normais (LUTOSLAWSKA et al., 2003). Da mesma forma, não foram encontradas correlações entre os níveis eritrocitário e plasmático de TBARS e o tempo de TRH (ÖZDEN et al., 2001). Por outro lado, alguns estudos observaram aumento nos níveis de proteína carbonilada (POLAC et al., 2012) e de peroxidação lipídica no plasma de mulheres na pós-menopausa, e redução destes parâmetros após terapia hormonal combinada (ÖZDEN et al., 2001; BEDNAREK-TUPIKOWSKA et al., 2004; NAZIROGLU & SIMSEK, 2004). Embora, nós não tenhamos encontrado diferenças nos níveis séricos de TBARS e proteína carbonilada, entre as mulheres na pré-menopausa e na pós-menopausa, nós

observamos uma correlação negativa entre os níveis séricos de progesterona e de produtos da oxidação de proteínas.

Nossos resultados indicam que a TEP foi efetiva para promover um aumento na capacidade antioxidante total do plasma de mulheres após a menopausa e que esse aumento esteve associado a uma maior atividade das isoformas sanguíneas da SOD (CuZn e MnSOD). A terapia composta apenas de estrogênios, aumentou a atividade sanguínea da CuZnSOD, mas não a atividade da MnSOD ou a atividade antioxidante total do plasma. Esses resultados indicam que as propriedades antioxidantes da TRH, *ex vivo*, são devido ao aumento nas defesas antioxidantes enzimáticas e mais pronunciadas em associação de estrogênios e progestinas. Nosso trabalho também é o primeiro a demonstrar o efeito de diferentes TRH sobre a atividade das isoformas da SOD presentes no sangue humano e esses resultados podem ser relevantes para doenças e alterações metabólicas subsequentes à menopausa.

Seguindo o valor das inovações no contexto terapêutico, estudos demonstram que o polimorfismo genético da MnSOD pode influenciar na atividade de remoção do superóxido mitocondrial (DUARTE et al., 2010; COSTA et al., 2012; MONTANO et al., 2012). Adicionalmente, COSTA et al. (2012) observaram em células mononucleares humanas, que o citrato de clomifeno (indutor de ovulação) exibe atividade antioxidante similar a observada com outros moduladores seletivos de receptores de estrogênios, como o tamoxifeno (GHAREHBAGNI et al., 2010; HERNÁNDEZ-ESQUIVEL et al., 2011) e o raloxifeno (WONG et al., 2008; OZBASAR et al., 2010), com intensidade de efeito dependente do polimorfismo da MnSOD, resultando em metabolismo oxidativo celular instável. Esta instabilidade do estatus oxidativo pode resultar em fragmentação celular por excesso de EROs. Sendo assim, outra perspectiva de novos estudos baseia-se na hipótese de que o polimorfismo da MnSOD (ALA16VAL) possa estar associado às diferentes respostas desta isoforma mitocondrial frente aos hormônios esteroides estudados *in vivo*.

CONCLUSÕES

As seguintes observações baseiam as conclusões deste trabalho:

- Os estrogênios e as progestinas, naturais e sintéticos, modulam diretamente, de forma não genômica, a atividade da CuZnSOD eritrocitária humana *in vitro*, apresentando um efeito bifásico e sinérgico. Em baixas concentrações hormonais (fisiológicas) eles causam um aumento dose-dependente da atividade enzimática, efeito desapareceu em concentrações mais elevadas de hormônio.
- A atividade da CuZnSOD presente no plasma rico em plaquetas humano é modulada apenas pelos hormônios esteróides naturais (17β-estradiol e progesterona).
- A atividade da MnSOD, presente no plasma rico em plaquetas, não é modulada diretamente por hormônios esteróides.
- A terapia hormonal com estrogênios mais progestinas aumenta a atividade da CuZnSOD e da MnSOD no sangue de mulheres na pós menopausa, levando a um aumento do poder antioxidante total do plasma (FRAP) em comparação com mulheres na menopausa sem TRH.
- A terapia hormonal somente com estrogênios aumenta apenas a atividade da CuZnSOD no sangue de mulheres na pós menopausa em comparação com mulheres na menopausa sem TRH.
- A atividade da CuZnSOD e o poder antioxidante total do plasma foram positivamente correlacionados com os níveis séricos de E_2 e progesterona e com o tempo de TRH.

Estas observações nos permitem concluir que o estatus antioxidante em mulheres, em especial a atividade das isoformas da SOD no sangue, está sob influência hormonal. Nossos resultados são particularmente importantes por demonstrarem *in vitro*, pela primeira vez, o efeito estimulatório direto e sinérgico dos hormônios esteróides sobre a atividade das isoformas da SOD no sangue. Além disso, demonstramos que as terapias hormonais, melhoram o estatus antioxidante após a menopausa, e que este efeito não se deve apenas aos estrógenos, mas também a progesterona, reforçando a importância deste hormônio na terapia.

7 REFERÊNCIAS BIBLIOGRÁFICAS

ADACHI T , WANG XL . Association of extracellular-superoxide dismutase phenotype with the endothelial constitutive nitric oxide synthase polymorphism . **FEBS Letters**. v. 433, p. 166-8, 1998.

AFRICANDER, D.; VERHOOG, N.; HAPGOOD, J.P. Molecular mechanisms of steroid receptor-mediated actions by synthetic progestins used in HRT and contraception. **Steroids.** v. 76:636–652, 2011.

ALZAMORA, R.; MICHEA, L.; MARUSIC, E.T. Role of 11beta-hydroxysteroid dehydrogenase in nongenomic aldosterone effects in human arteries. **Hypertension**. v. 35, n. 5, p. 1099–104, 2001.

AL-GUBORY, K.H.; BOLIFRAUD, P. GARREL, C. Regulation of key antioxidant enzymatic systems in the sheep endometrium by ovarian steroids. **Endocrinology.** v. 149, n. 9, p. 4428-4434, 2008.

AQUINO, E.M.; BARRETO, S.M.; BENSENOR, I.M.; CARVALHO, M.S.; CHOR, D.; DUNCAN, B.B.; LOTUFO, P.A.; MILL, J.G.; MOLINA, M.D.E.L.C.; MOTA, E.L.; PASSOS, V.M.; SCHMIDT, M.I.; SZKLO, M. Brazilian longitudinal study of adult health (ELSA-Brasil): Objectives and Design. **American Journal of Epidemiology**. v. 175, p. 315-24, 2012.

AUGUSTI, P.R.; RUVIARO, A.R.; QUATRIN, A.; SOMACAL, S.; CONTERATO, G.M.M.; VICENTINI, J.T.; DUARTE, M.M.F.; EMANUELLI, T. Imbalance in superoxide dismutase/thioredoxin reductase activities in hypercholesterolemic subjects: relationship with low density lipoprotein oxidation. **Lipids in Health and Disease**. v. 11, v. 79, p.1-9, 2012.

AZEVEDO, R.B.; LACAVA, Z.G.M.; MIYASAKA, C.K.; CHAVES, S.B.; CURI, R. Regulation of antioxidant enzyme activities in male and female rat macrophages by sex steroids. **Brazilian Journal of Medicine and Biology Reserach.** v**.** 34, n. 5, p. 683-687, 2001.

AYERS, S. et al. Mechanism involved in the protective effect of Estradiol-17β on lipid peroxidation and DNA damage. **American Journal of Physiology**. v. 274, E, p. 1002-1008, 1998.

BAMBINI-JUNIOR, V.; RODRIGUES, L.; BEHR, G.A.; MOREIRA, J.C.F.; RIESGO, R.; GOTTFRIED, C. Animal model of autism induced by prenatal exposure to valproate: behavioral changes and liver parameters. **Brain Research.** v. 1408, p. 8–16, 2011.
BASU, S. et al. Association between oxidative stress and bone mineral density. **Biochemical Biophysical Research Communication**. v. 288, p. 275-279, 2001.

BEDNAREK-TUPIKOWSKA, G. et al. Serum lipid peroxide levels and erythrocyte glutathione peroxidase and superoxide dismutase activity in premenopausal and postmenopausal women. **Gynecological Endocrinology**. v. 15, p. 298-303, 2001.

BEDNAREK-TUPIKOWSKA, G. et al. Serum lipid peroxides and total antioxidant status in postmenopausal women on hormone replacement therapy. **Gynecological Endocrinology**. v. 19, p. 57-63, 2004.

BEDNAREK-TUPIKOWSKA, G. et al. Effects of oestradiol and oestroprogestin on erythrocyte antioxidative enzyme system activity in postmenopausal women. **Clinical Endocrinology**. v. 64, p. 463-468, 2006.

BENZIE, I.F.F.; STRAIN, J.J. The ferric reducing ability of plasma (FRAP)as a measure of "antioxidant power": The FRAP assay. **Analytical Biochemistry**. v. 239:70-76, 1996.

BERAL, V.; BANKS, E.; REEVES, G. Million Women Study Collaborators. Effects of estrogen-only treatment in postmenopausal women. **JAMA.** v. 11; n. 292(6):685-6, 2004.

BOONYARATANAKORNKIT, V.; EDWARDS, D.P. Receptor mechanisms of rapid extranuclear signalling initiated by steroid hormones. **Essays Biochemistry**. v. 40, p. 105–20, 2004.

BORRÁS, C.; GAMBINI, J.; GÓMEZ-CABRERA, C.; SASTRE, J.; PALLARDÓ, F.; MANN, G.; VIÑA, J. 17β-oestradiol up-regulates longevity-related, antioxidant enzyme expression via the ERK1 and ERK2 [MAPK]/NFκ B cascade. **Aging Cell**. v. 4, p. 113–118, 2005.

BRUCE, D et al. Symtoms of the menopause. **Best Practice & Research Clinical Obstetric and Gynaecology**. v. 23, p. 25-32, 2009.

BUREAU, I. et al. Trace mineral status in postmenopausal women: impact of hormonal replacement therapy. **Journal of Trace Elements in Medicine and Biology**. v. 16, p. 9-13, 2002.

CAO, K. et al. Covalent binding of catechol estrogens to glutathione catalyzed by horseradish peroxidase, lactoperoxidase, or rat liver microsomes. **Chemical Research in Toxicology**. v. 11, p. 917-924, 1998.

CASTELAO, J.E.; GAGO-DOMINGUEZ, M. Risk factors for cardiovas-cular disease in women: Relationship to lipid peroxidation and oxidative stress.**Medical Hypotheses**. v. 71: 39–44, 2008.

CAULEY, J.A. et al. Effects of estrogen plus progestin on risk of fracture and bone mineral density. **JAMA-Journal of the American Medical Association**. v. 290, p. 1729-38, 2003.

CHAO, T.C.; VANALTEN, P.J.; WALTER, R.J. Steroid sex-hormones and macrophage function – modulation of reactive oxygen intermediates and nitrite release. **American Journal of Reproductive Immunology**. v. 32, n. 1, p. 43-52, 1994.

COSTA, F.; DORNELLES, E.; MÂNICA-CATTANI, M.F.; ALGARVE, T.D.; SOUZA FILHO, O.C.; SAGRILLO, M.R.; GARCIA, L.F.M.; CRUZ, I.B.M. Influence of Val16Ala SOD2 polymorphism on the in-vitro effect of clomiphene citrate in oxidative metabolismo. **Reproductive BioMedicine Online**. v. 24, p. 474–81, 2012.

CRIST, B.L.; ALEKEL, D.L.; RITLAND, L.M.; HANSON, L.N.; GENSCHEL, U.; REDDY, M.B. Association of oxidative stress, iron, and centralized fat mass in healthy postmenopausal women. **Journal Womens Health (Larchmt)**. v. 18, p. 795-801, 2009.

DALLE-DONNE, I.; ROSSI, R.; GIUSTARINI, D.; MILZANI, A.; COLOMBO, R. Protein carbonyl groups as biomarkers of oxidative stress. **Clinica Chimica Acta**. v. 329, p. 23–38, 2003.

DARLEY-USMAR, V.M. et al. Nitric oxide, free radicals and cell signaling in cardiovascular disease. **Biochemistry Society Hematology**. v. 25, p. 925-929, 1997.

DELIBASI, T.; KOCKAR, C.; CELIK, A.; KOCKAR, O. Antioxidant effects of hormone replacement therapy in postmenopausal women. **Swiss Medical Weekly**. v. 136, p. 510–14, 2006.

Disponivel em : < http://www.sigmaaldrich.com/catalog/oroduct/sigma>. Produtos : E8875 e P0130. Acessado em 27 de abril de 2013.

DUARTE, M.M.; MORESCO, R.N.; DUARTE, T.; SANTI, A.; BAGATINI, M.D.; DA CRUZ, I.B.; SCHETINGER, M.R.; LORO, V.L. Oxidative stress in hypercholesterolemia and its association with Ala16Val superoxide dismutase gene polymorphism. **Clinical Biochemistry**. v. 43, p. 1118–23, 2010.

EVANS, S.M.; FOLTIN, R.W. Exogenous Progesterone Attenuates the Subjective Effects of Smoked Cocaine in Women, but not in Men . **Neuropsychopharmacology**. v. 31, p. 659 – 74, 2006.

FENG, Z.; ZHANG, J. Long-term melatonin or 17h-estradiol supplementation alleviates oxidative stress in ovariectomized adult rats. **Free Radical Biology and Medicine**. v. 39, p. 195–204, 2005.

FINKEL, T.; HOLBROOK, N.J. Oxidants, oxidative stress and the biology of aging. **Nature**. v. 408, p. 239-47, 2000.

FRIDOVICH, I. The biology of oxygen radicals. **Science**. v. 201, p. 875-80, 1978.

FU, X.M.; WANG, P.; TING ZHU, B. Characterization of the estradiol-binding site structure of human protein disulfide isomerase (PDI) . **PLoS ONE**. v. 6, e27185, 2011.

FUJIMOTO, H.; KOBAYASHI, H.; OHNO, M. Age-Induced Reduction in Mitochondrial Manganese Superoxide Dismutase Activity and Tolerance of Macrophages Against Apoptosis Induced by Oxidized Low Density Lipoprotein. **Circulation Journal**. v. 74, p. 353 – 360, 2010.

GHAREHBAGNI, M.; SENDAG, F.; AKERCAN, F.; ZEYBEK, B.; SEZER, E.; TEREK, M.C.; KARADADAS, N. Effects of tamoxifen on tissue nitrite/nitrate levels and plasma lipid peroxidation in female rats. **Clinical & Experimental Obstetrics & Gynecology**. v. 37, p. 112–4, 2010.

GÖKKUŞU, C.; ÖZBEK, Z.; TATA, G. Hormone replacement therapy: relation to homocysteine and prooxidant-antioxidant status in healthy postmenopausal women. **Archives of Gynecology and Obstetrics.** v. 285, n. 3, p. 733-9, 2012.

GURDOL, F. et al. Change in enzymatic antioxidant defense system in blood and endometrial tissue of women after menopause. **Research Communications in Molecular Pathology and Pharmacology**. v. 97, p. 38-46, 1997.

GREENDALE, G.A.; LEE, N.P.; ARRIOLA, E.R. The menopause. **The Lancet**. v. 353, p. 571-580, 1999.

HALE, G.E.; BURGER, H.G. Hormonal changes and biomarkers in late reproductive age, menopausal transition and menopause. **Best Practice & Research Clinical Obstetric and Gynaecology**. v. 23, p. 7-23, 2009.

HALIWELL, B.; GROOTVELD, M. The measurement of free radical reactions in humans. Some thoughts for future experimentation. **FEBS Letters**. v. 213, p. 9-16, 1987.

HARMAN, D. Aging: a theory based on free radical and radiation chemistry. **Journal of Gerontology**. v. 11, p. 298-300, 1956.

HERNÁNDEZ-ESQUIVEL, L.; NATALIA-PAVÓN; ZAZUETA, C.; GARCÍA, N.; CORREA, F.; CHÁVEZ, E. Protective action of tamoxifen on carboxyatractyloside-induced mitochondrial permeability tran-sition. **Life Sciences**. v. 88, p. 681–7, 2011. .

HUANG, P.; FENG, L.; OLDHAM, E.A.; KEATING, M.J.; PLUNKETT, W. Superoxide dismutase as a target for the selective killing of cancer cells . **Nature**. v. 407, p. 390 – 395, 2000.

HUBER, L.A. et al. 17β-estradiol inhibits LDL oxidation and cholesteryl macrophages. **Free Radical Research Communication**. v. 8, p. 167-173, 1989.

HULLEY, S.; GRADY, D.; BUSH, T.; FURBERG, C.; HERRINGTON, D.; RIGGS, B.; VITTINGHOFF, E. Heart and Estrogen/progestin Replacement Study (HERS) Research Group. Randomized trial of estrogen plus progestin for secondary prevention of coronary heart disease in postmenopausal women. **JAMA**. v. 280, n. 7, p. 605-613, 1998.

HULLEY, S.; GRADY, D. Postmenopausal hormone treatment. **JAMA**. v. 301, p. 2493 – 2495, 2009 .

ÍNAL, M.E.; KANBAK, G.; SUNAL, E. Antioxidant enzyme activities and malondialdehyde levels related to aging. **Clinica Chimica Acta**. v. 305, p. 75– 80, 2001.

IÑARREA, P.; CASANOVA, A.; ALAVA, M.A.; ITURRALDE, M.; CADENAS, E. Melatonin and steroid hormones activate intermembrane Cu,Zn-superoxide dismutase by means of mitochondrial cyto-chrome P450 . Free Radical in Biology and Medicine. v. 150, p. 1575 – 81, 2011.

JOHNSON, F.; GIULIVI, C. Superoxide dismutases and their impact upon human health. **Molecular Aspects of Medicine.** v. 26, p. 340–352, 2005.

JINNO, M.; TAMURA, H.; YONEI, Y. Anti-Aging Medicine and Reproductive Health. **Anti-Aging Medicine** v. 9, n. 1, p. 6-13, 2012.

KASAPOGLU, M.; ÖZDEN, T. Alterations of antioxidant enzymes and oxidative stress markers in aging. **Experimental Gerontology**. v. 36, p. 209-220, 2001.

KASAPOVIC, J. et al. Effects of estradiol benzoate and progesterone o superoxide dismutase activity in the thymus of rats. **Physiological Research**. v. 50, p. 97-103, 2001.

KATTAN, Z. et al. Role of manganese superoxide dismutase on growth and invasive properties of human estrogen-independent breast cancer cells. **Breast Cancer Research Treatment**. v. 108, p. 203-215, 2008.

KE, R.W.; PACE, D.T.; AHPKAS, R.A. Effect of hormone therapy on oxidative stress and endothelial function in African American and Caucasian postmenopausal women. **Fertility and Sterility**. v. 79, p. 1118-1122, 2003.

KIM, H.T.; KIM, H.Y.; NAM, J.W.; LEE, H.J. RHO, H.M.; JUNG, G. Study of 5'-flanking region of human Cu/Zn superoxide dismutase. **Biochemical and Biophysical Research Communication**. v. 201, p. 1526-1533, 1994.

KUHL, H. Pharmacokinetics of oestrogens and progestogens. Maturitas. v. 12, n. 3, p. 171–97, 1990.

KUMAR, S. et al. Role of estrogen receptors in pro-oxidative and anti-oxidative actions of estrogens: A perspective. **Biochimica et Biophysica Acta.** v. 1800, p. 1127-1135, 2010.

LIEHR, J.G. Genotoxic effects of estrogens. **Mutation Research**. v. 238, p. 269-276, 1990.

LUTOSLCAWSKA, G.; TKACZYK, J.; PANCZENKO-KRESOWSKA, B.C.; et al. Plasma TBARS, blood GSH concentrations, and erythrocyte antioxidante enzyme activities in regularly menstruating women with ovulatory and anovulatory menstrual cycles. **Clinica Chimica Acta**. v. 331, p. 159–163, 2003.

MAILER, K.; ADDETIA, R.; LIVESEY, D.L. UV spectrocopic studies of human erythrocyte superoxide dismustase. **Journal of Inorganic Biochemistry**. v. 37, p. 151 – 161, 1989.

MARNETT, L.J. Oxyradicals and DNA damage. **Carcinogenesis**. v. 21, n. 3, p. 361-370, 2000.

MIQUEL, J.; RAMÍREZ-BOSCÁ, A.; RAMÍREZ-BOSCA, J.V.; ALPERI, J.D. Menopause: a review on the role of oxygen stress and favorable effects of dietary antioxidants. **Archives of Gerontology and Geriatrics**. v. 42, p. 289-306, 2006.

MINISTÉRIO DA SAÚDE. SECRETARIA DE ATENÇÃO À SAÚDE. Departamento de Ações Programáticas Estratégicas. Manual de Atenção à Mulher no Climatério/Menopausa / Ministério da Saúde, Secretaria de Atenção à Saúde, Departamento de Ações Programáticas Estratégicas. Editora do Ministério da Saúde. Brasília, Brasil, 2008.

MOORTHY, K. SHARMA, D. BASIR, S.F.; et al. Administration of estradiol and progesterone modulate the activities of antioxidant enzyme and aminotransferases in naturally menopausal rats. **Experimental Gerontology**. v. 40, p. 295-302, 2005.

MONTANO, M.A.E.; LERA, J.P.B.; GOTTLIEB, M.G.V.; et al. Association between manganese superoxide dismutase (MnSOD) gene polymorphism and elderly obesity. **Molecular and Cellular Biochemistry**. v. 328, p. 33–40, 2009.

NAZIROGLU, M.; SIMŞEK M, SIMŞEK H.; et al. The effects of hormone replacement therapy combined with vitamins c and e on antioxidant levels and lipid profiles in postmenopausal women with Type 2 diabetes. **Clinica Chimica Acta**. v. 344, p. 63-67, 2004.

NARAYANAN, R.; COSS, C.C.; YEPURU, M.; et al. Steroidal androgens and nonsteroidal, tissue-selective androgen receptor modulator, S-22, regulate androgen receptor function through distinct genomic and nongenomic signaling pathways. **Mollecular Endocrinology**. v. 22, n. 11, p. 2448–65, 2008.

NORDBERG, J.; ARNÉR, E.S.J. Reactive oxygen species, antioxidants, and the mammalian thioredoxin system. **Free Radical Biology & Medicine**. v. 31, n. 11, p. 1287-1312, 2001.

ORGANIZACION MUNDIAL DE LA SALUD (OMS). Investigaciones sobre la menopausia en los años noventa. Genebra, 1996.

ORGANIZAÇÃO MUNDIAL DA SAÚDE (OMS). Mulheres e saúde: evidências de hoje, agenda de amanhã. Genebra, 2011.

OTOMO-CORGEL J, STEINBERG BJ. IN: ROSE LF, GENCO RJ, MEALEY BL, COHEN DW, editor. Medicina periodontal e a mulher como paciente. Medicina Periodontal. São Paulo: Livraria Editora Santos; 2002. p.83-98.

OZBASAR, D.; TOROS, U.; OZKAYA, O.; et al. Raloxifene decreases serum malondialdehyde and nitric oxide levels in postmenopausal women with end-stage renal disease under chronic hemodialysis therapy. **Journal of Obstetrics and Gynaecology Research**. v. 36, p. 133–7, 2010.

ÖZDEN, S. et al. The effects of hormone replacement therapy on lipid peroxidation and antioxidant status. **Maturitas**. v. 38, p. 165-170, 2001.

PAJOVIĆ, S.B.; SAIČIĆ, Z.S. Modulation of antioxidant enzyme activities by sexual steroid hormones. **Physioogical Research**. v. 57, p. 801-11, 2008

PERRY, J.J.P.; SHIN, D.S.; GETZOFF, E.D.; TAINER, J.A. The structural biochemistry of the superoxide dismutases. **Biochimica et Biophysica Acta.** v. 1804, p. 245–262, 2010.

PINHO, R.A.; ANDRADES, M.E.; OLIVEIRA, M.R.; et al. Imbalance in SOD/CAT activities in rat skeletal muscles submitted to treadmill training exercise. **Cell Biology International**. v. 30, p. 848–853, 2006.

POLAC, I.; BOROWIECKA, M.; WILAMOWSKA, A.; NOWAK, P. Oxidative stress measured by carbonyl groups level in postmenopausal women after oral and transdermal hormone therapy. **American Journal of Obstetrics & Gynaecology**. v. 38, p.1177-81, 2012.

ROSSOUW, J.E. et al. Postmenopausal hormone therapy and risk of cardiovascular disease by age and years since menopause. **JAMA-Journal of the American Medical Association**.v. 297, n. 13, p. 1465-1477, 2007.

SÁNCHEZ-RODRÍGUEZ, M.A.; ZACARÍAS-FLORES, M.; ARRONTE-ROSALES, et al. Menopause as risk factor for oxidative stress. **Menopause.** v. 19, p. 361-7, 2012.

SANDUKJI, A. et al. Oxidative stress and bone markers in plasma of patients with long-bone fixative surgery: Role of antioxidants. **Human and Experimental Toxicology**. v. 30, n. 6, p. 435-442, 2011.

SCHUMACHER, M.; et al. Novel perspectives for progesterone in hormone replacement therapy, with special referece to the nervous system. Endocrune Reviews. v. 28, n. 4, p. 387-39, 2009.
SKOUBY, S.O.; JESPERSEN, J. Progestins in HRT: sufferance or desire? **Maturitas**. v. 20, n. 62(4), p. 371-5 2009.

SIES, H. Oxidative stress: introductory remarks. In: Sies, H., ed. **Oxidative stress**. London: Academic Press, 1985. p. 1-8.

SIES, H. What is oxidative stress? In: Keaney, J. F. Jr., ed. **Oxidative stress and vascular disease**. Boston: Kluwer Academic Publishers, 2000. p. 1-8.
SIGNORELLI, S.S. et al. Behavior of some indicators of oxidative stress in postmenopausal and fertile women. **Maturitas**, v.53, p. 77-82, 2005.

SITRUK-WARE, R. Pharmacological profile of progestins. **Maturitas.** v. 47, p. 277-83, 2004.

SOULES, M.R.; SHERMAN, s.; PARROT, E.; et al. Stages of Reproductive Aging Workshop (STRAW). **Journal of Womens Health Gender-Based Medical**. v. 10, p.843-48, 2001.

STADTMAN, E.R.; LEVINE, R.L. Protein oxidation. Reactive oxygen species: From radiation to molecular biology. **Annals of the New York Academy of Sciences**. v. 899, p. 191-208, 2000.

ST CLAIR, D.; ZHAO, Y.; CHAISWING, L.; OBERLEY, T. Modulation of skin tumorigenesis by SOD. **Biomedicine and Pharmacotherapy**. v. 59, n. 4, p. 209–214, 2005.

STEVENSON, J.C. et al. HRT and cardiovascular disease. **Best Practice & Research Clinical Obstetric and Gynaecology**. v. 23, p. 109-120, 2009.

STREHLOW, K. et al. Modulation of antioxidant enzyme expression and function by estrogen. **Circulation Research**. v. 93, p. 170-177, 2003.

SUBBIAH, M.T.R. et al. Antioxidant potential of specific estrogens on lipid peroxidation. **Journal of Clinical Endocrinology and Metabolism**. v. 77, p. 1095-1097, 1993.

SUGINO, N. et al. Differential regulation of copper-zinc superoxide dismutase and manganese superoxide dismutase by progesterone withdrawal in human endometrial stromal cells. **Molecular Human Reproduction**. v. 8, n. 1, p. 68-74, 2002.

TCHERNOF, A.; LABRIE, F.; LANGER, A.B.; PRUD'HOMME, D.; BOUCHAR, C.; TREMBLAY, A.; et al . Relationships between endog-enous steroid hormone, sex hormone-binding globulin and lipoprotein levels in men: contribution of visceral obesity, insulin levels and other metabolic variables . **Atherosclerosis.** v. 133, p. 235 – 244, 1997.

THIBODEAU, P.A. et al. *In vitro* pro- and antioxidant properties of estrogens. **Journal of Steroid Biochemistry and Molecular Biology**. v. 81, p. 227-236, 2002.

TONIOLO, P.G. et al. A prospective study of endogenous estrogens and breast cancer in postmenopausal women. **Journal of The National Cancer Institute**. v. 87, p. 190-197, 1995.

TRANQUILLI, A.L. et al. Transdermal estradiol and medroxyprogesterone antioxidants. **Gynecology and Endocrinology**. V. 9, p. 137-141, 1995.

TREVISAN, M. et al. Correlates of markers of oxidative status in the general population. **American Journal of Epidemiology**. v. 154, p. 348-356, 2001.

TURGEON JL, MCDONNELL DP, MARTIN KA, WISE PM. Hormone therapy: physiological complexity belies therapeutic simplicity. **Science**. v. 304, n. 5675, p.1269–73, 2004.

UNFER, T.C. et al. Influence of hormone replacement therapy on blood antioxidant enzymes in menopausal women. **Clinica Chimica Acta**. v. 369, p. 73-7, 2006.

VANKRIEKEN, L.; VAN DER, HORST FAL.; CASTRACANE, V.D. An international multicenter reference range study of normal men-strual cycles using IMMULITE ® reproductive hormone assays. **Clinical Chemistry**. v. 45, p. A92, 1999.

WAN, X.S.; DEVALARAJA, M.N.; St CLAIR, D.K. Molecular structure and organization of the human manganese superoxide dismutase gene. **DNA and Cell Biology**. V. 13, p. 1127-36, 1994.

WASSMANN, K.; WASSMANN, S.; NICKENIG, G. Progesterone antagonizes the Vasoprotective effect of estrogen on antioxidant enzyme expression and function. **Circulation Research**. v. 97, p. 1046-54, 2005.

WEATHERMAN, R.V.; FLETTERICK, R.J.; SCANLAN, T.S. Nuclear-receptor ligands and ligand-binding domains . **Annual Review of Biochemistry**. v. 68, p. 559 – 581, 1999.

WHI - Writing Group for the Women's Health Initiative Investigators (WGWHIII). Risk and benefits of estrogen plus progesterone in healthy postmenopausal women. **JAMA-Journal of the American Medical Association.** v. 288, p. 321-33, 2002.

WHI - Writing Group for the Women's Health Initiative Investigators (WGWHIII). Postmenopausal hormonal therapy and risk of cardiovascular disease by age and years since menopause. **JAMA - Journal of the American Medical Association.** v. 297, n. 13, p. 1465-77, 2007.

WHITE, R.E.; GERRITY, R.; BARMAN, S.A.; HAN, G. Estrogen and oxidative stress: A novel mechanism that may increase the risk for cardiovascular disease in women. **Steroids**. v. 7, p. 788–793, 2010.

WÓJCIK, M.; KOSIOR-KORZECKA, U.; BOBOWIEC, R. Cytoprotective action of 17β-oestradiol against iron-induced hepatic oxidative stress *in vitro*. **Bull Vet Inst Pulawy.**, v. 54, p. 259-263, 2010

WONG, C.M.; YUNG, L.M.; LEUNG, F.P.; TSANG, S.Y.; AU, C.L.; CHEN, Z.Y. Raloxifene protects endothelial cell function against oxidative stress. **British Journal of Pharmacology**. v. 155, p. 326–34, 2008.

YLÄ-HERTTUALA, S. Oxidized LDL and atherogenesis. **Heart in Stress Annals of The New York Academy of Sciences**. v. 874, p. 134-137, 1999.

7 ANEXOS

7.1 Anexo I

Questionário

Nome: _____

SAME (n° carteira do HUSM): _____

Endereço: _____

Cidade: _____ **Estado:** _____**CEP:** _____

Telefone para contato (pode ser de vizinho ou parente): _____

Olá, por favor, responda a todas as seguintes questões com muita atenção:

1. Qual sua data de nascimento (dd/mm/aaaa)? _____

2. Qual sua raça (etnia)? 1.( ) branca 2.( ) negra 3.( ) amarela

3. Como está sua menstruação:?

1.( ) normal 2.( ) irregular 3.( ) menopausa – quanto tempo (meses)?_____

4. Quantos filhos você têm? _____

5. Quanto tempo amamentou seus filhos? _____

6. Pressão arterial: _____

7. Fumante: 1.( ) Não 2.( ) Sim - nº de cigarros por dia? _____

8. Consume bebida alcoólica? 1.( ) Não 2.( ) Sim - nº de copos por semana:_____

9. Pratica exercícios físicos? 0.( ) Não Faz 1.( ) Leve 2.( ) Moderado 3.( ) Intenso

10. Você tem alguma doenças diagnosticadas:

( ) diabetes. Qual? _____( ) em tratamento

( ) doenças endócrinas (Tireóide). Qual (s)?_____ ( ) em tratamento

( ) doenças renais. Qual (s)? _____( ) já tratadas ( ) em tratamento

( ) doenças da medula óssea. Qual (s)? _____( ) em tratamento

( ) doenças cardíacas /ou vasculares (varizes)._____( ) já tratadas ( ) em tratamento

( ) doenças pulmonares. Qual (s)? _____( ) já tratadas ( ) em tratamento

( ) outras doenças. Qual (s)? _____( ) já tratadas ( ) em tratamento

Qual a medicação em uso (s)?

11. <u>Você faz uso de alguma Terapia de Reposição com Hormônios (ou Isoflavonas)</u>?

1. ( ) Não 2.( ) Sim – quanto tempo (meses)?_____

- Se for uso de tempo alternado especificar como faz o tratamento:_____
- **Nome comercial do medicamento de uso (s):** _____
- **Apresentação do medicamento de uso (s):**

1.( ) comprimido 2.( )cápsula 3.( ) gel vaginal 4.( ) adesivo 5.( ) injetável

Princípio ativo do medicamento de uso (s):

1.( ) estrogênios conjugados 2. ( ) estradiol 3.( ) estrog. + progesterona

4. ( ) progesterona 5.( ) isoflavonas

12. Você usa algum dos suplementos abaixo:

1.( )vitamina C 2.( )vitamina E 3.( )polivitamínicos e poliminerais 4.( )cálcio

5.( )cálcio+vit D 6. Sulfato ferroso

Quanto tempo?_____ Qual o nome do medicamento? _____

13. Usa ou usou medicamentos com Corticóides? 1.( ) Não 2.( ) Sim - Quanto tempo? ____

14. Tem algum dos problemas intestinais abaixo com frequência?

1. ( ) constipação – intestino preso 2.( ) diarréia

15. Consome regularmente algum (s) desses alimentos abaixo citados? Com que freqüência?

( ) Leite (n° copos por dia): _____( ) Leite em pó (nº de colheres por dia): _____

( ) Iogurte (unidades por dia): _____( ) Queijo (nº fatias por dia): _____

( ) Peixes (sardinha, linguado...) (porções por semana): _____

( ) Vegetais verdes escuros (porções por dia): _____

( ) Fibras como aveia, linhaça, granola (pães, biscoitos...) (porção por dia):_____

( ) Soja (porções por semana): _____

( ) laranja/limão/morango/abacaxi (n° por dia): _____ ( ) maçã/pêra(n° por dia): _____ ( )uva(n° por dia): _____ ( ) outras frutas, quais? _____

( )Chás de frutas (n° xíc/dia): _____ ( )Chá de ervas (n° xíc/dia): _____

( )Chimarrão (n° de cuias/dia): _____

Avaliações antropométricas (para cálculo do índice de massa corporal – IMC):

17. Qual o seu peso (kg)? _____

18. Qual a sua altura? _____

Muito obrigada por sua colaboração, entraremos em contato.

(Taís - 55 3220 8547).

7.2 Anexo II:

TERMO DE CONSENTIMENTO LIVRE E ESCLARECIDO

Você está sendo convidada a participar de uma pesquisa intitulada "INFLUÊNCIA DAS TERAPIAS DE REPOSIÇÃO HORMONAL SOBRE O ESTATUS OXIDATIVO EM MULHERES", que tem como objetivo geral avaliar a influência dos hormônios estrogênios e progesterona, sobre o estresse oxidativo no sangue de mulheres na menopausa. Este projeto é importante para verificar se esses hormônios poderiam evitar o estresse oxidativo que ocorre na menopausa, e que parece contribuir para a ocorrência de diversas doenças, tais como osteoporose, doenças cardiovasculares e neurodegenerativas, nessa fase da vida da mulher.

O estudo será desenvolvido nos Ambulatórios de Reumatologia e do Climatério (HUSM), no Laboratório de Análises Clínicas (LAC-HUSM), no Núcleo Integrado de Desenvolvimento de Análises Laboratoriais (NIDAL), do Departamento de Tecnologia e Ciência dos Alimentos e no Laboratório de Absorção Atômica (Departamento de Química) da Universidade Federal de Santa Maria, e envolverá mulheres do município de Santa Maria – RS.

O protocolo de estudo obedece à resolução CNS 196/96 e foi aprovado pelo Comitê de Ética em Pesquisa da Universidade Federal de Santa Maria, sob o n° 23081.015517/2006-40 e CAAE n° 0120.0.243.000-06.

Procedimentos a serem realizados

Você deverá responder a um questionário e realizar apenas uma coleta de sangue (cerca de 15 mL), a qual será marcada com antecedência e realizada em laboratório de análises clínicas, sem qualquer custo. O sangue será destinado para análise dos hormônios, da atividade de enzimas antioxidantes, do estatus oxidativo e de lipídios, entre outras medidas bioquímicas. Será mantida a confidencialidade dos dados obtidos com o questionário, e estes serão armazenados por um período de no máximo 48 meses, enquanto que o sangue será armazenado por no máximo 24 meses para a realização de todas as análises previstas. Após este período, os questionários e o material excedente serão descartados seguindo-se as normas de segurança adequadas. Você terá livre acesso aos resultados dos seus exames de creatinina, cálcio sanguíneo, ferritina, hematócrito, ácido úrico, HDL, LDL, colesterol total, triglicerídeos, atividade das enzimas aspartato aminotransferase, alanina aminotransferase, estradiol, progesterona e hormônio folículo estimulante, recebendo orientação para o encaminhamento médico em caso de resultados fora dos padrões de referência.

Riscos individuais e possibilidade de exclusão

As pacientes que voluntariamente se submeterem a responder o questionário e às coletas de sangue estarão sujeitas aos riscos mínimos, como os envolvidos em qualquer procedimento de coleta de sangue, isto é, poderão, em casos de coleta mal realizada, desenvolver flebite, flebotrombose, hematoma local e petéquias, que serão tratados, sem nenhum custo, pelo médico que a encaminhou.

Você poderá se recusar a participar do projeto ou retirar o seu consentimento em qualquer momento da pesquisa, sem penalização alguma e sem nenhum prejuízo ao seu cuidado médico habitual.

Confidencialidade

Será mantida a confidencialidade sobre toda e qualquer informação obtida a partir dos questionários e/ou das análises do sangue. A divulgação dos dados obtidos na presente pesquisa preservará a identidade dos (as) pacientes sujeitos (as) da pesquisa.

Telefones para contato com os pesquisadores

Prof. Dra. Tatiana Emanuelli – Departamento de Tecnologia e Ciência dos Alimentos – CCR

(55) 3220 8547

Dr. João Carlos Nunes da Silva – Dept. de Clínica Médica – CCS – UFSM

(55) 3220 8544

Dra. Clarice Mottecy – Dept. de Clínica Médica – CCS – UFSM

(55) 3220 8552

Farmacêutica Taís Cristina Unfer – Doutoranda do Programa de Pós Graduação em Farmacologia – UFSM

(55) 9933 4888 e (55) 3220 854

Identificação e consentimento do (a) paciente

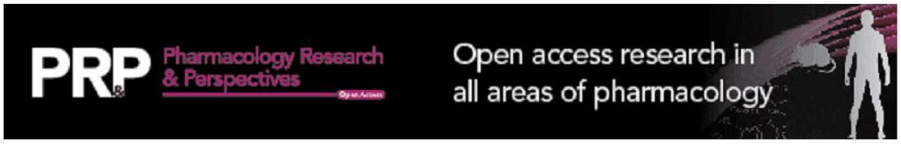
Nome: _____RG (número da identidade): _____

Assinatura: _____

Santa Maria, _____ de _____de_____.

7.3 Anexo III

Guia para autores – Basic & Clinical Pharmacology & Toxicology



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DOI reference
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