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Efeitos da suplementação com vitamina A sobre parâmetros bioquímicos, de estresse oxidativo e comportamentais em modelo animal de menopausa por ovariectomia bilateral

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“A vida ensina a todos,

poucos são aqueles que aprendem.”

(Guilherme A. Behr)

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PARTE I

RESUMO

Introdução: A caracterização de alterações metabólicas, associadas ao aumento nos níveis de estresse oxidativo, em mulheres na menopausa ganhou força na última década. Alterações no estado redox similares às observadas em mulheres durante o declínio da atividade hormonal do ovário podem ser obtidas experimentalmente pela ovariectomia bilateral de ratas. A busca por tratamentos alternativos que possibilitem uma melhora na qualidade de vida da mulher na menopausa é realmente de grande importância. **Objetivos**: Os objetivos desta tese foram: I – determinar os parâmetros de estresse oxidativo, bioquímicos e de comportamento que distingam ratas Wistar bilateralmente ovariectomizadas (OVX) de ratas *sham* (controle); II – investigar os efeitos da suplementação com palmitato de retinol (vitamina A) nas doses de 500 ou 1500 UI/kg/dia, durante 30 dias, nos parâmetros previamente citados, com foco no sistema nervoso central (SNC) e sangue/plasma. **Resultados I**: A ovariectomia causou aumento no ganho de peso corporal e pronunciada atrofia uterina. Diminuição de triglicerídeos, aumento nos níveis de colesterol total, e redução no teor de ácido úrico no plasma. Verificamos aumento nas atividades das enzimas glutationa peroxidase (GPx) e catalase (CAT) no sangue. Diminuição nas defesas antioxidantes totais não enzimáticas (TRAP e TAR) e nos níveis de tióis proteico e não-proteico no plasma, acompanhado pelo aumento no dano oxidativo às proteínas (carbonil). Observamos também que a relação nas enzimas superóxido dismutase/catalase (razão SOD/CAT) encontra-se aumentada no hipotálamo e córtex frontal, o conteúdo de tióis totais (SH) diminuído no hipocampo, acompanhado de aumento na lipoperoxidação (TBARS) em córtex frontal, nas ratas OVX. Além disso, as ratas OVX apresentaram atividade locomotor/exploratória diminuída nos testes de comportamento. **Resultados II**: Em uma primeira análise, a suplementação com vitamina A foi capaz de melhorar a capacidade antioxidante em ratas OVX, restaurando as defesas tanto enzimáticas quanto não enzimáticas no plasma, e promovendo a redução nos níveis de dano oxidativo às proteínas plasmáticas. Entretanto, em uma segunda análise, a suplementação com vitamina A diminuiu o comportamento exploratório e o teor de SH no hipocampo em ratas *sham*; promoveu o aumento na relação SOD/CAT no hipocampo, diminuiu o potencial antioxidante total no hipocampo em ambos os grupos *sham* e OVX, além de promover aumento nos níveis de TBARS em córtex frontal nas ratas OVX. **Conclusão**: Mesmo que a suplementação com vitamina A tenha apresentando efeitos antioxidantes em plasma, a mesma acaba por induzir um estado pró-oxidante em regiões cerebrais de ratas OVX. Este trabalho relata pela primeira vez que a vitamina A em doses relativamente baixas pode desencadear efeitos completamente distintos dependendo dos tecidos estudados, sugerindo que deve haver cautela em relação ao uso de suplementos com vitamina A durante a menopausa.

ABSTRACT

Introduction: Menopause has been reported to be associated with increased oxidative stress and metabolic disorders among women worldwide. Disarrangements in the redox state similar to those observed in women during the decline of ovarian hormonal activity can be obtained experimentally through rat bilateral ovariectomy. The search for alternative treatments to improve life quality in postmenopausal woman is really important. **Aims of study**: I – to evaluate the oxidative stress, biochemical and behavioral parameters that distinguish sham-operated female rats from Wistar rats bilaterally ovariectomized (OVX). II – to investigate the effects of retinol palmitate (a vitamin A) supplementation (500 or 1500 IU/kg/day, during 30 days) on behavioral parameters, brain structures and blood/plasma redox profiles. **Results I**: Ovariectomy caused an increase in body weight gain, pronounced uterine atrophy, decreased the plasma triglycerides and the uric acid content, but increased the total cholesterol levels. We found that blood peroxidase activities (catalase and glutathione peroxidase) where increased, plasma non-enzymatic antioxidant defenses (TRAP and TAR), and protein and non-protein SH levels where found to be decreased, which was accompanied by an enhancement on protein oxidative damage (carbonyl). Moreover, we observed that increased hypothalamic and frontal cortex superoxide dismutase/catalase (SOD/CAT) ratio where associated with decreased hippocampal thiol content, and accompanied by an increased frontal cortex lipid oxidative damage (TBARS) in OVX rats. Also, ovariectomy affected the locomotor/exploratory activity which was observed in the behavioral tests. **Results II**: In a first analysis, vitamin A supplementation was capable to ameliorate antioxidant status in OVX rats, restoring both enzymatic and non-enzymatic defenses, and decreasing protein oxidative damage levels in plasma. However, in a second analysis, vitamin A supplementation decreased locomotor/exploratory behavior and total hippocampal thiol content in sham-operated rats, increased hippocampal SOD/CAT ratio and decreased total antioxidant potential in the hippocampus on both sham and OVX groups, and increased cortical TBARS levels in OVX rats. **Conclusion**: Even though vitamin A supplementation can act as an antioxidant in plasma, it can also induce a pro-oxidant status in determined brain regions of OVX rats. This is the first research in the literature showing that relatively low vitamin A doses have completely different effects depending on the studied tissue, suggesting that some caution need to be taking when regarding the use of vitamin A supplementation during menopause.

LISTA DE ABREVIATURAS

ALT – Alanina aminotransferase

ASC – Área de superfície corporal

AST – Aspartato aminotransferase

CA – Campo aberto

CAT – Catalase

EC-SOD – Superóxido dismutase extra-celular

ERO – Espécies reativas de oxigênio

GPx – Glutationa peroxidase

HDL – Lipoproteína de alta densidade (do Inglês: high density lipoprotein)

iNOS – Óxido nítrico sintase induzível

LCE – Labirinto em cruz elevada

LDL – Lipoproteína de baixa densidade (do Inglês: low density lipoprotein)

MDA – Malondialdeido

$O_2^{\bullet-}$ – Radical superóxido

OVX – Ovariectomia, ovariectomizadas

SH – Sulfidril, tióis, tiol

SNC – Sistema nervoso central

SOD – Superóxido dismutase

TAR – Reatividade antioxidante total (do Inglês: Total antioxidant reactivity)

TBARS – Espécies reativas ao ácido tiobarbitúrico (do Inglês: Thiobarbituric acid reactive species)

TRAP – Potencial antioxidante total não enzimático (do Inglês: Total radical antioxidant potential)

TRH – Terapia de reposição hormonal

VLDL – Lipoproteína de baixíssima densidade (do Inglês: very low density lipoprotein)

APRESENTAÇÃO

Os dados aduzidos nesta tese sugerem alguma cautela quanto à suplementação com vitamina A em mulheres na menopausa. Além disso, demonstram que o perfil redox apresentado em tecidos periféricos nem sempre reflete no mesmo perfil obtido para o sistema nervoso central.

Neste estudo, avaliamos especificamente as alterações provocadas pela suplementação com vitamina A em modelo animal de menopausa, quanto a parâmetros de estresse oxidativo e comportamentais. Os "Materiais e Métodos" e os "Resultados" estão apresentados na forma de artigo científico publicado em revista internacional.

No 1º artigo, avaliamos alterações bioquímicas e no perfil redox de sangue e plasma. No 2º artigo, o foco foram as estruturas do sistema nervoso central, hipocampo, hipotálamo e córtex frontal, além da avaliação comportamental dos animais.

O capítulo "Discussão" apresenta a interpretação dos resultados obtidos nessa Tese, apoiada na literatura científica corrente. Por fim, são expostas as conclusões desta Tese.

No capítulo "Referências Bibliográficas" estão listadas aquelas citadas nos capítulos "Introdução" e "Discussão". Os anexos correspondem a dados gerados durante o período de desenvolvimento desta Tese e que não fazem parte do corpo principal da mesma.

1 – INTRODUÇÃO

1.1 – Menopausa: um processo inevitável

Ao longo dos últimos cem anos a expectativa de vida humana em países desenvolvidos aumentou significativamente e mais pessoas estão vivendo vidas longas. Portanto, é notável o aumento do número de mulheres que experienciam a menopausa, um período que se estende por quase um terço de suas vidas. A menopausa é um processo inevitável que afeta geralmente mulheres entre as idades de 40 e 60 anos e assinala o fim da fase fértil da vida da mulher. Após 12 meses de ausência permanente da menstruação a mulher é considerada na menopausa (Miquel *et al*., 2006). Mulheres na menopausa experimentam uma grande variedade de alterações fisiológicas (o climatério), principalmente associadas com a cessação da secreção de hormônios sexuais. É inequívoco o papel fundamental dos hormônios femininos estradiol, progesterona, LH e FSH, tanto por sua ação reguladora no ciclo reprodutivo feminino, como por diversas atividades específicas de cada hormônio. Na verdade, hormônios sexuais femininos têm importantes funções biológicas benéficas conhecidas, tais como, controlando episódios depressivos (Graziottin & Serafini, 2009), reduzindo o fator de risco para complicações da artéria coronária e doenças cardiovasculares (Phillips *et al*., 1997; Stice *et al*., 2009), e também na qualidade de antioxidantes endógenos (Yagi, 1997; Huang *et al*., 1999; Agarwal *et al*., 2008).

De acordo com o cenário descrito aqui, não é difícil visualizar a complexidade metabólica apresentada por mulheres menopáusicas, que exibem alterações importantes em cascatas de sinalização e vias metabólicas. Os sintomas comuns da menopausa incluem: distúrbios de humor e cognição, sintomas vasomotores (ondas

de calor, ou calorões), atrofia vaginal e de útero, e perturbações do sono (Pinkerton *et al*., 2009).

1.1.1 – Estresse oxidativo na menopausa

O estresse oxidativo tem sido definido como um desequilíbrio entre a produção de espécies reativas de oxigénio (ERO) e aumento inadequado nas defesas antioxidantes, um estado caracterizado por uma sobrecarga em oxidantes, podendo culminar em disfunção celular (Halliwell, 2007). Estas espécies reativas são geradas continuamente em condições fisiológicas e efetivamente controladas/eliminadas por sistemas antioxidantes intracelulares e extracelulares. A literatura fornece evidências do papel do estresse oxidativo afetando toda a vida reprodutiva da mulher, mesmo na menopausa. Estudos anteriores demonstram que o estresse oxidativo está envolvido em diversos processos relacionados com o aumento da idade e que muitas vezes acompanham a menopausa (Agarwal *et al*., 2008; Miquel *et al*., 2006; Sanchez-Rodriguez *et al*., 2011).

A transição da menopausa é um período de grande vulnerabilidade para episódios depressivos, particularmente entre as mulheres com histórico de transtorno de humor (Harlow *et al*., 2003). Para além da conhecida relação entre a menopausa e estresse oxidativo, transtornos de humor têm sido associados com grandes alterações no perfil redox (Berk *et al*., 2011; Moylan *et al*., 2012.). Também tem sido sugerido que o aumento no dano a lipídios, proteínas e DNA em estruturas cerebrais associadas à resposta ao stress e emoção podem contribuir para diversas patologias. De fato, evidências sugerem que a perda de regulação entre áreas do sistema nervoso central (SNC) pode contribuir para diversas doenças mentais (Frey

et al., 2010), e uma disfunção na sinalização redox do SNC pode apresentar grande influência sobre tais patologias.

1.1.2 – Tratamentos para a menopausa: tradicional

Notavelmente, um considerável número de estudos epidemiológicos demonstra que mulheres apresentam um risco elevado de desenvolver depressão e ansiedade durante a transição para a menopausa, e a reposição transdérmica de estradiol foi demonstrada ser eficaz no tratamento da depressão durante esta transição (Frey *et al*., 2008). No entanto, nem todas as mulheres na pós-menopausa são boas candidatas para fazer uso da terapia de reposição hormonal (TRH), como, por exemplo, aquelas com maior risco de coagulação sanguínea ou histórico de câncer de mama.

Mais importante, algumas mulheres são relutantes em utilizar a TRH devido ao possível risco aumentado de câncer de mama, derrame e/ou complicações cardiovasculares (Jensen *et al*., 2010). Diversas linhas de estudo sugerem que o estrogênio pode ter tanto efeitos protetores como nocivos, dependendo do momento na iniciação da TRH, idade, tipo de menopausa (natural versus cirúrgica), ou fase da menopausa (Rocca *et al*., 2011). Recentemente, um estudo demonstrou que uma em cada duas mulheres na pós-menopausa, que haviam interrompido a TRH, optaram por terapias alternativas para tratar sintomas relacionados à menopausa (Kupferer *et al*., 2009).

1.1.3 – Tratamentos para a menopausa: alternativas

Uma série de estratégias de tratamento têm sido propostas como alternativas à TRH tradicional para as mulheres na pós-menopausa, incluindo suplementos de vitaminas, minerais, antioxidantes, utilização de ervas (chás), além de antidepressivos (Borrelli & Ernst, 2010; Dennehy & Tsourounis, 2010; Freeman *et al*., 2011; Thompson, 2010; Wong *et al*., 2009). É reconhecido que uma dieta equilibrada com quantidades adequadas de vitaminas, minerais e outros nutrientes, representa um papel importante na prevenção e tratamento de doenças cardiovasculares, osteoporose, obesidade, diabetes, câncer, depressão e diversos outros sintomas relacionados com a menopausa (Hagey & Warren, 2008). Neste contexto, a ingestão adequada de vitaminas é essencial e a suplementação pode ser recomendada para algumas mulheres na pós-menopausa (Dennehy & Tsourounis, 2010; Ziaei *et al*., 2007).

A suplementação com vitaminas em mulheres na pós-menopausa pode ser benéfica para a saúde dos ossos, a saúde cardiovascular, o risco de câncer de mama, cognição e sintomas vasomotores (Dennehy & Tsourounis, 2010). A suplementação com vitamina C, D, K, e de cálcio tem sido recomendada em alguns casos para manutenção do metabolismo ósseo adequado. Até hoje, o único suplemento vitamínico estudado que auxilia na melhora dos sintomas vasomotores durante a menopausa é a vitamina E, entretanto seus benefícios clínicos ainda não estão bem conhecidos (Ziaei *et al*., 2007; Biglia *et al*., 2009).

1.1.4 – Tratamentos para a menopausa: por que utilizar a vitamina A?

A vitamina A pertence ao grupo das vitaminas lipossolúveis. Por definição, as vitaminas não são sintetizadas *de novo* em quantidades suficientes pelo nosso organismo, devendo assim serem obtidas através da dieta. Pode estar presente nos

alimentos tanto como pré-vitamina A, na forma de retinol ou como éster de retinol, provenientes de alimentos de origem animal; ou como pró-vitamina A proveniente de alimentos de origem vegetal, e neste caso encontra-se na forma de carotenóides que serão convertidos a vitamina A pelo organismo (Banaszak et al., 1994; Napoli, 1999). Entretanto, a vitamina A pré-formada é absorvida e utilizada de forma mais eficiente pelo nosso organismo, possuindo taxas de absorção entre 70-90%, enquanto a pró-vitamina A possui taxas de absorção entre 20–50%, podendo estas taxas dependerem dos níveis de vitamina A de cada indivíduo entre outros fatores nutricionais e não-nutricionais (Penniston & Tanumihardjo, 2006). A vitamina A e os retinóides, compostos naturais e sintéticos possuidores de uma estrutura química ou propriedades funcionais similares à vitamina A, são importantes em diversos processos biológicos, incluindo a reprodução, a regulação do sistema imune e a visão, assim como a manutenção do crescimento e da diferenciação celular (Wasserman & Corradino, 1971). Em um artigo bastante interessante, Koda *et al*. (2007) relatam os efeitos antiestrogênicos para o tratamento agudo por três dias com ácido retinóico (metabolito da vitamina A) em ratas ovariectomizadas.

A eficácia clínica da vitamina A no tratamento da menopausa ainda não é clara, em particular no que diz respeito à função cardiovascular, câncer da mama, e no desempenho cognitivo em mulheres pós-menopáusicas. Com relação à saúde óssea, a ingestão elevada de vitamina A por 18 anos está associada com aumento no risco de fraturas por osteoporose em mulheres na pós-menopausa (Feskanich *et al*., 2002). No entanto, em um estudo posterior não foi encontrada associação entre a ingestão de vitamina A ou retinol e o risco aumentado de fraturas de quadril ou totais (Caire-Juvera *et al*., 2009).

Tanto os efeitos benéficos e danosos da vitamina A, atribuíveis à sua atividade redox, vem sendo relatados. Trabalhos realizados pelo nosso grupo, e que posteriormente foram corroborados por outros autores, demonstram que a suplementação de vitamina A (ou sua deficiência) pode apresentar efeitos antioxidantes ou pró-oxidantes, dependendo da dose, do tecido-alvo, e do modelo experimental estudado (Behr *et al*., 2012a,b; de Oliveira *et al*., 2009a,b,c; Gatica *et al*., 2005; Pasquali *et al*., 2009; Schnorr *et al*., 2011a,b).

1.2 – Modelos animais para estudo da menopausa

Os modelos animais utilizados em pesquisa tentam mimetizar uma doença ou condição, pré-existente ou induzida, semelhante a um estado humano, com a finalidade de melhor entender a doença/condição ou estudar um novo tratamento, terapia, ou estratégia (Behr *et al*., 2008; da Rocha *et al*., 2010; Zapelini *et al*., 2008). Modelos experimentais de menopausa são amplamente utilizados para fins de pesquisa, e uma variedade de diferentes metodologias é apresentada na literatura. As duas formas mais comuns para induzir sintomas semelhantes à menopausa em animais experimentais são por procedimentos cirúrgicos (ovariectomia, com a interrupção drástica na secreção hormonal) e por indução química (com perda progressiva da função do ovário) (Acosta *et al*., 2009; Wright *et al*., 2008). Tem sido demonstrado também que cada modelo desenvolve características bioquímicas diferentes em roedores. Desta forma, é possível optar por um ou outro modelo de acordo com suas aplicações específicas dentro da pesquisa desejada.

1.2.1 – Procedimento cirúrgico: ovariectomia

O procedimento cirúrgico mais estudado e melhor caracterizado para induzir a menopausa experimental em ratos e camundongos é a ovariectomia bilateral. Este procedimento torna possível, em um curto espaço de tempo, a aquisição de ratos fêmea desprovidas de secreção hormonal ovariana. Além disso, ratas ovariectomizadas (OVX) mostram maior risco de apresentar sintomas de osteoporose (Muthusami *et al*., 2005), hipertrofia cardíaca (Bhuiyan & Fukunaga, 2010), disfunções cardiovasculares importantes (Lee *et al*., 2008), atrofia uterina (Goss *et al*., 2007), aumento na temperatura da pele da cauda (Bowe *et al*., 2006), diminuição na concentração plasmática de vitaminas A , C, e E (Dilek *et al*., 2010), e um desequilíbrio entre a produção de radicais livres e os níveis de defesas antioxidantes, o que resulta no aumento do estresse oxidativo e, consequentemente, uma aceleração do processo de envelhecimento em diferentes tecidos (Agarwal *et al*., 2005; Behr *et al*., 2012a,b; Lee *et al*., 2005; Muthusami *et al*., 2005).

O ciclo reprodutivo das ratas é chamado de ciclo estral, e a duração deste ciclo é em média de 4-5 dias (Marcondes *et al*., 2002). Dois meses na vida reprodutiva de ratas Wistar representam entre 12-15 ciclos estrais. Nas mulheres, este número de episódios de ovulação corresponde a aproximadamente um ano de vida reprodutiva. Após um ano de perda permanente da menstruação, a mulher é considerada na menopausa. Sendo assim, o modelo experimental utilizado em nosso trabalho é semelhante a um período da menopausa humana precoce, já que iniciamos o tratamento com vitamina A 60 dias após a ovariectomia bilateral realizada.

1.2.2 – Ratas OVX: seria este um bom modelo?

Até hoje, modelos experimentais para estudo da menopausa são em grande parte baseados na indução cirúrgica ou química, e nenhum deles representa a falha progressiva da função ovariana que ocorre na menopausa natural (Deecher *et al*., 2008; Miquel *et al*., 2006). A transição para a menopausa pode ocorrer ao longo de um período de 5 a 15 anos. Ainda no início, durante a perimenopausa, os ciclos menstruais são geralmente mais frequentes e caracterizam-se por flutuações mais extremas nos níveis de estrogênio. Mais tarde, na transição da menopausa, os ciclos tornam-se imprevisíveis e diminuem em número, expondo as mulheres a períodos progressivamente mais longos de supressão na secreção de estrogênio. Um ano de ausência de estrogénio marca o início do período da menopausa chamado menopausa precoce (Deecher *et al*., 2008).

Embora amplamente utilizadas, ratas OVX são problemáticas no que diz respeito à reprodução dos efeitos da transição na menopausa natural. A ovariectomia produz uma cessação rápida e dramática da função ovariana, em vez de um declínio gradual que ocorre durante a perimenopausa. Recentemente, a administração de produtos químicos como o 4-vinilciclohexano diepóxido foi descrita como indutor da perda de função dos ovários (Acosta *et al*., 2009). Mas este é um modelo experimental farmacológico e não fisiológico. Com o uso de quaisquer alterações fisiológicas induzidas por drogas, cuidados devem ser tomados para assegurar que a droga não venha a ter efeitos secundários sobre o tecido alvo e outros.

1.3 – Menopausa, estresse oxidativo e vitamina A

Alguns trabalhos na literatura têm focado nos efeitos da vitamina A em mulheres na menopausa, outros avaliam o perfil de estresse oxidativo observado durante a transição para a menopausa. Entretanto, até hoje, este trabalho é o primeiro que busca relacionar menopausa, parâmetros de estresse oxidativo e suplementação com vitamina A (Behr *et al*., 2012a,b).

Para isto, neste trabalho determinamos os parâmetros bioquímicos, de estresse oxidativo (marcadores de função antioxidante e de dano oxidativo) e comportamentais que distinguem ratas OVX de ratas controle, além de promover a investigação dos efeitos da suplementação com palmitato de retinol (vitamina A) nas doses de 500 ou 1500 IU/kg/dia, durante 30 dias, nos parâmetros previamente citados, com foco prioritário em sangue/plasma e estruturas do SNC (hipocampo, hipotálamo e córtex frontal).

2 – OBJETIVO

Considerando o exposto na Introdução desta tese, este trabalho tem como objetivo elucidar as possíveis alterações bioquímicas e comportamentais provenientes da suplementação com vitamina A (palmitato de retinol - Arovit®) nas doses de 500 ou 1500 UI/kg por 30 dias em modelo animal de menopausa.

2.1 – Objetivos específicos

1 – Avaliar as alterações bioquímicas em sangue que diferenciam ratas OVX de ratas controle (*sham*);

2 – Quantificar os parâmetros de estresse oxidativo (marcadores de função antioxidante e de dano oxidativo) e comportamentais (locomoção/exploração e ansiedade) que diferenciam ratas OVX de ratas controle, com foco em sangue/plasma e estruturas do SNC (hipocampo, hipotálamo e córtex frontal);

3 – Realizar o tratamento com palmitato de retinol (vitamina A) por 30 dias no modelo animal proposto e analisar as possíveis alterações bioquímicas e em parâmetros de estresse oxidativo, nas estruturas previamente citadas;

4 – Observar as alterações comportamentais provenientes do tratamento com vitamina A; a avaliação do comportamento dos animais após o período de tratamento, foi realizada através dos experimentos de campo aberto e labirinto em cruz elevada.

5 – Comparar os dados experimentais obtidos em sangue/plasma com os dados obtidos nas estruturas do SNC (hipocampo, hipotálamo e córtex frontal);

PARTE II

3 – ARTIGOS CIENTÍFICOS

3.1 – Artigo 1

Increased blood oxidative stress in experimental menopause rat model: the effects of vitamin A low-dose supplementation upon antioxidant status in bilateral ovariectomized rats.

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ORIGINAL ARTICLE

Increased blood oxidative stress in experimental menopause rat model: the effects of vitamin A low-dose supplementation upon antioxidant status in bilateral ovariectomized rats

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ABSTRACT

Menopause has been reported to be associated with increased oxidative stress and metabolic disorders among women worldwide. Disarrangements in the redox state similar to those observed in women during the decline of ovarian hormonal activity can be obtained experimentally through rat bilateral ovariectomy. The search for alternative treatments to improve life quality in postmenopausal woman is really important. The aim of this study was to evaluate biochemical and oxidative stress parameters that distinguish sham-operated female rats from Wistar rats bilaterally ovariectomized (OVX). Additionally, we have also investigated the effects of retinol palmitate (a vitamin A supplement) low-dose supplementation (500 or 1500 IU/kg/day, during 30 days) upon blood and plasma antioxidant status in OVX rats. Ovariectomy caused an increase in body weight gain, pronounced uterine atrophy, decreased plasma triglycerides and increased total cholesterol levels, and reduced acid uric content. Moreover, we found increased blood peroxidase activities (catalase and glutathione peroxidase), decreased plasma non-enzymatic antioxidant defenses total reactive antioxidant potential and total antioxidant reactivity, decreased protein and non-protein SH levels, accompanied by increased protein oxidative damage (carbonyl). In addition, vitamin A low-dose supplementation was capable to ameliorate antioxidant status in OVX rats, restoring both enzymatic and non-enzymatic defenses, promoting reduction in plasma SH content, and decreasing protein oxidative damage levels. This is the first work in the literature showing that vitamin A at low dose may be beneficial in the treatment of menopause symptoms. Further studies will be made to better understand the effects of vitamin A supplementation in menopause rat model.

INTRODUCTION

Over the last hundred years, the average human lifespan in developed countries has greatly increased and more people are living long lives. Therefore, an increased number of women experienced menopause, a period that extends for almost one-third of their lives. Menopause is an unavoidable process that usually affects women between the ages of 40 and 60 years and signals the end of the fertile phase of a woman's life. After 12 months of permanent loss of menstruation, a woman is considered to be in menopause [1]. Menopausal

women are going to experience a wide variety of physiological changes (the climacteric or climacterium) principally associated with the cessation in sexual hormone secretion. In fact, female sexual hormones have important known benefic biologic functions, such as, controlling depressive episodes [2], reducing risk factor for coronary artery and general cardiovascular diseases [3,4], and also acting as endogenous antioxidants [5–7].

According to the scenario described here, it is not difficult to visualize the metabolic complexity presented by menopausal women, showing important alterations in signaling cascades and metabolic pathways. Common menopausal symptoms include mood and cognition disturbances, vasomotor symptoms (i.e. hot flushes), vaginal and uterine atrophy, and sleep disruption [8]. The literature provides evidence of oxidative stress affecting the entire reproductive lifespan of a woman, even menopause. More recently, many studies suggested the involvement of free radicals and oxidative stress in aging and some age-related processes that often accompany menopause [1,9]. Increased production of reactive oxygen species (ROS) is considered to be one of the major causes of several age-related diseases. These species are continuously generated in physiological conditions and effectively controlled/eliminated by intracellular and extracellular antioxidant systems. Oxidative stress has been defined as an unbalance between increased ROS production and inadequate antioxidant activity [10].

In the recent years, diverse authors suggest the use of different treatments for women in menopause instead of the traditional hormonal replacement. First, not all postmenopausal women with menopausal symptoms are considered likely candidates to receive hormonal therapy; second, to avoid the increased risk to develop breast cancer, stroke, and/or cardiovascular complications associated with the hormonal replacement [11]. Alternative treatments include non-hormone drugs, herbal remedies, vitamins, minerals, antioxidant supplementation, and alternative therapies [12–15]. A balanced diet with adequate amounts of vitamins, minerals, and other nutrients, plays an important role in the prevention and treatment of cardiovascular disease, osteoporosis, obesity, diabetes, cancer, depression, and other menopause-related diseases [16]. Furthermore, in some cases postmenopausal woman appear to be healthier when taking specific supplementations, such as vitamins D and E, or minerals. [17,18]. Despite this, both basic science and clinical studies are still needed to elucidate the mechanisms and true effects of these treatments, which make it difficult to provide evidence-based recommendations [19,20].

Animal models serving in research may have an existing, inbred or induced disease or injury that is similar to a human condition, for the purpose of better understanding the disease/condition or to try a new treatment, therapy, or strategy [21–23]. Menopause experimental models are widely used for research purposes, and a variety of different methodologies are presented in the literature. The two most common ways to induce menopause-like symptoms in experimental animals are by surgical procedures (ovariectomy, with dramatic cessation in hormonal secretion) and by chemical induction (with progressive ovarian function loss) [24,25]. It has also been shown that each model develops different biochemical characteristics in rodents. In this way, it is possible to decide on one or other model according to specific research applications.

The best characterized and reported surgical procedure to induce experimental menopause in rats and mice is the bilateral ovariectomy. This procedure makes possible in a short period of time the acquisition of female rats without ovarian hormones secretion. In addition, ovariectomized (OVX) rats show higher risk to present osteoporoses symptoms [26], cardiac hypertrophy [27], important cardiovascular dysfunctions [28], uterine atrophy [29], increased tail skin temperature [30], decreased plasma vitamin A, C, and E concentrations [31], and an imbalance between free radical production and antioxidant defenses levels, with increased oxidative stress and consequently an acceleration of aging process in different tissues [9,26,32].

To date, rat models of menopause are largely based on surgical or chemical induction, and none of them represent the progressive failure of ovarian function that occurs in natural menopause [1,33]. The transition to menopause can occur over a 10- to 15-year period. Early in perimenopause, menstrual cycles are typically more frequent and characterized by more extreme fluctuations in oestrogen levels. Later in the menopausal transition, cycles become unpredictable and decrease in number, exposing women to progressively longer periods of oestrogen withdrawal. One year of oestrogen absence marks the initiation of the menopause period called early menopause [33]. Although widely used, OVX rats are problematic with regard to reproducing the effects of natural menopause transition. Ovariectomy produces a rapid, dramatic cessation of ovarian function, rather than

the gradual decline that occurs in perimenopause. In addition, the administration of chemicals like 4-vinylcyclohexene diepoxide has been described recently to induce ovarian function loss [24]. But this is a pharmacological rather than physiological rodent model. With the use of any drug-induced physiological changes, care will need to be taken to assure that the drug has no secondary effects on the target tissue and others.

Several works have been focused on the effects of vitamin A in menopausal woman, others focused on the oxidative stress profile established during the transition to menopause. But, to our knowledge, this paper is the first work linking menopause, vitamin A treatment and blood-related oxidative stress parameters. In this work, we evaluated biochemical and oxidative stress parameters that distinguish sham-operated female rats from Wistar rats bilaterally OVX. Additionally, we have also investigated the effects of retinol palmitate (a vitamin A supplement commercially available at drug stores) low-dose supplementation (500 or 1500 IU/kg/day, during 30 days) upon blood and plasma antioxidant status in OVX rats.

MATERIAL AND METHODS

All experimental procedures were performed in accordance with the National Institutes of Health Guide for Care and Use of Laboratory Animals (NIH publication number 80–23 revised 1996) and were carried out according to the determinations of the Brazilian College of Animal Experimentation, COBEA.

Animals and reagents

Thirty-two female Wistar rats (200–250 g) were obtained from our own breeding colony. They were caged in groups of five with free access to food and water and were maintained on a 12-h light–dark cycle (7:00–19:00 h), at a temperature-controlled colony room (23 ± 1 °C). These conditions were maintained constant throughout the experiments. Animals were supplied with commercial pellet food (Nuvilab® CR-1 type – Curitiba, PR, Brazil) and water ad libitum.

Arovit® (retinol palmitate, a commercial water-soluble form of vitamin A) was purchased from Roche (Rio de Janeiro, RJ, Brazil). Ketamine hydrochloride was purchased from Virbac Ltda (Jurubatuba, SP, Brazil) and xylazine hydrochloride from Vetbrands Ltda (Goiânia, GO, Brazil). All other chemicals used in the study were purchased from Sigma Chemical Co. (St Louis, MO, USA).

Surgical procedures

Rats were allowed 2 weeks to acclimatize to the surroundings before beginning any experimentation. Thirty-two 60-day-old female rats were randomly divided into either sham-operated group ($n = 8$) or OVX group ($n = 24$). All animal dissections were conducted by surgical procedures with aseptic technique. Rats were anesthetized by an intraperitoneal injection (ketamine, 100 mg/kg; plus xylazine 15 mg/kg, respectively). The ventral part of the abdominal region was shaved and then cleaned with ethanol. One small incision (1 cm) was made through the skin and the muscle wall on the center of peritoneal area. Ovaries were then located, a braided silk sterile suture (Shalon LTDA; São Luis de Montes Belos, GO, Brazil) was performed around the area of the uterine horns (closely to oviducts and to the cervical junction), and the ovaries were removed. The wound was closed in two layers, i.e. muscle and skin using sterile sutures. Sham animals were also anesthetized, the skin and muscle layers were opened, the uterus and ovaries were gently manipulated but not excised, and the wound was closed in two layers. After surgery, rats were housed individually for some hours to allow recovery and then re-grouped in their home cages. Sixty days after surgical procedures, we started the treatment.

The reproductive cycle of female rats is called the estrous cycle, and the mean cycle length is 4–5 days [34]. Two months into the rat reproductive life represents 12–15 estrous cycles. In women, this number of ovulation episodes represents approximately one reproductive life year. After 1 year of permanent loss of menstruation, a woman is considered to be in menopause. From that, the menopause experimental model used here is similar to a human early menopause period. Also, some related symptoms between human menopause and the experimental protocol used here were reported previously.

Treatment

Two months after surgical procedures, animals were treated once a day for 30 days. All treatments were carried out at night (i.e. when the animals are more active and take a greater amount of food) to ensure maximum vitamin A absorption, because this vitamin is better absorbed during or after a meal. Sham-operated and one OVX group were treated with vehicle (physiological saline – NaCl 0.9%, $n = 8$ each). Two other OVX groups ($n = 8$ each) were treated with retinol palmitate (vitamin A) 500 and 1500 IU/kg/day (OVX + 500 and OVX + 1500, respectively). Vitamin A

treatment was prepared daily while protecting vitamin A from light. Treatment was performed orally via a metallic gastric tube (gavage) in a maximum volume of 0.4 ml. Adequate measures were taken to minimize pain or discomfort. During treatment, the animals were weighted weekly and weight gain analyzed.

Samples acquisition

The animals were killed 90 days after surgical procedures (with treatment in the last 30 days). Rats were decapitated 18 h after last vitamin A administration with researchers blinded to group. Blood samples were collected for analysis, and the plasma was separated immediately. The uterus was cut above the cervical junction, visible fat removed, and the cleaned uterus was weighed. Whole blood was rapidly collected (and plasma separated) by one researcher, and another analyzed and weighed uteri. Blood and plasma samples were stored at −80 °C for posterior analyses. On the day of the experiment, a sample aliquot was used. Blood samples were frozen (−80 °C) and thawed (25 °C) two times and centrifuged (600 ***g***, 5 min). Supernatants were used for all biochemical assays described herein. Plasma samples were thawed (25 °C), mixed (vortex), and directly used for posterior assays. Plasma TBARS (thiobarbituric acid reactive species) measurements were normalized by the lipid content in the samples. Aminotransferase activities and redox parameters were normalized by the protein content using bovine albumin as a standard [35].

Plasma lipid profile and biochemical parameters

The plasma was separated to determine the levels of total cholesterol, high-density lipoprotein (HDL), and triglycerides. The plasma lipid profile was determined with commercial kits. Levels of triglycerides and total cholesterol were determined with commercial kits produced by Human do Brasil S/A (Itabira, MG, Brazil). Quantitation of HDL was determined with commercial kit produced by in vitro Diagnostica S/A (Barbacena, MG, Brazil). The concentrations of low-density lipoprotein (LDL) and very-low-density lipoprotein (VLDL) were assessed by using the Friedewald equation [36]. Plasmatic uric acid levels, aspartate aminotransferase (AST, E.C. 2.6.1.1) and alanine aminotransferase (ALT, E.C. 2.6.1.2) plasma activities were quantified with commercial kit produced by in vitro Diagnostica S/A. Iron concentrations in plasma were determined with commercial kit produced by Wiener Laboratórios S.A.I.C (Rosario, Argentina). Blood glucose levels were measured with Accu-Chek® (Roche Diagnostics GmbH, Mannheim, Germany) Active, blood glucose monitor, strips and lancing device produced by Roche Diagnostics GmbH, just before the killing. Animals were not fasted for the blood glucose measurement.

Antioxidant enzyme activities quantitation

Blood superoxide dismutase (SOD, EC 1.15.1.1) activity was assessed by quantifying the inhibition of superoxide-dependent adrenaline auto-oxidation in a spectrophotometer at 480 nm, as previously described [37]. The same protocol was used to assess the extracellular form of CuZn-SOD (EC-SOD) activity in plasma samples. Results are expressed as Units SOD/mg protein. Blood catalase (CAT, EC 1.11.1.6) activity was assayed by measuring the rate of decrease in H_2O_2 absorbance in a spectrophotometer at 240 nm [38]. CAT activity is expressed as Units CAT/mg protein. Blood glutathione peroxidase (GPx, EC 1.11.1.9) activity was determined by measuring the rate of NADPH oxidation in a spectrophotometer at 340 nm, as previously described [39]. GPx activity was expressed as Units (nmol NADPH oxidized/min)/mg protein.

Non-enzymatic antioxidant defenses measurement

We used the total reactive antioxidant potential (TRAP) test as an index of the non-enzymatic antioxidant capacity on plasma, based on the peroxyl radical (generated by AAPH solution, 2,20-azobis[2-amidinopropane], with luminol) quenching by sample compounds [40]. The reading is taken by chemiluminescence emission. Briefly, we prepared AAPH solution and added luminol (system); thereafter, we waited 2 h for the system to stabilize to do the first reading. After the addition of the sample, we analyzed the readings for nearly 60 min. The results were transformed in percentage, and the area under curve (AUC) was calculated by software GraphPad (San Diego, CA, USA) as described [41]. When the sample was more reduced AUC (in relation to the system AUC), more antioxidant is the sample. The total antioxidant reactivity (TAR) was also analyzed in the plasma and it is based on the same technical principles of TRAP, but TAR is more related to the quality of samples antioxidants. The TAR results were calculated as the ratio of light in the absence of samples (Io)/light intensity right after sample addition (I) [42]. A higher value means a higher antioxidant potential.

Measurement of total and non-protein thiol content

An assay that serves to analyze oxidative alterations in proteins was used to measure the level of reduced thiol

content (SH) in samples [43]. Briefly, for total SH content measurement, a 100-µg sample aliquot (blood or plasma) was diluted in PBS 10 and 10 mM 5,5′-dithionitrobis 2-nitrobenzoic acid and read in a spectrophotometer at 412 nm after 60 min incubation at 25 °C. For non-protein total SH content, a 1-mg sample aliquot was reacted with trichloroacetic acid (10% v/v), centrifuged (10 000 ***g***, 10 min), and the supernatants were used to measure the level of SH. All results are expressed as umol SH/mg protein.

Oxidative damage parameters

As an index to plasma lipoperoxidation, we used the thiobarbituric acid reactive species (TBARS) test, which is widely adopted as a method for measurement of lipid redox state, as previously described [44]. The TBARS consists of an acid-heating reaction of the lipid peroxidation end product, malondialdehyde (MDA), with thiobarbituric acid (TBA, 4,6-Dihydroxypyrimidine-2-thiol). The TBARS was determined at 532 nm and was expressed as nmol/mg lipid. The oxidative damage to plasma proteins was measured by the quantitation of carbonyl groups, as previously described [45]. Briefly, this method is based on the reaction of dinitrophenylhydrazine with protein carbonyl groups, and the absorbance read in a spectrophotometer at 370 nm. Results were expressed as nmol carbonyl/mg protein.

Statistical analyses

Results were expressed as mean ± SEM. All analyses were performed using the Statistical Package for the Social Sciences (SPSS Inc., Chicago, IL, USA) software (version 15.0), and GraphPad Prism (GraphPad Software Inc., San Diego, CA, USA) software (version 4.02). The influence of the surgery, the effect of vitamin A in OVX rats, and the possible vitamin A action restoring sham values were analyzed. In this way, we performed three different statistical analyses. First, differences between sham-operated and OVX saline-treated groups (Sham and OVX) were determined by the *t* test analysis. Second, differences among OVX experimental groups (OVX, OVX + 500, and OVX + 1500) were determined by one-way ANOVA followed by the post hoc Tukey's test. Finally, to better see whether Sham and OVX vitamin A-treated groups differed significantly, a one-way ANOVA was performed followed by the post hoc Tukey's test. Differences were considered statistically significant at $P \leq 0.05$. Statistical results were presented in the result section, as follows: first, the *P*, *t* and df values, of Sham vs. OVX; second, the ANOVA *P* and *F* values, and the Tukey's test *P* value for the selected OVX groups comparison; and finally, if necessary, the ANOVA *P* and *F* values, and the Tukey's test *P* value for the selected groups comparison. To better observe differences resulting from the surgery or from the treatment, we decided to use three different statistical tests. Also, a single test would probably mask important differences, for instance between sham-operated and OVX groups.

RESULTS

Body weight gain and uterine tissue weight

For weight gain analyses (*Figure 1a*), we monitored rat weights during the 30-day vitamin A treatment. In this period, weight gain (g) was significantly higher in the OVX saline-treated group (OVX = 13.50 ± 1.476) when compared to the Sham group (Sham = 7.500 ± 1.225) ($P = 0.0074$, $t = 3.128$, df = 14). Body weight gain in OVX rats was not significantly altered by vitamin A treatment (OVX + 500 = 7.875 ± 1.093, OVX + 1500 = 8.625 ± 1.194) ($P = 0.2722$, $F = 1.385$).

After killing, we collected and weighted the uterine tissue (*Figure 1b*). Sham rats presented different uterine morphology according to the estrous cycle phase upon the day of decapitation. Seven sham female rats presented uterine morphology related to non-proestrus phases, estrus, and diestrus (0.32–0.51 g, low fluid

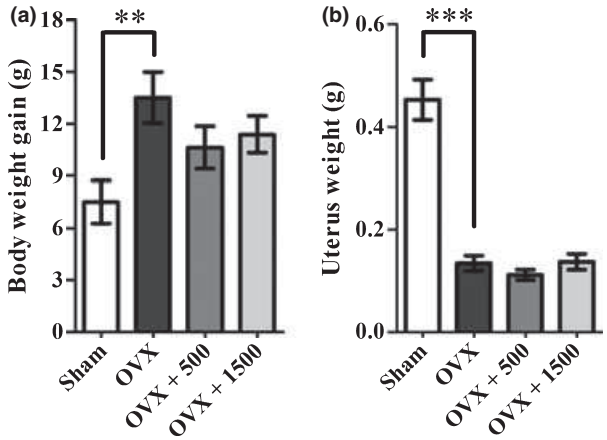

Figure 1 Effects of bilateral ovariectomy and vitamin A supplementation on body weight gain and uterus weight. The body weight gain (a) and the uterus weight (b) were analyzed after the end of the treatment. Data are mean ± SEM ($n = 8$ per group). Statistical difference between sham and ovariectomized (OVX) groups, $^{**}P < 0.01$, $^{***}P < 0.001$ (*t*-test). Statistically different from OVX group.

content and thin tissue), and one sham rat presented a characteristic proestrus uterus morphology (0.65 g, high fluid content and thick tissue). All OVX rats (with or without vitamin A treatment) presented a significant reduction in the uterine tissue weight (OVX = 0.134 ± 0.015, OVX + 500 = 0.112 ± 0.010, OVX + 1500 = 0.137 ± 0.016) when compared to the sham group (Sham = 0.452 ± 0.040) ($P < 0.001$, $t = 7.524$, df = 14; and $P = 0.3668$, $F = 1.052$). OVX uterine tissues were highly atrophied at the end of the study (90 days after ovariectomy procedure), showing the absence of ovarian hormones secretion, and also ovulation, during this period. OVX uterine weight was not altered by vitamin A treatment ($P = 0.3668$, $F = 1.052$) as well as visual morphology.

Plasma lipid profile and biochemical parameters

At the end of the experimental period, OVX groups (with or without vitamin A treatment) showed alterations in the plasma lipid content when compared to sham group (*Table I*). OVX saline-treated triglycerides levels were found to be reduced ($P < 0.001$, $t = 4.204$, df = 14), and total cholesterol content increased ($P = 0.0014$, $t = 3.966$, df = 14) when compared to sham-operated group. Also, LDL and VLDL estimations showed significant alterations when compared to sham group ($P = 0.0067$, $t = 3.177$, df = 14, and $P < 0.001$, $t = 4.204$, df = 14). Vitamin A treatment during 30 days was not capable of restoring the normal lipid profile in OVX rats (triglycerides $P = 0.1412$, $F = 2.152$, total cholesterol $P = 0.7432$, $F = 0.3010$, and HDL $P = 0.9919$, $F = 0.0081$).

In blood/plasma biochemical parameters tested (*Table II*), we found reduced plasmatic uric acid values in OVX saline-treated group when compared to the sham group ($P = 0.004$, $t = 3.441$, df = 14), and vitamin A was not capable of changing these levels ($P = 0.5057$, $F = 0.7044$). The other biochemical parameters did not differ significantly among all analyzed groups.

Blood antioxidant enzyme activities

Blood CAT activity showed an increase in the OVX saline-treated group (OVX = 19.12 ± 0.941) when compared with the sham group (Sham = 15.28 ± 1.204) ($P = 0.025$, $t = 2.510$, df = 14) (*Figure 2c*). CAT activity in OVX groups treated with vitamin A 500 and 1500 IU/kg/day (OVX + 500 = 16.21 ± 0.571, OVX + 1500 = 14.77 ± 0.800) differ significantly from OVX saline-treated group ($P = 0.0027$, $F = 7.936$, $P < 0.05$ OVX vs. OVX + 500 and $P < 0.01$ OVX vs. OVX + 1500); however, only the higher dose restored the CAT activity close to sham values. OVX saline-treated group also showed an increase in blood GPx activity (OVX = 15.37 ± 0.595) when compared with the sham group (Sham = 10.45 ± 0.520) ($P < 0.001$, $t = 6.228$, df = 14) (*Figure 2d*). Both vitamin A treatment doses were able to promote restoration in GPx activity near to the sham values (OVX + 500 = 12.93 ± 0.545, OVX + 1500 = 11.26 ± 0.522) differing significantly from OVX saline-treated group ($P < 0.001$, $F = 13.93$, $P < 0.05$ OVX vs. OVX + 500 and $P < 0.001$ OVX vs. OVX + 1500). The two SOD measures (total SOD activity in blood and EC-SOD in plasma) showed no differences among all four analyzed groups ($P = 0.9291$, $F = 0.1496$; and $P = 0.2065$, $F = 1.622$, respectively) (*Figure 2a* and *b*). We observed an increased blood CAT and GPx activities, in response to ovariectomy, but no changes in blood SOD activity, which led to SOD/CAT + GPx ratio imbalance (OVX = 0.672 ± 0.029) when compared to sham group (Sham = 1.000 ± 0.102) ($P = 0.0077$, $t = 3.107$, df = 14) (*Figure 3*). Vitamin A treatment with 1500 IU/kg/

Table I Plasma lipid profile.

	Sham	OVX	OVX + 500	OVX + 1500
Triglycerides (mg/dL)	66.89 ± 6.91	35.69 ± 2.71[a]	42.58 ± 4.67[b]	46.55 ± 3.61[b]
Total cholesterol (mg/dL)	73.80 ± 2.68	89.24 ± 2.83[a]	86.06 ± 4.41	90.39 ± 4.77[b]
HDL (mg/dL)	22.24 ± 2.25	23.27 ± 3.16	23.37 ± 1.55	22.98 ± 1.57
LDL (mg/dL)	38.18 ± 5.26	58.84 ± 3.82[a]	54.18 ± 4.91	58.10 ± 3.94[b]
VLDL (mg/dL)	13.38 ± 1.38	7.138 ± 0.54[a]	8.516 ± 0.93[b]	9.31 ± 0.72[b]

Triglycerides, total cholesterol, and HDL-cholesterol fraction were measured in plasma samples, LDL and VLDL values were indirectly obtained with the Friedwald methodology. Sham and OVX groups were treated with saline; OVX + 500 group treated with retinol palmitate 500 U/kg/day; and OVX + 1500 group were treated with retinyl palmitate 1500 U/kg/day. Animals were treated once a day for 30 days. Data are mean ± SEM ($n = 8$ per group). Statistically different from sham group, [a]$P < 0.05$ (*t*-test), [b]$P < 0.05$ (one-way ANOVA followed by the post hoc Tukey's test).

HDL, high-density lipoprotein; LDL, low-density lipoprotein; OVX, ovariectomized; VLDL, very-low-density lipoprotein.

Table II Blood/plasma biochemical data.

	Sham	OVX	OVX + 500	OVX + 1500
Aminotransferases				
AST activity (U/dL)	26.70 ± 1.374	29.83 ± 2.428	28.64 ± 1.083	28.75 ± 1.020
ALT activity (U/dL)	15.16 ± 0.812	15.70 ± 1.551	14.84 ± 1.051	14.05 ± 0.877
Uric acid (mg/dL)	0.477 ± 0.033	0.295 ± 0.042[a]	0.339 ± 0.037	0.368 ± 0.052
Iron (mg/dL)	0.339 ± 0.0125	0.341 ± 0.0243	0.290 ± 0.0243	0.303 ± 0.0332
Glycemia (mg/dL)	96.3 ± 7.1	99.5 ± 11.4	104.2 ± 9.2	106.9 ± 10.6

AST and ALT activities, uric acid content and iron content were measured in plasma samples. The glycemic level just before killing was measured in blood samples. Sham and OVX groups were treated with saline; OVX + 500 group was treated with retinol palmitate 500 U/kg/day; and OVX + 1500 group was treated with retinyl palmitate 1500 U/kg/day. Animals were treated once a day for 30 days. Data are mean ± SEM (n = 8 per group). Statistically different from sham group, [a]$P < 0.05$ (t-test).

ALT, alanine aminotransferase; AST, aspartate aminotransferase; OVX, ovariectomized.

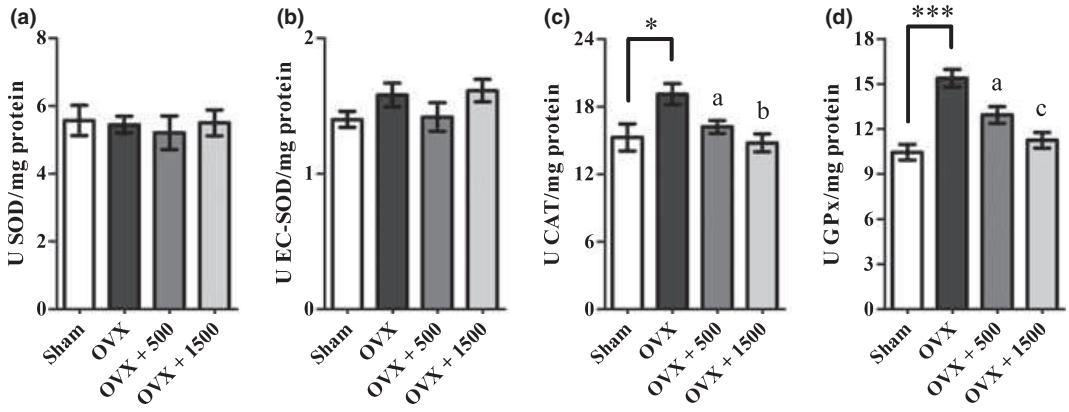

Figure 2 Effects of bilateral ovariectomy and vitamin A supplementation on blood antioxidant enzyme activities. Total superoxide dismutase (SOD) activity (a), catalase activity (c), and glutathione peroxidase activity (d) were measured in blood samples. Extracellular SOD activity (b) was measured in plasma samples. Data are mean ± SEM (n = 8 per group) and the experiments were performed in triplicate. Statistical difference between sham and ovariectomized (OVX) groups, *$P < 0.05$, ***$P < 0.001$ (t-test). Statistically different from OVX group, [a]$P < 0.05$, [b]$P < 0.01$, [c]$P < 0.001$. (one-way ANOVA followed by the post hoc Tukey's test).

day in OVX rats (OVX + 1500 = 1.001 ± 0.075) was able to restore this ratio near to sham value (P = 0.0061, F = 6.564, $P < 0.01$ OVX vs. OVX + 1500).

Plasma non-enzymatic antioxidant defenses

The plasmatic non-enzymatic potential was measured (*Figure 4*); the function of these substances (like polyphenols, vitamins, and protein sulfhydryl) is the main antioxidant defense system in blood plasma. A decreased non-enzymatic potential was observed in OVX saline-treated group when compared to sham-operated group, seen by increased AUC (*Figure 4b*) in TRAP (more related to the amount of the antioxidant) (Sham = 16.79 ± 2.712, OVX = 43.53 ± 3.638) ($P < 0.001$, t = 5.893, df = 14). Furthermore, the TAR (*Figure 4c*) (more related to the quality of the antioxidant, that is, the scavenger capacity) also showed significant difference between sham and OVX saline-treated group (Sham = 52.27 ± 3.358, OVX = 21.06 ± 3.071) ($P < 0.001$, t = 6.859, df = 14).

Vitamin A treatment was able to improve plasmatic non-enzymatic antioxidant defenses in OVX rats. The treatment with 500 IU/kg/day showed an improvement in both TRAP and TAR analyses (OVX + 500 = 26.56 ± 4.418, and 41.07 ± 3.044, respectively) when compared to OVX saline-treated group ($P < 0.001$, F = 12.25, $P < 0.01$; and $P < 0.001$, F = 24.62, $P < 0.001$, respectively). Moreover, the treatment with 1500 IU/kg/day presented a higher improvement in TRAP and TAR analyses (OVX + 1500 = 18.63 ± 2.626, and 51.89 ± 3.334, respectively) when compared to OVX saline-treated group ($P < 0.001$ and $P < 0.001$, respectively).

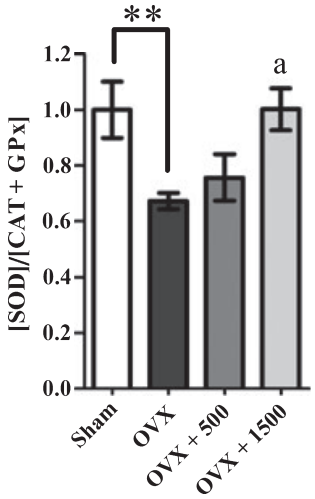

Figure 3 Effects of bilateral ovariectomy and vitamin A supplementation on blood antioxidant enzymatic defenses ratio. The ratio between superoxide dismutase and catalase plus glutathione peroxidase activities were analyzed (arbitrary units) with the blood results. Data are mean ± SEM (n = 8 per group). Statistical difference between sham and ovariectomized (OVX) groups, $^{**}P < 0.01$ (t-test). Statistically different from OVX group, $^{a}P < 0.01$. (one-way ANOVA followed by the post hoc Tukey's test).

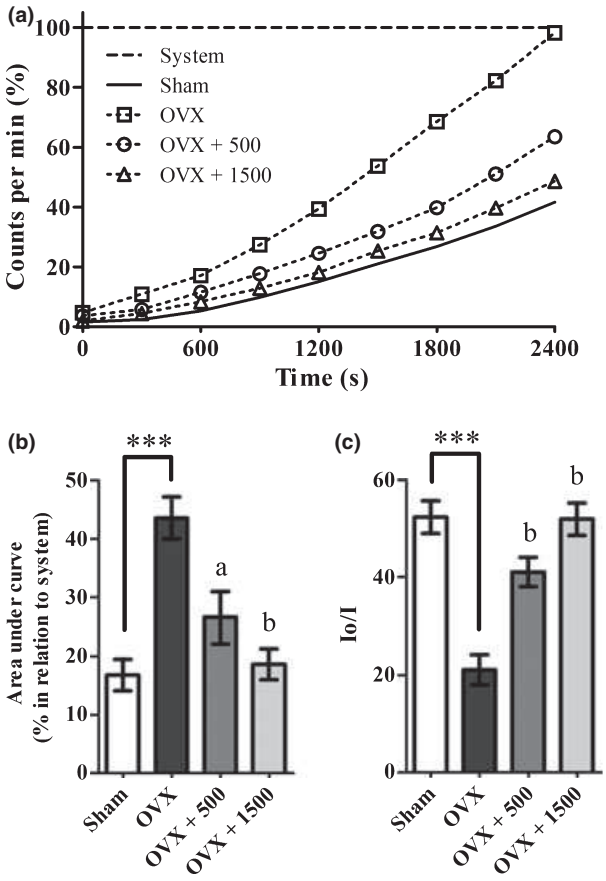

Figure 4 Effects of bilateral ovariectomy and vitamin A supplementation on plasma non-enzymatic antioxidant potential. An experiment's representative graphic (a), the area under curve of total reactive antioxidant potential (b), and the total antioxidant reactivity of plasma samples were analyzed. Data are mean ± SEM (n = 8 per group) and the experiments were performed in duplicate. Statistical difference between sham and ovariectomized (OVX) groups, $^{***}P < 0.001$ (t-test). Statistically different from OVX group, $^{a}P < 0.01$, $^{b}P < 0.001$ (one-way ANOVA followed by the post hoc Tukey's test).

Blood- and plasma-reduced sulfhydryl content

Blood-reduced sulfhydryl content (total SH content *Figure 5a* and non-protein SH content *Figure 5b*) and plasma-reduced sulfhydryl content (total SH content *Figure 5c* and non-protein SH content *Figure 5d*) presented decreased levels in OVX saline-treated rats (OVX = 40.25 ± 1.000, 0.396 ± 0.048, 1.070 ± 0.023, and 0.183 ± 0.013, respectively) when compared with the sham-operated group (Sham = 48.42 ± 1.815, 0.702 ± 0.103, 1.212 ± 0.028, and 0.346 ± 0.040, respectively) (P = 0.0015, t = 3.944; P = 0.0173, t = 2.697; P = 0.0016, t = 3.914; and P = 0.0016, t = 3.894, df = 14; respectively), showing an increase in thiol oxidation levels in OVX group. Vitamin A treatment at 1500 IU/kg/day in OVX rats was able to restore reduced thiol content in the plasma total SH content (OVX + 1500 = 1.228 ± 0.027) ($P < 0.001$, F = 10.95, $P < 0.001$ OVX vs. OVX + 1500) and in the plasma non-protein SH content (OVX + 1500 = 0.256 ± 0.024) (P = 0.0158, F = 5.082, $P < 0.05$ OVX vs. OVX + 1500) analyses.

Plasma oxidative damage levels

OVX saline-treated rats presented significant increase in the plasma protein carbonylation levels (OVX = 10.19 ± 0.376) when compared to the sham group (Sham = 7.767 ± 0.445) (P = 0.001, t = 4.165, df = 14) (*Figure 6b*). Vitamin A treatment with 1500 IU/kg/day in OVX rats (OVX + 1500 = 8.187 ± 0.353) was able to reduce plasma protein damage near to sham values (P = 0.0362, F = 3.901, $P < 0.05$ OVX vs. OVX + 1500). On the other hand, ovariectomy and/or vitamin A treatment did not alter plasma lipid peroxidation levels measured (P = 0.9036, t = 0.123, df = 14; and P = 0.5900, F = 0.5411) (*Figure 6a*).

DISCUSSION

Currently, there is no single experimental model that specifically represents the progressive failure of ovarian

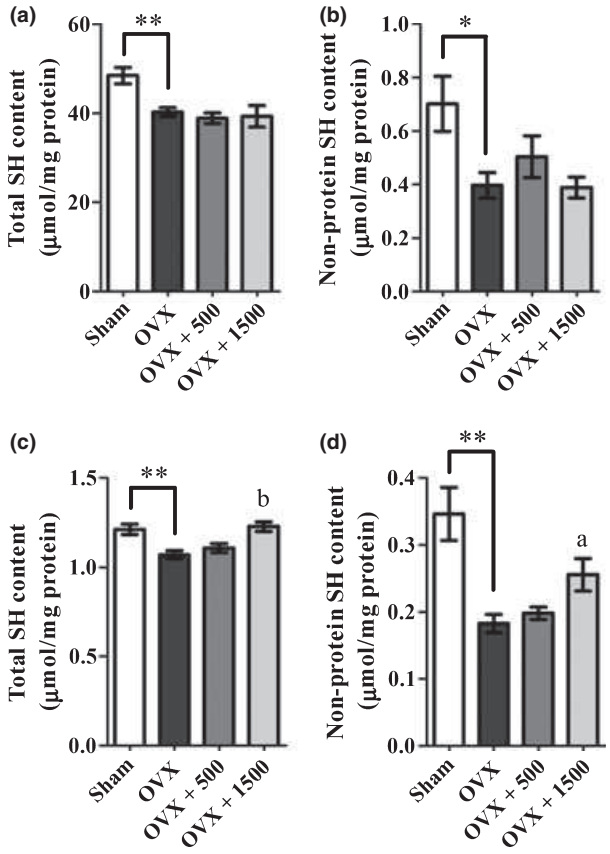

Figure 5 Effects of bilateral ovariectomy and vitamin A supplementation on blood and plasma reduced thiol content. Total reduced thiol content and non-protein thiol content on blood (a and b, respectively), and plasma (c and d, respectively) samples. Data are mean ± SEM (n = 8 per group), and the experiments were performed in triplicate. Statistical difference between sham and ovariectomized (OVX) groups, $^{*}P < 0.05$, $^{**}P < 0.01$ (t-test). Statistically different from OVX group, $^{a}P < 0.05$, $^{b}P < 0.001$ (one-way ANOVA followed by the post hoc Tukey's test).

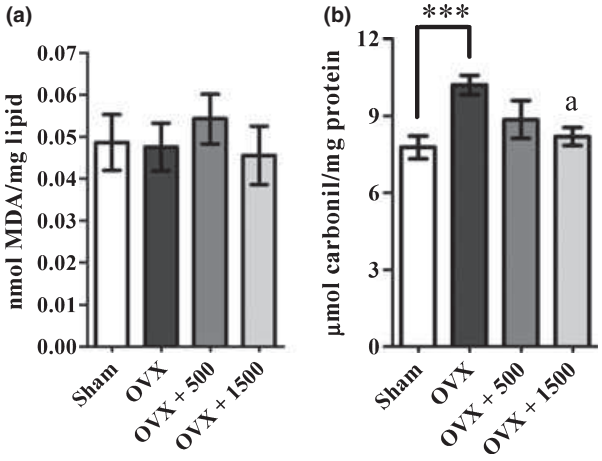

Figure 6 Effects of bilateral ovariectomy and vitamin A supplementation on plasma oxidative damage parameters. Lipid peroxidation (a) and protein carbonylation (b) were analyzed in plasma samples. Data are mean ± SEM (n = 8 per group) and the experiments were performed in duplicate. Statistical difference between sham and ovariectomized (OVX) groups, $^{***}P < 0.001$ (t-test). Statistically different from OVX group, $^{a}P < 0.05$. (one-way ANOVA followed by the post hoc Tukey's test).

function that occurs in natural menopause transition, and in the same time using the available models based on surgical or chemical induction to accurately recreate and study the erratic hormonal state that occurs during the natural menopause transition remains to be seen. With this caveat in mind, the OVX rat model remains the most popular choice as it has been proven to represent some of the most important clinical features of oestrogen deficiency-induced (or postmenopausal) bone loss, circulatory dysfunctions, and nervous system aging in the adult human [26,32,46]. Associated with the higher risk of showing menopause-related symptoms, OVX rats are able to present increased oxidative stress levels and consequently an accelerated aging process in different tissues [9,26,32]. In this scenario, oxidative stress has been proposed to explain the biologic side effects of experimental menopause. In the present work, our first objective was to evaluate the effects of bilateral ovariectomy upon biochemical and oxidative stress parameters in Wistar rats. Our results confirmed that OVX rats presented increased blood oxidative stress and considerable changes in the lipid profile, associated with an increase in body weight gain and expected uterine tissue atrophy.

The ovary of the premenopausal human female, as well as the ovary of various animal species, serves as the body's primary source of oestrogen (17β-estradiol or E2), the hormone associated with protection of the premenopausal woman from a variety of potential postmenopausal health problems, such as increased risk for a decline in cardiovascular, skeletal, and nervous system function, and for accelerated aging process. An important anti-atherosclerotic effect of oestrogen is probably its beneficial influence on lipid metabolism. Postmenopausal women usually exhibit increased levels of LDL, lipoprotein(a), and total cholesterol and decreased HDL level [47]. In the present study, OVX rats presented an increase in total cholesterol levels, but no alterations in HDL fraction, and an interesting decrease in triglycerides. According to the lipid profile performed, an increase in LDL levels of OVX rats may be suggested (*Table I*). Additionally, OVX rats presented reduced

triglycerides levels compared to sham-operated animals (*Table I*). However, the literature provides evidences that experimental menopause rodent models and menopausal woman exhibit increased plasmatic triglycerides levels [48,49]. According to another study comparing the group of patients in surgical menopause following bilateral oophorectomy with natural menopause group, that while total cholesterol and LDL levels were found higher in surgical menopause group, no statistical difference was found between the two groups in HDL, VLDL, and triglycerides levels [50]. Furthermore, the factors determining the level of triglycerides are not well defined, although diet, smoking, and physical activity are important factors [51]. The data also showed a decrease in plasmatic uric acid levels in OVX rats (*Table II*). Recently, increased uric acid levels were associated with increased risk for metabolic syndrome in both premenopausal and postmenopausal women [52]. In other hand, another recent study suggests that menopause explains a substantial portion, but not all, of the serum uric acid level age-associated increase among women [53]. In addition, we show that female rats bilaterally OVX presented increased body weight gain and extremely atrophied uterine tissue (*Figure 1*). These findings confirm the efficiency of ovariectomy surgery causes drastic cessation of sexual hormones secretion.

The increased risk of coronary heart disease in postmenopausal women is closely related with the fall in female hormones secretion. Accumulating evidence suggests that cardiovascular diseases are associated with increased oxidative stress in blood vessels. Increased ROS, such as superoxide ($O_2^{\bullet -}$) and hydrogen peroxide (H_2O_2), causes blood vessels to become thicker, produce inflammation in the vessel wall, and thus are regarded as risk factors for vascular disease. Whereas controlled ROS concentrations also act as signaling molecules in many aspects of growth factor-mediated physiological responses [54]. Oxidative stress is a particular state characterized by an overload in oxidants, which may culminate in cellular dysfunction [10,55]. In our study, OVX rats presented an increase in blood antioxidant peroxidase activities (*Figure 2c* and *d*), a decrease in plasma non-enzymatic defenses (*Figure 4*), a decrease in both protein and non-protein SH content (*Figure 5*), accompanied by increased plasma protein oxidative damage levels (*Figure 6b*).

The increase in blood peroxidase activities (CAT and GPx), without changes in blood SOD measures (*Figure 2*), suggests that other source of H_2O_2 instead of SOD presented in blood, is active. Blood vessels express the three isoforms of SOD: cytosolic (CuZn-SOD), mitochondrial SOD, and an EC-SOD [56]. A consequence of SOD activity is the formation of H_2O_2 that it is relatively stable and diffusible (including through cell membranes), compared with many other ROS. Although recognized as a key player in both oxidative damage and redox-regulated cellular processes, there are still many gaps in our knowledge of how it acts. H_2O_2 directly reacts with biologic molecules causing thiol redox transformations, cell signaling, and also oxidative damage [57,58]. In the current study, we have also shown a decrease in plasma non-enzymatic defenses, on both TRAP and TAR analyzes (*Figure 4*). Non-enzymatic antioxidants, such as vitamin C, vitamin E, selenium, zinc, taurine, glutathione (GSH), beta-carotene, and carotene, usually are obtained from dietary sources [7]. The literature reported concentrations of vitamins A, C, and E in the plasma of OVX rats are lower than in controls [31]. Thus, plasma concentrations of vitamins A, C, and E in OVX rats may be decreased as a result of their action in inhibiting free radicals insult. Also, the blood-reduced GSH pool may be depleted by the oxidative insult caused by ovariectomy. In agreement with this idea, a decrease in both protein and non-protein SH content (*Figure 5*) in OVX rats were showed, suggesting increased levels of oxidized protein and GSH. In addition, we found decreased plasmatic uric acid levels in OVX rats (*Table II*), which represents the main plasma non-protein antioxidant. The decreased non-enzymatic antioxidant potential observed in OVX saline-treated group (*Figure 4*) may be correlated with the lower uric acid levels found. Indeed, OVX vitamin A-treated groups showed higher uric acid values and increased non-enzymatic antioxidant potential. Different studies have shown a positive correlation between non-enzymatic potential and plasmatic uric acid levels [59]. Particularly in septic shock, non-enzymatic antioxidant levels are strongly influenced by uric acid levels [60].

Moreover, OVX rats presented increased plasma carbonyl levels, but no changes in lipid peroxidation (*Figure 6*). Most parts of the literature reported increased lipid peroxidation levels in OVX rats and in postmenopausal women [1,31,61,62]. On the other hand, it is well recognized that protein oxidative damage is closely involved with many menopause-related pathologies [63]. Of the many biologic targets of oxidative stress, lipids are the most involved class of biomolecules. One of several low-molecular-weight end products formed via the decomposition of certain primary and secondary lipid peroxidation products is the MDA. This molecule can be quantified by different approaches. The two most com-

mon are by chromatography (analyzing the MDA content) and spectrophotometry (reaction of sample with TBA solution, TBARS test) [64]. The utilization of MDA analysis and/or the TBARS test in studies of lipid peroxidation require caution, discretion, and correlative data from other indices of oxidative stress. In our work, we found no significant lipid peroxidation in animals with decreased antioxidant capacity. More than likely, the measurement of TBARS levels is not an accurate/specific approach to evaluate lipid peroxidation in blood/plasma samples. More recently, the measurement of F2-isoprostanes by methods utilizing mass spectrometry is gaining force. This test is widely regarded as the best currently available biomarker of lipid peroxidation [65]. F2-isoprostanes are certainly the most specific markers of lipid peroxidation but also the most difficult to measure. In summary, according to the biochemical and oxidative stress parameters presented, we emphasize that rat ovariectomy methodology to induce human menopause-related conditions is valid and suitable for research purposes, corroborating with the previous literature [26–29,32,46].

Our second objective was to evaluate the effects of vitamin A supplementation in OVX rats upon the previously described parameters. Our results suggest that vitamin A at doses of 500 and 1500 IU/kg have potentially beneficial effects in OVX rats, improving the blood antioxidant status after 30 days of supplementation. Regarding the vitamin A dose used in this study, we chose it according to the vitamin A quantity often supplemented in the pellet food (25 200 IU/kg of food, according to the manufacturer) and the amount that each adult rat eats per day (15–25 g of pellet food). Therefore, in our own breeding colony, the dietary consumption of vitamin A in food is around to 375–625 IU per rat. In addition, several works originating within our research group demonstrated that vitamin A supplementation in higher doses (up to 2500 IU/kg/day) potentially induces dysfunctions in the redox and bioenergetics states of different tissues in healthy male rats [66–69]. It is important to emphasize that here we tested the supplementation of vitamin A in a totally distinct rat model, with clearly noticeable gender and physiological differences being considered. Since we started the treatment 2 months after surgical procedures, the results lead us to believe vitamin A treatment probably acts more to reverse some of the changes induced by OVX surgery than it does to prevent it. However, this is not a simple phenomenon. It is not possible to discard that vitamin A treatment may also be important in prevention of some menopause-related symptoms during the last 30 days of the experiment. In addition, longer periods of treatment may induce different changes in metabolism, sometimes leading to hepatic toxicity. In our study, we did not observe changes on plasmatic AST and ALT activities (hepatic function markers) among all groups (*Table II*).

Moreover, when deciding to use vitamin A as an antioxidant therapy, the possible pro-oxidative effects elicited by such treatment may be taken into account, mainly in CNS tissues because of its low capacity to tolerate reactive species [70]. Today, there is no consensus on a definitively safe supplemental dose of retinyl palmitate during menopause for humans among literature [71]. Literature reports common safety factors applied to extrapolate animal data to the human. Approximate equivalence was obtained by the application of a 10-fold factor for difference in species and another 10-fold factor to account for interspecies difference [72]. Taking this information and comparing with the doses used in our work (500 and 1500 IU/day/kg), we observed sevenfold and 21-fold ratios. Our results suggest that vitamin A low-dose treatment (doses not significantly higher than the ingested diary amount) seems to be safe for menopausal women. However, further investigations need to be made in other structures to better determine these issues. In recent studies, higher doses of vitamin A have been associated with adverse effects on bone metabolism. A moderately high vitamin A intake (three times the adult recommended dietary intake – RDA) and high plasma vitamin A levels have been associated with a low bone mineral density in menopausal woman [73]. On the other hand, serum retinyl esters are not elevated in different populations of postmenopausal women with and without osteoporosis who have taken vitamin A supplements that are either higher or not than the RDA [74,75]. However, retinyl ester concentration (percentage of total vitamin A) was marginally associated with osteoporosis and should be further investigated. Thus, at present it is unclear whether a high vitamin A intake should be considered as a risk factor for osteoporosis and other menopause-related diseases.

Hormonal replacement therapy may be prescribed if severe side effects (menopause-related symptoms) caused by low levels of sexual hormones are experienced. However, not all postmenopausal women with menopausal symptoms are considered likely candidates to receive hormonal therapy. Thus, to avoid the increased risk of developing complications and diseases associated with the hormonal replacement, many women choose

alternative treatments [11–15]. It is well known that vitamin and mineral supplementation may play an important role in the management of menopausal symptoms [16]. Despite this fact, both basic scientific and clinical studies are still needed to elucidate the mechanisms and true effects of these treatments [19,20]. Our work suggests that the blood tissue is an important site of oxidative stress induced by ovariectomy, which is important because the blood and plasma parameters are good markers for whole organism conditions [23]. In addition, low-dose vitamin A treatment was capable of ameliorating the blood antioxidant profile. In the present study, we found that vitamin A was capable of promoting restoration in CAT and GPx activities near to sham-operated levels (*Figure 2*). Furthermore, vitamin A treatment at 1500 IU/kg/day was able to restore the blood SOD/CAT + GPx ratio near to sham value (*Figure 3*). More importantly, we have shown that plasma non-enzymatic antioxidant defenses were greatly improved, in a dose-dependent manner, after vitamin A treatment (*Figure 4*). As expected, this improvement in antioxidant defenses is shown by the visualization in a reduced protein oxidative damage in plasma samples (*Figure 6b*). Additionally, plasmatic reduced SH content was increased after treatment with vitamin A 1500 IU/kg/day (*Figure 5c* and *d*). On the other hand, this phenomenon was not observed in blood SH analyses. Instead of the variety of studies regarding the use of different vitamins (B6, C, D, E and K) in the treatment of menopausal symptoms [1,17,18,76,77], the vitamin A (or retinoids in general) is not studied as much [78]. More recently, low plasma retinol was reported to strongly predict a poorer prognosis in postmenopausal breast cancer patients [79]. Another study suggested that dietary vitamin A and beta-carotene are modestly protective against ovarian cancer, particularly among smokers [80]. Moreover, retinoids have important functions in the activation of many blood-related signaling pathways, regulating epithelial cell growth, immune system, and hematopoiesis [81,82].

Many vitamins inhibit nitric oxide (NO) production by inducible NO synthase (iNOS), as supported by their known antiatherogenic and antineuroinflammatory roles [83]. For example, vitamin A inhibits iNOS gene transcription in vascular smooth muscle cells [84], and endothelial cells [85]. By reducing NO generation by iNOS, vitamin A plays an important role in preventing radical induced cytotoxicity. Also, retinol and retinoic acid (an active metabolite of vitamin A) modulate different redox-dependent signaling pathways [86]. The beneficial effects of vitamin A against oxidative events found in our study could be related to the activation of these pathways. However, the molecular mechanisms related to these effects still need to be clarified. In addition, the restoration of plasma antioxidants could also indicate that vitamin A had scavenged specific oxidant radicals, shown by decreases in both CAT and GPx activities, and accompanied by restoration in non-enzymatic antioxidant potential and plasmatic SH level. Unfortunately, it is almost impossible to indicate which vitamin A metabolite is the responsible for the observed effects, given the vast number of existing vitamin A metabolites [87]. Indeed, different vitamins directly scavenge ROS. However, among them, vitamins E and C have been recognized as two of the most important antioxidants [88]. On the other hand, several studies suggested that β-carotene and retinoids could exert antioxidant effects through a mechanism of free radical scavenging and/or detoxification [88,89]. While substantial experimental evidence has been accumulated demonstrating the potency and nature of the biologic effects of retinoids, in most cases their underlying mechanisms of action remain uncertain.

Several works have been focused on the effects of ROS in pathophysiological changes in the skeleton, cardiovascular system, and thermoregulatory control mechanisms in OVX rats. But, to our knowledge, the present paper is the first work demonstrating that female blood oxidative profile (CAT and GPx activities; TRAP and TAR measures; protein and non-protein SH levels; and protein oxidative damage parameter) is altered by bilateral ovariectomy, thus suggesting that the cessation in sexual hormones secretion, accompanied by increased oxidative stress, may play an important role in the development of menopause-related symptoms. In addition, this is the first work to show that low-dose supplementation on vitamin A was capable of ameliorating antioxidant status in OVX rats. Further investigations will be made to better determine the influence of vitamin A supplementation in oxidative profile of other tissues and organs of OVX rats. Menopause is an inevitable milestone in the reproductive life of every woman. Traditionally, in developing countries, menopause and problems thereof are accepted as normal physiological phenomena. However, with increasing life expectancy among women in developing countries, the prevalence of osteoporosis, cardiovascular disease, and postmenopausal problems in women continue to increase substantially. Most women not only care about living long lives but also about living healthy lives. In this sense, both basic scientific and clinical studies are very important to elucidate the

mechanisms associated with menopause symptoms and to potentially prospect new alternative treatment.

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3.2 – Artigo 2

Increased cerebral oxidative damage and decreased antioxidant defenses in ovariectomized and sham-operated rats supplemented with vitamin A.

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ORIGINAL RESEARCH

Increased cerebral oxidative damage and decreased antioxidant defenses in ovariectomized and sham-operated rats supplemented with vitamin A

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Abstract Previous studies have linked oxidative stress with aging and aging-related processes, including menopause. Abnormalities in the redox state similar to those observed in menopausal women can be modeled experimentally with rat ovariectomy. The aim of the present study was to investigate the effects of vitamin A (retinol palmitate) supplementation (500 or 1,500 IU kg^{-1} day^{-1} for 30 days) on behavioral parameters and brain redox profile in ovariectomized (OVX) and sham-operated rats. Ovariectomy caused pronounced uterine atrophy and decreased locomotor/exploratory activity. Moreover, we found increased hypothalamic and frontal cortex superoxide dismutase/catalase (SOD/CAT) ratio and decreased hippocampal thiol content, accompanied by increased frontal cortex lipid oxidative damage (TBARS) in OVX rats. Vitamin A at 1,500 IUkg^{-1} day^{-1} decreased exploratory behavior and decreased total hippocampal thiol content in sham-operated rats, increased hippocampal SOD/CAT ratio and decreased total antioxidant potential in the hippocampus of both sham and OVX groups, and increased cortical TBARS levels in OVX rats. Thus, vitamin A may induce a pro-oxidant state in discrete brain regions of sham-operated and OVX rats. These results suggest some caution regarding the use of high doses of vitamin A supplementation during menopause.

Keywords Behavior · Central nervous system · Menopause · Ovariectomy · Redox profile · Vitamin A

Abbreviations

AUC	Area under curve
CAT	Catalase
CNS	Central nervous system
EPM	Elevated plus maze
H_2O_2	Hydrogen peroxide
HRT	Hormone replacement therapy
MDA	Malondialdehyde
O_2	Superoxide anion
OFT	Open-field test

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OVX	Ovariectomized
ROS	Reactive oxygen species
SH	Sulfhydryl
SOD	Superoxide dismutase
TBARS	Thiobarbituric acid reactive species
TRAP	Total radical antioxidant potential

Introduction

The menopause transition is a period marked by significant physical and psychological changes associated with cessation of sex hormone secretion. Female gonadal hormones can influence central nervous system (CNS) function in a variety of ways, such as enhancing plasticity at neural synapses in CNS structures (Flanagan-Cato 2000; Waters et al. 2009) and modulating mood and behavior (Graziottin and Serafini 2009). Ovarian hormones also have peripheral effects, such as modulating the risk for coronary artery and general cardiovascular diseases (Phillips et al. 1997). These central and peripheral effects may be related to the antioxidant properties of female hormones (Agarwal et al. 2008).

Increased production of reactive oxygen species (ROS) is considered to be one of the major causes of age-related diseases. Typically, ROS are continuously generated in physiological conditions and are effectively controlled/eliminated by intracellular and extracellular antioxidant systems. Oxidative stress has been defined as an imbalance between increased ROS production and inadequate antioxidant defense, a particular state characterized by an overload in oxidants, which may culminate in cellular dysfunction (Halliwell 2007). Previous studies have demonstrated that oxidative stress is involved in aging and age-related processes that often accompany menopause (Agarwal et al. 2008; Miquel et al. 2006; Sanchez-Rodriguez et al. 2011).

The menopause transition is a time of increasing vulnerability for depressive episodes, particularly among women with a history of mood disorder (Harlow et al. 2003). In addition to the well-known relationship between menopause and oxidative stress, mood disorders have been associated with altered redox profile (Berk et al. 2011; Moylan et al. 2012). It has also been suggested that increased lipid, protein, and DNA damage in brain structures associated with stress and emotion response can contribute to several pathologies. Indeed, evidence suggests that the loss of regulation between CNS areas contribute to different mental illnesses (Frey et al. 2010), and a dysfunction in the CNS redox signalling may have an influence on such pathologies.

Notably, a number of large epidemiological studies have demonstrated that women have an elevated risk to develop depression and anxiety during the menopause transition, and transdermal estradiol has been shown to be effective in the treatment of perimenopausal depression (Frey et al. 2008). However, not all postmenopausal women are good candidates to receive hormone replacement therapy (HRT; e.g. those with increased risk for blood clotting or personal history of breast cancer). Moreover, some women are reluctant to use HRT due to possible increased risk for breast cancer, stroke, and/or cardiovascular complications (Jensen et al. 2010). Several lines of evidence suggest that estrogen may have either protective or harmful effects depending on the timing of HRT initiation, age, type of menopause (natural versus surgical), or menopause stage (Rocca et al. 2011). Recently, a study showed that one in two postmenopausal women who had discontinued HRT have chosen alternative therapies to treat menopause-related symptoms (Kupferer et al. 2009). A number of treatment strategies have been proposed as alternatives to traditional HRT for postmenopausal women, including herbal supplements, vitamins, minerals, antioxidant supplementations, and antidepressants (Borrelli and Ernst 2010; Dennehy and Tsourounis 2010; Freeman et al. 2011; Thompson 2010; Wong et al. 2009).

It has been long recognized that a balanced diet with adequate amounts of vitamins, minerals, and other nutrients, plays an important role in the prevention and treatment of cardiovascular disease, osteoporosis, obesity, diabetes, cancer, depression, and menopause-related symptoms (Hagey and Warren 2008). In this context, adequate dietary vitamin intake is essential and supplementation is often recommended for some postmenopausal women (Dennehy and Tsourounis 2010; Ziaei et al. 2007). Vitamin supplementation in postmenopausal women may be beneficial for bone health, cardiovascular health, breast cancer risk, cognition, and vasomotor symptoms (Dennehy and Tsourounis 2010). Supplementation with vitamin C, D, K, and calcium have been recommended in some cases for adequate bone metabolism. To date, the only supplement studied for vasomotor symptoms has been vitamin E, but its clinical benefits are yet to be determined. The clinical efficacy of

vitamin A supplementation also is unclear, particularly in regard to cardiovascular function, breast cancer, and cognitive performance in postmenopausal women. In terms of bone health, high dietary intake of vitamin A for 18 years was associated with increased risk of osteoporotic fractures in postmenopausal women (Feskanich et al. 2002). However, no association between vitamin A or retinol intake and risk of hip or total fractures was found in a later study (Caire-Juvera et al. 2009). Both beneficial and hazardous effects of vitamin A attributable to its redox activity also have been reported. For instance, we and others have demonstrated that vitamin A supplementation/ deficiency can promote anti or pro-oxidant effects depending on the dose, the target tissue, and the experimental model (Behr et al. 2012; Gatica et al. 2005; Pasquali et al. 2009; Schnorr et al. 2011a).

Importantly, plasma vitamin A levels were reduced in ovariectomized (OVX) rats 30 days after surgery (Dilek et al. 2010). More recently, we showed that vitamin A supplementation at 500 or 1,500 IU kg^{-1} day^{-1} during 30 days reduced protein oxidative damage and increased total antioxidant potential in the plasma of OVX rats (Behr et al. 2012). Although vitamin A supplementation may have peripheral antioxidant effects in OVX rats, the effects in the CNS have not been examined to date. Thus, the main objective of the present was to investigate if vitamin A supplementation would prevent brain oxidative stress induced by ovariectomy in a rat model of menopause. As a secondary objective, we also investigated the effects of vitamin A on locomotor and anxiety-like behavior.

Methods

All experimental procedures were performed in accordance with the EC Directive 86/609/EEC on the protection of animals used for scientific purposes adopted on 2010 and were carried out according to the recommendations of the Brazilian Society for Science in Laboratory Animals (SBCAL-COBEA).

Animals and reagents

Female Wistar rats (*Rattus novergicus*, 200–250 g) were obtained from our breeding colony. They were caged in groups of five with free access to food and water and were maintained on a 12-h light–dark cycle (7:00–19:00 hours), in a temperature-controlled colony room (23±1 °C). These conditions were maintained constant throughout the experiments. Animals had free access to water and to commercial pellet food containing the following: total protein (22 %), vegetal fiber (8 %), minerals (10 %), calcium (1.4 %), and phosphorous (0.8 %). Enrichment by kilograms: vitamin A (12,000 IU), vitamin D3 (1,800 IU), vitamin E (30 IU), vitamin K3 (3 mg), vitamin B1 (5 mg), vitamin B2 (6 mg), vitamin B6 (7 mg), vitamin B12 (20 μg), niacin (60 mg), folic acid (1 mg), biotin (0.05 mg), choline (600 mg), iron (50 mg), copper (10 mg), zinc (60 mg), manganese (60 mg), cobalt (1.5 mg), iodine (2 mg), selenium (0.05 mg), lysine (100 mg), and methionine (300 mg)—(Nuvilab® CR-1 type; Curitiba, PR, Brazil).

Arovit® (retinol palmitate, a commercial water-soluble form of vitamin A) was purchased from Roche (Rio de Janeiro, RJ, Brazil). Ketamine hydrochloride was purchased from Virbac Ltd. (Jurubatuba, SP, Brazil) and xylazine hydrochloride from Vetbrands Ltd. (Goiânia, GO, Brazil). All other chemicals used in the study were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

Surgical procedures

Forty-two 60-day-old female rats were randomly divided into either sham-operated group (n=21) or OVX group (n=21). All surgical procedures on the animals were performed using aseptic techniques as previously described (Behr et al. 2012). Treatment with vitamin A started at 2 months after surgery.

Two months of the rat reproductive life is composed of 12–15 estrous cycles. In humans, 12–15 menstrual cycles represent approximately one reproductive year, and 1 year after permanent cessation of menstruation, a woman is considered to be postmenopausal (Gracia et al. 2005). Thus, the timing of the menopausal model used here is similar to the early postmenopausal period in humans (Soules et al. 2001).

Treatment

Beginning 2 months after surgery, animals were treated with vitamin A or vehicle (0.9 % NaCl) once a day for 30 days. All treatments were carried out at night (i.e., when the animals are more active and consume a greater amount of food) in order to ensure maximum vitamin A absorption, since this vitamin is better

absorbed during or after a meal. Sham-operated and OVX rats were given either vehicle (n=7, each), vitamin A (retinol palmitate; 500 IU/day; n=7, each), or vitamin A (1,500 IU/day; n=7, each) orally via gavage in a maximum volume of 0.4 ml. Vitamin A solutions were prepared daily and protected from light.

Behavioral tasks

The open-field test (OFT) and the elevated plus maze (EPM) test were performed to evaluate locomotor/exploratory activity and anxiety-like behavior, respectively. These behavioral tasks were analyzed with the ANY-maze software, version 4.72 (ANY-maze Video Tracking System; Wood Dale, IL, USA). This software is a flexible video tracking system designed to automate analysis of behavioral testing.

Open-field test

The OFT is one of the most traditional and widely used methods for the assessment of locomotor and exploratory behaviors as well as emotional state in rodents (Prut and Belzung 2003). In our study, the OFT was performed in the morning of the 27th day of treatment, 12 h after vitamin A intake. Our OFT apparatus consisted of a circular arena surrounded by 50-cm high walls. It was located in a separate brightly lit room illuminated with two, 40-W fluorescent overhead lights each. Two virtual circumferences divided its black floor into two concentric circles, diameters of which were 40 and 90 cm. All the animals were gently placed in the periphery of the arena to freely explore it for 5 min. Then, they were returned to their home cages. Total distance traveled, rearing episodes, total immobile time (2 s minimum), number of center entries, and the time spent in center area were registered.

Elevated plus maze test

The EPM is a well-described assay of anxiety-related behavior in rodents (Walf and Frye 2007a). In the present study, the EPM was performed in the morning of the 29th day of treatment, 12 h after vitamin A intake. Our EPM apparatus consisted of two open arms (45 cm long×10 cm wide, with 5 mm high railing) and two enclosed arms of equal length and width (45×10 cm with 50 cm high walls) forming a square cross with a 10-cm square center piece (60 cm from the floor). Female rats were placed in the center of the maze facing an open arm at the start of the test. They were allowed to freely explore the EPM for 5 min before being returned to their home cages. The plus maze experimental room was illuminated with only one red overhead light (14-W) to permit videotaping. Total distance traveled, total immobile time, open arms entries, open arm distance traveled, closed arms entries, and closed arm distance traveled were recorded.

Tissue extraction and sample preparation

Rats were euthanized by decapitation (without anesthesia) on the 30th day of experiment (18 h after the last vitamin A treatment). The uterus was cut at the cervix, removed, stripped of fat, and weighed in order to confirm the efficacy of ovariectomy. (Goss et al. 2007). The hypothalamus, frontal cortex, and hippocampus were dissected out in ice immediately after the rat was sacrificed and stored at −80 °C for later analyses. Samples were homogenized in PBS buffer with potter equipment (homogeniser tool suited for the disruption of soft tissues) and centrifuged (1,000×g, 10 min). Supernatants were used for all biochemical assays described herein. Biochemical results were normalized to protein content using bovine albumin as standard (Lowry et al. 1951).

Redox profile in CNS structures

Enzyme activity quantification

Superoxide dismutase (SOD; EC 1.15.1.1) activity was assessed by quantifying the inhibition of superoxide-dependent adrenaline auto-oxidation in a spectrophotometer at 480 nm, as previously described (Misra and Fridovich 1972). Results were expressed as units SOD/mg protein. Catalase (CAT; EC 1.11.1.6) activity was assayed by measuring the rate of decrease in hydrogen peroxide (H_2O_2) absorbance in a spectrophotometer at 240 nm (Aebi 1984). CAT activity was expressed as units CAT/mg protein. SOD/CAT ratio was also calculated (Halliwell 2007).

Non-enzymatic antioxidant measurement

We used the total reactive antioxidant potential (TRAP) test as an index of non-enzymatic antioxidant capacity in CNS structures. This assay is based on the

chemiluminescence emission produced by peroxyl radical (generated by 2,20-azobis[2-amidinopropane] (AAPH) solution with luminal) following radical quenching by sample compounds (Lissi et al. 1992). Briefly we prepared AAPH solution and added luminol (system solution); thereafter, we waited 2 h for the system to stabilize before conducting the first reading. After addition of the sample we analyzed the readings over approximately 40 min. The results were transformed to percentages and the areas under the curve (AUC) were calculated as described (Dresch et al. 2009). First, samples luminescence counts were transformed to percent of radical production (system was considered 100 % radical production), and then AUC was calculated between 0- and 24-min interval using GraphPad Prism software. The smaller the AUC (in relation to the system AUC), the more antioxidants in the sample, indicating less radical production.

Oxidative damage

We used the thiobarbituric acid reactive species (TBARS) test, a widely used method for measurement of lipid redox state, as an index of lipoperoxidation (Draper and Hadley 1990). The TBARS test consists of an acid-heating reaction of the lipid peroxidation end product, malondialdehyde (MDA), with thiobarbituric acid (4,6-dihydroxypyrimidine-2-thiol). TBARS was determined at 532 nm and was expressed as nanomoles MDA per milligram of protein.

Oxidative damage to proteins was measured by the quantification of carbonyl groups, as previously described (Levine et al. 1990). Briefly, this method is based on the reaction of dinitrophenylhydrazine with protein carbonyl groups, and the absorbance read in a spectrophotometer at 370 nm. Results were expressed as nanomoles carbonyl per milligram of protein.

Measurement of total thiol content

Oxidative alterations in proteins and non-protein sources (e.g., glutathione) were used to measure the level of reduced sulfhydryl (SH) content in samples (Ellman 1959). Briefly, for total SH content measurement, a 60ug sample aliquot was diluted in PBS and 10 mM 5,5′-dithionitrobis 2-nitrobenzoic acid, and read in a spectrophotometer at 412 nm after 60 min incubation at 25 °C. The results were expressed as micromoles SH per milligram of protein.

Statistical analysis

Results were expressed as mean±SEM. All analyses were performed using the Statistical Package for the Social Sciences version 15.0 (SPSS Inc., Chicago, IL, USA) software, and GraphPad Prism version 5.04 (GraphPad Software Inc., San Diego, CA, USA). In order to determine if the surgery and/or vitamin A treatment affected the behavioural or biochemical measures, a two-way analysis of variance (ANOVA) was performed with surgery (sham-operated or OVX) and treatment (saline, vitamin A at 500, or vitamin A at 1,500 IU kg^{-1} day^{-1}) as factors. For post-hoc comparisons, the Bonferroni test was conducted when ANOVA was significant. Significance was set at $p \leq 0.05$.

Results

Uterine tissue weight

Sham-operated rats displayed different uterine morphology depending on the estrous cycle phase on the day of decapitation. Seventeen sham female rats displayed estrus and diestrus uterine morphology (0.35–0.51 g, low fluid content and thin tissue), and four sham rats presented with proestrus uterine morphology (0.58–0.61 g, high fluid content and thick tissue). All OVX rats displayed a significant reduction in uterine tissue weight as compared with the sham groups (Table 1). There was a significant effect of surgery ($F_{(1,\ 36)}=57.0$; $p<0.001$) but no effect of

Table 1 Uterine tissue weight

	Sham	OVX
Saline	0.45 ± 0.06	0.13 ± 0.02*
500 IU kg^{-1} day^{-1}	0.41 ± 0.07	0.16 ± 0.04*
1,500 IU kg^{-1} day^{-1}	0.47 ± 0.05	0.15 ± 0.03*

Fresh uterine tissue weight from Sham and OVX groups. Data are presented as mean±SEM (n=7/group)

*$p<0.001$, different from Sham group (two-way ANOVA followed by Bonferroni's posttest)

treatment and no surgery X treatment interaction. Ovariectomy substantially reduced uterine weight.

Behavioral tasks

Open-field test

As shown in Fig. 1, there was a significant effect of surgery and treatment. OVX rats exhibited a decrease in total distance traveled ($F_{(1,\ 36)}$=28.3; p<0.001 (Fig. 1a)), increased immobile time ($F_{(1,\ 36)}$=54.7; p<0.001 (Fig. 1c)), and decreased number of rearing episodes ($F_{(1,\ 36)}$=15.02; p<0.001 (Fig. 1b)) as compared with sham-operated rats. Among sham-operated rats, supplementation with vitamin A 1,500 IU kg^{-1} day^{-1} reduced the number of rearing episodes ($F_{(2,\ 36)}$=5.62; p<0.001; post-hoc, p<0.001 (Fig. 1b)) and decreased time spent in open-field center area ($F_{(2,\ 36)}$=7.01; p<0.001; post-hoc, p<0.001 (Fig. 1e)) as compared with sham+saline. Vitamin A supplementation did not induce significant behavior alterations within OVX rats. Also, vitamin A supplementation at 500 IU kg^{-1} day^{-1} did not affect the OFT measures. We found surgery×treatment interaction effect associated with rearing behavior only ($F_{(2,\ 36)}$=3.69; p<0.05). After vitamin A supplementation, the sham+1,500 rats displayed decreased rearing compared with the control group (p<0.05). Ovariectomy surgery induced a 2-fold decreased rearing, whereas vitamin A treatment did not alter this parameter in the OVX group (Fig. 1b).

Elevated plus-maze test

As depicted in Fig. 2, there was a significant effect of surgery. OVX surgery decreased total distance traveled ($F_{(1,\ 36)}$=17.21; p<0.001 (Fig. 2a)), as well as open arm ($F_{(1,\ 36)}$=12.58; p<0.001 (Fig. 2e)) and closed arm distance traveled ($F_{(1,\ 36)}$=14.87; p<0.001 (Fig. 2f)), and increased total immobile time ($F_{(1,\ 36)}$=18.32; p<0.001 (Fig. 2d)) in EPM. In addition, the number of entries in both open and closed arms was reduced in OVX rats ($F_{(1,\ 36)}$= 10.11; p<0.01 (Fig. 2b) and $F_{(1,\ 36)}$=12.92; p< 0.001 (Fig. 2c)). There was no effect of treatment and no surgery×treatment interaction. Vitamin A supplementation at 500 or 1,500 IU kg^{-1} day^{-1} did not induce significant anxiety-like behavior in sham-operated or OVX groups.

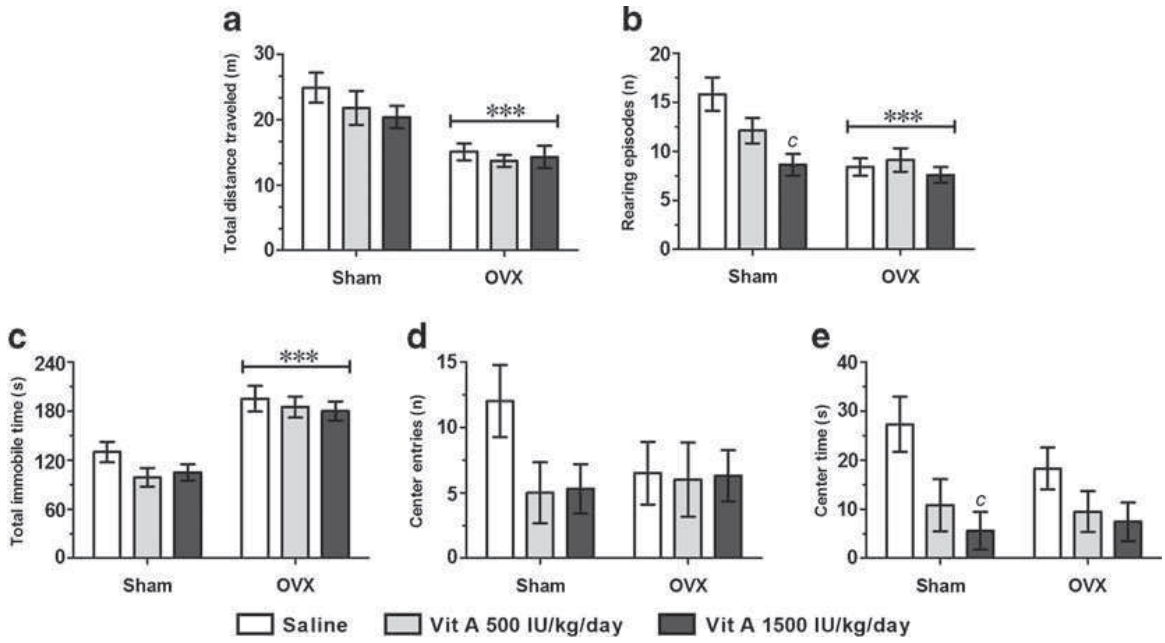

Fig. 1 OFT. **a** Total distance traveled (in meters). **b** Number of rearing episodes. **c** Total immobile time (in seconds). **d** Number of center entries. **e** Time in center area (in seconds). Sham and OVX groups were treated with saline, retinol palmitate at 500 IU kg^{-1} day^{-1}, or retinol palmitate at 1,500 IU kg^{-1} day^{-1}. The OFT was performed 12 h after the 26th day of treatment. Data are presented as mean±SEM (n=7/group). ^{c}p<0.001, different from respective saline group; ***p<0.001, different from Sham group (two-way ANOVA followed by Bonferroni's posttest)

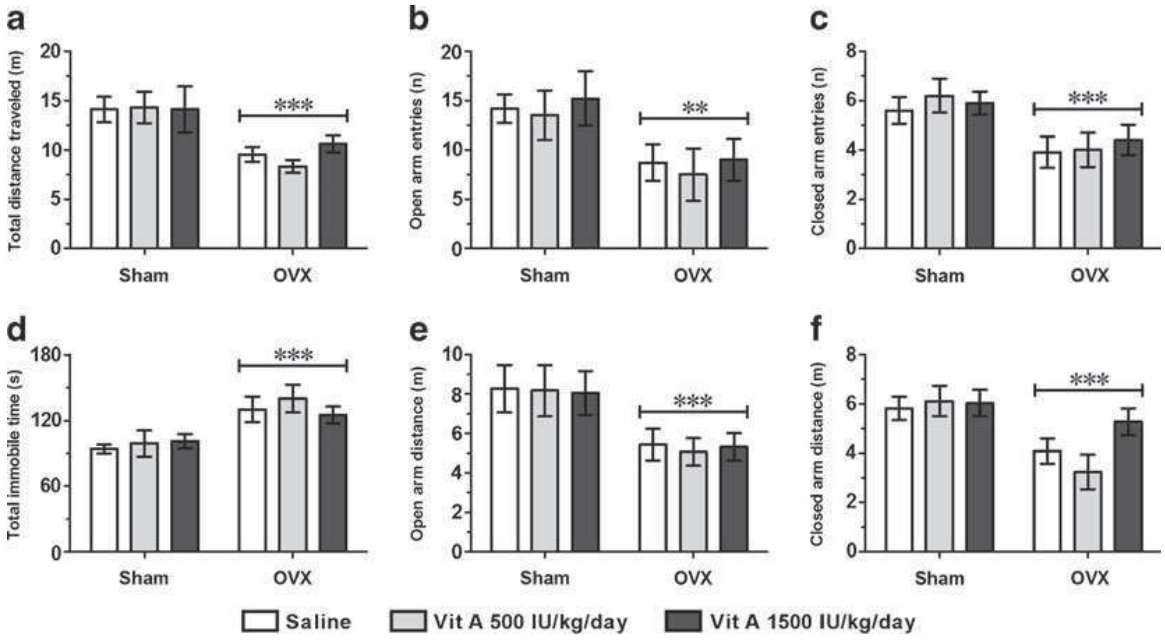

Fig. 2 Elevated plus-maze test. **a** Total distance traveled (in meters). **b** Open arm entries (number). **c** Closed arm entries (number). **d** Total immobile time (in seconds). **e** Open arm distance traveled (in meters). **f** Closed arm distance traveled (in meters). Sham and OVX groups were treated with saline, retinol palmitate at 500 IU kg^{-1} day^{-1}, or retinol palmitate at 1,500 IU kg^{-1} day^{-1}. The elevated plus-maze test was performed 12 h after the 28th day of treatment. Data are presented as mean±SEM (n=7/group). **p<0.01; ***p<0.001, different from Sham group (two-way ANOVA followed by Bonferroni's posttest)

Redox measures

Antioxidant enzyme activity

There was a significant effect of surgery and treatment in enzyme activities analyzes. Ovariectomy induced significant alterations in antioxidant enzyme activities in the hypothalamus and frontal cortex of female rats (Fig. 3). Specifically, ovariectomy increased SOD activity ($F_{(1,\ 36)}$=35.2; p<0.001 (Fig. 3a) and $F_{(1,\ 36)}$=21.9; p<0.001 (Fig. 3d)) and decreased CAT activity ($F_{(1,\ 36)}$=12.3; p<0.01 (Fig. 3b) and $F_{(1,\ 36)}$=7.31; p<0.01 (Fig. 3e)). Ovariectomy also increased SOD/CAT ratio in the hypothalamus and frontal cortex ($F_{(1,\ 36)}$=67.2; p< 0.001 (Fig. 3c) and $F_{(1,\ 36)}$=58.8; p<0.001 (Fig. 3f)). Ovariectomy had no effect on hippocampal SOD or CAT activity. However, vitamin A supplementation at 1,500 IU kg^{-1} day^{-1} reduced hippocampal SOD activity ($F_{(2,\ 36)}$=4.29; p<0.05; post-hoc, p<0.05 (Fig. 3g)) and CAT activity (Fig. 3H, $F_{(2,\ 36)}$=9.40; p<0.001; post-hoc, p< 0.001) in OVX group, which consequently increased SOD/CAT ratio (Fig. 3I, $F_{(2,\ 36)}$=13.9; p<0.001; post-hoc, p<0.001). Vitamin A supplementation at 1,500 IU kg^{-1} day^{-1} reduced CAT activity in sham-operated group (post-hoc, p<0.001 (Fig. 3h)), which consequently increased SOD/CAT ratio (post-hoc, p<0.001 (Fig. 3i)). Vitamin A supplementation at 500 IU kg^{-1} day^{-1} did not alter antioxidant enzyme activities in any of the brain areas. There was no surgery×treatment interaction.

Non-enzymatic antioxidant defenses

There was no significant effect of surgery in hypothalamus, frontal cortex, or hippocampus (Fig. 4). We found a significant treatment effect in hippocampus ($F_{(2,\ 36)}$=9.51; p<0.001 (Fig. 4c)). Vitamin A supplementation at 1,500 IU kg^{-1} day^{-1} reduced non-enzymatic antioxidant defenses in the hippocampus in both sham-operated (post-hoc, p<0.001) and OVX groups (post-hoc, p<0.001), as illustrated by an increased AUC in TRAP analysis (Fig. 4d). Vitamin A supplementation at 500 IU kg^{-1} day^{-1} did not alter non-enzymatic antioxidant defenses in any of the brain areas. Again, no surgery×treatment interaction was found.

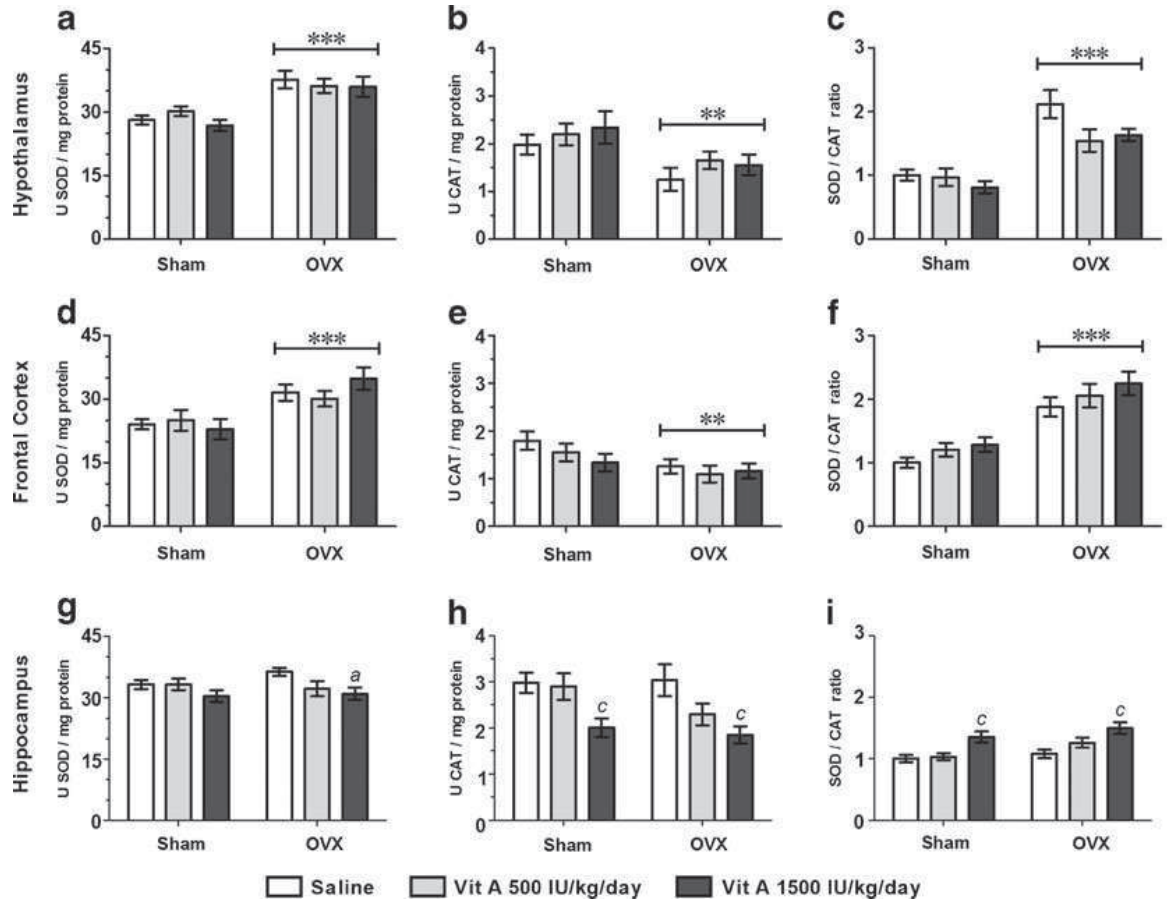

Fig. 3 Effects of bilateral ovariectomy and vitamin A supplementation on CNS antioxidant enzyme activities. **a**, **d**, **g** Total superoxide dismutase activity (*SOD*). **b**, **e**, **h** Catalase activity (*CAT*). **c**, **f**, **i** SOD/CAT ratio (arbitrary units). Measured in the hypothalamus (**a**–**c**), frontal cortex (**d**–**f**), and hippocampus (**g**–**i**). Data are presented as mean±SEM (n=7/group). $^{a}p<0.05$; $^{c}p<0.001$, different from respective saline group; $^{**}p<0.01$; $^{***}p<0.001$, different from Sham group (two-way ANOVA followed by Bonferroni's posttest)

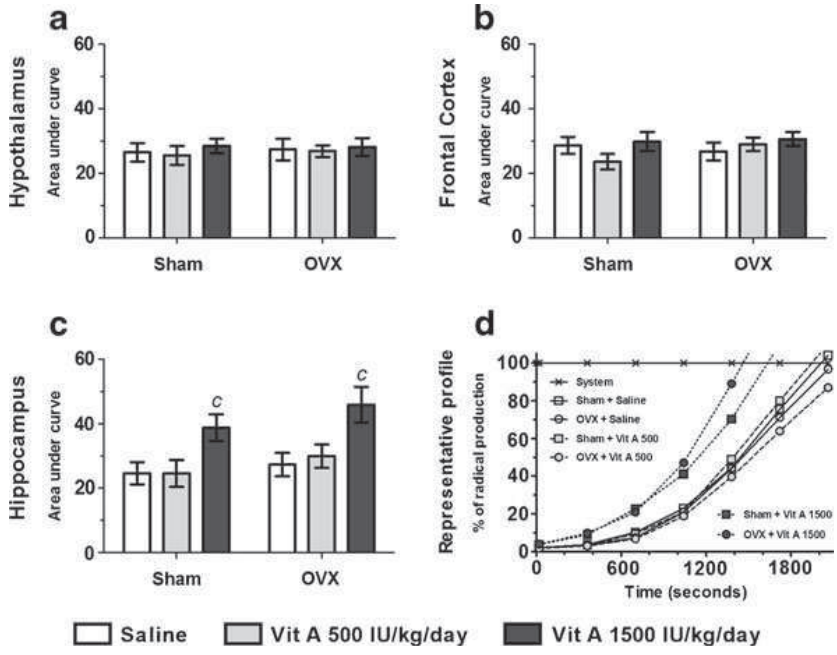

Fig. 4 Effects of bilateral ovariectomy and vitamin A supplementation on CNS non-enzymatic antioxidant potential. TRAP was measured in the hypothalamus (**a**), frontal cortex (**b**), and hippocampus (**c**). Hippocampal representative TRAP profile is described (**d**). Data are presented as mean±SEM (n=7/group). $^{c}p<0.001$, different from respectively saline group (two-way ANOVA followed by Bonferroni's posttest)

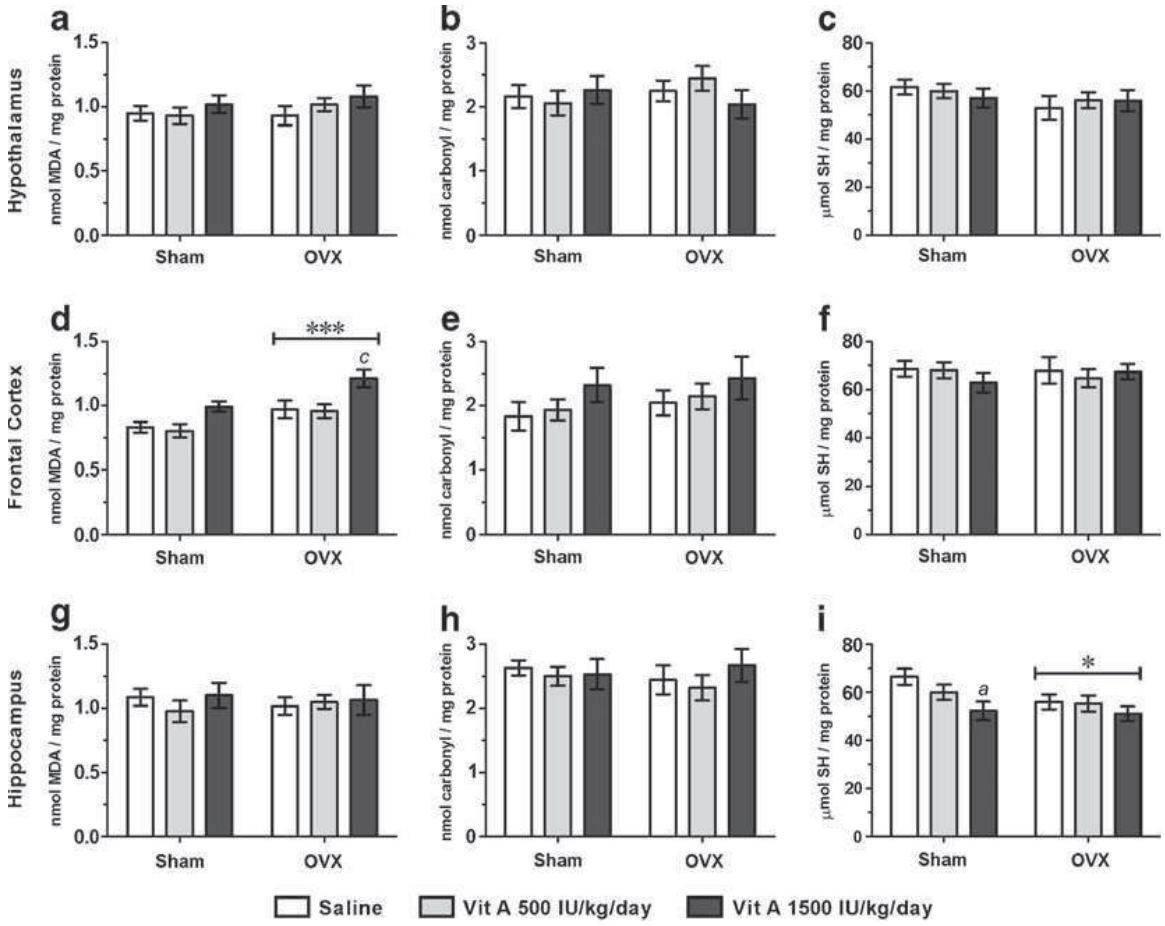

Fig. 5 Effects of bilateral ovariectomy and vitamin A supplementation on CNS oxidative damage parameters and total reduced thiol content (*SH*). **a**, **d**, **g** TBARS. **b**, **e**, **h** Carbonyl levels. **c**, **f**, **i** SH content. Measured in the hypothalamus (**a**–**c**), frontal cortex (**d**–**f**) and hippocampus (**g**–**i**). Data are presented as mean±SEM (n=7/group). $^{a}p<0.05$; $^{c}p<0.001$, different from respective saline group; $^{*}p<0.05$; $^{***}p<0.001$, different from Sham group (two-way ANOVA followed by Bonferroni's posttest)

Lipid and protein oxidative damage and total SH content

As depicted in Fig. 5, there was a significant effect of surgery and treatment in lipid damage and SH content parameters. OVX surgery increased frontal cortex TBARS ($F_{(1,\ 36)}$=14.3; $p<0.001$ (Fig. 5d)) and decreased hippocampal total SH content ($F_{(1,\ 36)}$=4.12; $p<0.05$ (Fig. 5i)). Vitamin A supplementation at 1,500 IU kg^{-1} day^{-1} increased TBARS in the frontal cortex of OVX rats ($F_{(2,\ 36)}$=9.87; $p<0.001$; post-hoc, $p<0.001$ (Fig. 5d)). Vitamin A supplementation at 1,500 IU kg^{-1} day^{-1} also decreased total SH content of hippocampus in sham-operated group ($F_{(2,\ 36)}$= 4.25; $p<0.05$; post-hoc, $p<0.05$ (Fig. 5i)). There was no effect of surgery or treatment in carbonyl index (protein damage). Vitamin A supplementation at 500 IU kg^{-1} day^{-1} did not induce oxidative damage and did not alter SH content in any of the brain areas. There was no surgery×treatment interaction in any of the redox profile measures, which suggests that the effects of vitamin A on brain redox profile were not sham or OVX specific.

Discussion

In the present study, we investigated the effects of vitamin A supplementation on behavior and brain redox profile in sham-operated and OVX female rats. Ovariectomy induced pronounced uterine atrophy, decreased locomotor/exploratory activity and increased anxiety-like behavior. Also, OVX rats displayed increased hypothalamic and frontal cortex SOD/CAT

ratio, decreased hippocampal total thiol content, and increased frontal cortex lipid oxidative damage. It has been long recognized that ovariectomy can cause significant changes in brain redox profile (Abbas and Elsamanoudy 2011; Huang and Zhang 2010; Martins et al. 2012), and our results suggest that vitamin A supplementation at 500 and 1,500 IU kg^{-1} day^{-1} did not prevent brain oxidative stress associated with ovariectomy.

Physiological changes after ovariectomy include osteoporosis (Muthusami et al. 2005), cardiac hypertrophy (Bhuiyan and Fukunaga 2010), abnormal myocardial architecture (Lee et al. 2008), uterine atrophy (Goss et al. 2007), increased tail-skin temperature (Bowe et al. 2006), decreased plasma vitamins A, C, and E (Dilek et al. 2010), and increased oxidative stress with acceleration of aging processes in bone (Muthusami et al. 2005), heart, aorta (Lee et al. 2005), and peripheral blood (Behr et al. 2012). In addition, OVX rats exhibit a number of behavioral alterations, such as decreased locomotor/exploratory activity (Roy et al. 1990), increased anxiety-like behavior (Walf and Frye 2007b), and accelerated memory decrements (Acosta et al. 2009). Our results are in line with ovariectomy-induced decreased locomotor and exploratory activity and alterations in cerebral redox profile. We observed decreased locomotor activity in both OFT and EPM protocols. In addition, in OFT analysis we observed a reduction in vertical exploratory activity (rearing) and in EPM analyzes we observed an increase in anxiety-like behavior (reduced number of entries and increased immobility time in open arms). However, in EPM analyzes we also observed decreased number of entries and distance traveled in closed arms. These results indicate that the OVX effects on behavioral measures were more pronounced in locomotor/exploratory activity rather than in anxiety-like behavior.

Along with decreased locomotor behavior, OVX rats displayed altered CNS redox profile as illustrated by increased hypothalamic and frontal cortical SOD activity, decreased CAT activity, and increased SOD/CAT ratio. Increased SOD/CAT ratio favors the maintenance of higher levels of H_2O_2, since SOD converts $O_2^{\cdot -}$ to H_2O_2, but CAT is not able to metabolize H_2O_2 to water (Halliwell 2007). H_2O_2 is one of the main contributors to oxidative damage, given that H_2O_2 can react with free radicals or transition metals and generate powerful and potentially dangerous ROS (Koppenol 2001). Our results indicate that increased brain oxidative stress in OVX rats may lead to oxidative damage as illustrated by increased frontal cortex lipid peroxidation and hippocampal thiol oxidation. Previous studies have reported increased lipid oxidative damage in total brain homogenate (Ozgonul et al. 2003) and isolated mitochondria (Feng and Zhang 2005; Irwin et al. 2011) of OVX rats. However, another study examining rat hippocampus did not observe changes in lipid damage levels in OVX rats but found increased CAT activity (Monteiro et al. 2005). To our knowledge, the present study is the first to report changes in the SOD/CAT ratio in CNS structures of OVX rats, which is indicative of oxidative stress.

In the present study, vitamin A at higher doses (1,500 IU kg^{-1} day^{-1}) decreased exploratory activity, increased hippocampal SOD/CAT ratio, decreased hippocampal thiol content (oxidation), and decreased total non-enzymatic antioxidant potential in the hippocampus of sham-operated rats. Moreover, vitamin A treatment at 1,500 IU kg^{-1} day^{-1} increased hippocampal SOD/CAT ratio, decreased hippocampal total non-enzymatic antioxidant potential, and increased frontal cortex lipid oxidative damage in OVX rats. These results suggest that vitamin A at higher doses induced oxidative stress in the rat brain in vivo. To our knowledge, this is the first study that investigated the effects of vitamin A supplementation on CNS redox profile in sham-operated and OVX female rats. Previous studies from our group revealed that vitamin A doses between 2,500–9,000 IU kg^{-1} day^{-1} for 4 weeks or more can induce abnormalities in the redox and bioenergetics states of various tissues in healthy male rats (de Oliveira et al. 2009a; de Oliveira et al. 2009b; de Oliveira et al. 2009c; Pasquali et al. 2009), as well as in female dams and their offspring when treated during pregnancy and nursing (Pasquali et al. 2010; Schnorr et al. 2011a; Schnorr et al. 2011b). For instance, acute and chronic vitamin A supplementation may induce oxidative/nitrosative stress in the adult rat hippocampus (de Oliveira et al. 2007), substantia nigra (de Oliveira et al. 2008), frontal cortex (de Oliveira et al. 2009b), and hypothalamus (de Oliveira et al. 2009d). Furthermore, vitamin A supplementation at different doses during pregnancy and nursing increases SOD/CAT ratio and oxidative damage in maternal and offspring striatum and hippocampus (Schnorr et al. 2011b).

In the only study that used lower doses of vitamin A in adult female rats (500 IU kg^{-1} day^{-1} for

2 months), de Oliveira et al. (2011) showed increased 3-nitrotyrosine levels in mitochondrial membranes and impaired respiratory chain activity in discrete brain regions. In the present study, vitamin A at 500 IU kg^{-1} day^{-1} for 1 month did not alter behavior or brain redox profile in sham-operated or OVX female rats, which suggests that longer vitamin A exposure (2 vs. 1 month) may trigger oxidative stress via protein nitration and mitochondrial dysfunction. We have also previously shown that vitamin A at 500–1,500 IU kg^{-1} day^{-1} for 1 month exerted antioxidant effects in the plasma of OVX rats, increasing total antioxidant potential (Behr et al. 2012). However, in the present study we found pro-oxidant effects with vitamin A at 1,500 IU kg^{-1} day^{-1} in the CNS of sham-operated and OVX rats. These results of pro-oxidant effects in the CNS contrast strikingly with those of antioxidant effects in peripheral blood previously reported. These latter findings are consistent with several clinical trials that investigated vitamin supplementation in postmenopausal women and showed that vitamin supplementation may be beneficial or harmful depending on the organ and treatment regimen (Dennehy and Tsourounis 2010; Hagey and Warren 2008). While the clinical efficacy of Vitamin A for menopausal women remains to be tested, long-term use of high-doses of vitamin A (>3,000 μg/day for 18 years) may increase the risk for osteoporotic fractures (Feskanich et al. 2002).

When translating results from pharmacological challenges in rat models to humans, one must take into account the differences between rat and human metabolism. Equivalent doses may be obtained with application of an uncertainty factor of 10-fold for within species differences and of 10-fold for interspecies differences (Boyes et al. 1997; EPA 1998). Thus, according to this model, Wistar rats can receive doses up to ×100 higher as compared with humans. More recently, Reagan-Shaw et al. (2008) explored the dose translation from animals to humans and advocated the use of a "body surface area (BSA) normalization method" as a factor when converting doses from animals to humans. The BSA-based dose calculation is considered the most appropriate method and is far superior to the simple conversion based on body weight. However, a concerted effort toward designing more appropriate dose conversions that eliminate some of the limitations associated with the BSA method is needed in order to improve therapeutic outcomes in pharmacological trials (Gao et al. 2008; Saadeh et al. 2011). According to the BSA normalization method, the doses used in our study would reflect 1,450 and 4,350 μg for humans (0.3 μg of retinol=1 IU), which are close to the recommended vitamin A dietary intake for older women (700–3,000 μg) (Chernoff 2005; Kennedy and Meyers 2005).

The sophisticated cross-talk among different CNS structures permit rapid and precise detection of food, danger, and mates, all crucial elements for survival and species maintenance. Additionally, the fine regulation in this cross-talk is mandatory for mood stabilization and modulation. A shift in emotional regulation circuitry might therefore occur in women during the menopausal transition and possibly contribute to the occurrence of mood and anxiety symptoms in women during/after this period in life (Frey et al. 2010). In these work, we focused on the hippocampus, frontal cortex, and hypothalamus. Together, these three brain areas are responsible to mood and locomotor brain-related functions. The acquisition of new memories for places and events requires synaptic plasticity in the hippocampus, and is critical for spatial learning, object recognition and memory (Cheng and Frank 2008; Vukovic et al. 2011). The role of frontal cortex, whether in rodents or humans, should be viewed within the context of its crucial role in brain wide orchestration of adaptive behavior, particularly through its involvement in executive functions (Kesner and Churchwell 2011). The hypothalamus is the brain control center of hormonal/endocrine functions, presenting connections with many parts of the CNS, including the hippocampus, and the frontal lobes of the cerebral cortex (Shepherd 2006). The active form of vitamin A, retinoic acid, modulates different CNS functions by regulating gene expression in the brain (McCaffery et al. 2003). Interesting, some retinoids approved for dermatological treatment carries a black box warning related to the risk of depression, suicide and psychosis (Bremner et al. 2012).

Some studies have reported behavioral changes in animals under oxidative stress conditions. Furthermore, we have previously shown redox impairment in CNS regions associated to altered behavior in Wistar rats after vitamin A supplementation at different doses. For instance, de Oliveira et al. (2007) showed decreased locomotor/exploratory activity associated with increased hippocampal oxidative stress in male Wistar rats supplemented with vitamin A at doses ranging from 1,000 to 9,000 IU/kg for 28 days. Schnorr et al. (2011a, b) showed decreased exploratory

activity in vitamin A-treated offspring and decreased locomotory activity in dams and male offspring associated with increased SOD/CAT ratio and oxidative damage in striatum and hippocampus. Furthermore, Merzoug et al. (2011) showed decreased locomotor/exploratory activity associated with increased brain oxidative stress in male Wistar rats after acute treatment with adriamycin. Finally, Cruz-Aguado et al. (2001) demonstrated several behavioral and biochemical changes after glutathione depletion in the rat brain, supporting the hypothesis that oxidant/antioxidant equilibrium is important in the regulation of brain function. In the present study, we found decreased locomotor/exploratory activity, accompanied by changes in the SNC redox profile of sham and OVX female rats. Even though a number of studies of rodents under various oxidative stress conditions have found behavioral changes, especially in locomotor/exploratory behaviors, the molecular underpinnings underlying the association between brain oxidative stress and changes in behavior have not been explored. This is a fruitful area for future research.

In conclusion, our study suggests some caution regarding the use of vitamin A supplementation at higher doses. We found that higher doses of vitamin A induced pro-oxidant effects in the brain in both OVX and sham-operated rats. Future studies investigating the influence of vitamin A supplementation at various doses in the redox profile of other tissues and organs, such as heart, kidney, and liver are warranted.

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Conflict of Interest Statement The authors declare no conflicts of interest related to the present report.

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PARTE III

4 – DISCUSSÃO

Atualmente não existe um único modelo experimental que, especificamente, represente a falha progressiva da função ovariana que ocorre durante a transição natural para a menopausa. Ao mesmo tempo, os modelos disponíveis com base na indução química ou cirúrgica precisam continuar sendo estudados e caracterizados quanto à possibilidade de recriar com maior precisão o estado hormonal irregular que ocorre durante a transição para a menopausa. Com essa advertência em mente, o modelo experimental de ovariectomia continua sendo a escolha mais popular, uma vez que desenvolve (mimetiza) algumas das características clínicas mais importantes da deficiência de estrogênio em mulheres adultas, como a perda óssea, disfunções do aparelho circulatório e envelhecimento do sistema nervoso (Baeza *et al*., 2010a; Lee *et al*., 2005; Muthusami *et al*., 2005). Associado ao maior risco de apresentar sintomas relacionados com a menopausa, as ratas OVX são capazes de apresentar aumento nos níveis de estresse oxidativo e, consequentemente, acelerar o processo de envelhecimento em diferentes tecidos (Agarwal *et al*., 2005; Behr *et al*., 2012a,b; Lee *et al*., 2005; Muthusami *et al*., 2005).

Neste cenário, o estresse oxidativo, aliado a uma possível disfunção redox, tem sido proposto como peça-chave para os efeitos biológicos secundários da menopausa experimental. No presente trabalho, nosso primeiro objetivo foi avaliar os efeitos da ovariectomia bilateral sobre os parâmetros de estresse oxidativo, bioquímicos e comportamentais em ratos Wistar. Nossos resultados confirmaram que as ratas OVX apresentaram aumento do estresse oxidativo em sangue/plasma e SNC, consideráveis mudanças no perfil lipídico plasmático, associados a um

aumento no ganho de peso corporal e esperada atrofia do tecido uterino, além de alterações comportamentais significantes.

Antes da menopausa, tanto o ovário da mulher quanto o ovário de várias outras espécies animais, serve como fonte primária de estrogénio (17β-estradiol ou E2). Este hormônio vem sendo associado com a proteção da mulher durante a pré-menopausa, mas também pode estar associado a uma grande variedade de potenciais problemas de saúde na pós-menopausa, tais como o aumento no risco de declínio das funções do sistema cardiovascular, esquelético e nervoso, e processo de envelhecimento acelerado. Um efeito antiaterosclerótico importante do estrogênio é, provavelmente, a sua influência benéfica sobre o metabolismo dos lipídeos. Mulheres na pós-menopausa geralmente apresentam um aumento dos níveis de LDL, lipoproteína (a) e colesterol total, e diminuição do nível de HDL (Wen *et al*., 2000). Em nosso estudo, as ratas OVX apresentaram aumento nos níveis de colesterol total, mas sem alterações na fração HDL, e uma diminuição interessante nos níveis de triglicerídeos. De acordo com o perfil lipídico observado, podemos também sugerir um aumento nos níveis de LDL nas ratas OVX.

Em nosso estudo as ratas OVX apresentaram níveis reduzidos de triglicerídeos em comparação com os animais controle. No entanto, a literatura fornece evidências de que tanto em modelos experimentais com roedores como em mulheres na pós-menopausa os níveis plasmáticos de triglicérides se apresentam aumentados (Saleh & Saleh, 2010; Tuna *et al*., 2010). Por outro lado, de acordo com outro estudo, comparando um grupo de pacientes que passaram pelo processo de menopausa cirúrgica por ooforectomia bilateral com um grupo de mulheres naturalmente menopáusicas, apesar do colesterol total e LDL se apresentarem maiores no grupo

menopausa cirúrgica, não foi encontrada diferença estatística entre os dois grupos nos níveis de HDL, VLDL e triglicerídeos (Suda *et al*., 1998).

Além disso, os fatores que determinam o nível de triglicerídeos não estão bem definidos, apesar da dieta, fumo, atividade física influenciarem os níveis de triglicerídeos (Castro Cabezas *et al*., 2001). Nossos dados também mostram uma diminuição nos níveis de ácido úrico plasmáticos em ratas OVX. Recentemente, o aumento nos níveis de ácido úrico foi associado com risco aumentado para síndrome metabólica em mulheres tanto na pré-menopausa como na pós-menopausa (Lee *et al*., 2011). Por outro lado, outro estudo recente sugere que a menopausa tem influencia parcial, mas não total, sobre o nível de ácido úrico associado à idade aumentada entre as mulheres (Hak & Choi, 2008). Além disso, em nosso estudo mostramos que ratas OVX apresentaram ganho de peso corporal aumentado e tecido uterino extremamente atrofiado. Estes resultados confirmam a eficiência da cirurgia de ovariectomia bilateral em provocar a cessação drástica da secreção de hormônios sexuais femininos.

O aumento no risco de doença coronariana em mulheres na pós-menopausa está intimamente relacionada com a queda na secreção de hormônios femininos. Evidências sugerem que as doenças cardiovasculares estão associadas com o estresse oxidativo aumentado nos vasos sanguíneos (Stirone *et al*., 2005). Níveis aumentados de ERO, tais como o radical superóxido ($O_2^{\bullet -}$) e o peróxido de hidrogénio (H_2O_2), fazem com que os vasos sanguíneos se tornam mais espessos, potencializando a inflamação das paredes do vaso e, portanto, são considerados fatores de risco para inúmeras doenças vasculares.

Quando em concentrações controladas as ERO também atuam como moléculas de sinalização, em muitos casos mediando importantes respostas fisiológicas (Ushio-Fukai & Alexander, 2004). O estresse oxidativo é um estado particular caracterizado por uma sobrecarga nos níveis de oxidantes, podendo culminar em disfunção celular (Halliwell & Gutteridge, 2007; Halliwell, 2007). No nosso estudo, as ratas OVX apresentaram aumento nas atividades das enzimas antioxidantes no sangue, uma diminuição nas defesas não enzimáticas no plasma, uma diminuição no conteúdo de SH proteico e não proteico, acompanhado do aumento nos níveis de dano oxidativo às proteínas plasmáticas.

O aumento na atividade das enzimas antioxidantes catalase (CAT) e glutationa peroxidase (GPx) no sangue, sem alterações na atividade da superóxido dismutase (SOD), sugere que outra fonte de H_2O_2, em vez da SOD, esteja ativa. Sabe-se que os vasos sanguíneos expressam as três isoformas de SOD: citosólica, mitocondrial, e extracelular (EC-SOD) (Faraci & Didion, 2004). Uma consequência na atividade da SOD é a formação de H_2O_2 que é relativamente estável e difusível (inclusive através de membranas celulares), em comparação com as demais ERO. Embora reconhecido como uma peça-chave tanto para o dano oxidativo como para regulação redox de processos celulares, ainda há muitas lacunas sobre como o H_2O_2 age. Além disso, o H_2O_2 reage diretamente com moléculas biológicas causando transformações redox-tiol, promovendo sinalização celular e corroborando para o dano oxidativo (Blanc *et al*., 2003; Trachootham *et al*., 2008).

No presente estudo, também mostramos um decréscimo nas defesas não enzimáticas plasmáticas, tanto no potencial antioxidante total não enzimático (TRAP) como na reatividade antioxidante total (TAR). Antioxidantes não enzimáticos, tais como a vitamina C, vitamina E, selénio, zinco, taurina, glutationa (GSH), beta-

caroteno, e caroteno, são geralmente obtidos a partir de fontes dietéticas (Agarwal *et al*., 2008). A literatura relata concentrações de vitaminas A, C, e E no plasma de ratas OVX mais baixas do que em animais controle (Dilek *et al*., 2010). Assim, as concentrações plasmáticas de vitaminas A, C e E em ratas OVX podem estar diminuídas como resultado da sua ação inibitória sobre os insultos desencadeados pelas ERO. Além disso, o *pool* sanguíneo de SH reduzido pode estar esgotado em decorrência do insulto oxidativo causado pela ovariectomia. De acordo com esta ideia, observamos um decréscimo no conteúdo de SH proteico e não proteico em ratas OVX, sugerindo aumento nos níveis de oxidação proteica e de compostos SH, como a GSH.

Além disso, verificamos a diminuição nos níveis de ácido úrico plasmáticos em ratas OVX, sendo este um dos principais antioxidantes plasmáticos não-proteico. A diminuição no potencial antioxidante não enzimático observada no grupo OVX pode estar correlacionada com os baixos níveis de ácido úrico encontrados. Diferentes estudos têm demonstrado uma correlação positiva entre o potencial antioxidante e níveis de ácido úrico plasmático (Moura-Nunes *et al*., 2009). Particularmente no choque séptico, os níveis de antioxidantes não enzimáticos estão fortemente correlacionados com os níveis de ácido úrico (Andresen *et al*., 2008).

Além disso, as ratas OVX apresentaram aumento nos níveis plasmáticos de grupamentos carbonil (indicativo de dano à proteína), mas não houve alterações na peroxidação lipídica. Grande parte da literatura relata aumento nos níveis de lipoperoxidação em ratas OVX e em mulheres na pós-menopausa (Baeza *et al*., 2010b; Dilek *et al*., 2010; Miquel *et al*., 2006; Signorelli *et al*., 2001). Por outro lado, é bem reconhecido que o dano oxidativo à proteína está intimamente associado a muitas patologias relacionadas com a menopausa (Halliwell, 2006).

Dos muitos alvos biológicos das ERO, os lipídios são a classe de biomoléculas mais suscetíveis. Um dos vários produtos finais de baixo peso molecular formados via decomposição de produtos primários e secundários da lipoperoxidação é o malondialdeido (MDA). Esta molécula pode ser quantificada através de diferentes técnicas. As duas mais comuns são, por cromatografia (analisando a quantidade de MDA) e espectrofotometria (teste das espécies reativas ao ácido tiobarbitúrico – TBARS) (Janero, 1990). A utilização da análise de MDA e/ou o teste de TBARS em estudos de peroxidação lipídica exige cautela, critério, e dados de correlação com outros índices de estresse oxidativo. Em nosso trabalho, não encontramos níveis aumentados de peroxidação lipídica em animais com capacidade antioxidante diminuída. Muito provavelmente, a quantificação dos níveis de TBARS não é a abordagem mais precisa e específica para fazer a avaliação da lipoperoxidação no sangue/plasma. Mais recentemente, a quantificação de F2-isoprostanos por métodos que utilizam espectrometria de massa vem ganhando força. Este teste é atualmente considerado como o melhor biomarcador disponível para avaliar os níveis de dano a lipídios (Halliwell & Lee, 2010). F2-isoprostanos certamente são os marcadores mais específicos de peroxidação lipídica, mas também um dos mais difíceis de medir.

Além dos dados obtidos em tecido periférico, as ratas OVX apresentaram aumento da relação SOD/CAT no hipotálamo e córtex frontal, diminuição do teor total de SH em hipocampo, e maior dano oxidativo a lipídios em córtex frontal. Diversos trabalhos na literatura relatam mudanças significativas no perfil redox do SNC em consequência da ovariectomia bilateral (Abbas & Elsamanoudy 2011; Huang & Zhang 2010; Martins *et al*., 2012). Além disso, ratas OVX apresentam alterações comportamentais, tais como diminuição da atividade

locomotor/exploratória (Roy *et al*., 1990), aumento da ansiedade (Walf & Frye, 2007), e acelerado decréscimo na memória (Acosta *et al*., 2009). Nossos resultados corroboram com as alterações descritas na literatura, quando observamos diminuição da atividade locomotora e exploratória, e alteração no perfil redox cerebral em ratas OVX.

Nós observamos diminuição da atividade locomotora em ambos protocolos, tanto no teste de Campo Aberto (CA) como no Labirinto em Cruz Elevada (LCE). Bastante interessante, no experimento de CA observamos uma redução na atividade exploratória vertical (*rearing*) e na análise de LCE observamos um aumento na ansiedade (diminuição do número de entradas e tempo de imobilidade aumentado nos braços abertos). Ainda, no protocolo de LCE também observamos diminuição do número de entradas e distância percorrida nos braços fechados. Estes resultados podem indicar que os efeitos sobre as medidas comportamentais em ratas OVX estão mais pronunciados na atividade locomotor/exploratória em vez do comportamento de ansiedade.

Juntamente com o comportamento locomotor diminuído, as ratas OVX exibiram alterações no perfil redox do SNC como observado através do aumento na atividade da SOD em hipotálamo e córtex frontal, diminuição na atividade da CAT, e aumento na relação SOD/CAT. O aumento na razão de SOD/CAT favorece a manutenção de níveis mais elevados de H_2O_2, uma vez que a SOD converte $O_2^{\bullet-}$ para H_2O_2, mas a CAT não é capaz de metabolizar todo H_2O_2 à água (Halliwell, 2007). H_2O_2 é um dos principais contribuintes para desencadear danos oxidativos, uma vez que o H_2O_2 pode reagir com outros radicais livres ou metais de transição, gerando ERO mais reativas e potencialmente danosas (Koppenol, 2001).

Os nossos resultados indicam que o estresse oxidativo cerebral aumentado em ratas OVX pode conduzir a danos oxidativos como observado através do aumento na peroxidação lipídica em córtex frontal e oxidação de tióis no hipocampo. Estudos anteriores relatam aumento no dano oxidativo lipídico em homogenato de cérebro total (Ozgonul *et al*., 2003.) e mitocôndrias isoladas (Feng & Zhang, 2005;. Irwin *et al*., 2011) de ratas OVX. No entanto, outro estudo, onde o hipocampo de ratas OVX foi examinado, não foram observadas alterações nos níveis de dano a lipídios, porém os autores relatam aumento na atividade da CAT (Monteiro *et al*., 2005). Na literatura, o nosso estudo é o primeiro a relatar mudanças na relação SOD/CAT em estruturas do SNC de ratas OVX, o que pode ser indicativo de estresse oxidativo.

Em resumo, de acordo com os parâmetros bioquímicos, comportamentais e de estresse oxidativo em sangue/plasma e SNC apresentados, podemos afirmar que o modelo animal de ratas OVX é válido e adequado para fins de investigação científica de condições relacionadas à menopausa humana, o que é corroborado pela literatura precedente (Baeza *et al*., 2010a; Bhuiyan & Fukunaga, 2010; Goss *et al*., 2007; Lee *et al*., 2005, 2008; Muthusami *et al*., 2005).

Nosso principal objetivo foi o de avaliar os efeitos da suplementação com vitamina A em ratas OVX sobre os parâmetros anteriormente descritos. Os nossos resultados sugerem que a vitamina A em doses de 500 e 1500 UI/kg tem efeitos potencialmente benéficos em ratas OVX, melhorando a capacidade antioxidante do sangue após 30 dias de suplementação. Por outro lado, nosso estudo também sugere cautela quanto da administração de vitamina A durante a menopausa, uma vez que a suplementação na dose de 1500 UI/kg/dia induz aumento no estresse oxidativo em estruturas do SNC, além de provocar a diminuição na atividade locomotora exploratória em ratas OVX.

Com relação à dose de vitamina A utilizada neste estudo, a mesma foi determinada de acordo com a quantidade de vitamina A complementada no alimento dos ratos (25200 UI/kg de alimento, de acordo com o fabricante) e da quantidade que cada rato adulto come por dia (15-25g de alimento). Portanto, na nossa própria colônia de animais, o consumo de vitamina A no alimento está entre 375-625 UI por rato/dia. Além disso, vários trabalhos originários dentro do nosso grupo de pesquisa demonstraram que a suplementação de vitamina A em doses potencialmente mais elevadas (acima de 2500 UI/kg/dia) leva a disfunções nos estados redox e nas características bioenergéticas de diferentes tecidos em ratos machos saudáveis (de Oliveira *et al*., 2009a,b,c; Pasquali *et al*., 2009). É importante enfatizar que aqui foi testada a suplementação com vitamina A em um modelo animal totalmente distinto (neste caso ratas OVX), com diferenças perceptíveis de sexo e fisiológicas a serem consideradas.

Ainda, ao interpretar os resultados obtidos em estudos farmacológicos utilizando modelos animais, devemos levar em conta as diferenças entre o metabolismo humano e o da espécie utilizada como modelo. Doses equivalentes podem ser obtidas com a aplicação de um fator de incerteza de 10 vezes para diferenças de espécie, e de 10 vezes para diferenças entre espécies (Boyes *et al*., 1997;. EPA, 1998). Assim, de acordo com este modelo, ratos Wistar podem receber doses até 100 vezes superiores em comparação com os seres humanos.

Mais recentemente, Reagan-Shaw *et al*. (2008) exploraram a conversão de doses utilizadas em modelo animal para doses em seres humanos, defendendo o uso da "área de superfície corporal" (ASC) como um método a ser utilizado para converter doses em animais para humanos. O cálculo da dose utilizando o modelo ASC é considerado o método mais adequado e é muito superior à simples

conversão com base no peso corporal. No entanto, ainda hoje são necessários maiores esforços para conceber as conversões de doses mais apropriadas que eliminem algumas das limitações associadas ao método de ASC, a fim de melhorar os resultados terapêuticos em ensaios farmacológicos (Gao *et al*., 2008; Saadeh *et al*., 2011). De acordo com o método de normalização ASC, as doses utilizadas no nosso estudo refletiriam 1450 e 4350 μg para os seres humanos (0,3 μg de retinol = 1 UI), doses estas que estão perto da ingestão dietética de vitamina A recomendada para mulheres na menopausa (700-3000 μg) (Chernoff, 2005; Kennedy & Meyers, 2005).

Observando que começamos o tratamento dois meses após o procedimento cirúrgico, os resultados obtidos em sangue/plasma nos levam a crer que o tratamento com vitamina A provavelmente age mais revertendo algumas das alterações induzidas pela ovariectomia do que impedindo. No entanto, este não é um fenómeno simples. Não é possível descartar que o tratamento com vitamina A também pode ser importante na prevenção de alguns sintomas relacionados com a menopausa, durante os últimos 30 dias do protocolo. Além disso, períodos longos de tratamento podem induzir diferentes alterações no metabolismo, algumas vezes levando a toxicidade hepática. Em nosso estudo, não observamos mudanças nas transaminases plasmáticas, aspartato aminotransferase (AST) e alanina aminotransferase (ALT) (ambas marcadoras de função hepática) entre os grupos estudados.

Ainda, quando se decide utilizar a vitamina A como uma terapia antioxidante, os possíveis efeitos pró-oxidantes induzidos por este tratamento devem ser levados em consideração, principalmente em regiões do SNC devido a sua baixa capacidade de tolerar espécies reativas (de Oliveira *et al*., 2009d). Interessantemente, este

aspecto foi constatado em nosso trabalho, onde observamos efeitos antioxidantes em sangue/plasma e efeitos pró-oxidantes em SNC. Hoje, não há consenso na literatura sobre uma dose suplementar definitivamente segura de palmitato de retinol durante a menopausa para os seres humanos (Dennehy & Tsourounis, 2010).

Em estudos recentes, doses mais elevadas de vitamina A vem sendo associadas a efeitos adversos no metabolismo ósseo. A ingestão moderadamente elevada de vitamina A (três vezes a ingestão dietética recomendada para adultos - RDA) e níveis plasmáticos elevados de vitamina A foram associados a uma baixa densidade mineral óssea em mulheres na menopausa (Promislow *et al*., 2002). Por outro lado, ésteres de retinil no soro não estão elevados em diferentes populações de mulheres na pós-menopausa com e sem osteoporose que tomaram suplementos de vitamina A, superiores ou não ao recomendado pela RDA (Penniston *et al*., 2006; Rejnmark *et al*., 2004). No entanto, a concentração de vitamina A total está marginalmente associada à osteoporose e deve ser mais bem investigada. Assim, atualmente não é claro se a ingestão elevada de vitamina A deve ser considerada um fator de risco para a osteoporose e outras doenças relacionadas à menopausa.

A terapia de reposição hormonal pode ser prescrita quando a mulher na pós-menopausa sofre com efeitos graves causados pelos baixos níveis de hormônios sexuais. No entanto, nem todas as mulheres na pós-menopausa são consideradas candidatas para receber a terapia hormonal. Assim, para evitar o aumento do risco de desenvolvimento de complicações e patologias associadas com a substituição hormonal, muitas mulheres escolhem tratamentos alternativos (Bolanos *et al*., 2010; Goudev *et al*., 2000; Jensen *et al*., 2010; Kang *et al*., 2002; Messina *et al*., 2010). A suplementação com vitaminas e minerais representa um importante tratamento alternativo para os sintomas da menopausa (Hagey & Warren, 2008). Apesar disso,

estudos científicos básicos e clínicos ainda são necessários para elucidar os mecanismos e efeitos reais desses tratamentos (Lloyd & Hornsby, 2009; Wong *et al*., 2009). Nosso trabalho demonstra que o sangue e o SNC são importantes tecidos marcadores de estresse oxidativo induzido pela ovariectomia. Entretanto, quando correlacionamos os resultados obtidos após o tratamento com vitamina A, o efeito pró-oxidante observado no SNC não é observado no sangue, onde observamos efeito antioxidante.

No presente estudo, verificamos que a vitamina A foi capaz de promover a restauração das atividades de CAT e GPx (do sangue) próximo a valores encontrados no grupo controle. Além disso, o tratamento com vitamina A 1500 IU/kg/dia, foi capaz de restaurar a relação SOD/CAT+GPx no sangue. Ainda, observamos o aumento nas defesas antioxidantes totais não enzimáticas do plasma após o tratamento com vitamina A por 30 dias. Além do aumento no nível de defesas antioxidantes mostrado, podemos observar a diminuição no dano oxidativo à proteína em amostras de plasma, com o nível plasmático de SH reduzido aumentado após o tratamento com vitamina A 1500IU/kg/dia.

Apesar do grande número de estudos sobre a utilização de diferentes vitaminas (B6, C, D, E e K), no tratamento dos sintomas da menopausa (Gennari *et al*., 2009; Iwamoto *et al*., 2009; Miquel *et al*., 2006; Perez-Lopez, 2009; Ziaei *et al*., 2007), a vitamina A (ou retinóides em geral) não é tanto estudada (Caire-Juvera *et al*., 2009). Recentemente, níveis diminuídos de retinol plasmático foram fortemente associados com um pior prognóstico em pacientes com câncer de mama na pós-menopausa (Formelli *et al*., 2009). Outro estudo sugere que a vitamina A e o betacaroteno na dieta são modestamente protetores contra câncer de ovário, especialmente entre os fumantes (Tung *et al*., 2005). Além disso, os retinóides têm funções importantes na

ativação de muitas vias de sinalização relacionadas ao sangue, na regulação do crescimento das células epiteliais, no sistema imunológico, e na hematopoese (Evans, 2005; Pino-Lagos *et al*., 2008).

Muitas vitaminas inibem o óxido nítrico (NO) produzido pela NO sintase induzível (iNOS), apresentando conhecidas funções antiaterogênicas e antineuroinflammatórias (Wu & Meininger, 2002). Por exemplo, a vitamina A inibe a transcrição do gene de iNOS em células musculares lisas vasculares (Hirokawa *et al*., 1994) e em células endoteliais (Grosjean *et al*., 2001). Ao reduzir a geração de NO por iNOS, a vitamina A desempenha um papel importante na prevenção da citotoxicidade induzida por radicais livres. Além disso, o retinol e o ácido retinóico (um metabolito ativo da vitamina A) modulam diferentes vias de sinalização redox-dependentes (Zanotto-Filho *et al*., 2008).

A restauração nos níveis de antioxidantes plasmáticos pode indicar que o tratamento com vitamina A atua eliminando alguns radicais oxidantes específicos, observado por uma diminuição tanto na atividade de CAT quanto de GPx, acompanhado da restauração do potencial antioxidante não enzimático total e nível plasmático de SH. Infelizmente, é quase impossível indicar qual metabólito da vitamina A é o responsável pelos efeitos observados, dado o grande número de metabólitos de vitamina A existente (Ross, 1993). De fato, diferentes vitaminas tem atividade *scavenger* de ERO. No entanto, entre elas, as vitaminas E e C são reconhecidas como as duas que apresentam atividades antioxidantes mais importantes (Fang *et al*., 2002). Por outro lado, estudos sugerem que o betacaroteno e o ácido retinóico podem exercer efeitos antioxidantes através da atividade de *scavenger* de ERO e por mecanismos de desintoxicação (Fang *et al*., 2002; Hix *et al*., 2004). Embora evidências experimentais consideráveis venham sendo

acumuladas demonstrando a natureza dos efeitos biológicos de retinóides, em alguns casos os mecanismos celulares da ação permanecem incertos.

Quando observamos os resultados do tratamento com vitamina A no SNC das ratas tratadas, um perfil pró-oxidante é observado. A vitamina A em doses mais elevadas (1500 IU/kg/dia) reduz a atividade exploratória, aumenta a relação SOD/CAT no hipocampo, reduz o nível de SH do hipocampo (sugerindo oxidação) e diminui o TRAP no hipocampo de ratas controle tratadas. Além disso, a vitamina A no tratamento de 1500 IU/kg/dia aumenta a relação SOD/CAT no hipocampo, diminui o TRAP no hipocampo, e promove aumento no dano oxidativo a lipídios no córtex frontal de ratas OVX.

Este é o primeiro estudo que investigou os efeitos da suplementação de vitamina A no perfil redox do SNC em ratas OVX. Estudos anteriores do nosso grupo revelaram que doses de vitamina A entre 2500-9000 UI/kg/dia durante quatro semanas ou mais pode induzir anormalidades nos estados redox e na bioenergética de vários tecidos de ratos machos (de Oliveira *et al*., 2009a,b,c; Pasquali *et al*., 2009), bem como em ratas grávidas e seus filhotes, quando tratadas durante a gravidez e o período de amamentação (Pasquali *et al*., 2010; Schnorr *et al*., 2011a,b). Sabemos que a suplementação aguda ou crônica com vitamina A pode induzir estresse oxidativo e nitrosativo no hipocampo (de Oliveira *et al*., 2007), substância negra (de Oliveira *et al*., 2008), córtex frontal (de Oliveira *et al*., 2009c) e hipotálamo (de Oliveira *et al*., 2009e) de ratos adultos. Além disso, a suplementação com vitamina A em diferentes doses durante a gravidez e amamentação aumenta a razão SOD/CAT e promove aumento no dano oxidativo de estriado e hipocampo em mães e filhotes (Schnorr *et al*., 2011b).

Apenas outro único estudo administra doses mais baixas de vitamina A em ratas adultas (500 UI/kg/dia, durante dois meses). de Oliveira *et al*. (2011) demonstra um aumento em 3-nitrotirosina de membranas mitocondriais e comprometimento da atividade da cadeia respiratória em regiões cerebrais específicas. Em nosso estudo, o tratamento com vitamina A 500 IU/kg/dia por um mês não altera o comportamento ou o perfil redox do cérebro das ratas, o que sugere que a exposição por tempos mais prolongados à vitamina A pode ser o desencadeador do estresse oxidativo via nitração de proteínas e disfunção mitocondrial. Os resultados pró-oxidantes observados no SNC após a suplementação com vitamina A são consistentes com vários ensaios clínicos que investigaram a suplementação de vitamina em mulheres na pós-menopausa e mostram que a suplementação com vitaminas pode ser benéfica ou prejudicial, dependendo do órgão e do tratamento (Dennehy & Tsourounis, 2010; Hagey & Warren, 2008).

A sofisticada comunicação entre as diferentes estruturas do sistema nervoso central permite a detecção rápida e precisa de comida, perigo, companheiros, todos estes elementos cruciais para a sobrevivência e manutenção da espécie. Além disso, a regulação fina desta rede é obrigatória para estabilização e modulação do humor. Pode ser que uma mudança na regulação destes circuitos emocionais ocorra em mulheres durante a transição da menopausa o que, eventualmente, pode contribuir para a ocorrência de sintomas de transtorno de humor e de ansiedade em mulheres durante e após esse período da vida (Frey *et al*., 2010).

No presente trabalho, nos concentramos no hipocampo, córtex frontal, e hipotálamo. Juntas, essas três áreas do cérebro são responsáveis pela regulação do humor e manutenção de funções locomotoras relacionadas ao cérebro. A aquisição

de novas memórias de lugares e eventos requer plasticidade sináptica no hipocampo, e é fundamental para a aprendizagem espacial, reconhecimento de objetos e memória (Cheng & Frank 2008; Vukovic *et al*., 2011). O papel do córtex frontal quer nos roedores ou em seres humanos, deve ser visto no contexto de seu papel crucial na orquestração cerebral do comportamento adaptativo, através particularmente de seu envolvimento em funções executivas (Kesner & Churchwell, 2011). O hipotálamo é o centro de controle cerebral de funções endócrino-hormonais, apresentando conexões com muitas partes do SNC, incluindo o hipocampo, e os lobos frontais do córtex cerebral (Shepherd, 2006). Além disto, as estruturas escolhidas para análise são reconhecidas por apresentarem receptores para hormônios femininos, assim como a relação entre metabolitos da vitamina A e o metabolismo do estradiol está descrita (Koda *et al*., 2007). A forma ativa da vitamina A, ácido retinóico, modula várias funções do SNC, regulando a expressão de genes no cérebro (McCaffery *et al*., 2003). Interessante, alguns retinóides aprovados para tratamento dermatológico apresentam advertências relacionadas ao aumentado risco de suicídio, depressão e psicoses (Bremner *et al*., 2012).

Alguns estudos relatam mudanças de comportamento em animais que apresentam estresse oxidativo aumentado. Além disso, demonstramos previamente alterações redox do SNC em regiões associadas ao comportamento alterado de ratos Wistar após suplementação com vitamina A em diferentes doses. de Oliveira *et al*. (2007) demonstraram uma diminuição na atividade locomotor/exploratória associada a estresse oxidativo aumentado no hipocampo de ratos Wistar machos suplementados com vitamina A em doses que variaram de 1000 a 9000 UI/kg durante 28 dias. Schnorr *et al*. (2011a,b) mostraram diminuição na atividade exploratória em filhotes de mães tratadas com vitamia A e diminuição na atividade

locomotora em mães e filhotes do sexo masculino associado ao aumento na razão SOD/CAT e dano oxidativo em estriado e hipocampo.

Além disso, Merzoug *et al*., (2011) demonstraram diminuição na atividade locomotor/exploratória associada a estresse oxidativo cerebral aumentado em ratos Wistar machos após tratamento agudo com adriamicina. Ainda, Cruz-Aguado *et al*., (2001) relataram diversas alterações comportamentais e bioquímicas após depleção de GSH em cérebro de ratos, suportando a hipótese de que é importante o equilíbrio entre oxidantes e antioxidantes para a regulação da função cerebral. No presente estudo, encontramos que a atividade locomotor/exploratória está diminuída, e é acompanhada de mudanças no perfil redox do SNC em ratas OVX. Apesar do grande número de estudos em roedores sob várias condições de estresse oxidativo onde se observam mudanças de comportamento, especialmente em comportamentos locomotores e exploratórios, as bases moleculares subjacentes à associação entre o estresse oxidativo cerebral e as mudanças de comportamento ainda não foram plenamente exploradas. Uma hipótese sugerida é a de que a vitamina A esteja interferindo no metabolismo do estradiol nas ratas sham, e assim provocando o efeito comportamental observado.

Em nosso estudo observamos resultados completamente distintos entre SNC e sangue quanto a parâmetros de estresse oxidativo, onde observamos efeitos antioxidantes no sangue e pró-oxidantes no SNC após suplementação com vitamina A. Podemos sugerir alguns motivos para este achado um tanto interessante. Por diversas razões, o SNC é especialmente sensível ao estresse oxidativo. Primeiramente, o SNC tem capacidade reduzida de regeneração celular em comparação com outros órgãos. A reposição de células neuronais é um processo muito mais lento que a regeneração de outros tipos celulares, desta forma células

neuronais sofrem mais com os insultos provenientes do estresse oxidativo durante o processo de envelhecimento (Andersen, 2004). Em segundo lugar, características intrínsecas do SNC e de seu metabolismo, o tornam mais propenso a danos causados por espécies oxidantes. O consumo de oxigênio (O_2) pelo cérebro é muito elevado. Em humanos, o cérebro é responsável por cerca de 20% do consumo total de O_2. Isto significa que os neurônios dependem muito da eficiência das mitocôndrias. Todavia, um aumento nos níveis de estresse oxidativo pode danificar a mitocôndria, forçando a maior produção de ERO pela mitocondria lesada (Halliwell & Gutteridge, 2007).

O tecido sanguíneo, por outro lado, parece ser menos sensível ao estresse oxidativo que o SNC. Um dos motivos possíveis poderia ser o fato do sangue apresentar como característica fundamental o elevado turnover de biomoléculas, além da grande quantidade de proteínas. Uma das mais abundantes proteínas circulantes é a albulmina, com propriedades antioxidantes conhecidas (Roche *et al*., 2008). Além disso, quando comparamos o tempo de vida de células do SNC e células sanguíneas fica claro a diferença entre os tipos celulares, com neurônios podendo existir por uma vida inteira e celulas sanguíneas em torno de 4 meses. Por outro lado, se pode argumentar que o SNC é mais "protegido" contra o estresse oxidativo, em comparação ao sangue, devido a barreira hematoencefálica, e que além disso, o sangue recebe metabólitos oxidados de todo o organismo, assim estando mais suceptivel ao dano oxidativo. De qualquer forma, os resultados apresentados nesta tese são de bastante interesse e ressaltam que nem sempre o sangue é um bom indicador do estado redox do SNC.

Vários trabalhos têm focado nos efeitos de ERO em alterações fisiopatológicas do esqueleto, sistema cardiovascular, e mecanismos de controle da termorregulação

em ratas OVX. Porém, o presente trabalho é o primeiro a demonstrar um perfil oxidativo mais completo (atividades de SOD, CAT e GPx; TRAP e TAR; níveis de SH, e dano oxidativo) para o sangue e SNC de ratas OVX, sugerindo que a cessação da secreção de hormônios sexuais, acompanhado pelo aumento no estresse oxidativo, podem desempenhar um papel importante no desenvolvimento de sintomas relacionados com a menopausa. Além disso, este é o primeiro trabalho que evidencia que a suplementação com doses relativamente baixas de vitamina A é capaz de promover a melhora na capacidade antioxidante no sangue de ratas OVX. Por outro lado, após os 30 dias de tratamento com vitamina A na dose de 1500 UI/kg/dia o SNC das ratas tratadas apresenta níveis de estresse oxidativo aumentados, além de alteração no comportamento exploratório. Investigações adicionais devem ser feitas para melhor determinar o efeito da suplementação de vitamina A no metabolismo oxidativo de outros tecidos e órgãos de ratas OVX.

A menopausa é um marco inevitável na vida reprodutiva de cada mulher. Tradicionalmente, nos países em desenvolvimento a menopausa e os problemas da mesma são aceitos como fenômenos fisiológicos normais. No entanto, com a expectativa de vida cada vez maior entre as mulheres, a prevalência de osteoporose, doenças cardiovasculares e problemas de saúde em mulheres na pós-menopausa continuam a aumentar substancialmente. A maioria das mulheres não só se preocupa em viver uma vida longa, mas também sobre como viver uma vida saudável. Neste sentido, os estudos científicos básicos e clínicos são muito importantes para elucidar os mecanismos associados com os sintomas da menopausa e buscar tratamentos alternativos à reposição hormonal. Estudos futuros que investiguem o efeito da suplementação de vitamina A em doses baixas no perfil redox de outros tecidos e órgãos, tais como coração, rim, e fígado continuam sendo

necessários, assim como a investigação dos mecanismos moleculares responsáveis pelo papel redox da vitamina A em modelo animal de menopausa.

5 – CONCLUSÃO

Quanto aos objetivos específicos propostos nesta tese:

1 – Avaliar as alterações bioquímicas em sangue que diferenciam ratas OVX de ratas controle (sham); Foram determinados diferentes parâmetros bioquímicos que distinguem ratas OVX de ratas controle. As ratas OVX apresentaram aumento no ganho de peso corporal, pronunciada atrofia uterina, diminuição nos triglicerídeos e aumento nos níveis de colesterol total, e redução no teor de ácido úrico no plasma.

2 – Quantificar os parâmetros de estresse oxidativo (marcadores de função antioxidante e de dano oxidativo) e comportamentais (locomoção/exploração e ansiedade) que diferenciam ratas OVX de ratas controle, com foco em sangue/plasma e estruturas do SNC (hipocampo, hipotálamo e córtex frontal); Os parâmetros objetivados foram quantificados. Encontramos um grande número de alterações no perfil redox de sangue, bem como nas estruturas do SNC, provocadas pela ovariectomia bilateral em ratas Wistar. As ratas OVX apresentaram aumento nas atividades de CAT e GPx no sangue, diminuição nas defesas antioxidantes plasmáticas e nos níveis de SH, acompanhado de aumento no dano oxidativo a proteínas. Além disso, as ratas OVX apresentaram aumento na razão SOD/CAT em hipotálamo e córtex frontal, redução dos níveis de SH no hipocampo, e aumento no dano lipídico em córtex frontal. Além disso, foi observado comportamento alterado para as ratas OVX quando comparado ao comportamento das ratas controle, onde as ratas OVX apresentaram diminuição no comportamento locomotor/exploratório.

3 – Realizar o tratamento com palmitato de retinol (vitamina A) por 30 dias no modelo animal proposto e analisar as possíveis alterações bioquímicas e em parâmetros de estresse oxidativo, nas estruturas previamente citadas; O tratamento

com vitamina A promoveu alterações tanto nos parâmetros bioquímicos como nos parâmetros de estresse oxidativo analisados. A suplementação com vitamina A foi capaz de melhorar a capacidade antioxidante total, restaurando as defesas tanto enzimáticas como não-enzimática, promovendo a redução no teor SH, e diminuição nos níveis de dano a proteínas em sangue de ratas OVX. Entretanto, no SNC a suplementação com vitamina A promoveu efeitos pró-oxidantes, evidenciados pelo aumento na relação SOD/CAT e diminuição no potencial antioxidantes total em hipocampo, e aumento nos níveis de dano a lipídios em córtex frontal de ratas OVX.

4 – Observar as alterações comportamentais provenientes do tratamento com vitamina A; O tratamento com vitamina A promoveu alterações apenas no comportamento dos animais controle. Observamos diminuição no comportamento locomotor de ratas controle suplementadas com vitamina A. Não observamos alterações comportamentais provenientes do tratamento em ratas OVX.

5 – Comparar os dados experimentais obtidos em sangue/plasma com os dados obtidos nas estruturas do SNC (hipocampo, hipotálamo e córtex frontal); Interessantemente, o tratamento com vitamina A em ratas OVX apresentou resultados bastante distintos em sangue/plasma e estruturas do SNC. Mesmo que a suplementação com vitamina A tenha apresentando efeitos antioxidantes em plasma, a mesma acaba por induzir um estado pró-oxidante em regiões cerebrais de ratas OVX.

Este trabalho relata, pela primeira vez, que a suplementação com vitamina A em doses relativamente baixas pode desencadear efeitos completamente distintos dependendo dos tecidos estudados, sugerindo que deve haver mais cautela em relação ao uso de suplementos com vitamina A durante a menopausa.

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ANEXOS

Anexo 1

Preclinical and clinical evidence of antioxidant effects of antidepressant agents: implications for the pathophysiology of major depressive disorder.

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Review

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Review Article

Preclinical and Clinical Evidence of Antioxidant Effects of Antidepressant Agents: Implications for the Pathophysiology of Major Depressive Disorder

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Major depressive disorder (MDD) is a common mental disorder associated with a significant negative impact on quality of life, morbidity/mortality, and cognitive function. Individuals who suffer with MDD display lower serum/plasmatic total antioxidant potentials and reduced brain GSH levels. Also, F2-isoprostanes circulatory levels are increased in MDD subjects and are correlated with the severity of depressive symptoms. Urinary excretion of 8-OHdG seems to be higher in patients with MDD compared to healthy controls. Despite the fact that antidepressant drugs have been used for more than 50 years, their mechanism of action is still not fully understood. This paper examines preclinical (*in vitro* and animal model) and clinical literature on oxidative/antioxidant effects associated with antidepressant agents and discusses their potential antioxidant-related effects in the treatment of MDD. Substantial data support that MDD seems to be accompanied by elevated levels of oxidative stress and that antidepressant treatments may reduce oxidative stress. These studies suggest that augmentation of antioxidant defences may be one of the mechanisms underlying the neuroprotective effects of antidepressants in the treatment of MDD.

1. Introduction

Despite the fact that antidepressant drugs have been used for more than 50 years, their mechanism of action is still not fully understood. The hypothesis that antidepressants restore noradrenergic and serotoninergic neurotransmitter systems has been dominant [1]. Recently, a new concept of antidepressants action has been suggested, based on growing evidence demonstrating antioxidant effects of antidepressants in the treatment of major depressive disorder (MDD) (Table 1). This paper examines preclinical (*in vitro* and animal models) and clinical literature on oxidative/antioxidant effects of antidepressant agents and discusses the relevance of intracellular oxidative pathways in the pathophysiology of MDD.

2. Oxidative Stress and Antioxidants: Background

Reactive oxygen species (ROS) are continuously generated in physiological conditions and are effectively controlled/eliminated by intracellular and extracellular antioxidant systems [2]. ROS are products of normal cellular metabolism and are well recognized for their dual role as deleterious and essential compounds, given that ROS can be harmful or beneficial [3]. Beneficial effects of ROS occur at low levels and involve cell signalling and signal transduction [4]. ROS also play an essential role in the human immune system helping killing and eliminating infectious organisms. However, elevated or chronic inflammations are major determinants of disease later in the human lifespan, and ROS

Table 1: Antioxidant effects of antidepressant agents: preclinical and clinical studies.

Antidepressant	Oxidative/Antioxidant-related effects			Drug class
	In vitro	Animal models	Human data	
Amitriptyline	+	+		TCA
Bupropion		+		NDRI
Citalopram			+	SSRI
Desipramine	+			TCA
Duloxetine				SNRI
Escitalopram		+	+	SSRI
Fluoxetine	+	+	+	SSRI
Fluvoxamine	+		+	SSRI
Imipramine	+	+		TCA
Maprotiline	+			TCA
Milnacipran			+	SNRI
Mirtazapine	+			NaSSA
Moclobemide			+	MAOI
Nefazodone			+	SNDRI
Nortriptyline	+			TCA
Paroxetine			+	SSRI
Reboxetine	+		+	NRI
Sertraline			+	SSRI
Tianeptine			+	SSRE
Trazodone			+	SARI
Venlafaxine		+	+	SNRI

MAOI: monoamine oxidase inhibitor; NaSSA: noradrenergic and specific serotonergic antidepressant; NDRI: norepinephrine-dopamine reuptake inhibitor; NRI: norepinephrine reuptake inhibitor; SARI: serotonin antagonist and reuptake inhibitor; SNDRI: serotonin-norepinephrine-dopamine reuptake inhibitor; SNRI: serotonin-norepinephrine reuptake inhibitor; SSRE: selective serotonin reuptake enhancer; SSRI: selective serotonin reuptake inhibitor; TCA: tricyclic or tetracyclic antidepressant.

play a critical role in several age-related diseases, particularly cancer, cardiac and neurodegenerative disorders [5]. The major source of ROS in humans is the leakage of superoxide anion ($O_2^{\bullet -}$) from mitochondria during oxidative phosphorylation. Another minor source of ROS is cytoplasmatic, including the $O_2^{\bullet -}$generating enzymes such as xanthine oxidase (XO), NADPH oxidases, and cytochromes P450 (CytP450). The main ROS include $O_2^{\bullet -}$, hydrogen peroxide (H_2O_2), and hydroxyl radical ($OH^{\bullet}$). $OH^{\bullet}$ is a strong oxidant formed during Fenton ($Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^{\bullet} + OH^{-}$) and Haber-Weiss ($H_2O_2 + OH^{\bullet} \rightarrow H_2O + O_2^{\bullet -} + H^{+}$ and $H_2O_2 + O_2^{\bullet -} \rightarrow O_2 + OH^{-} + OH^{\bullet}$) reactions. Additionally, some nitrogen species can be potentially dangerous to the cell, such as peroxynitrite (ONOO–), which is formed in a rapid reaction between $O_2^{\bullet -}$ and nitric oxide (NO) [3].

The main enzymatic antioxidant defences include superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx). SOD enzymes are highly efficient in the catalytic dismutation of $O_2^{\bullet -}$ and generation of H_2O_2 which, in turn, can be removed by two types of enzymes—the catalases (CAT) and peroxidases (e.g., GPx). Importantly, the activity of GPx is closely dependent on glutathione reductase (GR), glutathione tripeptide (GSH), and others cofactors. Moreover, virtually all cells contain nonenzymatic defenses, like GSH, vitamins C (ascorbate) and E (alpha-tocopherol), and metal-binding and related protective proteins [37].

The term "oxidative stress" has been defined as an imbalance between the generation of ROS and antioxidant defenses, favouring the former [3]. In situations of oxidative stress, several biomolecules (e.g., lipid membrane, proteins, and DNA) can be damaged. Because ROS have extremely short half-lives, they are difficult to measure. Therefore, most studies measure products of the damage induced by oxidative stress. For instance, malondialdehyde (MDA) is one of the low-molecular-weight end products formed via the decomposition of primary and secondary lipid peroxidation products [38]. MDA and other thiobarbituric reactive substances (TBARS) condense with two equivalents of thiobarbituric acid that can be assayed spectrophotometrically [39]. Another compound commonly used as a biomarker of oxidative stress is 4-Hydroxynonenal (4-HNE). 4-HNE is generated in the oxidation of lipids containing polyunsaturated omega-6 acyl groups, such as arachidonic or linoleic groups, and the corresponding fatty acids [40]. Perhaps the most accurate markers of lipid peroxidation are the isoprostanes (i.e., F2-isoprostanes). Isoprostanes are prostaglandin-like compounds formed *in vivo* from the free radical-catalyzed peroxidation of essential fatty acids (primarily arachidonic acid) [41]. Proteins are possibly the most immediate targets of cellular oxidative damage. Carbonyl groups (aldehydes and ketones) are produced in protein side chains (especially of Pro, Arg, Lys, and Thr) when they are oxidized, which can be measured by specific techniques [42]. Another method to evaluate levels of oxidation/reduction content in biological samples is the total reduced thiol (–SH) quantification [43]. ROS can also attack and damage the DNA, thereby generating 8-hydroxydeoxyguanosine (8-oxodG) and 8-hydroxyguanosine (8-oxoG) [37].

Additionally, total antioxidant potentials can be measured using various methods such as TAC, total antioxidant capacity; TRAP, total-radical nonenzymatic antioxidant potential; OSI, oxidative stress index; TOS, total oxidant status. Low total antioxidant capacity could be indicative of oxidative stress or increased susceptibility to oxidative damage [44].

3. Oxidative Stress in Major Depressive Disorder

MDD is one of the most common mental disorders among humans and it is associated with a significant negative impact on quality of life, morbidity/mortality, and cognitive function. The pathophysiology of depression is multifactorial and includes changes in brain monoaminergic transmission (e.g., 5-HT, NE, DA), abnormalities in neurotransmitter receptors function (e.g., AC-cAMP pathway), reduced neurotrophic factors (e.g., BDNF), dysregulation of HPA axis (cortisol), increased proinflammatory cytokines (e.g., IL-6, TNF-α, NF-κB), increased NO (e.g., L-arginine-NO-cGMP pathway), and increased oxidative stress (e.g., lipid and DNA damage) [45–47].

Individuals who suffer with MDD display lower serum/plasmatic total antioxidant potentials [28, 32, 48] and reduced brain GSH levels [31] as compared to matched controls. Plasmatic coenzyme Q10 (CoQ10), a strong antioxidant and a key molecule in the mitochondrial electron transport chain, is significantly lower in major depressive patients [34], which indicates lower antioxidant defenses against oxidative stress. Moreover, increased serum XO levels observed in MDD subjects suggest increased systemic ROS production [29]. XO is a widely distributed enzyme involved in later stages of purine catabolism, which catalyzes the oxidation of hypoxanthine to xanthine and of xanthine to uric acid, both reactions with potential to generate $O_2^{\bullet -}$ and H_2O_2 [49]. A recent post-mortem study found increased XO activity in the thalamus and putamen patients with recurrent MDD [35].

Dimopoulos et al. (2008) have found that F2-isoprostanes (F2-iso) circulatory levels were increased in major depressive patients and were significantly correlated with the severity of depressive symptoms [50]. The presence of detectable quantities of F2-iso in human biological fluids implies ongoing lipid peroxidation [51]. Furthermore, urinary excretion of 8-OHdG, a marker of oxidative damage to DNA, was found to be higher in patients with MDD than healthy controls [52].

4. Antioxidant Effects of Antidepressants

4.1. Studies In Vitro. The main findings of *in vitro* assays using rat mitochondria and cell culture protocols are depicted in Table 2. Kolla et al. (2005) have demonstrated that pretreatment with amitriptyline and fluoxetine protects against oxidative stress-induced damage in rat pheochromocytoma (PC12) cells. Both drugs attenuated the decrease in cell viability induced by H_2O_2 in PC12 cells. Also, pretreatment with amitriptyline and fluoxetine was associated with increased SOD activity, and no signs of cell death were observed in the treated cells [10]. In another study, pretreatment with imipramine, fluvoxamine, or reboxetine inhibited NO production in a dose-dependent manner in an activated microglia cell culture protocol [11]. The authors suggested that these antidepressant drugs have inhibitory effects on IFN-γ-activated microglia and that these effects are, at least in part, mediated by cAMP-dependent PKA pathway.

Schmidt et al. (2008) examined the effects of desipramine, imipramine, maprotiline and mirtazapine on mRNA levels of various antioxidant enzymes in human monocytic U-937 cells [12]. In this study, short-term treatment with these drugs decreased mRNA levels of SOD and CAT. However, long-term treatment increased mRNA levels of SOD, GST, and GR. These results suggest that the effects of these antidepressants on the expression of antioxidant enzymes are dependent on the duration of the treatment regimen. Zhang et al. (2008) showed for nortriptyline some antioxidant effects using isolated rat liver mitochondria or PCN cell culture. Nortriptyline was able to inhibit loss of mitochondrial membrane potential and the activation of caspase 3 in isolated rat liver mitochondria and decrease cell death induced by oxygen/glucose deprivation on PCN cells [9].

The antioxidant effects of fluoxetine on isolated rat brain and liver mitochondria have been extensively studied. Curti et al. (1999) reported that fluoxetine can indirectly and nonspecifically affect electron transport and F_1F_0-ATPase activity, thereby inhibiting oxidative phosphorylation in rat brain [6]. Two studies that evaluated the effects of fluoxetine in rat liver mitochondria revealed mixed results. Souza et al. (1994) reported that fluoxetine may be potentially hepatotoxic at high doses [7]. However, Nahon et al. (2005) demonstrated that fluoxetine was able to inhibit the opening of the mitochondrial permeability transition (MPT) pore, the release of cytochrome c (cytC) and protected against staurosporine-induced apoptotic cell death [8]. An important difference between these two studies is the fact that Souza et al. used isolated liver mitochondria and tested fluoxetine at different concentrations in order to establish potential toxic doses. On the other hand, Nahon et al. challenged isolated mitochondria against staurosporine-induced damage and showed protective effects of fluoxetine in this model.

In summary, studies *in vitro* not only revealed antioxidant-related effects for antidepressant drugs, but also some potential prooxidant effects specifically in rat liver with fluoxetine at higher dosages. Cell culture and isolated tissues studies are used extensively in research and drug development; however, these techniques have some limitations and studies using live organisms (i.e., rodents) are necessary to better evaluate safety as well as behavioural effects.

4.2. Animal Models. Several animal model protocols have been used to investigate oxidative/antioxidant-related effects of antidepressant drugs. Table 3 summarizes the studies conducted with acute and chronic antidepressant treatments in control and stressed animals.

Réus et al. (2010) reported increased SOD and CAT activity and decreased lipid and protein damage in male rat prefrontal cortex and hippocampus after both acute and chronic treatment with imipramine [17]. Additionally, imipramine treatment increased brain creatine kinase and increased activity of mitochondrial respiratory chain complexes [18, 53]. Katyare and Rajan (1995) showed that long-term administration of imipramine to female rats resulted in significant stimulation of the states 3 and 4 respiration rates. This effect was evident within a week of imipramine administration and was sustained through the second week of treatment [20]. These results suggest that imipramine treatment may induce changes in substrate oxidation pattern, increase rate of ATP synthesis, and can potentially increase mitochondrial ROS production.

Xu et al. (2003) examined dose-dependent effects of amitriptyline and venlafaxine on neuroprotective proteins in male rats. In this study, low dose (5 mg/kg) of amitriptyline and venlafaxine increased the intensity of BDNF immunostaining in hippocampal pyramidal neurons and the intensity of Bcl-2 immunostaining in hippocampal mossy fibers, but did not alter the Cu/Zn-SOD immunoreactivity. High

Table 2: *In vitro* studies with antidepressants.

Method		Antidepressant drugs tested	Main findings	Reference
In vitro	Rat brain mitochondria	Fluoxetine	Indirectly and nonspecifically affects electron transport and F_1F_0-ATPase activity inhibiting oxidative phosphorylation	Curti et al., 1999 [6]
In vitro	Rat liver mitochondria	Fluoxetine	Multiple effects on the energy metabolism of rat liver mitochondria; potentially toxic in high doses	Souza et al., 1994 [7]
In vitro	Rat liver mitochondria	Fluoxetine	Inhibits the opening of the MPT pore, the release of cytC, and protected against staurosporine-induced apoptotic cell death	Nahon et al., 2005 [8]
In vitro	Rat liver mitochondria	Nortriptyline	Inhibits loss of mitochondrial membrane potential and the activation of caspase 3	Zhang et al., 2008 [9]
Cell culture	PCN cells oxygen/glucose deprived	Nortriptyline	Decrease cell death	Zhang et al., 2008 [9]
Cell culture	PC12 cells exposed to H_2O_2	Amitriptyline, fluoxetine	Both agents attenuated cell death induced by H_2O_2, fluoxetine pretreatment increased SOD activity	Kolla et al., 2005 [10]
Cell culture	IFN-γ-activated microglia	Fluvoxamine, imipramine, reboxetine	All drugs inhibited IL-6 and NO production in a dose-dependent manner	Hashioka et al., 2007 [11]
Cell culture	Human monocytic U-937 cells	Desipramine, imipramine, maprotiline, and mirtazapine	Short-term treatment decreased mRNA levels of SOD and CAT after treatment with these drugs; long-term treatment increased mRNA levels of SOD, GST, and GR	Schmidt et al., 2008 [12]

CAT: catalase; cytC: cytochrome C; GR: glutathione reductase; GST: glutathione S-transferase; H_2O_2: hydrogen peroxide; IFN-γ: interferon-gamma; IL-6: interleukin 6; MPT: mitochondrial permeability transition; NO: nitric oxide; SOD: superoxide dismutase.

Table 3: Animal studies with antidepressant drugs.

Animal model		Antidepressant drugs tested	Main findings	Reference
Male albino mice	Acute treatment	Bupropion (10–40 mg/kg), i.p., once, 30 min before brain sample acquisition	Modulated the L-arginine-NO-cyclic cGMP signalling pathway in rat brain	Dhir and Kulkarni, 2007 [13]
Female Swiss mice	Acute treatment	Escitalopram (3 mg/kg), p.o., once, 30 min before behavioural tests	Antidepressant-like effect was mediated by an inhibition of either the NMDA receptor activation or NO-cGMP synthesis	Zomkowski et al., 2010 [14]
Male C57Bl/6J mice	Acute treatment	Imipramine (15 mg/kg), venlafaxine (6 mg/kg), both drugs, i.p., once only	Decreased brain $NO_2 + NO_3$ levels in control mice	Krass et al., 2011 [15]
Male Wistar rats	Acute treatment	Amitriptyline (10 mg/kg), i.p., once only, 3 h before analyses	Drug did not alter $NO_2 + NO_3$ serum levels in control rats	Vismari et al., 2012 [16]
Male Wistar rats	Acute and chronic treatment	Fluoxetine (20 mg/kg once or 10 mg/kg/day), i.p., once only or once a day for 12 days	Showed stimulation of mitochondrial respiration in state 4 in acute or prolonged treatments, indicating uncoupling of oxidative phosphorylation in rat liver mitochondria	Souza et al., 1994 [7]
Male Wistar rats	Acute and chronic treatment	Imipramine (10, 20 and 30 mg/kg), i.p., once only or once a day for 14 days	Decreased MDA and carbonyl content and increased SOD and CAT activity in prefrontal cortex and hippocampus	Réus et al., 2010 [17]
Male Wistar rats	Acute and chronic treatment	Imipramine (10, 20 and 30 mg/kg), i.p., once only or once a day for 14 days	Increased brain creatine kinase and mitochondrial respiratory chain activities	Réus, et al., 2012 [18]
Male Wistar rats	Acute and chronic treatment	Imipramine (10, 20 and 30 mg/kg), i.p., once only or once a day for 14 days	Altered respiratory chain complexes and CK activities; these alterations were different with relation to protocols (acute or chronic), complex, dose, and brain area	Réus, et al., 2012 [53]
Female Swiss mice	Acute and chronic treatment	Fluoxetine (10 mg/kg), p.o., once only or once a day for 28 days	Acute treatment reduced GPx activity in hippocampus; chronic treatment increases GSH in both hippocampus and prefrontal cortex	Lobato et al., 2010 [19]

Table 3: Continued.

Animal model		Antidepressant drugs tested	Main findings	Reference
Female Wistar rats	Chronic treatment	Imipramine (10 mg/kg) twice daily, i.p., 1 or 2 weeks	Promoted stimulation of the states 3 and 4 respiration rates (1 and 2 week treatments) on rat brain mitochondria	Katyare and Rajan, 1995 [20]
Male Sprague-Dawley rats	Chronic treatment	Amitriptyline (5, 10 mg/kg/day), venlafaxine (5, 10 mg/kg/day), both drugs. i.p., for 3 weeks	Both drugs increased SOD immunostaining in the hippocampal neurons	Xu et al., 2003 [21]
Male Wistar Han rats	Chronic treatment	Fluoxetine, 8 and 24 mg/kg/day, p.o., for 4 weeks	Increased levels of carbonyl groups, TBARS, and the uric acid content in the liver, effects more pronounced at high dose	Inkielewicz-Stêpniak, 2011 [22]
Male Swiss albino mice	Acute treatment, with or without previous restraint stress protocol	Fluoxetine, 5 mg/kg/day, i.p., 30 min before restraint stress protocol	Partially reversed the adverse effects of stress (restraint stress significantly increases the generation of ROS in the peripheral defence cells) restoring SOD, CAT, and GSH levels	Novio et al., 2011 [23]
Swiss Albino rats	Chronic treatment, with or without previous restraint stress protocol	Fluoxetine (20 mg/kg/day), imipramine (10 mg/kg/day), venlafaxine (10 mg/kg/day), all drugs, p.o., for 3 weeks	All drugs restored SOD, CAT, GST, and GR activity, increased GSH and decreased MDA and carbonyl in brain samples of stressed animals	Zafir et al., 2009 [24]
Male Wistar rats	Chronic treatment, with or without previous chronic social isolation stress	Fluoxetine, 5 mg/kg/day, i.p., for 3 weeks	Decreased SOD and increased GPx activity in both groups, increased TAC in stressed animals, also induced several hallmarks of apoptosis in the liver of stressed animals	Djordjevic et al., 2011 [25]
Male Swiss-Webster mice	Chronic treatment, stress induced by FST and TST	Venlafaxine (5, 10, and 20 mg/kg/day), i.p. for 3 weeks	Decreased MDA and NO and increased hippocampal GSH and TAC levels and GST activity in the stressed animals, also, reduced both serum and hippocampal 8-OHdG levels	Abdel-Wahab and Salama, 2011 [26]

8-OHdG: 8-hydroxydeguanosine; CAT: catalase; cGMP: cyclic guanosine monophosphate; CK: creatine kinase; FST: forced swimming test; GPx: glutathione peroxidase; GR: glutathione reductase; GSH: glutathione; GST: glutathione S-transferase; MDA: malondialdehyde; NO: nitric oxide; $NO_2 + NO_3$, total nitrite + nitrate; ROS: reactive oxygen species; SOD: superoxide dismutase; TAC: total antioxidant capacity; TBARS: thiobarbituric acid reactive species; TST: tail suspension test.

dose (10 mg/kg) of venlafaxine, however, decreased the intensity of BDNF immunostaining in all subareas of the hippocampus and increased the intensity of Cu/Zn-SOD immunostaining in the dentate granular cell layer [21]. More recently, Abdel-Wahab and Salama (2011) showed that long-term venlafaxine treatment at effective antidepressant dosages can protect against stress-induced oxidative cellular and DNA damage in male mice. At all doses tested, venlafaxine decreased MDA and total nitrite levels, increased total antioxidant potential and GSH content, and restored GST activity in hippocampus of stressed animals. Venlafaxine also promoted increased total antioxidant potential and GSH levels in the control, nonstressed group. Finally, this treatment was able to reduce serum and hippocampal levels of 8-OHdG (a marker of DNA damage) in stressed animals [26] showing potential antioxidant effects related to these antidepressant agents.

The effects of chronic (one month) fluoxetine treatment on lipid and protein oxidative damage, uric acid concentration in the liver and the activity of transaminases and transferases in the serum have been investigated in male rats. Chronic fluoxetine treatment increased the levels of TBARS, carbonyl groups, and the uric acid content in the liver. The activities of alanine transaminase (ALT), aspartate transaminase (AST), and GST were increased in the serum. The overall effects are more pronounced in the higher dose (24 versus 8 mg/kg) [22]. More recently, Djordjevic et al. (2011) showed altered antioxidant status and increased apoptotic signalling in male rat liver after 21 days of fluoxetine treatment. Control animals and stressed animals displayed decreased activity of SOD and increased activity of GPx. In addition, in both experimental groups, fluoxetine altered several markers of apoptosis in the liver, including decreased Bcl-2 expression and increased DNA fragmentation [25]. These effects seemed to be associated with liver toxicity induced by high-dose fluoxetine treatment in rats.

Novio et al. (2011) investigated the effects of fluoxetine on intracellular redox status in peripheral blood cells obtained from male mice exposed to restraint stress. They found that restraint stress significantly increased the generation of ROS in the peripheral blood and that acute treatment with fluoxetine partially reversed this effect, possibly through normalization of SOD and CAT activity and GSH content [23]. Using a depression-like rat model, Zafir et al. (2009) examined antioxidant effects of fluoxetine and venlafaxine in the rat brain. The results evidenced a significant recovery in the activities of SOD, CAT, GST, GR, and GSH levels by these antidepressants after restraint stress. Also, fluoxetine and venlafaxine treatment prevented lipid and protein oxidative damage induced by stress [24]. In another study, acute fluoxetine treatment reduced GPx activity in the hippocampus, whereas chronic treatment increased GSH in both hippocampus and prefrontal cortex of female mice [19].

Recent data support that some antidepressants are able to modulate NO synthesis and nitrosative stress-associated signalling cascades. Dhir and Kulkarni (2007) tested different dosages of bupropion in male rats. The antidepressant-like effect of bupropion was prevented by pretreatment with L-arginine (a substrate of nitric oxide synthase, NOS). Pretreatment with 7-nitroindazole (a specific neuronal NO synthase, nNOS inhibitor) potentiated bupropion's effects. In addition, treatment with methylene blue (a direct inhibitor of NOS and soluble guanylate cyclase, sGC) potentiated the effect of the drug in the forced swim test [13]. This study suggests that bupropion possesses antidepressant-like activities in different animal models possibly through dopaminergic and L-arginine-NO-cyclic guanosine monophosphate (cGMP) signaling pathways. This is consistent with a study by Zomkowski et al. (2010) showing similar effects with escitalopram in female mice. The antidepressant-like effect of escitalopram in the forced swim test (FST) was prevented by pretreatment with N-methyl-D-aspartic acid (NMDA), L-arginine, and sildenafil (a phosphodiesterase inhibitor). Also, the administration of 7-nitroindazole, methylene blue or ODQ (i.c.v., a soluble sGC inhibitor) in combination with escitalopram reduced the immobility time in the FST. This study highlights the role of NMDA receptors and L-arginine-NO-cGMP pathway in the mechanism of action of antidepressant agents [14]. Recently, Krass et al. (2011) reported that imipramine decreased brain nitrite + nitrate (NO_2 + NO_3) levels, a marker of nitrosative stress, in male rat brain. This result supports the idea that antidepressants are able to inhibit NO synthesis in the rat brain [16], an effect that could be mechanistically related to the ability of L-arginine to counteract their antidepressant-like effects [15]. In summary, studies in animal models suggest that antidepressant agents modulate antioxidant enzyme activities and decrease oxidative stress markers on liver, brain, and peripheral tissues. In addition, there is a clear association between high dosages of antidepressants and increased hepatic oxidative stress. However, a major limitation of the studies above mentioned is that not all studies measured oxidative stress markers (i.e., MDA, carbonyl); therefore, these prooxidant effects need further investigation.

Consistent with the above-mentioned studies, changes in the blood/brain antioxidant profile have been associated with changes in depressive-like behaviour. More specifically, it has been demonstrated that some classic antioxidants induce antidepressant-like effects in rodents. In one study, treatment with *Ginkgo biloba* extract (10 mg/kg) reduced recorded immobility time in the forced swimming test (FST) to the same extent as imipramine (39% versus 38%). No differences in locomotor activity were observed, suggesting a selective antidepressant-like effect. This antidepressant-like effect of *Ginkgo biloba* extract was associated with a reduction in lipid peroxidation and superoxide radical production (as indicated by a downregulation of SOD activity) [54]. In rats displaying depressive-like behaviour induced by chronic mild stress, administration of liquiritin, an antioxidant derived from *Glycyrrhiza uralensis*, decreased immobility time, increased sucrose consumption, increased SOD activity, and attenuated MDA production in the peripheral blood [55]. These findings are further corroborated by a study showing that Ebselen (2-phenyl-1,2-benzisoselenazol-3[2H]-one), a substance that mimics the activity of the antioxidant enzyme GPx [56], decreased immobility time

in rodents, an effect that was dependent on its interaction with the noradrenergic and dopaminergic systems [57]. Additionally, alpha-tocopherol (vitamin E) administration produced antidepressant-like effects in animal models of depression. Along with antidepressant-like effects, long-term treatment with alpha-tocopherol enhanced antioxidant defences in the mouse hippocampus and prefrontal cortex, two structures closely implicated in the pathophysiology of depression [19].

4.3. Post-Mortem Studies. A number of *post-mortem* studies reported altered oxidative stress parameters in individuals with MDD (Table 4). Michel et al. (2010) showed increased XO activity in the thalamus and putamen of seven individuals with an ante-mortem diagnosis of recurrent MDD (age range = 61–93 y.o.). Four of these subjects received SSRI and one was medicated with clomipramine in the 6 months before death, while two of them were not antidepressant treatments [35]. These results suggest increased ROS production in brain samples of depressive patients due to increased XO activity. Two recent studies showed reduced oxidized and total GSH in the prefrontal cortex of MDD subjects as compared to controls [31, 36]. In addition, GPx levels were reduced in MDD subjects [31]. Because 10 in 14 patients have taken antidepressants at time of death, we can speculate that antidepressants had limited or no effects on GSH and GPx levels. In a subsequent study with the same cohort, GST levels were also reduced in MDD patients and no effects of antidepressant treatment were observed [36].

In summary, while some changes in antioxidant enzymes have been observed in MDD, these *post-mortem* studies are not conclusive mostly because of small sample sizes, lack of control groups, and lack of relevant information (i.e., treatment duration, specific drugs used).

5. Clinical Data: Human Studies

In the last decade, an increasing number of studies have addressed the potential effects of antidepressant treatments on oxidative stress and antioxidant potential in humans (Table 4). Corroborating with animal data, the majority of these studies revealed that antidepressant agents possess antioxidant properties when used in the treatment of MDD. Increased serum SOD and MDA levels have been found in a cohort of 62 major depressive patients (age 43.8 ± 12.9, mean ± SD; 34/28, female/male ratio) [27]. In another study, plasmatic vitC levels were reduced in patients with MDD compared with age- and sex-matched healthy volunteers ($n = 40$). Oxidative stress markers (SOD, vitC, lipid peroxidation) were reversed after 4 weeks of treatment with fluoxetine (20 mg/day, $n = 32$) and citalopram (20 mg/day, $n = 30$). Notably, these antioxidant effects were persistent after 12 weeks of treatment [27].

Bilici et al. (2001) reported increased oxidative stress in major depressive patients ($n = 32$), indexed by higher antioxidant enzyme activities (erythrocyte SOD, GPx, and plasmatic GR) and MDA levels (erythrocyte and plasmatic). After treatment with four different SSRIs drugs (fluoxetine 20 mg/day, $n = 7$; sertraline 50 mg/day, $n = 13$; fluvoxamine 100 mg/day, $n = 5$; or citalopram 20 mg/day, $n = 5$), for 12 weeks, antioxidant enzyme activities (plasmatic GPx) and MDA levels (plasma and erythrocyte) were restored to control levels. Plasmatic GR and erythrocyte SOD were also significantly decreased in MD patients after 12-week antidepressant treatment [30]. In another study, a group of 50 MDD patients (age 36.7 ± 5.2; 22/28 F/M ratio) who had achieved remission from their first episode of depression after 3 months of treatment with 20 mg of fluoxetine were tested before and after remission [48]. Before treatment, MDD patients displayed increased erythrocyte SOD and CAT activities, increased MDA levels, and decreased plasmatic total antioxidant status (TAS) level. After three months of fluoxetine treatment, MDA levels were normalized [48]. Decreased serum SOD and increased XO were found in 20 individuals with MDD (age range 17–62 years, 19/17 F/M ratio) [29]. Although increased XO levels indicate increased free radical production, no difference was observed in serum total nitrite levels (a marker of nitrosative stress, possible associated to ONOO–) between control and MDD patients before treatment. Also, the authors did not find a significant relationship between the duration of illness and SOD, XO activities, or nitrite levels in this cohort. Treatment with citalopram (20 mg/day, $n = 10$), fluoxetine (20 mg/day, $n = 11$), fluvoxamine (150 mg/day, $n = 7$), or sertraline (50 mg/day, $n = 8$) for 8 weeks increased SOD activity whereas decreased XO levels suggesting that normalization of these enzymes was associated with symptomatic improvement [29].

Cumurcu et al. (2009) investigated whether 3 different total antioxidant parameters (TAC, TOS, and OSI) were associated with MDD and evaluated the impact of antidepressant treatment on these oxidative/antioxidant parameters in a cohort of 57 major depressive patients (age 35.5 ± 12.1, 46/11 F/M ratio). TOS and OSI were higher and TAC level was lower in the MDD group compared with controls ($n = 40$). Furthermore, the authors found a positive correlation between the severity of the disease and serum TOS and OSI ($r = 0.58$, and $r = 0.63$, resp.). Also, a negative correlation was found between the severity of the disease and serum TAC ($r = -0.553$) at the pretreatment stage. After 3 months of treatment with escitalopram, 10–20 mg/day, $n = 10$; paroxetine, 20–40 mg/day, $n = 20$; or sertraline, 50–100 mg/day, $n = 27$, TOS and OSI were decreased and TAC was increased compared with pretreatment values [32]. These further suggest that recovery from a major depressive episode may be associated with normalization of antioxidant potential induced by antidepressants.

More recently, a 24-week follow-up study evaluated the effects of long-term antidepressant treatment on oxidative/antioxidant status in a cohort of 50 MDD subjects (age 33.1 ± 10.0, 39/11 F/M ratio) [33]. Antidepressant treatments included venlafaxine (125 ± 43.3 mg/day, $n = 21$), milnacipran (100 mg/day, $n = 2$), paroxetine 25 ± 7.6 mg/day, $n = 8$, escitalopram 16.3 ± 5.2 mg/day, $n = 8$, sertraline 80 ± 27.4 mg/day, $n = 5$, citalopram 33.3 ± 11.5 mg/day, $n = 3$, fluoxetine 20 mg/day, $n = 1$, tianeptine 37.5 mg/day, and moclobemide 600 mg/day. Plasmatic MDA, serum oxidized LDL (OxLDL) levels, and erythrocyte SOD

Table 4: Antidepressant treatment and oxidative stress markers in major depressive disorder.

Sample (F/M)	Altered oxidative stress markers in MD[a]	Treatment duration	Antidepressant drugs tested	Effect for antidepressants	Reference
34/28	↑MDA ↑SOD ↓VitC	4 weeks and 12 weeks	Citalopram ($n = 30$), fluoxetine ($n = 32$)	↓MDA ↓SOD ↑VitC (effects in both 4 and 12 weeks treatment)	Khanzode et al., 2003 [27]
72/24	↑MDA ↑SOD ↓TAC ↑VitE	6 weeks	Reboxetine, sertraline, venlafaxine	No effects	Sarandol et al., 2007 [28]
19/17	↑XO ↓SOD	8 weeks	Citalopram ($n = 10$), fluoxetine ($n = 11$), fluvoxamine ($n = 7$), sertraline ($n = 8$)	↓XO ↑SOD ↓nitrite	Herken et al., 2007 [29]
21/9	↑MDA ↑SOD ↑GPx ↑GR	12 weeks	Citalopram ($n = 5$), fluoxetine ($n = 7$), fluvoxamine ($n = 5$), sertraline ($n = 13$)	↓MDA ↓SOD ↓GPx ↓GR	Bilici et al., 2001 [30]
28/22	↑MDA ↑SOD ↑CAT ↓TAC	12 weeks	Fluoxetine ($n = 50$)	↓MDA	Galecki et al., 2009 [48]
46/11	↓TAC ↑TOS ↑OSI	12 weeks	Escitalopram ($n = 10$), paroxetine ($n = 20$), sertraline ($n = 27$)	↑TAC ↓TOS ↓OSI	Cumurcu et al., 2009 [32]
39/11	↑MDA ↑OxLDL ↑SOD	24 weeks	Citalopram ($n = 3$), escitalopram ($n = 8$), fluoxetine ($n = 1$), milnacipran ($n = 2$), moclobemide ($n = 1$), paroxetine ($n = 8$), sertraline ($n = 5$), tianeptine ($n = 1$), venlafaxine ($n = 21$)	↓MDA ↓SOD ↓TAC	Kotan et al., 2011 [33]
20/15	↓CoQ10	? weeks	? ($n = 15$)	No effects*	Maes et al., 2009 [34]
5/2	↑XO	Post-mortem study	SSRI ($n = 4$), TCA ($n = 1$)	No effects#	Michel et al., 2010 [35]
6/9	↓GPx ↓GSH	Post-mortem study	Trazodone ($n = 1$), nefazodone ($n = 2$, one together SSRI), TCA and/or SSRI ($n = 7$)	No effects#	Gawryluk et al., 2011 [31]
	↓GST	Post-mortem study		No effects*	Gawryluk et al., 2011 [36]

Sample (female/male) from MD group; [a]Compared to respective control group. *Compared to unmedicated MD group; #speculative. CAT, catalase; CoQ10, coenzyme Q-10; GPx, glutathione peroxidase; GSH, glutathione; GST, glutathione S-transferase; MDA, malondialdehyde; OSI, oxidative stress index; OxLDL, oxidized LDL; SOD, superoxide dismutase; SSRI, selective serotonin reuptake inhibitor; TAC, total antioxidant capacity; TCA, tricyclic or tetracyclic antidepressant; TOS, total oxidant status; VitC, vitamin C; VitE, vitamin E; XO, xanthine oxidase.

activity were increased in MDD patients before treatment, and MDA levels were positively correlated with the severity of MDD. After 24-weeks of treatment, MDA and SOD levels decreased. However, TAC was also found decreased after 24-week treatment with antidepressants, indicating that the oxidative stress observed in depressed patients was partly improved during 24 weeks of antidepressant treatment. Patients on venlafaxine were also compared with patients on SSRIs in the aspect of oxidative stress parameters in the follow-up period, but no significant differences were found [33].

Sarandol et al. (2007) found that MDD was accompanied by increased peripheral oxidative stress; however, short-term antidepressant treatment (6 weeks) did not alter oxidative/antioxidant systems in a cohort of 96 MDD patients (age 40 ± 11, 72/24 F/M ratio). In this study, MDD patients had increased plasmatic MDA levels and increased susceptibility of red blood cells (RBCs) to oxidation. Also, SOD activity was increased in patients with MDD, and there was a positive correlation between the severity of depressive symptoms and SOD activity ($r = 0.419$). After 6 weeks of treatment with venlafaxine 75–150 mg/day, sertraline 50 mg/day, or reboxetine 4–8 mg/day, these oxidative parameters were not altered [28].

Maes et al. (2009) investigated plasma concentrations of CoQ10 in 35 depressed patients (age 42.1 ± 10.5, 20/15 F/M ratio) and 22 sex-, age-matched controls. Plasmatic CoQ10 was lower in depressed patients than controls. However, there was no correlation between plasma CoQ10 and the severity of illness or the number of depressive episodes. During the study, part of the depressed patients were on antidepressant treatment at the time of blood sampling ($n = 15$), while the remaining were unmedicated ($n = 20$). There were no differences in plasma CoQ10 between depressed patients who were taking antidepressants and those without [34].

6. Concluding Remarks

This paper examined preclinical (*in vitro* and animal models) and clinical literature on oxidative/antioxidant effects of antidepressant agents. Overall, most animal and human data support that antidepressant drugs exert antioxidant effects during treatment for MDD.

In vitro and animal studies also suggest that some antidepressants may be prooxidant at high doses. The antioxidant effects of antidepressant drugs seem to vary depending on the dose, treatment regimen, and duration. Notably, a number of clinical trials revealed that treatment with antidepressants can reverse the increased oxidative stress observed in individuals with MDD. Short-term treatments (4 to 8 weeks) do not seem to alter antioxidant/oxidative parameters in MDD patients, whereas longer treatments (12 to 24 weeks) seem to induce robust antioxidant effects.

Overall, the literature reviewed does not support differences in antioxidant potential between different antidepressant agents/classes. However, many of these studies were short in duration and likely underpowered to address the question of differences in antioxidant potential amongst particular drugs and larger studies are warranted.

Brain imaging studies have suggested that MDD may be associated with decreased volumes of various brain regions [58–60]. For instance, MDD subjects have smaller normalized frontal lobe volumes when compared with the nondepressed controls after controlling for age, gender and "total cumulative illness rating scale score" [61]. Presence of temporal lobe atrophy and moderate-to-severe white matter lesions can predict occurrence of major depression during a 5-year followup in a population-based sample of elderly [62]. Considering that the presence of oxidative (and nitrosative) stress may cause neurodegeneration and reduced neurogenesis [63, 64], the relationship between oxidative stress and changes in brain structure and function in MDD is a promising area for future studies.

An important issue in biomarker research is the fact that peripheral markers may not necessarily correlate with changes in the central nervous system. For instance, Teyssier et al. (2011) demonstrated that the expression of oxidative stress-response genes was not altered in the prefrontal cortex of individuals with MDD. They concluded that the pathogenic role of oxidative stress in the neurobiology of depression could not be inferred from alterations in the periphery [65]. However, in this *post-mortem* study all of the patients had received antidepressant treatment, which may have normalized oxidative stress parameters. Furthermore, there is also evidence suggesting that BDNF, oxidative stress, and inflammation tend to be abnormal among individuals with multiple mood episodes and correlate with length of illness [51, 66, 67]. Peripheral biomarkers detected during acute mood episodes could in fact constitute markers of disease activity [68]. Studies of peripheral biomarkers in large randomized, placebo-controlled trials will ultimately confirm whether or not normalization of oxidative stress parameters is associated with treatment response.

In conclusion, there is increasing body of evidence supporting that MDD may be associated with changes in oxidative stress markers and that antidepressant agents (especially long-term treatment) may increase antioxidant defences. It is possible that augmentation of antioxidant defences may be one of the mechanisms underlying the neuroprotective effects of antidepressants observed in the treatment of MDD.

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Anexo 2

Mitochondrial Dysfunction in Bipolar Disorder: Lessons from Brain Imaging and Molecular Markers

Minuzzi L, Behr GA, Moreira JC, Frey BN.

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Review

Tradução do título para a Língua Portuguesa:

Disfunção mitocondrial na doença bipolar: Lições acerca de imagem cerebral e marcadores moleculares.

Mitochondrial Dysfunction in Bipolar Disorder: Lessons from Brain Imaging and Molecular Markers

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Abstract

Bipolar disorder (BD) is a chronic major mental illness characterized by extreme mood episodes, cognitive impairment, and high rates of disability. Several lines of evidence suggest that BD may be associated with abnormalities in mitochondrial function. Here we critically review findings from brain imaging and from preclinical studies that investigated markers of energy metabolism in BD. Research with postmortem brain and peripheral tissue revealed changes in size and distribution of mitochondria, as well as decreased mitochondrial electron transport chain function, increased oxidative stress, and increased lipid and protein damage. PET imaging studies revealed decreased glucose metabolism in sub-areas of the prefrontal cortex, amygdala, and hippocampus structures in BD. On the other hand, increased lactate levels in BD have been found in cerebrospinal fluid and in gray matter by magnetic resonance spectroscopy, which suggest that distinct pathophysiological processes may be region-specific. Resting state fMRI studies have demonstrated decreased functional connectivity between fronto-limbic circuits. In conclusion, these results support the hypothesis of mitochondrial dysfunction in BD and suggest that BD is associated with decreased energy production and a shift towards anaerobic glycolysis. Such changes in energy metabolism can potentially decrease cell plasticity and ultimately disrupt brain circuits associated with mood and cognitive control.

Key words: Mitochondrial dysfunction, bipolar disorder, PET, biomarkers.

Título: Disfunción mitocondrial en el trastorno bipolar: lecciones de las imágenes cerebrales y los marcadores moleculares

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Resumen

El trastorno bipolar (TB) es una enfermedad mental crónica grave caracterizada por episodios de ánimo extremo, trastornos cognitivos y altas tasas de discapacidad. Varias líneas de evidencia sugieren que el TB puede estar asociado con anormalidades en la función mitocondrial. Aquí analizamos críticamente los hallazgos de las imágenes cerebrales y de los estudios preclínicos que han investigado los marcadores del metabolismo de energía en TB. Las investigaciones post mórtem basadas en tejidos cerebrales y tejidos periféricos revelaron cambios en el tamaño y en la distribución de las mitocondrias, además de una disminución en la funcionalidad de la cadena de transporte de electrones de las mitocondrias, un mayor estrés oxidativo y mayores daños lipídicos y proteínicos. Estudios con imágenes TEP revelan un metabolismo de glucosa disminuido en las subáreas de la corteza prefrontal, la amígdala y el hipocampo en TB. Por otro lado, se han hallado concentraciones mayores de lactato en TB en el líquido cefalorraquídeo cerebral y en la materia gris utilizando la espectroscopia con resonancia magnética, lo cual sugiere que los procesos fisiopatológicos individuales pueden ser específicos de las distintas regiones. Los estudios con resonancias magnéticas funcionales han demostrado una menor conectividad funcional entre los circuitos frontolímbicos. En conclusión, estos resultados apoyan la hipótesis de una disfunción mitocondrial en el TB y sugieren que el TB está asociado con una menor producción de energía y un cambio hacia la glicólisis anaeróbica. Estos cambios en el metabolismo energético pueden disminuir potencialmente la plasticidad celular y, en últimas, perturbar los circuitos cerebrales asociados con el estado de ánimo y el control cognitivo.

Palabras clave: Disfunción mitocondrial, trastorno bipolar, tomografía por emisión de positrones, biomarcadores.

Introduction

Bipolar disorder (BD) is a chronic and disabling major mental illness characterized by episodic disturbances in mood reactivity (e.g. mania, depression) and cognitive impairment. Even though BD is associated with structural and functional brain changes, the fact that BD is also associated with increased mortality due to general medical conditions such as heart disease and cancer (1), as well as higher risk for metabolic syndrome, suggests that BD is a systemic rather than a brain-limited disorder (2). Over the past decade, there has been converging evidence supporting the hypothesis of mitochondrial dysfunction as a key element in the pathophysiology of BD (3-5). The brain metabolizes approximately 20% of all of the body's oxygen because it works under a constant high energy demand. Considering that most of the energy produced in the brain is used to replenish the ATP consumed by the Na-K-ATPase pump and in the production/metabolism of neurotransmitters, mitochondrial dysfunction may disrupt the membrane's ionic gradient and the glutamatergic clearance, thereby leading to abnormal neuronal firing. Furthermore, persistent glutamatergic activity may lead to a state of excitotoxicity and potentially cell death.

Here we critically review studies conducted with postmortem brain tissue, peripheral blood and studies in vitro that provide evidence of mi-

tochondrial dysfunction in BD, as well as data from positron emission tomography, resting state functional magnetic resonance imaging and magnetic resonance spectroscopy that assessed energy metabolism in individuals with BD in vivo.

Molecular Markers of Mitochondrial Dysfunction

Evidence of mitochondrial dysfunction in BD is suggested by altered mitochondrial morphology, impaired brain energy metabolism, altered mitochondria-dependent Ca2+ signaling, the effects of mood stabilizers on mitochondria, increased mitochondrial DNA deletion in the neural tissue of BD patients, and the association of mitochondrial DNA mutations and/or polymorphisms with BD (4-6).

Morphological changes of mitochondria have been observed in the central nervous system (CNS) as well as in peripheral tissue in individuals with BD. Mitochondria from patients with BD exhibited size and distributional abnormalities compared with psychiatrically- healthy age-matched controls. Specifically, in the prefrontal cortex, individual mitochondria profiles had significantly smaller areas in individuals with BD (7). In two peripheral cell types (fibroblasts and lymphocytes) the mitochondria from BD individuals exhibited alterations in distribution and morphology, showed by dense or bulky networks with perinuclear clustering profile. This study was the first to demonstrate that brain cortical and also peripheral cells from BD patients display abnormalities in the morphology (size and shape) and in the intracellular distribution of mitochondria. Given that abnormal mitochondrial morphology is linked to altered energy metabolism, changes in mitochondrial size and distribution may lead to energy deficits and, therefore, may have consequences for cell plasticity, resilience, and survival in patients with BD.

Abnormalities in mitochondrial function have also been observed in BD. A recent postmortem study reported a reduction in the activity of the complex I of the mitochondrial electric transport chain (ETC) in the prefrontal cortex in BD, but not in schizophrenia (SZ) or major depressive disorder (MDD), and this reduction was associated with increased protein oxidative damage (8). Prolonged impairment in oxidative phosphorylation activity causes accumulation of lactate and unprocessed glucose. Consistent with this, a study found increased lactate in the cerebrospinal fluid of BD patients, which indicates a shift from mitochondrial respiration to anaerobic glycolysis (9). Some data suggest that cellular response to metabolic stress may be impaired in BD, and such impairment may be closely linked to mitochondrial function. For instance, the molecular response to glucose deprivation in lymphocytes of BD patients was significantly re-

duced as compared to cells of normal controls (10). Microarray analysis of the peripheral blood mononuclear cells revealed that control subjects upregulated expression of ETC genes in response to glucose deprivation, while cells from BD patients failed to show any changes in mitochondrial gene expression (10). Together, these studies suggest that alterations in the ETC within the mitochondria may be associated with a shift from oxidative phosphorilation to anaerobic glycolysis in BD. In this context, a preliminary study revealed that the mood stabilizer lithium can increase the activity of mitochondrial ETC complexes I/II and II/III in human brain tissue (11), which suggests that lithium may stabilize mitochondrial function.

Creatine kinase (CK) is a key enzyme involved in the transport of ATP from mitochondria to cytosol. An elegant study conducted by MacDonald et al (2006) found that CK mRNA were downregulated in the hippocampus and dorsolateral prefrontal cortex in BD (12). Considering the central role of CK in the transport of intracellular high-energy phosphates, this study revealed another indicative of altered energy metabolism in specific brain areas in BD. However, one study conducted in rats reported that neither lithium nor valproate were able to reverse or prevent the inhibition of CK activity induced by amphetamine (13).

Several lines of evidence suggest that BD may be associated with altered Ca2+ signaling. Since mitochondria buffer cytosolic Ca2+, abnormal Ca2+ homeostasis may induce detrimental effects on mitochondrial and cell function and viability (14,15). In addition, the interplay between elevated intracellular Ca2+ concentration, reactive oxygen species (ROS) formation, and mitochondrial dysfunction has been long associated with brain pathology (3, 14). Studies with peripheral blood found elevated Ca2+ levels in platelets and lymphocytes of BD patients (16-18). Also, lymphoblastoid cell lines derived from BD patients showed higher Ca2+ peaks after thapsigargin, thrombin- or lysophosphatidic acid-mediated stimulation (17,19). Chronic lithium treatment attenuates intracellular calcium mobilization in B lymphoblast cell lines (20). In this context, it has been hypothesized that elevated intracellular Ca2+ levels when associated with mitochondrial dysfunction and subsequent decreased ATP production may reduce the ability of neuronal cells to appropriately respond to temporary peaks in stressful stimuli such as increased glutamate release during emotional distress (21).

Oxidative stress refers to the cytotoxic consequences of excessive generation of ROS – superoxide anion (O2-·), hydrogen peroxide (H2O2), and hydroxyl radical (OH·) (22). Under normal circumstances, ROS are eliminated by enzymatic (i.e. superoxide dismutase, SOD; catalase, CAT; glutathione peroxi-

dase, GPx) and non-enzymatic antioxidant defences (i.e. vitamins; glutathione, GSH). When these antioxidant systems are outweighed by extreme levels of ROS, there is a higher potential for damage to DNA, lipids (i.e. cell and organelle membranes) and proteins (i.e. receptors, transcription factors and enzymes) (23). A number of studies reported increased oxidative damage in both peripheral blood and postmortem brain of BD patients (6,24). For instance, one study found a remarkably high frequency of DNA damage (as measured by DNA fragmentation using the comet assay) in peripheral blood of BD subjects relative to controls and that the frequency of DNA damage correlated with the severity of manic and depressive symptoms (25). Consistent with this finding, a postmortem study found that DNA fragmentation is also increased in non-GABAergic neurons in the anterior cingulate cortex in individuals with BD (26). Another consistent finding that has been replicated by several laboratories is increased lipid peroxidation (lipid damage) in BD. Increased levels of 4-hydroxynonenal, a product of lipid peroxidation, were found in the anterior cingulate cortex of subjects with BD and SZ (27). Several studies have reported increased levels of thiobarbituric acid reactive substances (TBARS) in both plasma (28) and serum samples (29-33). Notably, treatment with lithium and valproate at therapeutically relevant concentrations significantly inhibited glutamate-induced increased intracellular Ca+2, lipid peroxidation, protein oxidation, DNA fragmentation, and cell death in primary cultured rat cerebral cortical cells (34,35). In addition, both of these mood stabilizers can also enhance mitochondrial function and protect against mitochondrially-mediated toxicity in SH-SY5Y neuron cell culture (36). Animal models using increased oxidative stress induced by amphetamine exposure found that lithium and valproate reversed and prevented amphetamine-induced TBARS formation in vivo (37). Consistent with this finding, another rat study showed that lithium and valproate were able to modulate the oxidative balance and prevent cerebral DNA damage (38).

Studies looking at antioxidant activity profile in BD patients have been less consistent. For instance, there are reports of increased SOD and CAT activities (31), unaltered SOD and decreased CAT activity (32,39), an imbalance between increased SOD and decreased CAT activities (29), increased GPx activity (29), and also decreased GPx activity (32) in the peripheral blood of BD subjects. A postmortem study showed decreased total GSH levels in prefrontal cortex of patients with BD, SZ and major depression (40). Furthermore, a case study that investigated the oxidative stress profile in two monozygotic twins during a manic episode showed that bipolar twins had higher TBARS, SOD and

DNA damage, and lower CAT. TBARS and SOD were normalized after mood stabilization, whereas CAT and DNA damage remained unaltered after 6 weeks of treatment (41). A recent meta-analysis confirmed that a consistent finding in BD patients is the presence of increased TBARS across all mood states (42). As far as the potential use of antioxidant agents, there are some encouraging data suggesting that N-acetyl cysteine (NAC), a precursor of glutathione, may be a useful adjunctive treatment in reducing oxidative stress, and improving clinical symptoms, quality of life, and functioning in individuals with BD (43, 44).

In summary, there is converging evidence from animal, clinical, and preclinical studies suggesting that BD is associated with altered Ca2+ signaling and gene expression, increased oxidative stress and DNA damage, and mitochondrial dysfunction. Studies in vitro and in vivo support that both lithium and valproate can stabilize mitochondrial function and prevent oxidative stress. Perhaps more importantly, some initial clinical trials support the use of antioxidants, such as NAC, as adjunctive in the treatment of BD.

Brain Imaging and Energy Metabolism in BD

Positron Emission Tomography (PET)

Brain metabolism can be measured by PET technique using a radiolabelled glucose analogue, [18F]-fluorodeoxyglucose (FDG). When FDG is taken up into the cell, it is metabolized by phosphorylation to FDG-6 phosphate and accumulates in the cell, providing an indirect measurement of the local glucose metabolism.

An early study with euthymic and drug-free BD patients and electrical stimulation showed a reduction of FDG uptake in frontal cortex, occipital cortex and basal ganglia compared to healthy controls (HC) (45). Using the subgenual region as region-of-interest (ROI), a population of depressive BD patients presented a reduction of the glucose metabolism in that area compared to HC (46). In the same sample, manic patients (n=4) revealed higher global FDG uptake. Subsequently, this finding has been independently replicated in a sample of depressed BD patients (47). In the amygdala no differences in glucose metabolism between depressive BD, HC and MDD patients were observed. However, individuals that were off-medication presented higher FDG uptake, which suggests that treatment may normalize over activity in the amygdala (48). A study with FDG and cerebral blood flow (CBF) in BD type I and type II patients showed a positive correlation between glucose metabolism and blood flow, but such correlation was less robust in comparison to HC. Also there was a negative correlation between glucose metabolism and CBF in the left pregenual ACC, indicating a local uncoupling flow-metabolism disrup-

tion that might suggest vasomotor or mitochondrial dysfunction (49). A recent FDG-PET study examined a population of depressed and euthymic women with BD type I, as compared to MDD and HC. This study showed a decrease of glucose metabolism in frontal gyri, right cingulate, inferior parietal cortex and angular gyri in BD patients. However, the euthymic BD patients did not display differences in these brain regions (50), which suggested that some metabolic changes may be state-related depending on the brain area. Another study found a reduction in bilateral dorsolateral prefrontal cortex (DLPFC) and left amygdala, and an increase in the left occipital cortex and right temporal cortex in manic subjects with psychosis. However this study used a "clinical diagnosis" rather than the DSM classification for the selection of the sample (51). Benson and col. reported a FDG study with treatment refractory depressed BD patients. In that study, correlation analysis across different brain regions showed higher metabolic association between the left DLPFC, left inferior parietal cortex, thalamus and insula and other brain regions in the BD sample compared to HC (52). These results are in conflict with previous report of global decrease in metabolism in depressed BD patients in comparison HC in DLPFC, insula, striatum, ACC, PFC and subgenual (53).

In summary, measurements of the brain metabolism with PET reported mostly a decrease in metabolic activity in several brain regions of BD patients in comparison to HC. Even though methodological limitations such as low sample size, heterogeneity of the BD population and lack of control of baseline glucose level may have skewed the results, the consistent finding of low FDG levels in the brain of BD cannot be ignored and suggest that a decrease in energy metabolism may be part of the pathophysiology of BD.

Resting State fMRI

Resting state functional MRI is a technique based on the study of low frequency blood oxygen level-dependent oscillations in the absence of any mental task while the subject rests quietly (54). Although the underlying brain activity revealed by this technique has been debatable, it has been suggested that it would identify brain regions that present functional connectivity across each other (55). To date, only four studies have looked at resting state fMRI technique in BD. In a study that included both manic and depressive BD patients, connectivity between pregenual anterior cingulate (pACC) and amygdala, and between thalamus and pallidostriatum were decreased in BD compared to HC, but were similar to MDD patients (56). Another study of manic and mixed BD I patients showed decreased activity in medial prefrontal cortex (MPFC) and the hippocampus as compared to HC (57). In a heterogeneous BD

population of rapid cyclers, euthymic, mixed, and depressive patients, BD subjects showed decreased connectivity between left ventral prefrontal cortex (VPFC) and left amygdala as well as between left VPFC and dorsofrontal and pariental regions. The same study revealed increases in connectivity between left VPFC and contralateral hemisphere (58). In an euthymic pediatric population of BD patients, resting state fMRI revealed a decrease in the connectivity between left DLPFC and contra-lateral temporal gyrus. Using the superior temporal gyrus (STG) as seed point, BD pediatric patients showed decrease in the connectivity between STG and frontal gyri (superior and middle), and between STG and thalamus. However it was observed an increase in the connectivity between STG and parahippocampal gyrus in the patients in comparison to HC (59).

Overall, the preliminary studies of resting state fMRI suggest that individuals with BD present a decrease in the connectivity between a number of corticolimbic regions associated with mood and cognitive control. Whether such abnormal functional connectivity are part of the pathophysiology of the disease or part of a compensatory mechanism it remains to be determined.

Magnetic Resonance Spectroscopy

Magnetic resonance spectroscopy (MRS) is a non-invasive imaging technique that provides in vivo quantification of a number of metabolites in the brain. Single proton MRS (1H-MRS) enables measurement of glutamate (Glu), glutamine (Gln), N-acetyl-L-aspartate (NAA), choline compounds (Cho), creatine and phosphocreatine (Cre), myoinositol (mI) and lactate. For the purpose of reviewing brain energy metabolism status we will focus on the studies reporting absolute levels of these metabolites in the brain. Results describing differences in ratio of the metabolites (e.g., NAA/Cre, NAA/Cho, etc) will not be reviewed.

In mammals, glutamate is the main excitatory neurotransmitter in the CNS. It is synthetized in glutamatergic neurons and astrocytes (60). Measurements of glutamate (Glu), glutamine (Gln), and the combined Glu-Gln (Glx) provide information about the local glutamate metabolism. Higher or lower glutamate metabolites may indicate abnormalities in the local glutamatergic metabolism. A MRS study with a sample of manic and depressive BD patients found a decrease in Glx levels in the frontal cortex and basal ganglia, while there was no alteration in the thalamus (61). In contrast, another study revealed some brain areas with high levels of Glx such as MVPFC, insular cortex and cortical grey matter (62). BD patients in a manic phase did not display differences in Glu, Gln or Glx levels compared to HC in the ACC, parietal-occipital cortex (63) or OFC (64). However, the ratio Gln/

Glu was reported to be higher in the ACC and the parietal-occipital cortex of manic BD patients (63). To date, only one study examined BD patients during depressive state. Depressive BD patients presented an increase of Glu and Glx but not Gln in the ACC (65). Children with BD showed low Gln but not Glu in the ACC compared to HC (66). In asymptomatic children with one parent presenting BD, the Glu and Glx levels were compared to controls in the cerebellar vermis (67). Different treatments for BD have been tested to reveal changes in the glutamate levels in the brain. Pharmacotherapy with riluzole (68) and lithium (69) did not change the Gln/Glu and Glu and Gln levels levels in the ACC after treatment. However, lithium decreased Glx levels in grey matter of BD patients in comparison to valproate (70). The mood-stabilizer lamotrigine showed to increase Gln in the ACC and the MPFC of depressed BD patients (65). Treatment with quetiapine did not change Glx levels in the medial frontal cortex of rapid cyclic BD patients (71). In a sample of depressed BD patients, treament with cytidine decreased Glu/Gln levels in the ACC in comparison to placebo (72).

Glutamate and glutamine levels as biomarkers for local glutamatergic function have shown higher activity in some cortical regions associated with mood and cognitive control (for instance, ACC and OFC). Considering that the glutamate-glutamine cycle is closely linked to energetic metabolism, these results further support that changes in energy metabolism are part of the pathophysiology of BD.

N-acetyl-L-aspartate (NAA) is produced in the mitochondria in the neuronal cell bodies and it is considered a marker of neuronal integrity (73). In heterogeneous samples of manic and depressive subjects, NAA has been found diminished only in the basal ganglia (61). Measurements in thalamus, frontal cortex white matter (61), anterior cingulate (ACC), insular cortex and grey matter (62) did not reveal differences in NAA levels compared to healthy controls (HC). In euthymic BD patients, it has been reported a decrease in NAA levels in hippocampus (74) and an increase in thalamus (75). Proton MRS studies on medial prefrontal cortex (MPFC) (76), frontal cortex (77), and basal ganglia (78) did not find differences in NAA levels in comparison to HC. BD patients, while in manic phase, showed decrease of NAA levels in the orbital frontal cortex (OFC) (64). However, other brain regions such as ACC (63,78), parietal-occipital cortex (63), dorsolateral prefrontal cortex (DLPFC) (79), and frontal cortex (78) did not reveal differences in NAA levels during manic phase in comparison to HC. Only two MRS studies have focused on NAA levels and BD patients during the depressive phase. These studies found no differences in NAA levels in the ACC (65) and frontal cortex (77). Children diagnosed with BD showed lower

levels of NAA in the DLPFC, MPFC (80) and cerebellum (81) compared to HC. No differences were found in NAA levels in the frontal cortex (81,82), posterior cingulate (PC) and occipital cortex (80) in the pediatric population with BD. Two studies in children focused on the ACC presented different results: NAA levels were found unchanged in an earlier study (83). Recently, in another study, it has been shown a decrease of NAA in the ACC of children with BD (80). Pharmacotherapy seems to affect the NAA levels in the brain of BD patients. Lithium (69), riluzole (68), and ethyl-EPA (84) showed to increase NAA concentration in cortical regions after treatment. Lamotrigine (65) produced a reduction in the NAA levels in the ACC of BD patients. Also in a pediatric BD sample, NAA levels in MVPFC but not in VLPFC decreased after treatment with lithium (85). Some pharmacological studies with quetiapine (71), valproate (70), and lithium (70,85) did not show any effect on NAA levels in different cortical regions.

Low NAA levels in the brain have been described in several neurodegenerative disorders (86). Hence a local decrease of NAA in the brain might be indicative of neuronal damage or death. In BD, some brain regions such as hippocampus, OFC and basal ganglia presented a reduction in NAA levels compared to HC, suggesting decreased neuronal integrity. Interestingly, studies in the pediatric population with BD have shown more brain regions with low NAA levels compared to HC, supporting the idea of BD as a neurodevelopmental disorder.

Creatine and phosphocreatine (Cre) are abundant in the brain. Creatine is producted in the liver and kidneys and it is transported into the brain, where it is converted to phosphocreatine in the mitochondria. Phosphocreatine is then transported to the cytosol and donates a phosphate group to ADP to replenish ATP. Hence, Cre levels may be used as a measure of energy utilization in the brain. Two MRS studies investigated Cre levels in heterogeneous groups of BD patients with both depressive and manic states. As with NAA, Cre presented diminished in the basal ganglia (61). Thalamus, frontal cortex white matter (61), anterior cingulate (ACC), insular cortex and grey matter (62) did not show differences in Cre levels in this population compared to HC. In euthymic BD patients, the results of Cre brain levels also followed the NAA levels: in the hippocampus, Cre levels were found diminished (74), whereas in thalamus Cre levels were found increased compared to HC (75). In euthymic BD, Cre levels were compared to HC in the frontal cortex and MPFC (76,77). While in manic BD patients, no changes in Cre levels were found in several brain regions (63, 64, 79), depressive BD patients presented higher Cre levels in the ACC (65) and lower levels in the frontal cortex (77). In a study

with medication-free individuals with BD at various mood states, it has been reported a decrease in Cre levels in the left DLPFC (87). In pediatric BD, Cre levels have been found mostly decreased compared to HC in the ACC, DLPFC, MPFC (80) and cerebellum (81). In the ACC, another study failed to find differences in Cre levels in the same population (66). Also in children and adolescents with BD, Cre levels were normal in the frontal cortex (81), posterior cingulate and occipital cortex (80). Light therapy (88), lithium (61,70,85,89), ethyl-EPA (84), quetiapine (71), and risperidone (83) all failed to show any effect on Cre levels after treatment.

Creatine and phosphocreatine levels as biomarkers for energy consumption in the brain have demonstrated close relationship with NAA in the same brain regions. Although no alterations in Cre levels were found in manic subjects, BD depressive patients showed opposite changes in Cre levels in distinct areas of the PFC (higher Cre in ACC, lower Cre in frontal cortex). Lower Cre levels have been reported in the DLPFC in BD subjects free of medications but this finding needs replication. In early stages of brain development, studies with children diagnosed with BD consistently showed reduced levels of Cre in frontal cortical regions and cerebellum. Together these results suggest that the abnormalities in the Cre metabolism in the frontal regions in BD may be more pronounced during depression and early in the course of the disease.

Lactate is continuously produced during normal metabolism and may serve as a source of energy in conditions of decreased oxidative phosphorilation. Thus, increased levels of lactate may be an indicative of metabolic dysfunction. Lactate levels were higher in the grey matter of depressed or mixed BD type I and II. In the same sample, the analysis of subpopulation of BD type I patients revealed increase levels of lactate compared to HC (62). Lactate levels in ACC were unchanged after treatment with lithium (70). However, after treatment with quetiapine, manic BD patients showed a reduction in the lactate levels in the medial frontal cortex, with patients who responded to the treatment presenting higher decrease (71).

In vivo quantification of lactate in BD brain has been limited. The only study in medication-free patients suggests that increased lactate may be associated with a shift towards anaerobic glycolysis in the grey matter of BD type I.

Results from PET and resting state fMRI suggest that there are local metabolic abnormalities in the brain of BD patients. Most of the cortical brain regions have shown decrease in markers for energy metabolism in BD compared to HC. MRS studies suggest that BD is associated with impaired oxidative phosphorilation and a shift towards anaerobic energy production with subsequent

decrease in total energy production (5). In addition, markers for neuronal integrity have shown regional low levels in BD. Together, these studies suggest that a decrease in energy metabolism associated with mitochondrial dysfunction may impair the connectivity between brain regions (e.g. fronto-limbic circuit) which may, in turn, lead to mood and cognitive impairment observed in BD.

Conclusions

The data reviewed above provide strong support to the hypothesis of mitochondrial dysfunction in BD. Considering that the main physiological roles of mitochondria are related to oxidative phosphorilation, Ca2+ metabolism and apoptosis, it is remarkable that all of these components have been found abnormal in BD. Notably, abnormalities in the mitochondrial ETC, altered Ca2+ signaling, increased oxidative stress and DNA damage have been observed in peripheral blood and in postmortem brain tissue in BD. These findings suggest that, at least in some brain areas of individuals with BD (most notably sub-areas of the prefrontal cortex), there may be a decrease in mitochondrial oxidative phosphorylation, which may, in turn, cause a decrease in ATP production. If this hypothesis is true, then it expected an increase in the production of lactate and a shift towards anaerobic glycolysis. Indeed, increased lactate levels have been reported both in the CSF as well as in the brain (gray matter) of BD subject as measured by MRS in vivo. Studies with FDG-PET have demonstrated that a number of brain regions are associated with a decrease in glucose metabolism. But perhaps more importantly, initial results from resting state fMRI revealed abnormalities in functional connectivity between fronto-limbic areas associated with emotional and cognitive control.

Preliminary data suggest that lithium and, in a much less extent, valproate may help stabilizing mitochondrial function. There is also new data suggesting that the antioxidant agent NAC may be a useful adjunctive option in the treatment of BD. Whether or not the effects on mitochondrial function and oxidative stress are associated with their clinical response it remains to be determined. In the search for biomarkers of disease and treatment response, markers of energy metabolism and oxidative damage are promising candidates to understand the underlying neurobiology of BD. Ultimately, research in this field may help in the development of new treatment agents for this devastating major mental illness.

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