UNIVERSIDADE FEDERAL DE SANTA MARIA
CENTRO DE CIÊNCIAS RURAIS
PROGRAMA DE PÓS-GRADUAÇÃO EM MEDICINA VETERINÁRIA

17-β ESTRADIOL E RESVERATROL NOS PARÂMETROS BIOQUÍMICOS E HEMATOLÓGICOS DE RATAS OVARIECTOMIZADAS SUBMETIDAS A DESMIELINIZAÇÃO PELO BROMETO DE ETÍDIO

TESE DE DOUTORADO

Danieli Brolo Martins

Santa Maria, RS, Brasil

2012

17-β ESTRADIOL E RESVERATROL NOS PARÂMETROS BIOQUÍMICOS E HEMATOLÓGICOS DE RATAS OVARIECTOMIZADAS SUBMETIDAS A DESMIELINIZAÇÃO PELO BROMETO DE ETÍDIO

por

Danieli Brolo Martins

Tese apresentada ao Curso de Doutorado do Programa de Pós-graduação em Medicina Veterinária, Área de Concentração em Cirurgia Veterinária, Sub-área de Patologia Clínica Veterinária, da Universidade Federal de Santa Maria (UFSM, RS), como requisito parcial para obtenção do grau de **Doutor em Medicina Veterinária**

Orientadora: Profa. Dra. Sonia Terezinha dos Anjos Lopes

Co-orientadora: Cinthia Melazzo Mazzanti

Santa Maria, RS, Brasil

2012

Universidade Federal de Santa Maria
Centro de Ciências Rurais
Programa de Pós-Graduação em Medicina Veterinária

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

17-β ESTRADIOL E RESVERATROL NOS PARÂMETROS BIOQUÍMICOS E HEMATOLÓGICOS DE RATAS OVARIECTOMIZADAS SUBMETIDAS A DESMIELINIZAÇÃO PELO BROMETO DE ETÍDIO

elaborada por
Danieli Brolo Martins

como requisito parcial para obtenção do grau de
Doutor em Medicina Veterinária

COMISSÃO EXAMINADORA:

_____
Sonia Terezinha dos Anjos Lopes, Dra.
(Presidente/ Orientadora)

_____
Cristina W. Nogueira, Dra. (UFSM)

_____
Margarete D. Bagatini, Dra. (UFFS)

_____
Maria Rosa C. Schetinger, Dra. (UFSM)

_____
Roselia M. Spanevello, Dra. (UFPEL)

Santa Maria, 05 de março de 2012.

Dedico este trabalho à minha família.

AGRADECIMENTOS

A Deus, por ter me dado esta vida.

À minha família, que sempre me orientou pelo melhor caminho e soube compreender minha ausência em prol dos estudos. Obrigada pelo apoio, incentivo, enfim, por tudo!

Ao Demetrius, por ser a pessoa mais paciente que conheço. Obrigada por todos estes anos de carinho, compreensão, e muitas viagens longas! Saiba que esta conquista também é sua...

À pessoa que simplesmente transformou um sonho em realidade... de uma palestra sobre hematologia assistida no quarto semestre da faculdade, essa pessoa deu a oportunidade de uma graduanda ser uma estagiária de ACG, depois monitora de Patologia Clínica, após bolsista de iniciação científica, depois mestranda, doutoranda... Obrigada pela oportunidade, confiança...por tudo, professora Sonia!!! Com certeza, palavras de agradecimento são pouco, perto da profunda gratidão que sinto pela senhora nesses 10 anos que passamos juntas.

À amiga e co-orientadora Cinthia, uma pessoa admirável que transmite em todos os seus atos uma extrema responsabilidade e determinação. Obrigada por me auxiliar nas pesquisas desde a época do mestrado. Você e a profa. Sonia contribuíram para que este momento se tornasse realidade!

Aos amigos que conquistei no LacVet-UFSM. Em especial, a Dona Deva, a Rosangêla, a Dani Rodrigues, aos residentes e a tantos outros.... Aos estagiários, em especial aos meus bolsistas do doutorado, Estevan e Thais, pessoas muito esforçadas e queridas. Também quero agradecer muito aos

pós-graduandos Raqueli, Márcio, Roberto, Marci, Helo..... Obrigada pela amizade e companherismo!

À amiga Vera, por ter me ajudado em casa todos esses anos e por ter um carinho muito especial pela Eva!

Ao pessoal da Bioquímica Toxicológica, em especial à amiga Roberta Schmatz, e as professoras Rosinha e Vera por permitirem o uso do laboratório.

À equipe do LACE, especialmente ao professor Mazzanti, Diego e Nelci.

Também quero agradecer ao Silvandro e ao Elton pelo uso do biotério.

Aos professores, funcionários e alunos do Centro de Ciências da Saúde da UNICRUZ, por terem compreendido minha correria semanal entre Santa Maria e Cruz Alta!

Aos funcionários e professores do Hospital Veterinário – UFSM, especialmente à professora Dominguita Graça e ao prof. Marcelo Cecim, e ainda ao PPGMV, principalmente à Maria.

Aos meus amigos de quatro patas de Santiago, Burka, Bandy, Zuka, Gatones e Dedé. E como não podia faltar a minha amigona de apê, a Bichon mais linda do mundo, a Eva, companheira de cursinho, graduação, especialização, mestrado e doutorado... Te amo pequetita!

Finalmente, quero agradecer a Universidade Federal de Santa Maria, por ter contribuído significativamente para minha formação profissional.

À CAPES pela bolsa concedida.

“*A vida é uma peça de teatro que não permite ensaios.*

Por isso, cante, chore, dance, ria e viva intensamente,

antes que a cortina se feche e a peça termine sem aplausos.”

Charles Chaplin

RESUMO

Tese de Doutorado
Programa de Pós-graduação em Medicina Veterinária
Universidade Federal de Santa Maria

17-β ESTRADIOL E RESVERATROL NOS PARÂMETROS BIOQUÍMICOS E HEMATOLÓGICOS DE RATAS OVARIECTOMIZADAS SUBMETIDAS A DESMIELINIZAÇÃO PELO BROMETO DE ETÍDIO

AUTORA: Danieli Brolo Martins
ORIENTADORA: Profa. Dra. Sonia Terezinha dos Anjos Lopes
CO-ORIENTADORA: Profa. Dra. Cinthia Melazzo Mazzanti
Data e Local da Defesa: Santa Maria, 05 de março de 2012.

Dentre as doenças neurodegenerativas, as que causam desmielinização do sistema nervoso central tem grande importância, como a esclerose múltipla (EM) que acomete adultos jovens e de meia-idade. Neste sentido, o 17-β estradiol e o resveratrol surgem como substâncias promissoras devido as suas ações antioxidante, antiinflamatória e neuroprotetora. Assim, o objetivo do presente estudo foi verificar o efeito do 17-β estradiol e do resveratrol nos parâmetros bioquímicos e hematológicos em ratas ovariectomizadas submetidas ao modelo de desmielinização pelo brometo de etídio (BE). No primeiro artigo, os animais foram separados em 3 grupos experimentais para cada idade. Os grupos consistiram de controles adultos (Sham-A) e de meia-idade, ratas ovariectomizadas adultas e de meia-idade e ratas ovariectomizadas com reposição por 30 dias com 17-β estradiol adultas e de meia-idade. Após este período, a atividade da AChE foi mensurada no encéfalo,sangue total e linfócitos, e a peroxidação lipídica foi mensurado apenas no encéfalo. Os resultados obtidos demonstraram que a atividade da acetilcolinesterase (AChE) aumentou no estriado (ST) de ratas adultas ovariectomizadas sem e com reposição estrogênica, e de meia-idade sem reposição. No hipocampo (HP) também foi observado um aumento na atividade desta enzima apenas nas ratas de meia idade sem reposição do hormônio. A inibição na atividade da AChE ocorreu no ST e no córtex cerebral (CC) de ratas adultas com reposição estrogênica e nas ratas de meia-idade com e sem reposição hormonal. A atividade da AChE aumentou no sangue total de ratas adultas com reposição estrogênica e diminuiu nas ratas de meia-idade sem 17-β estradiol. Nos linfócitos foi observado um aumento na atividade da AChE de ratas adultas com ou sem reposição de estrógeno, e inibida no grupo de meia-idade sem reposição estrogênica.Houve um aumento na peroxidação lipídica em todas as estrituras encefálicas dos animais adultos ovariectomizados e córtex cerebral e cerebelo dos animais de meia-idade ovariectomizados.. Nos manuscritos, os animais foram divididos em 5 grupos para avaliar a fase de desmielinização e outros 5 grupos para avaliar a fase de remielinização. Em cada fase, os grupos consistiram de ratas controle; ratas ovariectomizadas; ratas ovariectomizadas e desmielinizadas; ratas ovariectomizadas tratadas com 17-β estradiol (maunscrito 1) ou resveratrol (manuscrito 2); e ratas ovariectomizadas e desmielinizadas tratadas com 17-β estradiol (manuscrito 1) ou resveratrol (manuscrito 2). No manuscrito 1, investigou-se os efeitos do 17-β estradiol sobre os parâmetros relacionados a atividade da AChE encefálica, do sangue total e dos linfócitos, e atividade da butirilcolinesterase (BuChE) por um período de 7 ou 21 dias. Quando a ovariectomia foi associada a desmielinização pelo BE foi observado um aumento na atividade da AChE em todas as estruturas estudadas, na desmielinização e na remielinização A suplementação estrogênica estabilizou a atividade da AChE na desmielinização e inibiu a atividade da enzima no CC, ST e cerebelo (CE) na remielinização, além de conseguir prevenir o prejuízo cognitivo induzido pela ovariectomia. A atividade da butirilcolinesterase (BuChE) aumentou na fase de desmielinização e esteve inibida na fase de remielinização nas ratas tratadas com 17-β estradiol. Já a AChE sanguínea apresentou elevação de sua atividade nas ratas desmielinizadas, não-desmielinizadas com reposição estrogênica e desmielinizadas com reposição estrogênica em ambas as fases. A atividade da AChE dos linfócitos aumentou nas ratas desmielinizadas, enquanto nos demais grupos houve uma inibição na atividade desta enzima na fase de desmielinização. No manuscrito 2, avaliou-

se o efeito do resveratrol no hemograma e na atividade da AChE de linfócitos. Apenas o uso do resveratrol por 7 dias diminuiu a atividade da AChE nos linfócitos de ratas submetidas a ovariectomia e nas ratas com ovariectomia e desmielinização, sem alterar o número dos linfócitos na circulação. Assim, os resultados obtidos neste estudo demonstraram que o 17-β estradiol e o resveratrol modulam a atividade da AChE neuronal e não-neuronal. Os efeitos da terapia estrogênica são dependentes da idade e da estrutura encefálica a ser analisada. Além disso, a queda estrogênica é prejudicial ao sistema colinérgico. Os efeitos danosos da ovariectomia podem ser potencializados na presença de desmielinização, sendo estes em grande parte revertidos com o uso do 17-β estradiol. Assim, este estudo colabora para uma melhor compreensão do uso da terapia de reposição estrogênica e terapias alternativas, como o resveratrol, em condições menopáusicas e, principalmente, em doenças neurodegenerativas que cursem com o processo de desmielinização, como a EM. Se ratificados estes resultados na espécie humana, estes compostos poderão ser considerados novas estratégias terapêuticas para as mulheres.

Palavras-chave: Terapia estrogênica. Fitoestrógeno. Colinesterases. Estresse oxidativo. Ovariectomia.

ABSTRACT

Doctoral Thesis
Postgraduate Program in Veterinary Medicine
Universidade Federal de Santa Maria

17-β ESTRADIOL AND RESVERATROL IN BIOCHEMISTRY AND HEMATOLOGY OF OVARIECTOMIZED RATS SUBMITTED TO DEMYELINATION BY ETHIDIUM BROMIDE

AUTHOR: Danieli Brolo Martins
ADVISER: Prof. Dr. Sonia Terezinha dos Anjos Lopes
CO-ADVISER: Prof. Dr. Cinthia Melazzo Mazzanti
Place and Date of Defense: Santa Maria, March 5th, 2012.

Among the neurodegenerative diseases those involving the demyelination of the nervous system, such as multiple sclerosis (MS) that affects young and middle-aged adults, have great importance. Thus, the goal of this study was to evaluate 17-β estradiol and resveratrol as promising substances due to their antioxidant and anti-inflammatory actions, in the neuronal and non-neuronal cholinergic system, hematologic and behavioral parameters in ovariectomized rats under or not the demyelination by ethidium bromide (EB). In the article, animals were randomly assigned into three experimental groups of each age. Control groups consisted of adult (sham-A) and middle-aged (sham-MA) female rats, ovariectomized adult (OVX-A) and middle-aged (OVX-MA) rats without estrogen therapy reposition, and ovariectomized adult (OVX+E2-A) and middle-aged (OVX+E2-MA) rats treated with 17-β estradiol for 30 days. After this period, AChE activity and lipid peroxidation were measured in the brain, blood and lymphocytes.The results showed that the acetylcholinesterase (AChE) activity increased in the striatum (ST) of adult ovariectomized animals with and without estrogen replacement and middle-aged animals without replacement, as well as in the hippocampus (HP) of middle-aged animals without hormone replacement. The inhibition of the AChE activity occurred in the ST of adult animals with estrogen replacement, in the cerebral cortex (CC) of adult animals with estrogen replacement, as well as in middle-aged animals with and without hormone replacement. The AChE activity of whole blood increased in adult animals with estrogen supplementation and decreased in middle-aged animals without 17-β estradiol. The AChE activity of lymphocytes was stimulated in adult animals with or without estrogen replacement, and it was inhibited in the middle-aged animals without estrogen replacement. There was an increase in lipid peroxidation in brain structures of all adult ovariectomized animals and cerebellum and cerebral cortex of middle-aged ovariectomized animals. In the manuscripts, the animals were divided into 5 groups to evaluate the demyelination phase and 5 groups to evaluate the remyelination phase. In each phase the groups were: control sham rats; ovariectomized rats, not demyelinated; demyelinated ovariectomized rats; ovariectomized rats, not demyelinated, treated with 17-β estradiol; and demyelinated ovariectomized rats treated with 17-β estradiol. In the manuscript 1, we investigate the effects of 17-β estradiol supplementation under the parameters related to the AChE activity in the brain, total blood and lymphocytes, as well as serum butyrylcholinesterase (BuChE) activity for a period of 7 or 21 days.Ovariectomy associated with EB increased the AChE activity in all structures in demyelination and remyelination phases. Estrogen supplementation stabilized the AChE activity in demyelination and inhibited the enzyme activity in the CC, ST and cerebellum (CE) in the remyelination, besides preventing cognitive impairment induced by ovariectomy. The BuChE activity showed elevation in demyelination and inhibition in remyelination in demyelinated animals treated with 17-β estradiol. The blood AChE activity showed an increase in demyelinated, non-demyelinated with estrogen replacement and demyelinated with estrogen replacement animals in both phases. In the demyelination phase, the AChE activity of lymphocytes showed an increase in the demyelinated group, while other groups have shown inhibition of this enzyme activity. In the manuscript 2, we evaluated the effect of resveratrol on

The complete blood count and AChE activity of lymphocytes. The use of resveratrol for seven days decreased the AChE activity in ovariectomized animals and animals with ovariectomy and demyelination, without changing the number of circulating cells, demonstrating that there is no correlation between the circulating lymphocytes and the AChE activity of these cells. Taken together, these results show that 17-β estradiol and resveratrol modulate the AChE activity. The effects of estrogen therapy are dependent on the age and brain structure to be analyzed. Low levels of estrogen are detrimental to the cholinergic system. The damaging effects of ovariectomy can be potentiated in the presence of demyelination, which are generally reversed by the use of 17-β estradiol. This study therefore contributes to a better understanding for the use of estrogen replacement therapy and alternative therapies, such as resveratrol, in menopausal conditions and, especially, neurodegenerative diseases, such as MS, which pursue the process of demyelination. If these results are ratified in humans, these substances can be considered new therapeutic strategies for women.

Keywords: Estrogen. Phytoestrogen. Cholinesterases. Oxidative stress. Ovariectomy.

LISTA DE ILUSTRAÇÕES

INTRODUÇÃO

ARTIGO

MANUSCRITO 1

MANUSCRITO 2

LISTA DE TABELAS

ARTIGO

MANUSCRITO 2

LISTA DE ABREVIATURAS

AChE – acetilcolinesterase

ACh - acetilcolina

ADP – adenosina 5’ difosfato

ATP - adenosina 5’ trifosfato

BuChE – butirilcolinesterase

BE – brometo de etídio

CHT – transportador de colina

ChAT - Colina acetil-transferase

EM – esclerose múltipla

GFAP – proteína ácida fibrilar glial

SNC – sistema nervoso central

SNP – sistema nervoso periférico

VAChT – transportador de acetilcolina vesicular

SUMÁRIO

APRESENTAÇÃO

Esta tese apresenta os resultados encontrados durante a realização do doutorado em Medicina Veterinária do Programa de Pós-graduação em Medicina Veterinária da Universidade Federal de Santa Maria. Inicialmente, é apresentada uma breve introdução sobre os temas a serem abordados. Logo após, os achados das pesquisas foram organizados na forma de artigo e manuscritos. Os itens Materiais e Métodos, Resultados, Discussão e Referências Bibliográficas, encontram-se no próprio artigo e nos manuscritos e representam a íntegra deste estudo. Os trabalhos estão estruturados de acordo com as normas das revistas científicas para as quais foram submetidos.

A parte final da tese apresenta a Discussão e a Importância do Trabalho e Perspectivas obtidas a partir da análise dos resultados como um todo. As Conclusões são apresentadas de acordo com cada artigo/manuscrito, bem como, suas correlações. Na sequência, as Referências Bibliográficas referem-se somente às citações que aparecem nos itens Introdução e Discussão.

1. INTRODUÇÃO

Dado a importância do encéfalo como um órgão alvo para o estrógeno, não é de se estranhar que muitas das queixas das mulheres que procuram tratamento relacionado a menopausa sejam de origem neurológica. Nesta perspectiva, a neurociência básica tem muito a contribuir para as questões clínicas em torno da reposição estrogênica, menopausa e envelhecimento (MORRISON et al., 2006).

As doenças neurológicas, ou que envolvem o sistema nervoso central (SNC), constituem a maior causa de morbidade e mortalidade em todo o mundo (ANDERSEN, 2004). Muitos indivíduos acometidos por doenças neurodegenerativas apresentam deficiências cognitivas, principalmente com prejuízo na memória, atenção e processamento de informações, reduzindo desta maneira, a qualidade de suas vidas (McCABE et al., 2009).

Nesse contexto, as doenças desmielinizantes resultam de uma grave consequência da destruição das bainhas de mielina presentes no SNC e no sistema nervoso periférico (SNP) (SIEGEL, 1999). Desta forma, na desmielinização, há a perda da bainha de mielina, mas com a preservação dos axônios. Tais alterações prejudicam a transmissão do impulso nervoso, levando ao aparecimento de sinais neurológicos (LOVE, 2006).

O modelo de desmielinização pelo brometo de etídio (BE) mimetiza mudanças enzimáticas e celulares que podem ocorrer em doenças que promovam a destruição das bainhas de mielina dos neurônios, como a esclerose múltipla (EM) em humanos (LEVINE & REYNOLDS, 1999; FUSHIMI

& SHIRABE, 2004; MAZZANTI et al., 2009; NASSAR et al., 2009) e a cinomose em caninos (ORSINI et al., 2007).

O BE, uma substância intercalante gliotóxica, tem sido usado em vários estudos a fim de induzir, experimentalmente, desmielinização focal no SNC, com posterior remielinização (GRAÇA et al., 2001; BONDAN et al., 2006; MAZZANTI et al., 2009; NASSAR et al., 2009; RAMOS et al., 2009). Tal fato permite o estudo da capacidade regenerativa desse órgão (SALLIS et al., 2006).

Nesse modelo, observa-se o desaparecimento oligodendroglial e astrocitário, com consequente perda primária das bainhas de mielina, além da ruptura da membrana limitante glial e da barreira hematoencefálica. A ausência dos processos astrocitários em muitas áreas de lesão induzida pelo BE permite a entrada de linfócitos, de células meníngeas infiltrantes e de células de Schwann, as últimas contribuindo para o reparo mielínico central (BONDAN, 1997; BONDAN et al., 2006; SALLIS et al., 2006).

O mecanismo de ação do BE consiste em alterar o DNA mitocondrial levando a uma respiração anormal da célula com consequente necrose (BAUMANN & PHAM-DINH, 2001). Essa substância tem sido empregada em diversos locais para a avaliação dos eventos relacionados à desmielinização e remielinização, tais como o tronco encefálico (GRAÇA et al., 2001; BONDAN et al., 2002; BONDAN et al., 2006; SALLIS et al., 2006; MAZZANTI et al., 2009; NASSAR et al., 2009), o nervo ciático (RIET-CORREA et al., 2002; RAMOS et al., 2009), a medula espinhal (FUSHIMI & SHIRABE, 2002; FUSHIMI & SHIRABE, 2004) e o nervo óptico (GUAZZO, 2005).

As análises histológicas têm demonstrado que a injeção intracisternal de BE causa mudanças degenerativas nos oligodendrócitos e astrócitos após 48-72 horas da indução (YAJIMA et al., 1979; BONDAN, 1997; BONDAN et al., 2002), apresentando infiltração de macrófagos e ausência de células GFAP (proteína ácida fibrilar glial) positivas (MAZZANTI et al., 2009).

O processo de desmielinização está completo em torno do sexto ao décimo dia pós contato com o BE (YAJIMA et al., 1979; LEVINE & REYNOLDS, 1999; BONDAN et al., 2002). Por outro lado, os primeiros sinais de remielinização surgem por volta dos 12 aos 15 dias pós-intoxicação pelo aparecimento de oligodendrócitos (BONDAN, 1997; LEVINE & REYNOLDS, 1999; GRAÇA et al., 2001; BONDAN et al., 2002; SALLIS, 2005) e aos 21 dias após a injeção do BE, a remielinização encontra-se em um estágio bastante avançado (SALLIS et al., 2006), demonstrando algumas áreas císticas e processos astrocitários reativos (MAZZANTI et al., 2009). Há ainda, relatos que indicam que o processo de remielinização pode ultrapassar os 35 dias (BONDAN et al., 2006) ou até mais que 40 dias (LEVINE & REYNOLDS, 1999).

Assim, o modelo do BE consiste em uma ferramenta importante para a compreensão dos mecanismos patogênicos de muitas doenças desmielinizantes que, atualmente, são pouco compreendidos (MAZZANTI, 2007; MAZZANTI et al., 2009; NASSAR et al., 2009).

Dentre as desordens neurodegenerativas de caráter desmielinizante, a de maior destaque é a esclerose múltipla (EM) (LOVE, 2006). A EM é uma doença desmielinizante crônica e inflamatória, que comumente acomete mulheres adultas jovens do ocidente (KIPP et al., 2009). No Brasil, a doença tem de baixa a média incidência, acometendo principalmente mulheres

brancas, com média de idade de 40,2 anos de idade (intervalo de 19 a 57 anos), e média de idade do primeiro surto em torno de 33,8 anos (GRZESIUK, 2006).

A maior prevalência de mulheres para a doença leva a suspeitas que fatores hormonais possam estar envolvidos, já que seu aparecimento ocorre após a maturidade sexual (BEBO et al., 2001). Soma-se a isso, a redução no número de surtos após o aumento no nível dos hormônios sexuais durante a gravidez, e a seguir um aumento na severidade da doença no período pós-parto, onde há redução destes hormônios, além de diminuição nos graus da doença quando comparado multíparas à nulíparas (DWOSH et al., 2003; VOSKUHL, 2003; KAAJA & GREER, 2005). Desta maneira, tem-se sugerido nos últimos anos que os hormônios sexuais possam ser utilizados como terapia efetiva para a EM (Figura 1) (SICOTTE et al., 2002; KAAJA & GREER, 2005; ACS et al., 2009).

Diversos hormônios sexuais são capazes de modular a atividade do SNC, sendo chamados de esteroides neuroativos (FALKENSTEIN et al., 2000). Nas mulheres, citam-se como exemplos, o estradiol, a progesterona e a testosterona, que possuem efeitos modulatórios na produção de múltiplos neurotransmissores como a acetilcolina (ACh), o glutamato e a dopamina (ZHENG, 2009).

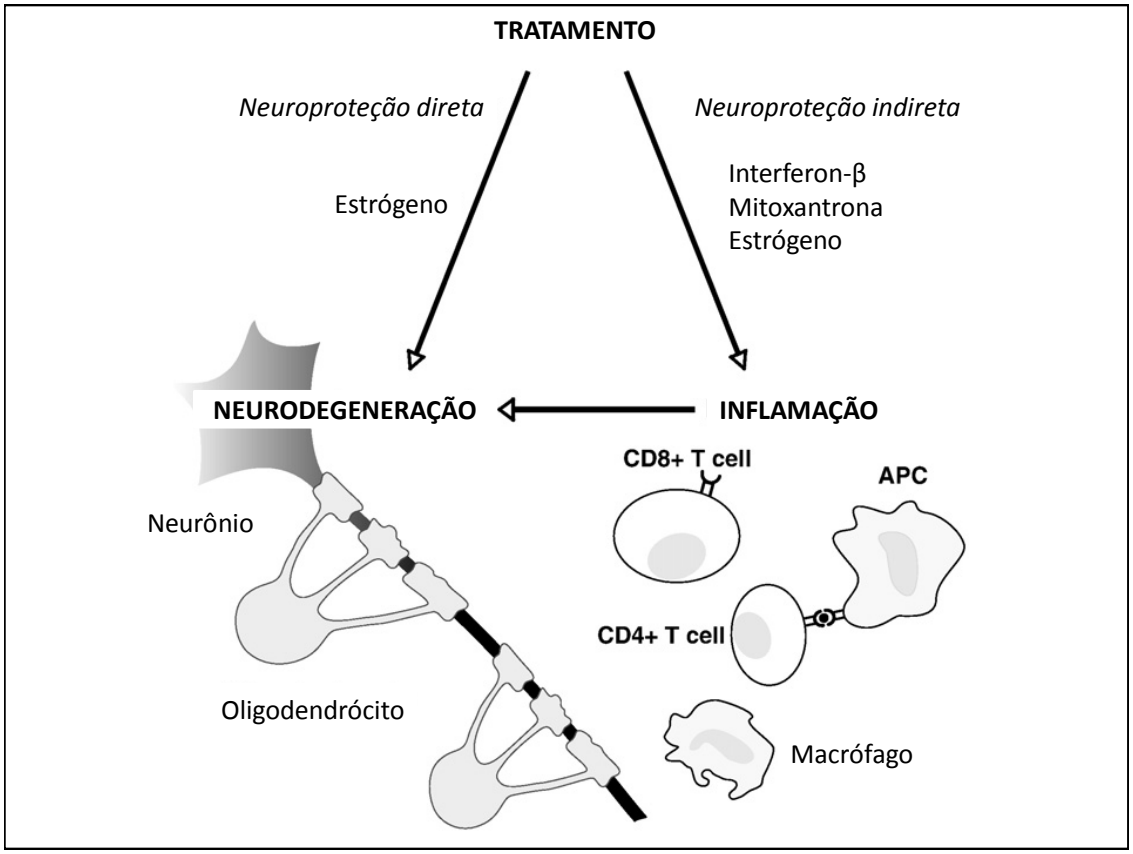


Figura 1 – Neuroproteção direta e indireta na esclerose múltipla (EM). Drogas aprovadas para o tratamento do surto-remissão da EM (interferon-β e mitoxantrona) agem no sistema imune conferindo propriedades antiinflamatórias. Estudos utilizando sistemas *in vitro* e modelos *ex vivo* indicam que o tratamento estrogênico tem o potencial de ser anti-inflamatório e diretamente neuroprotetor (Adaptado e modificado de GOLD & VOSKUHL, 2009).

Vários tecidos do corpo possuem diferentes sensibilidades ao 17-β estradiol (Figura 2) (YASUI et al., 2001). A privação hormonal deste esteroide pode interferir com o curso de várias neuropatias (VALENTE et al., 2002; SICOTTE et al., 2002; PEREIRA JUNIOR et al., 2009).

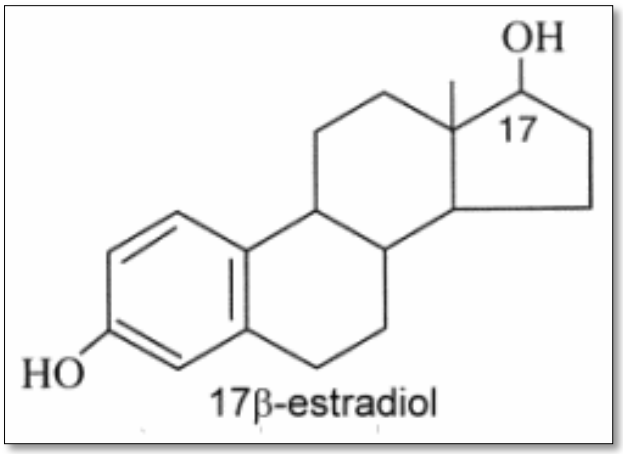


Figura 2 – Estrutura molecular do 17-β estradiol (www.cyberlipid.org/simple/simple0008.htm)

A administração sistêmica desse hormônio previne, de forma tempo e dose dependente, a ativação da neuroinflamação que pode ocorrer pela injeção intraventricular experimental de lipopolissacarídeos no encéfalo de cobaias (VEGETO et al., 2003). Contudo, a literatura é conflitante no aspecto de quando iniciar a terapia estrogênica, alguns autores apoiam o uso após o dano neurológico instalado (ACS et al., 2009) enquanto há os que discordam de sua eficácia neste momento (MARDER & SANO, 2000), defendendo seu uso de forma preventiva (HEINZ et al., 2006).

A reposição hormonal também tem sido relatada como benéfica frente a diversas reações biológicas do organismo, como a manutenção ou o aumento na capacidade antioxidante e a redução nos níveis de estresse oxidativo (AGUIAR et al., 2008), melhora nos padrões bioquímicos séricos (SCHMIDT et al., 2004; AGUIAR et al., 2008), na densidade mineral óssea (YASUI et al., 2001; SCHMIDT et al., 2006), e neuroproteção (ACS et al., 2009). Além disso, exerce função modulatória em diversos sistemas, além do nervoso, como o sistema imune e o cardíaco (CRAFT, 2007).

Um possível fator que pode explicar o potencial neuroprotetor do estrógeno contra algumas doenças do sistema nervoso é através do aumento ou da preservação da neurotransmissão colinérgica (NORBURI et al., 2003). Contudo, os mecanismos através dos quais esse composto exerce seu efeito neuroprotetor ainda não estão bem estabelecidos, mas acredita-se que suas propriedades antioxidantes possam contribuir para suas ações benéficas no SNC (SAIKO et al., 2008; FAROOQUI & FAROOQUI, 2009). Todavia, uma desvantagem farmacológica para o efeito antioxidante desse hormônio consiste em sua passagem limitada pela barreira hemato-encefálica (GILGUN-SHERKI et al., 2001).

Já os efeitos da queda desse hormônio, obtidos através da ovariectomia em ratas, são caracterizados por perda progressiva da memória, degeneração do sistema nervoso colinérgico central e desequilíbrio apoptótico (SATO et al., 2003). Por isso, é necessário que se conheça os efeitos da terapia por estrógenos (YASUI et al., 2001), já que pouco se sabe sobre os mecanismos moleculares envolvidos nas ações benéficas deste esteroide (MONTEIRO, 2007) nas doenças desmielinilizantes que acometem a população feminina.

Com o passar dos anos, o encéfalo normal sofre mudanças na sua estrutura, função e metabolismo (MOORTHY et al. 2005; THAKUR e SHARMA 2006). No entanto, a depleção estrogênica culmina em uma aceleração do processo de envelhecimento nas funções nervosa e imune (BAEZA et al. 2010). O 17-β estradiol influencia neurobiologia do envelhecimento, já que a senescência endócrina e neuronal se sobrepõe no tempo e são mecanicamente interligadas em ciclos de feedbacks complexos. Assim, com o envelhecimento, todas as mulheres irão experimentar uma queda dramática

nos estrógenos circulantes caso alcancem o período de transição para a menopausa (MORRISON et al., 2006). No entanto, nem todas as mulheres podem fazer uso da terapia de reposição estrogênica devido aos seus efeitos colaterais ou a presença de tumores responsivos ao estrógeno. Neste aspecto, é necessário formas alternativas para suprir a carência das ações benéficas deste hormônio no corpo (MONTEIRO, 2007; SAKAMOTO et al., 2010).

Os fitoestrógenos são substâncias usadas no tratamento complementar da mulher na pós-menopausa (TEMPFER et al., 2009). Um exemplo bastante conhecido, é o resveratrol, um fitoestrógeno atóxico, do grupo das fitoalexinas, que tem funções anti-oxidante e anti-inflamatória promissoras (LABINSKYY et al., 2006). O resveratrol faz parte de um amplo grupo de componentes derivados de plantas que estruturalmente ou funcionalmente imitam os estrógenos dos mamíferos, mostrando assim, potenciais benefícios para a saúde (OSOSKI & KENNELLY, 2003).

O resveratrol (3,5,4'-trihydroxy-trans-stilbene) é um polifenol encontrado em uvas (50-100mg/g), vinho tinto (2-7 mg/L) (LI et al., 2008; MARQUES et al., 2009) e suco de uva (0,19 a 0,90 mg/L) (SAUTTER et al., 2005), entre outras plantas. Este composto possui duas formas isômeras: trans e cis. O isômero trans-resveratrol é convertido para cis-resveratrol em presença da luz visível, pois esta forma é mais estável, entretanto, com perda na função biológica (Figura 3) (SAUTTER et al., 2005; BAUR & SINCLAIR, 2006).

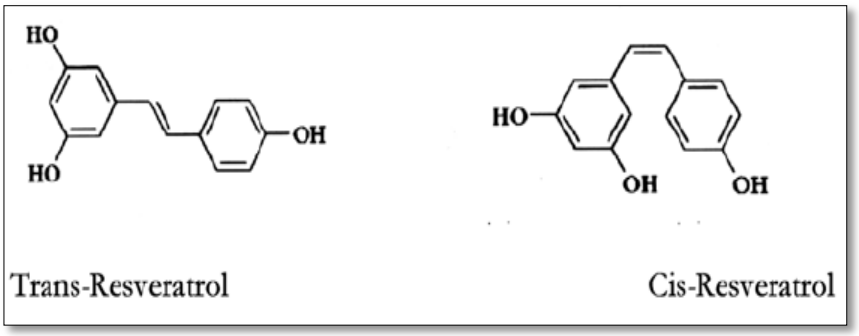


Figura 3 – Formas moleculares trans e cis do resveratrol (supplementscience.org/resveratrol.html)

Apesar do uso de fitoestrógenos ser uma alternativa para terapia de reposição hormonal, já que esta última pode apresentar efeitos colaterais graves como o câncer de mama (ELLIS et al., 2009) e distúrbios hemostáticos (GALLO et al., 2005), a literatura atual ainda é conflitante sobre seu uso (TEMPFER et al., 2009; SAKAMOTO et al., 2010).

O mecanismo primário pelo quais os fitoestrógenos influenciam os sistemas responsivos ao estrógeno dá-se através da ligação aos receptores estrogênicos (ER) α e β. Um exemplo disso é o receptor ERβ que tem um nível mais alto de expressão do que o ERα no hipocampo e córtex do cérebro adulto (SHUGHRUE et al., 1998; ZHANG et al., 2002), regiões cerebrais relacionadas a memória e aos processos cognitivos, vulneráveis a EM (McCABE et al., 2009). A afinidade relativa destas substâncias aos receptores de estrógeno é cerca de mil a sete mil vezes mais baixa se comparada ao estradiol (BOWERS et al., 2000; ALBERTAZZI & PURDIE, 2002). Por isso, o tratamento sistêmico com fitoestrógenos pode exercer um discreto efeito agonista estrogênico em diversos tecidos do organismo. Contudo, pouco se conhece se esse tratamento alternativo é capaz de exercer danos adversos nestes locais (TEMPFER et al., 2009).

Os principais órgãos alvos dos fitoestrógenos são o fígado, os rins, o coração, os ovários, os pulmões e o encéfalo (VITRAC et al., 2005). O resveratrol protege o organismo de diversos processos como o dano isquêmico, supressão da agregação plaquetária, propriedades anti-ateroescleróticas, além da inibição da oxidação das lipoproteínas de baixo peso molecular, redução da inflamação, melhora na função endotelial, renal (HUNTLEY, 2007; SAIKO et al., 2008), e cognitiva, além de amenizar os sinais clínicos motores e de perda da memória (FAROOQUI & FAROOQUI, 2009). Por ser considerado um fraco agonista estrogênico (MUELLER et al., 2004), o resveratrol também é recomendado à mulheres menopáusicas (HUNTLEY, 2007).

De forma semelhante à exercida pelo estrógeno, esta substância também é capaz de se ligar ao ERβ e ao ERα (HENRY & WITT, 2002), com afinidade similar para ambos os receptores *in vitro* (BOWERS et al., 2000). Todavia, é importante ressaltar que, quando utilizado em altas doses, o resveratrol pode tornar-se um antagonista dos receptores estrogênicos, agindo como um interruptor endócrino (MUELLER et al., 2004). Ainda, HENRY & WITT, (2002) verificaram que o resveratrol induziu alterações comportamentais em ratas não castradas, porém, não provocou o mesmo efeito nas ratas castradas.

A neurotoxicidade está frequentemente relacionada a disfunção mitocondrial, tanto na desmielinização espontânea quanto na desmielinização experimental pelo BE (BAUMANN & PHAM-DINH, 2001; SAIKO et al., 2008). Esta alteração causada na mitocôndria pode ser melhorada através da inclusão de modificadores metabólicos e/ou antioxidantes, como o resveratrol que pode

promover uma intervenção alternativa e precoce que auxiliaria na prevenção de maiores danos ao tecido nervoso (SAIKO et al., 2008). O resveratrol pode penetrar na barreira hemato-encefálica e exercer grandes efeitos neuroprotetores *ex vivo*, mesmo em doses baixas (0,1-1µg/kg) (BAUR & SINCLAIR, 2006). Este componente tem mostrado efeito neuroprotetor em diversos modelos experimentais de dano cerebral, observando-se uma importante recuperação neurológica, com redução das lesões nos neurônios após o tratamento com este composto (WANG et al., 2003; WANG et al.,2004; GAO et al., 2005).

Além disso, o resveratrol tem mostrado ser eficiente na prevenção de déficits de memória em modelos experimentais de Alzheimer e também no envelhecimento, sugerindo o envolvimento deste composto na neurotransmissão colinérgica (SAIKO et al., 2008; FAROOQUI & FAROOQUI, 2009). Por isso, pesquisas que enfoquem os efeitos deste fitoestrógeno, são necessárias, a fim de maximizar os benefícios do resveratrol no organismo (HUNTLEY, 2007). Neste sentido, tem-se atribuído uma boa ação do resveratrol (SCHMATZ et al., 2009) e do 17-β estradiol (FENG et al., 2004) sobre o sistema colinérgico, enquanto que a ovariectomia poderia prejudicar o correto funcionamento desse sistema (MONTEIRO et al., 2005; MONTEIRO et al., 2007).

O sistema colinérgico tem um papel fundamental em várias funções vitais, como o aprendizado, a memória e a organização cortical do movimento, o que faz desse sistema, alvo de inúmeras pesquisas (MESULAM et al., 2002), em especial em modelos de doenças inflamatórias neurodegenerativas, como é o caso da EM (MAZZANTI et al., 2007; MAZZANTI et al., 2009). As diferentes

regiões cerebrais envolvidas nas transmissões colinérgicas estão descritas na Figura 4.

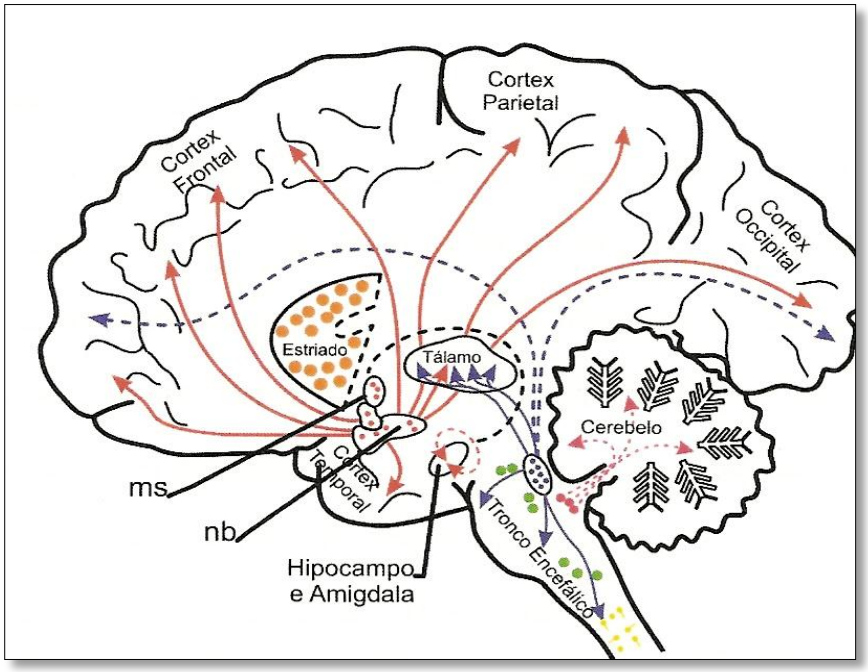


Figura 4 – Sistema colinérgico (encéfalo humano). As localizações dos principais grupos de corpos celulares e tratos de fibras colinérgicas são mostrados em vermelho. Os núcleos septo medial (ms) e núcleo basal (nb) estão em vermelho. O núcleo pedúnculo pontino apresenta-se em azul escuro e os interneurônios estriatais em laranja (Adaptado de PERRY et al. (1999), modificado por MAZZANTI, 2007).

A ACh (Figura 5) foi o primeiro composto a ser identificado como um neurotransmissor e passou a ser amplamente estudado nas sinapses do SNC e sistema nervoso periférico (SNP) (DESCARRIES et al., 1997). Sua síntese é realizada nos neurônios pré-sinápticos, pela colina-acetiltransferase (ChAT, E.C. 2.3.1.6), e armazenada em vesículas que liberam seu conteúdo por exocitose, após influxo de cálcio no terminal nervoso. Ao ser liberada, a ACh

interage com receptores específicos causando despolarização e propagação do potencial de ação na célula pós-sináptica. Sua rápida metabolização ocorre de forma enzimática (ODA, 1999). Os efeitos da ACh são mediados pela ativação de receptores nicotínicos e muscarínicos (DESCARRIES et al., 1997).

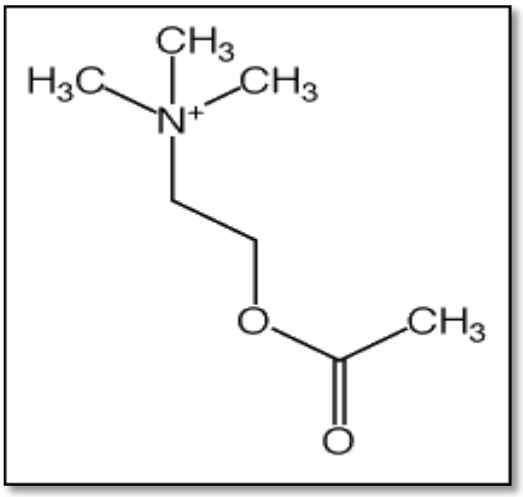


Figura 5 – Estrutura química da acetilcolina.Fonte: http://www.daviddarling.info/encyclopedia/A/acetylcholine.html

A ACh que permanece na fenda sináptica é hidrolisada por uma colinesterase específica em ácido acético e colina. Grande parte da colina resultante é captada pelo terminal do axônio colinérgico por um transportador de colina (CHT) e reutilizada na síntese de nova ACh (Figura 6) (MESULAM et al., 2002).

Existem dois tipos de colinesterases no sistema colinérgico: AChE (E.C. 3.1.1.7) ou colinesterase verdadeira, que hidrolisa preferencialmente ésteres com grupamento acetil (como a ACh) e a butirilcolinesterase ou pseudocolinesterase (BuChE; E.C. 3.1.8) que hidrolisa outros tipos de ésters

como a butirilcolina. A AChE é predominantemente encontrada no cérebro (10 vezes mais abundante que a BuChE), junção neuromuscular e eritrócitos (COKUGRAS, 2003). Já a BuCHE é principalmente encontrada no plasma, rins, fígado, intestino, coração, pulmão e tem uma distribuição neuronal muito mais restrita do que a AChE (MESULAM et al., 2002).

A AChE é uma serina hidrolase que hidrolisa rapidamente o neurotransmissor ACh tanto na sinapse colinérgica (Figura 6) quanto na junção neuromuscular, finalizando, deste modo, a transmissão do impulso nervoso (GRISARU et al., 1999). Além de seu papel clássico na transmissão colinérgica, a AChE tem um potente efeito na adesão celular (JOHNSON & MOORE, 1999), no crescimento dos neuritos (DAY & GREENFIELD, 2002), participa na regulação estrutural da diferenciação pós-sináptica, na osteogênese e também foi proposto a atividade hematopoética pela presença desta enzima em células progenitoras do sangue (SOREQ & SEIDMAN, 2001). A AChE já foi localizada e identificada nos linfócitos onde provavelmente representa um papel importante na regulação de funções imunes (KAWASHIMA & FUJII, 2000). Por todos esses fatores, uma inibição dessa enzima pode ter consequências devastadoras no cérebro e em outros órgãos (MESULAM et al., 2002).

No SNC a secreção de AChE tem sido encontrada no hipotálamo, substância nigra, estriado, hipocampo, cerebelo, além do fluido cerebroespinhal (PAXINOS, 1985). Essa enzima é secretada de ambos os terminais axonais dos neurônios, sendo esta taxa de secreção modulada pela estimulação neuronal, nível de neurotransmissor na fenda sináptica e pelo tratamento com fármacos (DESCARRIES, 1997).

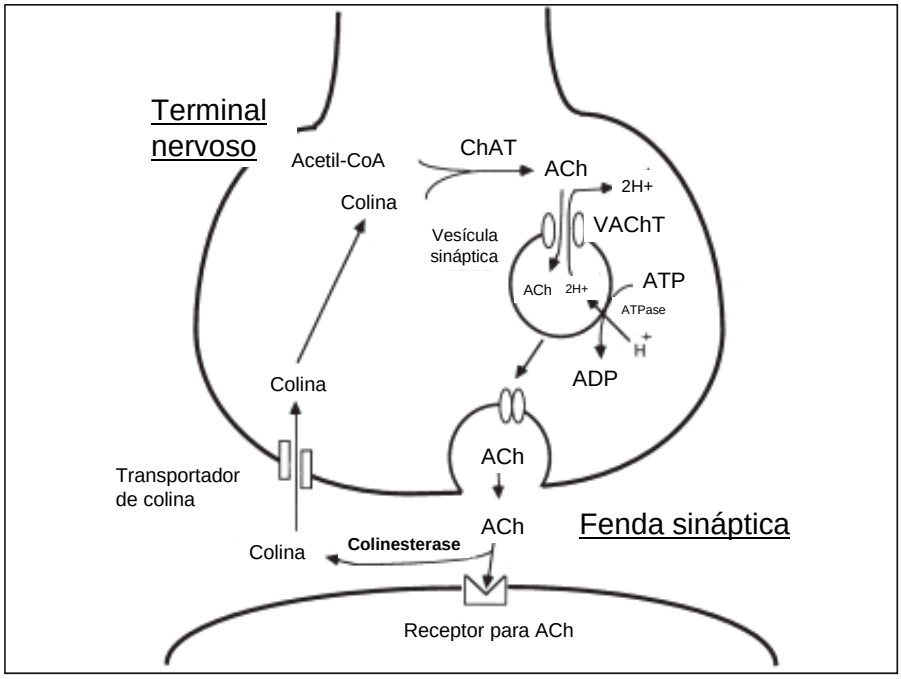


Figura 6 – Esquema da sinapse colinérgica. A acetilcolina (ACh) que está contida na vesícula sináptica do terminal nervoso é liberada na fenda sináptica por exocitose. Liga-se no neurônio pós-sináptico através de receptores específicos e é hidrolisada por colinesterases específicas, que a degradam em colina e acetil-CoA. O transportador de colina recolhe a colina resultante da reação que está livre na fenda sináptica e a leva novamente para o neurônio pré-sinático para sua reutilização (Adaptado de ODA, 1999).

Por ser uma das mais eficientes e conhecidas catálises biológicas, a AChE tem sido investigada como um importante alvo terapêutico em várias doenças neurodegenerativas (APPLEYEARD, 1994; DAS et al., 2001). Sabe-se que os estrógenos podem afetar a atividade da AChE no hipocampo, promovendo um estímulo a sua síntese, principalmente de suas formas globulares G_1, G_2 e G_4 (Figura 7) (PEREIRA et al., 2008).

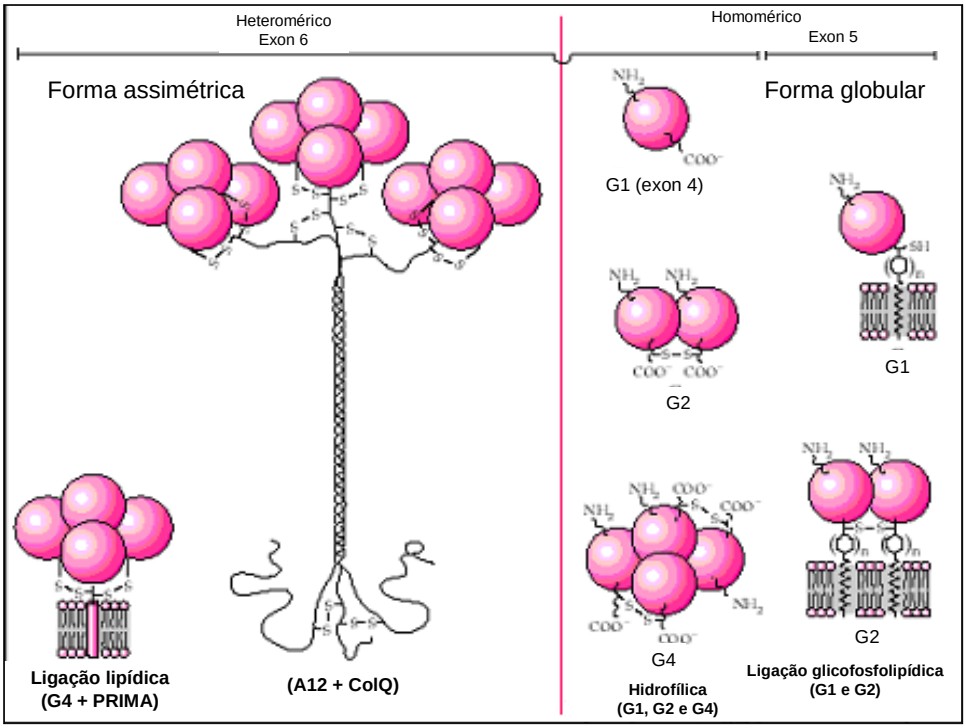


Figura 7 – Estruturas moleculares da acetilcolinesterase (AChE). Formas globulares e assimétricas (Adaptado de Feldman & Quenzer, 1984).

DAS et al., (2002) relataram que a ovariectomia por oito dias reduz a atividade da AChE hipocampal em ratas. Contudo, os autores não observaram mudança neste parâmetro após a terapia de reposição hormonal por estrógeno. Ainda FENG et al. (2004) não observaram mudanças na atividade desta enzima após 17 semanas de ovariectomia, ou mesmo após a reposição por 16 semanas com o 17-β estradiol. Assim, sugere-se que a duração e/ou o período crítico de início da terapia estrogênica devem ser considerados importantes para que haja influência na atividade da AChE cerebral (PEREIRA et al., 2008).

Já o resveratrol parece ser capaz de modular a neurotransmissão colinérgica e consequentemente, melhorar a cognição (SCHMATZ et al., 2009). Desta forma, substâncias que modulem a atividade da AChE podem

representar um alvo de pesquisa importante e promissor em patologias que envolvam eventos desmielinizantes e inflamatórios associados a disfunções cognitivas, como a EM (MAZZANTI, 2007; MAZZANTI et al., 2007; MAZZANTI et l., 2009).

Por sua vez, a BuChE é expressa por distintas populações de neurônios, sendo considerada um co-regulador da neurotransmissão colinérgica (DARVESH et al., 1998). É provável que esta enzima seja estruturalmente similar a AChE (Figura 8) (DARVESH et al., 2003) e esteja envolvida em alguns aspectos do desenvolvimento do sistema nervoso (KOSTOVIC & GOLDMAN-RAKIC, 1983).

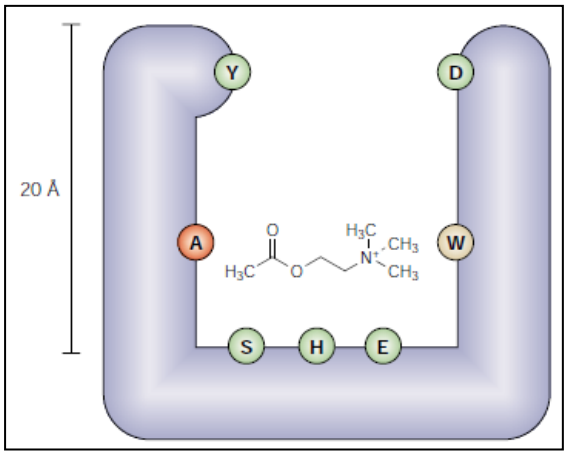


Figura 8 – Sítio ativo da butirilcolinesterase localizado a 20 Å ao fundo do gorge (Adaptado de DARVESH et al., 2003).

Assim, esta enzima está ganhando reconhecimento por seu possível papel em funções fisiológicas normais e seu envolvimento em patologias do SNC (DARVESH et al., 2003). Um indício para isso, é que as propriedades

bioquímicas da BuChE estão alteradas, principalmente no hipocampo e córtex, nas doenças neurodegenerativas (DARREH-SHORI et al., 2006).

Além disso, tem-se estudado a interação dos receptores estrogênicos associados ao estrógeno na atividade da BuChE em casos da doença de Alzheimer (COMBARROS et al., 2005; COMBARROS et al., 2007).

A AChE e a BuChE são enzimas que contribuem para a manutenção da integridade da membrana e possíveis mudanças na permeabilidade local pode ocorrer durante a transmissão e a condução sináptica (DAS et al., 2001, DAS, 2007). Neste sentido, diversos processos podem causar danos a membrana celular através da produção de espécies reativas de oxigênio (EROS) e de nitrogênio (ERNs) tais como o envelhecimento (AGUIAR et al., 2008) e a ovariectomia (HA et al., 2006).

As EROs incluem radicais livres, como: superóxido ($O_2^{\cdot -}$), hidroxil ($OH^{\cdot}$), peroxil ($^{\cdot}RO_2$), hidroperoxil ($^{\cdot}HRO_2^{-}$), assim como espécies não radicais, que apesar de não possuírem elétrons desemparelhados são muito instáveis, como por exemplo: peróxido de hidrogênio (H_2O_2) e ácido hidrocloroso (HOCL). As ERNs incluem radicais livres que incluem o óxido nítrico ($^{\cdot}ON$) e dióxido de nitrogênio ($^{\cdot}NO_2^{-}$), assim como espécies não radicais, por exemplo: peroxinitrito ($ONOO^{-}$), óxido nitroso (HNO_2) e peroxinitrato (RONOO) (TURKO et al., 2001; EVANS et al., 2002).

O estresse oxidativo é definido como o excesso de formação e/ou remoção insuficiente destas moléculas reativas (EROs e ERNs) (Figura 9) (SIES, 1993; TURKO et al., 2001; MARITIM et al., 2003). Durante a redução do oxigênio molecular, as EROs são formadas e existe a necessidade permanente de inativá-las. Os danos induzidos por estas moléculas reativas podem afetar

os lipídios, as proteínas, os carbohidratos, e o DNA e RNA (BIANCHI & ANTUNES, 1999). O estresse oxidativo está diretamente ligado às doenças neurodegenerativas, como a EM e a doença de Alzheimer. Suspeita-se que a quantidade de EROs e ERNs produzida no organismo possa estar relacionada à severidade dos sinais clínicos de cada paciente (MOSSBERG et al., 2009).

Também é interessante ressaltar que substâncias com ações estrogênicas, como o resveratrol e o próprio 17-β estradiol poderiam exercer atividades antioxidantes no encéfalo (FENG & ZHANG, 2005; MOKNI et al., 2007).

Figura 9 – Produção de espécies reativas de oxigênio como um papel central no ciclo de eventos que levam a neurodegeneração (Adaptado e modificado de ANDERSEN, 2004).

O SNC é um tecido que consiste substancialmente de membranas e ácidos graxos, o que aumenta a vulnerabilidade dos constituintes da membrana

lipídica aos danos oxidativos e a ação direta dos radicais livres (VEDDER et al., 1999). Assim, a peroxidação lipídica é considerada um evento fisiopatológico importante no estudo de substâncias farmacológicas, como os estrógenos, em diversas doenças neurodegerativas, e em danos isquêmicos e traumáticos (BRAUGHLER et al., 1987; VEDDER et al., 1999; MOSSBERG et al., 2009). Assim, o excesso ou acúmulo de EROs e ERNs no organismo, além de iniciar a peroxidação das membranas lipídicas (permitindo o acúmulo de peróxidos de lipídios), também pode prejudicar as proteínas e o DNA celular, acelerando processos como o envelhecimento e o câncer (CHEN et al., 2007; HALLIWELL & GUTTERIDGE, 2007).

O processo oxidativo processo ocorre em diferentes condições neurotóxicas e/ou neurodegenerativas, e em grau bem menor nas atividades fisiológicas normais dos circuitos neurais. Os radicais livres podem modificar a produção e o recaptação de neurotransmissores, a atividade dos canais de íons, e a função de diversos transportadores de substâncias para a célula e mitocôndrias, além dos receptores de superfície (MATTSON & LIU, 2002).

FENG & ZHANG (2005) observaram uma redução do estresse oxidativo com o uso a longo prazo do estradiol no encéfalo de ratas ovariectomizadas. Assim, dada a importância do processo de lipoperoxidação no SNC e a relevância clínica de sua interação com os estrógenos, é interessante que se conheça os possíveis efeitos deste hormônio no organismo (KUMAR et al., 2011).

O hemograma é coadjuvante indispensável no diagnóstico e no controle evolutivo de diversos tipos de doenças (FAILACE, 2003), incluindo as enfermidades inflamatórias desmielinizantes. Neste sentido, além da ação

antiinflamatória, o 17-β estradiol e o resveratrol tem demonstrado também ação antioxidante (GAO et al., 2003; MOORTHY et al., 2005), o que poderia contribuir como uma influência positiva para as células sanguíneas.

Vários tipos de leucócitos, como granulócitos e células mononucleares, fazem parte da patofisiologia das doenças neurodegenerativas, tais como a EM (MOSSBERG et al., 2009). Contudo, os dados de literatura demonstraram que o resveratrol é capaz de reduzir a ação exagerada de neutrófilos polimorfonucleares (ROTONDO et al., 1998), bem como, modular a produção de substâncias proinflamatórias por estas células (RICHARD et al., 2005).

Além disso, devido ao fato das hemácias serem carreadoras de oxigênio com uma alta concentração de gorduras poliinsaturadas em sua membrana e alta concentração de hemoglobina, estas ficam especialmente expostas a danos oxidativos. A hemoglobina produzida nos eritrócitos é potencialmente perigosa devido a sua reação com H_2O_2, podendo se converter em formas oxidadas, como a metahemoglobina, uma grande promotora do processo oxidativo (TEDESCO et al., 2000).

As mulheres com baixos níveis estrogênicos são mais susceptíveis às doenças neurodegenerativas, e considerando isso, as alterações bioquímicas e hematológicas são fatores que podem ser afetados nestas condições. Soma-se a este fato, diversos relatos dos efeitos neuroprotetores do 17-β estradiol (FENG et al., 2004; MOORTHY et al., 2005; AGUIAR et al., 2008) e do resveratrol (FRÉMONT, 2000; SCHMATZ et al., 2009) na prevenção de patologias degenerativas do SNC. Desta forma, assumindo a relevância das doenças neurológicas de ordem desmielinizante que podem ocorrer tanto em humanos quanto em animais, é importante que se investigue as alterações

bioquímicas e hematológicas que ocorrem em ratas de diferentes idades, submetidas a desmielinização experimental pelo BE e tratadas com 17-β estradiol ou resveratrol.

2. OBJETIVOS

2.1 Objetivo geral

Verificar o efeito do 17-β estradiol e do resveratrol em parâmetros bioquímicos e hematológicos em ratas ovariectomizadas submetidas ao modelo experimental de desmielinização pelo BE.

2.2 Objetivos específicos

Artigo:

a) Investigar os efeitos da suplementação estrogênica e da ovariectomia na atividade da AChE encefálica (estriado, cerebelo, córtex cerebral e hipocampo), do sangue total e dos linfócitos em ratas ovariectomizadas adultas e de meia-idade.

b) Determinar os efeitos da suplementação estrogênica e da ovariectomia sobre o nível de peroxidação lipídica no encéfalo (estriado, cerebelo, córtex cerebral e hipocampo) de ratas ovariectomizadas adultas e de meia-idade.

c) Verificar o efeito da suplementação estrogênica e da ovariectomia na diferença de peso obtida em ratas adultas e de meia idade.

Manuscrito 1:

a) Determinar o efeito do 17-β estradiol na atividade da AChE encefálica (estriado, cerebelo, córtex cerebral e hipocampo), no sangue total e nos linfócitos em ratas jovens ovariectomizadas e experimentalmente desmielinizadas pelo BE.

b) Investigar o efeito da reposição estrogênica na atividade da BuChE sérica em ratas jovens ovariectomizadas e experimentalmente desmielinizadas pelo BE.

Manuscrito 2:

a) Avaliar e correlacionar o hemograma à atividade da AChE nos linfócitos de ratas jovens ovariectomizadas e experimentalmente desmielinizadas pelo BE e submetidas ao tratamento com resveratrol.

3. DESENVOLVIMENTO

Os resultados que perfazem esta tese estão apresentados na forma de artigo e manuscritos. Os itens de desenvolvimento encontram-se dentro dos respectivos artigo/manuscritos. As normas apresentadas estão de acordo com o periódico ao qual o artigo foi aceito ou os manuscritos encaminhados, estando estas anexadas ao final da tese.

3.1 ARTIGO:

17-β estradiol in the acetylcholinesterase activity and lipid peroxidation in the brain and blood of ovariectomized adult and middle-aged rats

Danieli Brolo Martins, Cinthia Melazzo Mazzanti, Raqueli Teresinha França, Marciélen Pagnoncelli, Márcio Machado Costa, Estevan Martins de Souza, Jamile Gonçalves, Roselia Spanevello, Roberta Schmatz, Pauline da Costa, Alexandre Mazzanti, Diego Vilibaldo Beckmann, Marcelo da Silva Cecim, Maria Rosa Schetinger, Sonia Terezinha dos Anjos Lopes

Artigo publicado pela Life Sciences, v.90, p.551-359, 2012.

Life Sciences 90 (2012) 351–359

Contents lists available at SciVerse ScienceDirect

Life Sciences

journal homepage: www.elsevier.com/locate/lifescie

17-β estradiol in the acetylcholinesterase activity and lipid peroxidation in the brain and blood of ovariectomized adult and middle-aged rats

Danieli Brolo Martins [a], Cinthia Melazzo Mazzanti [a], Raqueli Teresinha França [a], Marciélen Pagnoncelli [a], Márcio Machado Costa [a], Estevan Martins de Souza [a], Jamile Gonçalves [b], Roselia Spanevello [b], Roberta Schmatz [b], Pauline da Costa [b], Alexandre Mazzanti [a], Diego Vilibaldo Beckmann [a], Marcelo da Silva Cecim [c], Maria Rosa Schetinger [b], Sonia Terezinha dos Anjos Lopes [a,*]

[a] Departamento de Clínica de Pequenos Animais, Universidade Federal de Santa Maria, Brazil
[b] Departamento de Química, Universidade Federal de Santa Maria, Brazil
[c] Departamento de Grandes Animais, Universidade Federal de Santa Maria, Brazil

ARTICLE INFO

Article history:
Received 12 June 2011
Accepted 13 December 2011

Keywords:
Hormone therapy reposition
Estrogen
Acetylcholinesterase
Central nervous system
Oxidative stress

ABSTRACT

Aims: To investigate the 17-β estradiol in the acetylcholinesterase activity and lipid peroxidation in the brain and blood of ovariectomized rats of different ages.
Main methods: Animals were randomly assigned into three experimental groups of each age ($n=6$). Control groups consisted of adult (sham-A) and middle-aged (sham-MA) female rats, ovariectomized adult (OVX-A) and middle-aged (OVX-MA) rats without estrogen therapy reposition, and ovariectomized adult (OVX + E2-A) and middle-aged (OVX + E2-MA) rats treated with 17-β estradiol for 30 days. After this period, AChE activity and lipid peroxidation were measured in the brain and blood.
Key findings: The AChE activity increased ($p<0.05$) in striatum (ST) in OVX-A, OVX + E2-A and OVX-MA, and hippocampus (HP) in OVX-MA. The enzyme activity decreased ($p<0.05$) in ST of OVX + E2-MA, and cerebral cortex (CC) in OVX + E2-A, OVX-MA and OVX + E2-MA. Blood AChE activity increased ($p<0.05$) in OVX + E2-A and decreased ($p<0.05$) in OVX-MA. Lymphocyte AChE activity increased ($p<0.05$) in OVX-A and OVX + E2-A and decreased ($p<0.05$) in OVX-MA. Lipid peroxidation increased ($p<0.05$) in ST of OVX-A, CC of OVX-A and OVX-MA, HP of OVX-A, and cerebellum (CE) of OVX-A, OVX-MA, and OVX + E2-MA. Lipid peroxidation decreased ($p<0.05$) in ST, CC and CE of OVX + E2-A, and ST and HP of OVX + E2-MA. Similar values of lipid peroxidation to control groups were found in ST and HP of OVX-MA, HP of OVX + E2-A and CC of OVX + E2-MA.
Significance: 17-β estradiol is able to modulate the AChE activity and non-neuronal cholinergic response as well as to reduce lipid peroxidation. Its response is dependent on the age and brain structure analyzed.

Introduction

The aging of the normal brain is accompanied by changes in its structure, function, and metabolism (Moorthy et al., 2005; Thakur and Sharma, 2006). Most of these changes are regulated by the estrogen which is derived from either circulation or steroidogenesis in the brain (McEwen and Alves, 1999). Post-menopausal women are more vulnerable than young women to neurodegenerative diseases, resulting in memory and cognitive dysfunction (Wise et al., 2001). However, the risk for dementia is increased in young women who did bilateral oophorectomy as well as in women who discontinued estrogen therapy before 50 years of age. In this context, the ovarian hormone 17-β estradiol has shown a protective effect in many neurodegenerative conditions. It decreases the risk and delays the onset and progression of Alzheimer's disease (AD) (García-Segura et al., 2001; Vegeto et al., 2008). Moreover, it enhances the recovery from traumatic neurological injury such as cerebral ischemia (Cimarosti et al., 2005).

Different ages and ovariectomy produce a progressive damaging effect on the nervous and immune functions (Baeza et al., 2010). Recent investigations have suggested that estrogens exert a protective effect against brain disorders from the evidence that menopause, which is characterized by the drastic drop in estrogen levels, results in an increased incidence of inflammatory pathologies of brain (Pozzi et al., 2006). However, ovariectomy in younger women (prior to menopause) significantly increases the risk for the development of memory problems and neurodegenerative disorders. However, the biological basis underlying these cognitive changes is still poorly understood (Craig et al., 2010). The onset-age of estrogen deficiency

* Corresponding author at: Hospital Veterinário Universitário, Centro de Ciências Rurais, Universidade Federal de Santa Maria, Av. Roraima, 97105-900, Santa Maria, RS, Brazil. Tel.: +55 55 3220 8814; fax: +55 55 3220 8978.
E-mail address: soniatldosanjos@hotmail.com (S.T.A. Lopes).

0024-3205/$ – see front matter © 2012 Elsevier Inc. All rights reserved.
doi:10.1016/j.lfs.2011.12.006

appears to be an important determinant of long term health (Rocca et al., 2009). Thus, some of the mechanisms underlying these effects may be independent of the classically defined estrogen receptors (ER) and may involve direct modulation of neurotransmitter receptor function or anti-oxidant activities of estrogen (García-Segura et al., 2001; Li and Shen, 2005).

Beneficial effects of estrogens on brain aging and cognition are related to interactions with cholinergic projections emanating from the basal forebrain. These cholinergic projections play an important role in the learning and memory processes as well as in neuronal plasticity and cognitive performance. Moreover, their function is known to decline with advanced age and in association with neurodegenerative diseases (Gibbs, 2010). Cholinergic neurons are significantly affected by changes in circulating levels of estrogen (Gibbs and Aggarwal, 1998) suggesting that the effectiveness of estrogen therapy decreases with age (Gibbs, 2010).

Acetylcholinesterase enzyme (AChE; E.C. 3.1.1.7), found in the cholinergic terminal, is a specific choline esterase, which hydrolyzes predominantly choline esters (acetylcholine — ACh) and is characterized by being present in high levels in the brain, nerve and red blood cells (Das, 2007). Until recently, neurons were the only identified source of ACh. It is now known that cells, other than neurons, express the proteins required for ACh metabolism. ACh is synthesized, amongst others, by immune system (lymphocytes, dendritic cells, neutrophils) and endothelial cells (Kawashima and Fujii, 2000). It is possible that ACh derived from these sources be involved in the modulation of local inflammatory processes and regulation of immune functions (Das, 2007).

Cholinergic signaling in non-neuronal cells is comparable to cholinergic neurotransmission (Wessler et al., 2003). In this aspect, cell activation is a key modulator of the non-neuronal cholinergic system. It is likely that a lymphocytic cholinergic system is involved in regulating immune function (Razani-Boroujerdi et al., 2008). Changes in lymphocyte AChE activity, therefore, reflect immune deficiency related to cell dysfunction (Battisti et al., 2009).

The 17-β estradiol is able to influence the development, regulation, and functioning of the immune system (Baeza et al., 2010). This hormone can influence many aspects of the central nervous system (CNS) and immune function. Moreover, understanding the cellular and molecular mechanisms that underlie these protective actions is essential to prevent the deleterious consequences of prolonged hypoestrogenicity and to improve women's health (Wise et al., 2001). The mechanism of neuroprotection remains still to be understood (Cimarosti et al., 2005; Sales et al., 2010). However, it is still debated whether estrogen treatment could result in improved cognitive function and other aspects in women after the menopause (Hogervorst and Bandelow, 2010).

Estrogen replacement has also been reported as beneficial against several biological reactions in the body, such as maintenance or increase in the antioxidant capacity and decreased levels of oxidative stress (Aguiar et al., 2008), especially during menopause (Moorthy et al., 2005). Also, the lack of estrogen can induce changes in lipid metabolism, with an increase of oxidative status (Signorelli et al., 2006) and fat accumulation in the body resulting in weight gain (Mittal and Kant, 2009).

Although hormonal replacement therapy may be one of the greatest conquests in the battle for quality of life for women, many factors should be considered regarding its use (Aguiar et al., 2006). Estrogen replacement therapy has showed a reduction in risk factors related to aging, oxidative stress (Moorthy et al., 2005; Aguiar et al., 2006, 2008), and neurological disorders (Wise et al., 2001; Pozzi et al., 2006; Vegeto et al., 2008). Thus, we hypothesized that 17-β estradiol replacement could improve parameters related to the AChE activity in the brain, total blood and lymphocytes, as well as lipid peroxidation in the brain and weight gain in adult and middle-aged ovariectomized female rats.

Materials and methods

Chemicals

The hormone 17-β estradiol, substrate, and buffers were obtained from Sigma Chemical Co (St. Louis, MO, USA). All the other reagents used in the experiments were of analytical grade and of the highest purity.

Animals

Thirty six female Wistar rats were used. Eighteen animals were 5 months old (adults) and the other 18 were 10 months old (middle-aged). The rats were maintained at a constant temperature (21 ± 2 °C) on a 12 h light/dark cycle with free access to food and water. The study was performed in accordance with the Federal University of Santa Maria Ethics Committee Guidelines for Experiments with Animals (process 86/2009). A soy-free rat chow (Supra®, Alisul, Carazinho, RS, Brazil) was used to avoid phytoestrogen interference.

After a week of habituation, the animals were submitted to vaginal smear for 2 weeks to verify the normal estrous cycle (Marcondes et al., 2002; Yener et al., 2007). Just rats with normal cycle entered in the experiment. The animals were weighted and, then, immediately submitted to the ovariectomy (OVX) procedures.

Surgical procedure

We chose the OVX to mimic the estrogen deprivation in animals since this is a model commonly used for this purpose (Monteiro et al., 2005; Acosta et al., 2009). The animals were anesthetized initially with 4% halothane in a 40% O_2/60% N_2O mixture and the anesthesia was maintained during the bilateral OVX with 1.5–2.5% halothane breathing spontaneously via facemask. Control groups underwent a sham surgery with an incision in the Alba line, but without gonad removal. After a recovery period of 15 days, all animals were submitted again to vaginal smear to confirm the efficacy of the surgical procedures (4 days) and then the treatment started.

Experimental groups and treatments

Adult and middle-aged rats were randomly assigned into three experimental groups of each age ($n = 6$), where control groups consisted of adult (sham-A) and middle-aged (sham-MA) female rats in proestrus, ovariectomized adult (OVX-A) and middle-aged (OVX-MA) rats without estrogen therapy reposition, and ovariectomized adult (OVX + E2-A) and middle-aged (OVX + E2-MA) rats treated with 17-β estradiol. Sham and OVX groups received just canola oil vehicle, while OVX + E2 groups received 0.1 μg/g of body weight of 17-β estradiol (according to Moorthy et al., 2005).

We started the hormonal reposition 15 days after OVX, time needed to bring down estrogen levels and absence of estrous cycle by vaginal cytology. Intervals of months between the OVX and the beginning of the hormonal treatment should be avoided because estrogen replacement therapy has no action when started after a long-term hormone deprivation (Daniel et al., 2006). Moreover, our choice for the oral administration of estrogens is because that is a practical and widely used method by women (Aguiar et al., 2008).

Canola oil vehicle and 17-β estradiol were administered with the same volume, by oral gavage, once a day. The administration was continuous for 30 days, and in the following day (euthanasia day) there was no treatment. On day 30, the animals were weighted again to compare the weight of the rats in the beginning and in the end of the experimental period.

Serum determination of 17-β estradiol

We used the enzymatic method of automated immunochemiluminescence with Immulite 2000 analyzer (Diagnostic Products Corporation, Los Angeles, CA, USA) and specific hormone kit (Immulite Estradiol).

Brain tissue preparation

The animals were submitted to euthanasia being previously anesthetized with halothane and brain structures were immediately removed, separated into striatum (ST), hippocampus (HP), cerebral cortex (CC) and cerebellum (CE), placed in a solution of 10 mM Tris–HCl, pH 7.4, on ice at a proportion of 1:10 (w/v) and after homogenized. The homogenate was centrifuged at 1800 rpm for 10 min and the resulting supernatant (S_1) was stored at −30 °C until use. Protein was determined previously in a strip that varied for each structure: ST (0.4 mg/mL), HP (0.8 mg/mL), CC (0.7 mg/mL), and CE (0.6 mg/mL) as determined by the Coomassie blue method according to Bradford (1976) using bovine serum albumin as standard solution.

Cerebral AChE enzymatic assay

The AChE enzymatic assay was determined by a modification of the spectrophotometric method of Ellman et al. (1961) as previously described by Rocha et al. (1993). The reaction mixture (2 mL final volume) was composed of 100 mM K+-phosphate buffer, pH 7.5 and 1 mM 5,50-dithiobisnitrobenzoic acid (DTNB). The method is based on the formation of yellow anion, 5,50-dithio-bis-acidnitrobenzoic, measured by absorbance at 412 nm during 2-min incubation at 25 °C. The enzyme (40–50 mg of protein) was pre-incubated for 2 min. The reaction was initiated by adding 0.8 mM acetylthiocholine iodide (AcSCh). All samples were run in duplicate or in triplicate, and the enzyme activity was expressed in μmoles AcSCh/h/mg of protein.

Blood sample collection

The blood was collected in vaccutainer tubes using EDTA 10% as anticoagulant. For AChE activity in whole blood, the samples were hemolyzed with phosphate buffer, pH 7.4 containing Triton X-100 (0.03%) and stored at −20 °C for 1 week. For AChE activity in lymphocytes, the peripheral lymphocytes were isolated and AChE activity was measured immediately.

Isolation of the cells

The peripheral lymphocytes were isolated using Ficoll Hypaque density gradient as described by Böyum (1968). After separation, only samples with at least 95% of lymphocytes, as verified in the coulter STKS (Miami—USA), were used. Lymphocyte viability and integrity were confirmed by determining the percentage of cells, excluding 0.1% trypan blue and measuring lactate dehydrogenase (LDH) activity (Bergmeyer, 1983).

Determination of AChE activity in whole blood

The AChE enzymatic assay was determined by the method of Ellman et al. (1961) modified by Worek et al. (1999). The specific activity of whole blood AChE was calculated from the quotient between AChE activity and hemoglobin content and the results are expressed as mU/μmol of whole blood.

Determination of AChE activity in lymphocytes

After the isolation of the lymphocytes, AChE activity was determined according to the method described by Ellman et al. (1961) and modified by Fitzgerald and Costa (1993). Briefly, proteins of all samples were adjusted to 0.1–0.2 mg/ml. 0.2 ml of intact cells was added to a solution containing 1.0 mM acetylthiocholine (ATC), 0.1 mM 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB), and 0.1 M phosphate buffer (pH 8.0). Immediately before and after incubation for 30 min, at 27 °C, the absorbance was read on a spectrophotometer at 412 nm. AChE was calculated from the quotient between lymphocyte AChE activity and protein content, and the results were expressed as μmol/h/mg of protein.

Thiobarbituric acid reactive substances (TBARS) measurement

Brain TBARS levels were determined by the method described previously by Ohkawa et al. (1979). In short, the reaction mixture contained 200 μL of brain homogenates or standard (MDA—malondialdehyde 0.03 mM), 200 μL of 8.1% sodium dodecyl sulfate (SDS), 500 μL of acetic acid solution (2.5 M HCl, pH 3.5) and 500 μL of 0.8% thiobarbituric acid (TBA). The absorbance was measured at 532 nm. TBARS tissue levels were expressed as nmol MDA/mg of protein.

Statistical analysis

The statistical analysis used was one-way ANOVA, followed by Duncan's multiple range tests. All the data are expressed as the mean ± standard error. Differences were considered significant when the probability was $p<0.05$.

Results

Adult and middle-aged control animals showed a normal estrous cyclic activity throughout the experimental period (cycle duration: 4 to 5 days), being in proestrus on the day of euthanasia. Ovariectomized rats without estrogen treatment presented continuous diestrus cytology confirming the reduction of circulating hormone. Ovariectomized rats

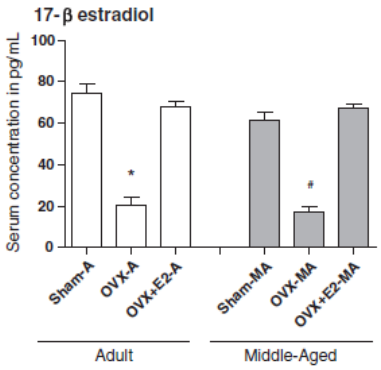


Fig. 1. Serum 17-β estradiol levels evaluated by immuno-chemiluminescence of female adult and middle-aged rats. Sham-A (adult female rat in proestrus), OVX-A (adult female ovariectomized rat without estrogen therapy reposition) and OVX + E2-A (adult female ovariectomized rat treated with 0.1 μg/g of 17-β estradiol) are distributed in the white columns. Sham-MA (middle-aged female rat in proestrus), OVX-MA (middle-aged female ovariectomized rat without estrogen therapy reposition) and OVX + E2-MA (middle-aged female ovariectomized rat treated with 0.1 μg/g of 17-β estradiol) are distributed in the gray columns. Bars represent mean ± SEM.*Denotes $p<0.05$ when compared with adult control group (Sham-A) (one-way analysis of variance/Duncan's multiple range test, $n=6$). #Denotes $p<0.05$ when compared with the middle-aged control group (Sham-A) (one-way analysis of variance/Duncan's multiple range test, $n=6$).

with 17-β estradiol supplementation showed daily typical proestrus cells. Estradiol levels were measured in all groups (Fig. 1) and revealed a significant difference ($p<0.05$) on the hormonal reduction of both ovariectomized age groups (OVX-A and OVX-MA) when compared to the control and supplemented groups of the same age. The estrogen reposition groups (OVX + E2-A and OVX + E2-MA) showed similar physiological values to those exhibited on proestrus by the control group.

AChE activity was evaluated in the supernatant (S_1) in all cerebral structures (CC, ST, HP and CE) (Fig. 2). In the ST, the ovariectomy significantly increased ($p<0.05$) the activity of the enzyme for OVX-A and for OVX-MA. However, AChE behavior varied according to hormonal reposition. While the adult group OVX + E2-A showed a significant increase in the AChE activity ($p<0.05$) in the middle-aged group OVX + E2-MA demonstrated a significant decrease ($p<0.05$) in the enzyme activity. In relation to the CC in the material S_1, in the adult group, the ovariectomy (OVX-A) did not modify AChE activity, but with the hormonal replacement (OVX + E2-A) there was a significant decrease ($p<0.05$) in the activity of the enzyme. In the middle-aged animals, a significant decrease ($p<0.05$) occurred in both ovariectomized group (OVX-MA) and in the group with estrogenic reposition (OVX + E2-MA). In the HP, there was no significant alteration among the adult groups. However, in the middle-aged animals, the ovariectomy induced a significant increase ($p<0.05$) in the activity of AChE in the OVX-MA group. In the CE of adult and middle-aged animals, there was no significant alteration in the activity of AChE among the groups of both ages.

The AChE activity in total blood (Fig. 3) of the adult animals showed no significant alteration when compared to sham-A group

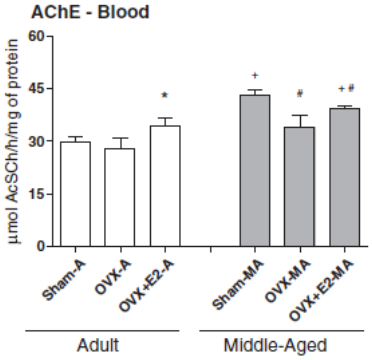


Fig. 3. Acetylcholinesterase (AChE) activity in the total blood of female adult and middle-aged rats. Sham-A (adult female rat in proestrus), OVX-A (adult female ovariectomized rat without estrogen therapy reposition) and OVX + E2-A (adult female ovariectomized rat treated with 0.1 µg/g of 17-β estradiol) are distributed in the white columns. Sham-MA (middle-aged female rat in proestrus), OVX-MA (middle-aged female ovariectomized rat without estrogen therapy reposition) and OVX + E2-MA (middle-aged female ovariectomized rat treated with 0.1 µg/g of 17-β estradiol) are distributed in the gray columns. Bars represent mean ± SEM. *Denotes $p<0.05$ when compared with the adult control group (Sham-A) (one-way analysis of variance/Duncan's multiple range test, $n=6$). #Denotes $p<0.05$ when compared with the middle-aged control group (Sham-A) (one-way analysis of variance/Duncan's multiple range test, $n=6$).

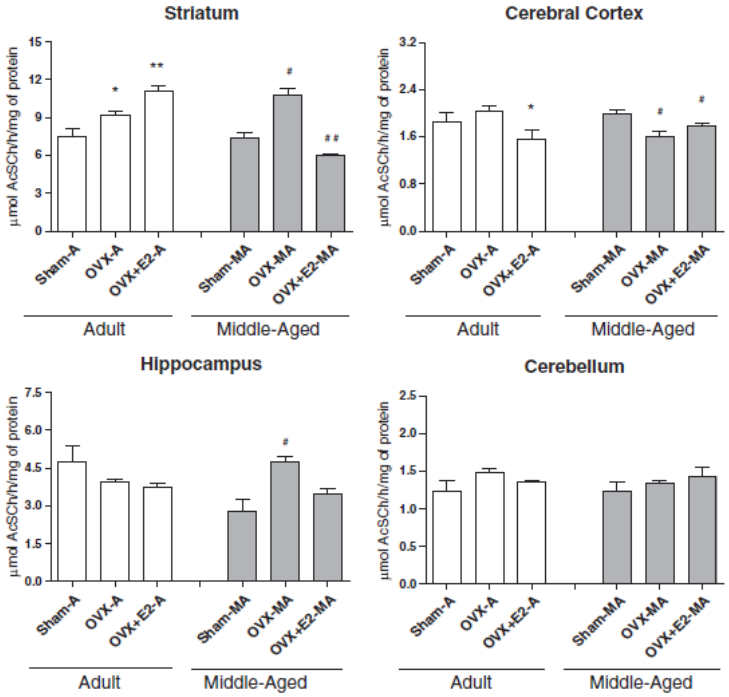


Fig. 2. Acetylcholinesterase (AChE) activity in different structures (striatum, cerebral cortex, hippocampus, and cerebellum) of the brain of female adult and middle-aged rats. Sham-A (adult female rat in proestrus), OVX-A (adult female ovariectomized rat without estrogen therapy reposition) and OVX + E2-A (adult female ovariectomized rat treated with 0.1 µg/g of 17-β estradiol) are distributed in the white columns. Sham-MA (middle-aged female rat in proestrus), OVX-MA (middle-aged female ovariectomized rat without estrogen therapy reposition) and OVX + E2-MA (middle-aged female ovariectomized rat treated with 0.1 µg/g of 17-β estradiol) are distributed in the gray columns. Bars represent mean ± SEM. *,**Denote $p<0.05$ when compared with the adult control group (Sham-A) (one-way analysis of variance/Duncan's multiple range test, $n=6$). #,##Denote $p<0.05$ when compared with the middle-aged control group (Sham-A) (one-way analysis of variance/Duncan's multiple range test, $n=6$).

to OVX-A group. In spite of that, the supplementation with 17-β estradiol induced a significantly increased ($p<0.05$) in the OVX-E2-A. In the middle-aged animals, the ovariectomized group (OVX-MA) had a significant decrease ($p<0.05$) in the activity of the enzyme. But interestingly, at this same age, the group treated with estrogen (OVX + E2-MA) showed no significant alteration when compared to sham-MA and OVX-MA.

Lymphocyte AChE activity (Fig. 4) was significantly increased ($p<0.05$) in OVX-A and OVX + E2-A of adult animals. In the middle-aged animals, there was a decrease ($p<0.05$) in lymphocyte AChE activity influenced by the ovariectomy in the OVX-MA group. However, the supplementation with 17-β estradiol, as seen in the OVX + E2-MA group, had a significant increase ($p<0.05$) when compared to the control group (sham-MA).

TBARS production in the ST, CC, HP and CE of the adult and middle-aged animals is shown in Fig. 5. In the ST, the ovariectomy increased significantly ($p<0.05$) the TBARS production only in the adult castrated animals (OVX-A), while in the middle-aged castrated animals (OVX-MA) the values were similar to those showed by the middle-aged control group (sham-MA). However, both age groups treated with 17-β estradiol (OVX + E2-A and OVX + E2-MA) showed a significant decrease ($p<0.05$) of the TBARS production, indicating a reduction in the lipid peroxidation. TBARS production of CC had a significant increase ($p<0.05$) in both ovariectomized groups (OVX-A and OVX-MA) when compared to their control age groups (sham-A and sham-MA, respectively). The adult group supplemented with 17-β estradiol (OVX + E2-A) demonstrated a significant reduction ($p<0.05$) of the TBARS production, while in the middle-aged group supplemented with 17-β estradiol (OVX + E2-MA) the group showed TBARS values similar to those presented by the sham-MA group. In the HP, OVX-A showed a significant increase ($p<0.05$) of the lipid peroxidation, while OVX-MA presented similar values to sham-MA. The adult group OVX + E2-A demonstrated similar TBARS production to sham-A, and the middle-aged group OVX + E2-MA showed a significant decrease ($p<0.05$) in TBARS production. In the CE, there was a significant increase ($p<0.05$) in the TBARS production in the ovariectomized groups of both ages (OVX-A and OVX-MA) and in the middle-aged group treated with 17-β estradiol (OVX + E2-MA). On the other hand, OVX + E2-A showed a significant decrease ($p<0.05$) of TBARS production.

Post hoc comparisons by Duncan's multiple range test revealed that the weight of the adult animals was higher in OVX-A group ($p<0.05$) and OVX + E2-A ($p<0.01$) when compared to sham-A control group (Table 1). On the other hand, the weight of the middle-aged animals was higher just in OVX-MA group ($p<0.05$) when compared to sham-MA control group, while there was no significant alteration between OVX + E2-MA and sham-MA (Table 2).

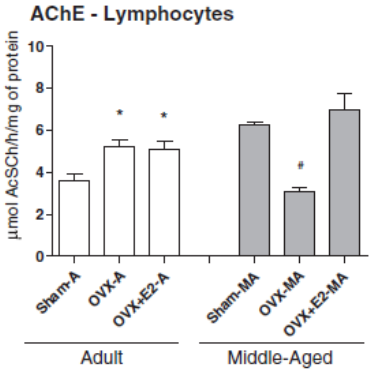


Fig. 4. Acetylcholinesterase (AChE) activity in the lymphocytes of female adult and middle-aged rats. Sham-A (adult female rat in proestrus), OVX-A (adult female ovariectomized rat without estrogen therapy reposition) and OVX + E2-A (adult female ovariectomized rat treated with 0.1 µg/g of 17-β estradiol) are distributed in the white columns. Sham-MA (middle-aged female rat in proestrus), OVX-MA (middle-aged female ovariectomized rat without estrogen therapy reposition) and OVX + E2-MA (middle-aged female ovariectomized rat treated with 0.1 µg/g of 17-β estradiol) are distributed in the gray columns. Bars represent mean ± SEM. *Denotes $p<0.05$ when compared with the adult control group (Sham-A) (one-way analysis of variance/Duncan's multiple range test, $n=6$). #Denotes $p<0.05$ when compared with the middle-aged control group (Sham-A) (one-way analysis of variance/Duncan's multiple range test, $n=6$).

Discussion

In the present study we verified the effect of 17-β estradiol on the AChE activity of brain (ST, CC, HP, and CE), blood, and lymphocyte, as well as lipid peroxidation and weight gain of ovariectomized adult and middle-aged female rats. We have chosen ovariectomy because this model is considered the best tool to mimic human ovarian hormone loss, being able to cause a premature aging of the nervous and immune system (Baeza et al., 2010). 17-β estradiol is considered to be the major ovarian hormone and its deficits may be related to the modulatory role on cholinergic innervations in brain, especially in areas associated to the memory processes such as hippocampus (McEwen, 2001; Craig et al., 2010). Furthermore, AChE is likely to play an important role in regulating immune functions, since this enzyme is present in blood and lymphocytes (Kawashima and Fujii, 2000; Kawashima and Fujii, 2003). There is scarce information on the AChE activity of cholinergic system related to the non-cholinergic system in the estrogenic reposition in females of different ages. Moreover, we seek to understand whether some of the changes observed in the activity of this enzyme in our study could be attributed to lipid peroxidation, an index of oxidative stress.

About 2% of the striatal neurons are cholinergic (Zhou et al., 2002). Our ST results showed that the activation of the enzyme in this area occurs in both ovariectomized groups (OVX-A and OVX-MA) and in the adult group with hormone replacement (OVX + E2-A). AChE activation leads to a fast ACh degradation and the subsequent downstimulation of ACh receptors (Grisaru et al., 1999). In this study, the ST was the only brain structure in the adult animals that demonstrated elevation of the activity of AChE. In adult animals, estrogen replacement (OVX + E2-A) has further heightened the activity of AChE compared with the ovariectomized group (OVX-A). The high concentration of AChE or an increase of enzymatic activity as seen in this case, in the ST, could rapidly terminate the ACh signal, and thereby minimize desensitization of nAChR (Zhou et al., 2002). Additionally to this process, the absence of estrogen can reduce in 50% ChAT production (Tam et al., 2002). ACh is also considered a neurotransmitter with anti-inflammatory effect (Das, 2007), and its low availability could facilitate damaging processes in this part of the brain. However, the estrogenic effect on AChE caused an inhibition of this enzyme activity in middle-aged animals (OVX + E2-MA), showing that in the ST, adult and middle-aged animals may have distinct AChE activity although having the same treatment. ER-α or ER-β subtypes have not been found extensively in the striatum (Shughrue et al., 1997) and a decrease of ER-β may occur with aging (Chakraborty et al. 2003). Thus, despite ST has its cholinergic system altered by the estrogenic depletion in a similar way in both adult and middle-aged animals, 17-β estradiol supplementation may cause an increase or a decrease in the AChE activity according to the age analyzed.

Our findings in the CC, one of the areas commonly affected in several neurodegenerative diseases (Kasa et al., 1997), showed that despite OVX in adult rats has no influence in the AChE activity, the OVX in the middle-aged animals and estrogenic supplementation in both ages can cause an inhibition of the AChE activity. Unlike the present study, Monteiro et al. (2005) studying the effect of OVX

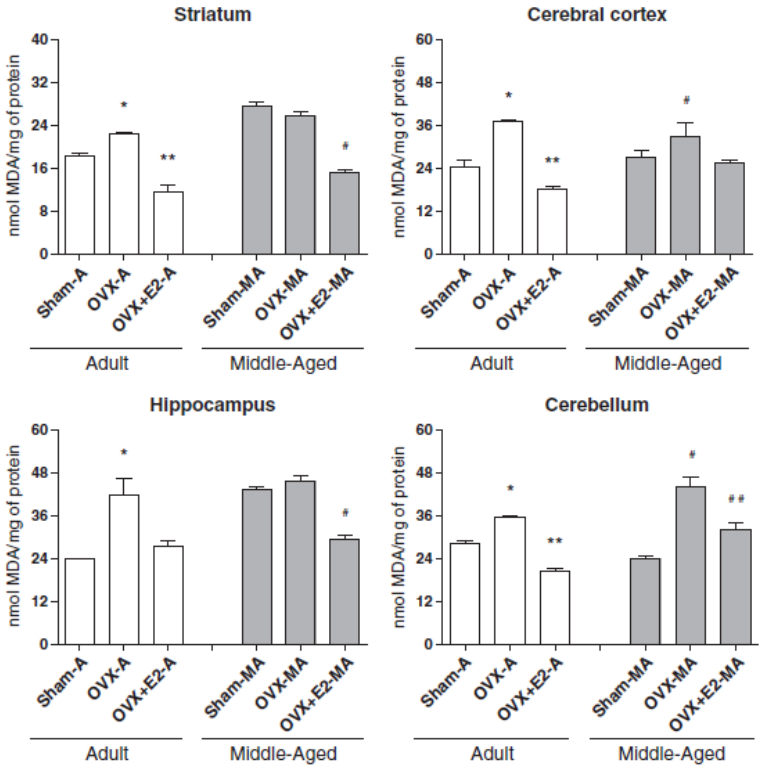


Fig. 5. Thiobarbituric acid reactive substances (TBARS) measurement in different structures (striatum, cerebral cortex, hippocampus, and cerebellum) of the brain of female adult and middle-aged rats. Sham-A (adult female rat in proestrus), OVX-A (adult female ovariectomized rat without estrogen therapy reposition) and OVX + E2-A (adult female ovariectomized rat treated with 0.1 μg/g of 17-β estradiol) are distributed in the white columns. Sham-MA (middle-aged female rat in proestrus), OVX-MA (middle-aged female ovariectomized rat without estrogen therapy reposition) and OVX + E2-MA (middle-aged female ovariectomized rat treated with 0.1 μg/g of 17-β estradiol) are distributed in the gray columns. Bars represent mean ± SEM. *,**Denote $p<0.05$ when compared with the adult control group (Sham-A) (one-way analysis of variance/Duncan's multiple range test, $n=6$). #,##Denote $p<0.05$ when compared with the middle-aged control group (Sham-A) (one-way analysis of variance/Duncan's multiple range test, $n=6$).

in 3 month-old rats found an increase of the AChE activity in CC, while Acosta et al. (2009), who studied 12 month-old female rats, found no significant AChE changes in this area due to ovarian hormone loss. An inhibition of this enzyme may have many consequences on the brain, as AChE is involved in many neuronal events (Mesulam et al., 2002).

In relation to HP, a brain area associated with memory and cognition, adult animals showed no difference between treatments. Feng et al. (2004) also observed no changes in the activity of this enzyme after 17 weeks of OVX, or even after the replacement for 16 weeks with 17-β estradiol in this brain region. Thus, the lack of ovarian hormones as well as exogenous estrogen replacement does not seem to influence the behavior of AChE in adult female rats. However, other studies found an increase of the enzyme activity in younger ovariectomized animals (Monteiro et al., 2005). However, the HP of the OVX-MA animals showed a stimulation of AChE activity, which agrees with the results reported by Acosta et al. (2009). In this aspect, there is an important impairment in the ACh concentration, which is a neurotransmitter that regulates the levels and activities of serotonin, dopamine, and other neuropeptides involved in many neurodegenerative diseases; thus, ACh modulates both immune response and neurotransmission (Das, 2007). We suggest that the decrease in the estrogenic levels in middle-aged rats may

Table 1
Average weights and standard deviation of female adult sham rats (Sham-A), female ovariectomized adult rats without hormone therapy reposition (OVX-A) and female ovariectomized adult rats supplemented with 0.1 μg/g of 17-β estradiol (OVX + E2-A).

Groups	Average weight (g) and standard deviation
Sham-A	0.232 ± 0.009
OVX-A	0.304* ± 0.01
OVX + E2-A	0.271* ± 0.012

Bars represent mean ± SD. Duncan's multiple range test, ($n=6$).
* Indicates a significant difference at $p<0.05$.

Table 2
Average weights and standard deviation of female middle-aged sham rats (Sham-MA), female ovariectomized middle-aged rats without hormone therapy reposition (OVX-MA) and female ovariectomized middle-aged rats supplemented with 0.1 μg/g of 17-β estradiol (OVX + E2-MA).

Groups	Average weight (g) and standard deviation
Sham-MA	0.301 ± 0.01
OVX-MA	0.334# ± 0.007
OVX + E2-MA	0.309 ± 0.02

Bars represent mean ± SD. Duncan's multiple range test, ($n=6$).
# Indicates a significant difference at $p<0.05$.

predispose to a greater inactivation of ACh in the synaptic cleft. On the other hand, the middle-aged ovariectomized animals that received 17-β estradiol (OVX + E2-MA) were able to normalize the activity of AChE. Thus, estrogen has a neuroprotective role in the HP in this age group, since its presence resulted in AChE activity similar to the control group. The inhibited AChE activity to values similar to the control group could contribute to learning and memory by the possible increasing of ACh level (Das et al., 2005).

The estrogenic hormone can improve learning skills by potentiating cerebellar plasticity and synapse formation, especially in periods of high estradiol levels of the estrous cycle (Andreescu et al., 2007). In our study, the CE was the only brain structure that remained unchanged in all groups studied. There was no influence of the OVX or estrogen replacement on the activity of AChE in adult, as well as in middle-aged female rats. However, Moorthy et al. (2005) observed that AChE activity in CE can decay in 12 month-old naturally menopausal rats and this alteration could be associated to several factors such as concentration of the circulating ovarian hormones, number of receptors, and affinity for the steroid. Thus, age and OVX may be important factors to influence the AChE activity in this brain area, since our animals were ovariectomized and were 5 and 10 months old. In addition, CE is a structure with few cholinergic pathways (Zimmerman and Soreq, 2006) that can explain its low AChE activity in relation to the other structures studied.

The duration and/or the critical period of initiation of estrogen therapy should be considered important for an influence on the brain AChE activity (Pereira et al., 2008). Another important aspect is that HP and CC, which receive cholinergic projections from the nucleus basalis of Meynert and ST (which has an intrinsic cholinergic circuit), did not present similar results in our research. Heterogeneous localization of estrogen receptors in the brain may be a possible reason for the varied influence of OVX and estrogen treatment on the AChE activity in different brain areas (Das et al., 2001). Although adult HP and CE of the different ages studied were not influenced by our treatments, OVX impaired AChE activity in adult ST and middle-aged ST, HP and CC. However, 17-β estradiol was not able to revert AChE activity in adult ST and middle-age CC, it decreased the enzyme activity in adult CC and middle-aged ST, and finally it normalized the AChE activity in middle-aged HP. Thus, estrogen has age- and brain region-specific selective beneficial effects on cholinergic system (Browne et al., 2009).

ACh has an important participation in several neurodegenerative diseases, particularly in those that affect cognition. Measuring the AChE activity can provide evidence, at least indirectly, of how the concentration of ACh is in the brain (Das, 2007). Thus, the evaluation of the non-neuronal cholinergic system is a relevant parameter that should be related to different ages, menopausal status, estrogenic therapy, as well as brain cholinergic system.

An important aspect of the present study is related to the evaluation of the non-neuronal AChE activity in the blood and lymphocytes that showed distinct behavior among the groups. Our results in the AChE activity of whole blood and lymphocytes of adult animals indicate a high activity of this enzyme, reflecting an activation of the cholinergic immune system in this age. The AChE activity in lymphocytes of adult animals remained high regardless of whether or not they received estrogen supplementation (OVX-A and OVX + E2-A). In whole blood, OVX did not affect AChE activity, but the estrogenic reposition increased its activity. Our results may indicate that an increased AChE activity could hydrolyze ACh more quickly, thereby reducing its anti-inflammatory effects due to the absence of negative feedback control exerted by this neurotransmitter. Additionally, ACh binds to nAChR in lymphocyte surfaces and inhibits the proliferation of many inflammatory substances, such as cytokines and reactive oxygen species (ROS) (Kawashima and Fujii, 2003; Das, 2007; Rao et al., 2007). For this, AChE could be used as a marker of low-grade systemic inflammation, since its activity can be elevated in initial stages of some neuronal disorders, such as AD (Das, 2007).

On the other hand, there was a low activity of AChE in whole blood and in lymphocytes of the middle-aged ovariectomized animals without estrogen replacement (OVX-MA). A decrease in AChE activity can also affect the normal function of the immune system (Battisti et al., 2009), demonstrating that the absence of ovarian hormones in females of older age can be injurious in this aspect. Our findings show the impairment that the estrogen reduction can cause in the non-neuronal cholinergic system of the middle-aged animals. Interestingly, Das (2007) and Rao et al. (2007) reported that a lower activity of the enzyme can occur in patients with terminal stages of AD or diabetes mellitus, which may indicate a serious risk of death.

Thus, we suspect that age is a key component in the relationship of non-neuronal cholinergic system and estrogen replacement therapy. It is possible that the reduced activity of this enzyme in middle-aged animals could be as a result of exhaustion of its stores associated or not with low synthesis (Das, 2007), whereas in adult animals the enzyme activity is still high. However, our findings indicate a better action of the estrogenic reposition in the extra cholinergic immune response of the middle-aged animals when compared to the adult ones. It was possible to observe that the supplementation with 17-β estradiol (OVX + E2-MA) was able to normalize lymphocyte AChE activity from animals of this age group. Regarding the fraction of whole blood, we observed that the administration of 17-β estradiol normalized just partially AChE activity, since its value was statically similar to the control group but also to the ovariectomized animals. Thus, AChE is an important therapeutic target and its pattern may differ on the basis of age (Das et al., 2001), as we observed in lymphocytes and whole blood of adult and middle-aged female rats.

It is known that decreased levels of estrogen may contribute to possible damage to the CNS (Schumacher et al., 2003). In this aspect, estrogens may also protect brain attenuating the formation of free radicals (Wise et al., 2001; Aguiar et al., 2008). Interestingly, AChE activity responds to various insults including oxidative stress, an important event that has been related to the pathogenesis and progression of a variety of CNS disorders (Mattson and Pedersen, 1998; Chauhan and Chauhan, 2006), OVX (Monteiro et al., 2005) and age (Moorthy et al., 2005; Aguiar et al., 2008). Thus, we also investigated the lipid peroxidation in order to explain a possible mechanism that contributes to the AChE activity alteration in our study.

The generation of ROS, which may happen with aging (Aguiar et al., 2008), and OVX (Ha et al. 2006) induce cellular membrane damage. AChE is an enzyme that contributes to the maintenance of cell membrane integrity and possible changes in permeability may occur during synaptic transmission and conduction (Das et al., 2001). Among the globular forms of this enzyme, G1 is cytosolic and G4 is membrane bound; G4 is also the most abundant form in several brain regions (Das et al., 2001; Aldunate et al., 2004; Das et al., 2005). Hormonal influences, such as replacement or estrogen depletion, on the cell membrane from adult or middle-aged animals could interfere in an oxidative process, leading to a change in conformation of AChE, thus, modifying its activity.

In our study, when comparing the results obtained with adult and middle-aged animals, we can suppose that age may interfere in the production of free radicals that cause lipid peroxidation in OVX or estrogenic hormone reposition. The intense action of lipid peroxidation in the adult ovariectomized animals (OVX-A) observed in the four brain structures studied reflects the susceptibility and vulnerability of the CNS of females of this age with a decline in ovarian hormone levels. Interestingly, the effect of 17-β estradiol supplementation was able to reduce lipid peroxidation values in most brain structures or even match it to normal levels, as in the case of HP. This fact can be explained because the HP of adult animals have a rich lipid content and high metabolic activity, which is more characteristic of younger ages than those of middle-aged (Aguiar et al., 2008). In adult animals, despite estrogen supplementation has been beneficial both in HP and

in other brain structures analyzed in our study, the 17-β estradiol acted more intensively at CC, ST, and CE.

Contrary to what happened in adult ovariectomized animals, the advanced age in HP and ST of the middle-aged OVX-MA may have reduced the lipid content and the metabolic activity of these structures (Aguiar et al. 2008), preventing the increase in lipid peroxidation in this condition. The different responses obtained in the four brain structures examined in middle-aged animals undergoing estrogen therapy (OVX + E2-MA) may be due to a variation in the concentration of antioxidant compounds in different brain regions. CE is an example that shows the lowest concentration of antioxidants (Vatassery 1992). Unlike what happened in adult animals, in the middle-aged animals the estrogen supplementation in the CE failed to reverse the action of lipid peroxidation.

It has been reported an increased local oxidative stress in fat accumulation in the body (Gower et al. 1998). These findings are important when adult and middle-aged ovariectomized animals without hormone reposition (OVX-A and OVX-MA) are analyzed. In this point, weight gain was more intense in the adult group compared to the middle-aged group. Despite that estrogen therapy have reversed lipid peroxidation in all brain structures of adult animals, this hormone failed to match the weight of the supplemented group (OVX + E2-A) to the control group. We can suggest that the use of 17-β estradiol was beneficial for the middle-aged animals where the average weight was equal to the control group. The fact that weight gain in OVX-MA was not so intense when compared to adult animals may have favored the action of the estrogen therapy in this age group. In this aspect, our study reveals that the lack of circulating estrogen may induce weight gain as well as increase lipid peroxidation especially in the adult animals. This is relevant, since oxidative stress increases when an increase in weight gain is observed (Mittal and Kant 2009).

In conclusion, the present results show that 17-β estradiol replacement is able to modulate the AChE activity and reduce lipid peroxidation and its response is dependent of age and brain structure analyzed. We also showed that OVX impaired more intensively the non-cholinergic system of middle-aged animals but estrogenic reposition can reverse this condition on this age. Furthermore, this study suggests that this hormone may influence the local immune response, enhancing the action of estrogen. Estrogen supplementation also normalizes body weight in middle-aged ovariectomized rats. These data provide new perspectives to estrogen treatment in the neurodiseases in women, since this hormone effects vary according to the age and different parts of the brain.

Conflicts of interest statement

The authors declare that there are no conflicts of interest.

Acknowledgments

This study was supported by CAPES (Coordenação de Aperfeiçoamento de Pessoal de Nível Superior) and Programa de Pós-Graduação em Medicina Veterinária — UFSM. The data presented is part of the Doctorate thesis of DBM.

References

Acosta JI, Mayer L, Talboom JS, Tsang CWS, Smith CJ, Enders CK, et al. Transitional versus surgical menopause in a rodent model: etiology of ovarian hormone loss impacts memory and the acetylcholine system. Endocrinology 2009;150:4248–59.
Aguiar RB, Dickel OE, Cunha RW, Monserrat JM, Barros DM, Martinez PE. Estradiol valerate and tibolone: effects on memory. Pharmacol Biochem Behav 2006;85:689–96.
Aguiar RB, Dickel OE, Cunha RW, Monserrat JM, Barros DM, Martinez PE. Estradiol valerate and tibolone: effects upon brain oxidative stress and blood biochemistry during aging in female rats. Biogerontology 2008;9:285–98.
Aldunate R, Casar JC, Brandan E, Inestrosa NC. Structural and functional organization of synaptic acetylcholinesterase. Brain Res Rev 2004;47:96–104.
Andreescu CE, Milojkovic BA, Haasdijk ED, Kramer P, De Jong FH, Krust A, et al. Estradiol improves cerebelar memory formation by activating estrogen receptor β. J Neurosci 2007;27:10832–9.
Baeza I, De Castro NM, Giménez-Llort L, De La Fuente M. Ovariectomy, a model of menopause in rodents, causes a premature aging of the nervous and immune systems. J Neuroimmunol 2010;219:90–9.
Battisti V, Schetinger MRC, Maders LDK, Santos KF, Bagatini MD, Correa MC, et al. Changes in acetylcholinesterase (AChE) activity in lymphocytes and whole blood in acute lymphoblastic leukemia patients. Clin Chim Acta 2009;402:114–8.
Bergmeyer HU. Methods of enzymatic analysis, vol. 3. London: Academic Press; 1983. p. 118–33.
Böyum A. Isolation of mononuclear cells and granulocytes from human blood. Isolation of mononuclear cells by one centrifugation, and of granulocytes by combining centrifugation and sedimentation at 1 g. Scand J Clin Lab Invest Suppl 1968;97:77–89.
Bradford M. A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 1976;72:248–54.
Browne C, Tobin JR, Voytko ML. Effects of two years of conjugated equine estrogens on cholinergic neurons in young and middle-aged ovariectomized monkeys. Brain Res 2009;1264:13–23.
Chakraborty TR, Ng L, Gore AC. Age-related changes in estrogen receptor β in rat hypothalamus: a quantitative analysis. Endocrinology 2003;144:4164–71.
Chauhan V, Chauhan A. Oxidative stress in Alzheimer's disease. Pathophysiology 2006;13:195–208.
Cimarosti H, Zamin LL, Frozza R, Nassif M, Horn AP, Tavares A, et al. Estradiol protects against oxygen and glucose deprivation in rat hippocampal organotypic cultures and activates Akt and inactivates GSK-3β. Neurochem Res 2005;30:191–9.
Craig MC, Brammer M, Maki PM, Fletcher PC, Daly EM, Rymer J, et al. The interactive effect of acute ovarian suppression and the cholinergic system on visuospatial working memory in young women. Psychoneuroendocrinology 2010;35:987–1000.
Daniel JM, Hulst JL, Berbling JL. Estradiol replacement enhances working memory in middle-aged rats when initiated immediately after ovariectomy but not after a long-term period of ovarian hormone deprivation. Endocrinology 2006;147:607–14.
Das A, Dikshit M, Nath C. Profile of acetylcholinesterase in brain areas of male and female rats of adult and old age. Life Sci 2001;68:1545–55.
Das A, Dikshit M, Nath C. Role of molecular isoforms of acetylcholinesterase in learning and memory functions. Pharmacol Biochem Behav 2005;81:89–99.
Das UN. Acetylcholinesterase and butyrylcholinesteraseas possible markers of low-grade systemic inflammation. Med Sci Monit 2007;13:214–21.
Ellman GL, Courtney DK, Andres V, Featherstone RM. A new and rapid colorimetric determination of acetylcholinesterase activity. Biochem Pharmacol 1961;7:88–95.
Feng Z, Cheng Y, Zhang J. Long-term effects of melatonin or 17 beta-estradiol on improving spatial memory performance in cognitively impaired, ovariectomized adult rats. J Pineal Res 2004;37:198–206.
Fitzgerald BB, Costa LG. Modulation of muscarinic receptors an acetylcholinesterase activity in lymphocytes and brain areas following repeated organophosphate exposure in rats. Fundam Appl Toxicol 1993;20:210–6.
García-Segura LM, Azcoitia I, DonCarlos LL. Neuroprotection by estradiol. Prog Neurobiol 2001;63:29–60.
Gibbs RB, Aggarwal P. Estrogen and basal forebrain cholinergic neurons: implications for brain aging and Alzheimer's disease-related cognitive decline. Horm Behav 1998;34:98-111.
Gibbs RB. Estrogen therapy and cognition: a review of the cholinergic hypothesis. Endocr Rev 2010;31:224–53.
Gower BA, Nagy TR, Goran MI, Toth MJ, Poehlman ET. Fat distribution and plasma lipid–lipoprotein concentrations in pre- and postmenopausal women. Int J Obes Relat Metab Disord 1998;22:605–11.
Grisaru D, Sternfeld M, Eldor A, Glick D, Soreq H. Structural roles of acetylcholinesterase variants in biology and pathology. Eur J Biochem 1999;264:272–86.
Ha BJ, Lee SH, Kim HJ, Lee JY. The role of Salicornia herbacea in ovariectomy-induced oxidative stress. Biol Pharm Bull 2006;29:1305–9.
Hogervorst E, Bandelow S. Sex steroids to maintain cognitive function in women after the menopause: a meta-analyses of treatment trials. Maturitas 2010;66:56–71.
Kasa P, Rakonczay Z, Gulyat K. The cholinergic system in Alzheimer's disease. Prog Neurobiol 1997;52:511–35.
Kawashima K, Fujii T. Extraneuronal cholinergic system in lymphocytes. Pharmacol Ther 2000;86:29–48.
Kawashima K, Fujii T. The lymphocytic cholinergic system and its contribution to the regulation of immune activity. Life Sci 2003;74:675–96.
Li R, Shen Y. Estrogen and brain: synthesis, function and diseases. Front Biosci 2005;10:257–67.
Marcondes FK, Bianchi FJ, Tanno AP. Determination o the estrous cycle phases of rats: some helpful considerations. Braz J Biol 2002;62:609–14.
Mattson MP, Pedersen WA. Effects of amyloid precursor protein derivatives and oxidative stress on basal forebrain cholinergic systems in Alzheimer's disease. Int J Dev Neurosci 1998;16:737–53.
McEwen BS, Alves SE. Estrogen actions in the central nervous system. Endocrinol Rev 1999;20:279–307.
McEwen BS. Estrogens effects on the brain: multiple sites and molecular mechanisms. J Appl Physiol 2001;91:2785–801.

Mesulam MM, Guillozet A, Shaw P, Levey A, Duysen EG, Lockridge O. Acetylcholinesterase knockouts establish central cholinergic pathways and can use butyrylcholinesterase to hydrolyse acetylcholine. Neuroscience 2002;110:627–39.
Mittal PC, Kant R. Correlation of increased oxidative stress to body weight in disease-free post-menopausal women. Clin Biochem 2009;42:1007–11.
Monteiro SC, Stefanello FM, Vianna LP, Matté C, Barp J, Belló-Klein A, et al. Ovariectomy enhances acetylcholinesterase activity but does not alter ganglioside content in cerebral cortex of female adult rats. Metab Brain Dis 2005;20:35–44.
Moorthy K, Yadav UCS, Siddiqui MR, Mantha AK, Basir SF, Sharma D, et al. Effect of hormone replacement therapy in normalizing age related neuronal markers in different age groups of naturally menopausal rats. Biogerontology 2005;6:345–56.
Ohkawa H, Ohishi N, Yagi K. Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. Anal Biochem 1979;95:351–8.
Pereira RTS, Porto CS, Godinho RO, Abdalla FMF. Effects of estrogens on intracellular signaling pathways linked to activation of muscarinic acetylcholine receptors and on acetylcholinesterase activity in rat hippocampus. Biochem Pharmacol 2008;75:1827–34.
Pozzi S, Benedusi V, Maggi A, Vegeto E. Estrogen action in neuroprotection and brain inflammation. Ann N Y Acad Sci 2006;1089:302–23.
Rao AA, Gumpeny RS, Das UN. Elevated butyrylcholinesterase and acetylcholinesterase may predict the development of type 2 diabetes mellitusand Alzheimer's disease. Med Hypotheses 2007;69:1272–6.
Razani-Boroujerdi S, Behl M, Hahn F, Pena JC, Hutt J, Sopori ML. Role of muscarinic receptors in the regulation of immune and inflammatory responses. J Neuroimmunol 2008;194:83–8.
Rocca WA, Shuster LT, Grossardt BR, Maraganore DM, Gostout BS, Geda YE, et al. Long-term effects of bilateral oophorectomy on brain aging: unanswered questions from the Mayo Clinic Cohort Study of Oophorectomy and Aging. Womens Health 2009;5:39–48.
Rocha JBT, Emanuelli T, Pereira ME. Effects of early undernutrition on kinetic parameters of brain acetylcholinesterase from adult rats. Acta Neurobiol Exp 1993;53:431–7.
Sales S, Ureshino RP, Pereira RTS, Luna MSA, Oliveira MP, Yamanouye N, et al. Effects of 17β-estradiol replacement on the apoptotic effects caused by ovariectomy in the rat hippocampus. Life Sci 2010;86:832–8.
Schumacher M, Weill-Engerer S, Liere P, Robert F, Franklin RJM, Garcia-Segura LM, et al. Steroid hormones and neurosteroids in normal and pathological aging of the nervous system. Prog Neurobiol 2003;71:3-29.
Signorelli SS, Neri S, Sciacchitano S, Di Pino L, Costa MP, Marchese G, et al. Behaviour of some indicators of oxidative stress in postmenopausal and fertile women. Maturitas 2006;53:77–82.
Shughrue PJ, Lane MV, Merchenthaler I. Comparative distribution of estrogen receptor-α and -β mRNA in the rat central nervous system. J Comp Neurol 1997;388:507–25.
Tam J, Danilovich N, Nilsson K, Sairam MR, Maysinger D. Chronic estrogen deficiency leads to molecular aberrations related to neurodegenerative changes in follitropin receptor knockout female mice. Neuroscience 2002;114:493–506.
Thakur MK, Sharma PK. Aging of brain: role of estrogen. Neurochem Res 2006;31:1389–98.
Vatassery GT. Vitamin E: neurochemistry and implication for Parkinson's disease. Ann N Y Acad Sci 1992;669:92-110.
Vegeto E, Benedusi V, Maggi A. Estrogen anti-inflammatory activity in brain: a therapeutic opportunity for menopause and neurodegenerative diseases. Front Neuroendocrinol 2008;29:507–19.
Wessler I, Kilbinger H, Bittinger F, Unger R, Kirkpatrick CJ. The non-neuronal cholinergic system in humans: expression, function and pathophisiology. Life Sci 2003;72:2055–61.
Wise PM, Dubal DB, Wilson ME, Rau SW, Bottner M. Neuroprotective effects of estrogen — new insights into mechanisms of action. Endocrinology 2001;142:969–73.
Worek F, Mast U, Kiderlen D, Diepold C, Eyer P. Improved determination of acetylcholinesterase activity in human whole blood. Clin Chim Acta 1999;288:73–90.
Zhou FM, Wilson CJ, Dani JA. Cholinergic interneuron characteristics and nicotinic properties in the striatum. J Neurobiol 2002;53:590–605.
Zimmerman G, Soreq H. Termination and beyond: acetylcholinesterase as a modulator of synaptic transmission. Cell Tissue Res 2006;326:655–69.
Yener T, Tunc AT, Aslan H, Aytan H, Caliskan AC. Determination of oestrous cycle of the rats by direct examination: how reliable? Anat Histol Embryol 2007;36:75–7.

MANUSCRITO 1:

Cholinesterases activities in ovariectomized rats experimentally demyelinated with ethidium bromide and treated with 17-β estradiol

Danieli B. Martins, Cinthia M. Mazzanti, Dominguita Graça, Marciélen Pagnoncelli, Raqueli França, Roberto M. Maciel, Márcio M. Costa, Naiara Stefanello, Michelle M. Rosa, Maribel Rubin, Estevan M. Souza, Heloisa E. Palma, Marta F. Duarte, Alexandre Mazzanti, Diego Beckmann, Maria R. Schetinger, Sonia T. A. Lopes

Manuscrito a ser submetido para Experimental Neurology

Cholinesterases activities in ovariectomized rats experimentally demyelinated with ethidium bromide and treated with 17-β estradiol

Danieli B. Martins[a*], Cinthia M. Mazzanti[a], Dominguita Graça[a], Marciélen Pagnoncelli[a], Raqueli França[a], Roberto M. Maciel[a], Márcio M. Costa[a], Naiara Stefanello[b], Michelle M. Rosa[b], Maribel Rubin[b], Estevan M. Souza[a], Heloisa E. Palma[a], Marta F. Duarte[b], Alexandre Mazzanti[a], Diego Beckmann[a], Maria R. Schetinger[b], Sonia T. A. Lopes[a*]

[a]Departamento de Clínica de Pequenos Animais, [b]Departamento de Química, Universidade Federal de Santa Maria, Brazil.

*Corresponding authors:

Danieli Brolo Martins

Email: vetdanielimartins@yahoo.com.br

Sonia Terezinha dos Anjos Lopes

Email: soniatldosanjos@hotmail.com

Hospital Veterinário Universitário, Centro de Ciências Rurais, Universidade Federal de Santa Maria, Av. Roraima 1000, 97105-900, Santa Maria, RS, Brazil.

Telephone number: + 55 -55 -3220-8814

Fax number: +55 -55 -3220 -8978

Highlights

Demyelinated ovariectomized rats has an increase in the activity of acetylcholinesterase (AChE) in different structures of the brain, in the demyelination and remyelination phases.

Estrogen supplementation normalized the AChE activity in the demyelination phase and inhibited the enzyme activity in the cerebral cortex, striatum and cerebellum at the remyelination phase.

Demyelinated ovariectomized rats demonstrated an increase in total boold AChE in both phases, and in the lymphocyte AChE in the demyelination phase.

17-β estradiol modulates the neuronal and non-neuronal AChE activity in the experimental demyelination by ethidium bromide

Keywords

Acetylcholinesterase

Butyrylcholinesterase

Estrogen

Neuroprotection

Demyelination

Remielination

Abbreviations:

ACh, acetylcholine; AChE, acetylcholinesterase; BuChE, butyrylcholinesterase; CNS, central nervous system; PNS, peripheral nervous system; MS, multiple sclerosis; AD, Alzheimer's disease; EB, ethidium bromide; OVX, ovariectomy; ST, striatum; HP, hippocampus; CC, cerebral cortex; CE, cerebellum.

Abstract

The aim of this study was to investigate the effects of 17-β estradiol supplementation under the parameters related to the acetylcholinesterase (AChE) activity in the brain, total blood and lymphocytes, as well as serum butyrylcholinesterase (BuChE) activity in experimental demyelinating ovariectomized rats. The animals were divided into 5 groups (n = 10) to evaluate the demyelination phase and 5 groups (n = 10) to evaluate the remyelination phase. In each phase the groups were: control sham rats (Sham); ovariectomized rats, not demyelinated (OVX); demyelinated ovariectomized rats (OVX+EB); ovariectomized rats, not demyelinated, treated with 17-β estradiol (OVX+E2), and demyelinated ovariectomized rats treated with 17-β estradiol (OVX+EB+E2). Our results demonstrated that the OVX+EB group presented an increase in the activity of AChE in different structures of the brain, in the demyelination and remyelination phases. Estrogen supplementation normalized the AChE activity in the demyelination phase and inhibited the enzyme activity in the cerebral cortex, striatum and cerebellum at the remyelination phase. Demyelinated ovariectomized rats demonstrated an increase in total boold AChE in both phases, and in the lymphocyte AChE in the demyelination phase, what may indicate an inflammatory process. However, estrogen reposition reduces the AChE activity in total blood and lymphocytes. Thus, 17-β estradiol modulates the neuronal and non-neuronal AChE activity in the experimental demyelination by ethidium bromide, suggesting that this hormone can be a good alternative for a new therapeutic strategy in neurodegenerative diseases in women.

Introduction

Demyelinating diseases are a serious consequence of the destruction of myelin sheaths present in the central nervous system (CNS) and peripheral nervous system (PNS) (Siegel, 1999). Such changes affect the transmission of nerve impulses, leading to the onset of neurological signs. Among the demyelinating neurological disorders, the most prominent is the multiple sclerosis (MS), a chronic inflammatory disease that commonly affects young adult women in the Occident (Kipp et al., 2009).

Estrogens, such as the ovarian hormone 17-β estradiol, have been considered a potential therapeutic tool to combat the inflammation present in demyelinating diseases, such as MS (Zhu et al., 2007; Zhu and Glaser, 2008; El-Etr et al., 2011). This statement is based on the fact that women with MS have an improvement in clinical symptoms of the disease during pregnancy, period in which there is an increased level of circulating estrogen, and later in the postpartum when estrogen levels fall, there is a worsening of the disease (Sicotte et al., 2002; Confravreux et al., 2003). However, the immunomodulating role of estrogens related to inflammatory demyelinating diseases remains unclear (Straub, 2007).

The presence or absence of estrogen can lead to neurochemical changes in different brain systems, such as the cholinergic system (Sato et al., 2003; Takur and Sharma 2006). Cholinergic neurons and their projections are widely distributed throughout the CNS with an essential role in regulating several vital functions, such as learning and memory (Mesulam et al. 2002). However, a better understanding of the mechanisms that underlie the effects of estrogens on cognition may lead to the development of more effective and targeted therapies that benefit the brain (Gibbs et al., 2010).

Cholinesterases are enzymes present in cholinergic and non-cholinergic tissues as well as in blood and other body fluids. They are divided into two classes according to their catalytic properties and specificity for substrates, sensitivity to inhibitors and tissue distribution (Schetinger et al., 2000). The enzyme acetylcholinesterase (AChE; E.C. 3.1.1.7), found in the cholinergic terminal, is a specific choline esterase, hydrolyzing predominantly choline esters (acetylcholine - ACh) and characterized by high levels in the brain, nerve and red blood cells (Das, 2007). On the other hand, butyrylcholinesterase (BuChE, EC 3.1.1.8), or pseudocholinesterase, hydrolyzes other esters such as butyrilcoline (Cokugras, 2003). It is found in plasma and CNS, and has a much more restricted neuronal distribution when compared to AChE (Mesulam et al., 2002; Das, 2007). Another important function of the cholinesterase is involved in the regulation of immune function (Kawashima and Fujii, 2003, 2004; Das, 2007). The "cholinergic anti-inflammatory pathway" represents a physiological mechanism by which the nervous system interacts with the innate immune system to restrain systemic inflammatory responses (Gallowitsch-Puerta and Pavlov, 2007).

AChE has also been investigated as an important therapeutic target in various neurodegenerative diseases such as Alzheimer's disease (Greig et al., 2002; Vezenkova et al., 2012) and other neurologic disorders involved in events of experimental demyelination and remyelination by ethidium bromide (EB) (Mazzanti et al., 2006a; Mazzanti et al., 2007).

Toxic demyelination by EB is one of the most commonly used models to explore the reparative capacity of the CNS (Stangel and Hartung, 2002; Guazzo 2005). EB selectively destroys glial cells (oligodendrocytes and astrocytes), which control the processes of demyelination and remyelination (Levine and Reynolds, 1999; Mazzanti et al., 2009).

Thus, several studies have suggested that steroid hormones could be used as effective therapy for MS (Sicotte et al., 2002; Kaaja and Greer, 2005), since steroids participate in regulating demyelination and remyelination in the CNS (Zhu and Glaser, 2008; Acs et al., 2009; Taylor et al., 2010). Therefore, we hypothesized that 17-β estradiol supplementation could improve parameters related to the AChE activity in the brain, total blood and lymphocytes, as well as serum BuChE. Also, we investigated the effects on learning and memory in a model of toxically induced CNS demyelination in ovariectomized female rats in order to verify the participation of this steroid hormone in the modulation of cholinergic neurotransmission and immune response.

Materials and Methods

Chemicals and reagents

The hormone 17-β estradiol, substrate and buffers were obtained from Sigma Chemical Co (St. Louis, MO, USA). All the other reagents used in the experiments were of analytical grade and of the highest purity.

Animals

Fifty adult female Wistar rats (60 days, 200-220 g) for demyelination phase and fifty adult female Wistar rats (60 days, 200-220 g) for remyelination were used in this experiment. The animals were kept in a constant temperature (21 ± 2°C) with light cycle 12h light / 12h dark with free access to food and water. The study was conducted in accordance with the Federal University of Santa Maria (UFSM) Ethics Committee Guidelines for Experiments with Animals (process 86/2009). A soy-free rat chow (Supra®, Alisul, Carazinho, RS, Brazil) was used to avoid phytoestrogen interference.

After a week of adaptation, the animals were submitted to vaginal cytology for 2 weeks to verify the presence of normal estrous cycle (Marcondes et al. 2002; Yener et al. 2007). Only rats with normal estrous cycle entered the experiment.

Experimental groups and treatments

The animals were divided randomly into 5 groups (n = 10) to evaluate the demyelination phase (seven days after EB surgery) and 5 groups (n = 10) to evaluate the remyelination phase (21 days after EB surgery).

In each phase, the animals were divided in sham rats (Sham); ovariectomized rats, not demyelinated, treated just with vehicle (canola oil) (OVX); demyelinated ovariectomized rats treated just with vehicle (canola oil) (OVX+EB); ovariectomized rats, not demyelinated, treated with 17-β estradiol (OVX+E2) and demyelinated ovariectomized rats treated with 17-β estradiol (OVX+EB+E2).

On the same day of the surgical procedure of demyelination, 0.1µg/g of body weight of 17-β estradiol (according to Moorthy et al., 2005) was administered by gavage; afterwards, the animals were given once a day. We started the hormonal reposition in the same day of the experimental demyelination procedure. Intervals of months between the ovariectomy (OVX) and the beginning of the hormonal treatment should be avoided because estrogen supplementation therapy has no action when it is started after a long-term hormone deprivation (Daniel et al., 2006). Moreover, our choice for the oral administration of estrogens was because it is a practical and widely used method by women (Aguiar et al., 2008). Canola oil vehicle and 17-β estradiol were administered with the same volume. The administration of vehicle or hormone was continuous for 7 days to the groups of demyelination phase, in the following day (euthanasia day) there was no treatment. In the same way, the administration of vehicle

or hormone was continuous for 21 days to the groups of remyelination phase; in the following day (euthanasia day) there was no treatment.

Surgical Procedures

Anesthesic procedure

The animals were anesthetized initially with 4% halothane in a 40% O_2/60% N_2O mixture and the anesthesia was maintained during both surgeries with 1.5–2.5% halothane breathing spontaneously via facemask.

Ovariectomy (OVX)

The control group underwent a sham surgery, with an incision in the Alba line, but with no gonad removal, while in the other groups the gonads were completely removed. After a recovery period of 10 days, all animals were submitted again to vaginal smear to confirm the efficacy of the surgical procedures (4 days). In the day 15 after ovariectomy the animals were submitted to the demyelination procedure. This time we needed to bring down estrogen levels and thus we could observe the absence of estrous cycle by vaginal cytology in the ovariectomized animals.

Experimental demyelination

With the aid of a roof motor of orthodontic use and a drill number 2, a hole was made 0.85 cm to the right of the bregma until exposing the duramater. With the use of a Hamilton syringe with a removable needle of caliber 26 s, the solutions were injected 2 mm deep into the subcortical white water. Ten microliters of EB (0.1%) was injected in the animals from the OVX+EB and OVX+EB+E2 groups, and the same volume of 0.9% saline solution was injected in the animals from the Sham, OVX and OVX+E2

groups. The duramater was left open and the skin, together with the remainder of the subcutaneous tissue, was sutured with a 4.0 nylon thread.

Laboratorial analysis

Serum determination of 17-β estradiol

We used the enzymatic method of automated immuno-chemiluminescence with Immulite 2000 analyzer (Diagnostic Products Corporation, Los Angeles, CA, USA) and specific hormone kit (Immulite Estradiol).

Brain tissue preparation

The animals were submitted to euthanasia being previously anesthetized with halothane, and the brain structures were immediately removed, separated into striatum (ST), hippocampus (HP), cerebral cortex (CC) and cerebellum (CE), placed in a solution of 10 mMTris–HCl, pH 7.4, on ice at a proportion of 1:10 (w/v) and after homogenized. The homogenate was centrifuged at 1800 rpm for 10 min and the resulting supernatant (S_1) was stored at -30°C until use. Protein was determined previously in a strip that varied for each structure: ST (0.4 mg/ml), HP (0.8 mg/ml), CC (0.7 mg/ml), and CE (0.6 mg/ml) as determined by the Coomassie blue method according to Bradford (1976) using bovine serum albumin as standard solution.

Cerebral AChE enzymatic assay

The AChE enzymatic activity was determined by a modification of the spectrophotometric method of Ellmann et al. (1961) as previously described by Rocha

et al. (1993). The reaction mixture (2 ml final volume) was composed of 100 mM K+-phosphate buffer, pH 7.5 and 1 mM 5,50-dithiobisnitrobenzoic acid (DTNB). The method is based on the formation of yellow anion, 5,50-dithio-bis-acidnitrobenzoic, measured by absorbance at 412 nm for 2-min incubation at 25°C. The enzyme (40–50 mg of protein) was pre-incubated for 2 min. The reaction was initiated by adding 0.8 mM acetylthiocholine iodide (AcSCh). All samples were run in duplicate or triplicate, and the enzyme activity was expressed in µmoles AcSCh/h/mg of protein.

Blood sample collection

The blood was collected in vaccutainer tubes using EDTA 10% as anticoagulant. For the AChE activity in whole blood, the samples were hemolysed with phosphate buffer, pH 7.4 containing Triton X-100 (0.03%) and stored at -20 °C for one week. For the AChE activity in lymphocytes, the peripheral lymphocytes were isolated and AChE activity was measured immediately.

Isolation of the cells

The peripheral lymphocytes were isolated using Ficoll Hypaque density gradient as described by Böyum (1968). After separation, only samples with at least 95% of lymphocytes, as verified in the coulter STKS (Miami—USA), were used. Lymphocyte viability and integrity were confirmed by determining the percentage of cells, excluding 0.1% trypan blue and measuring lactate dehydrogenase (LDH) activity (Bergmeyer, 1983).

Determination of AChE activity in lymphocytes

After the isolation of the lymphocytes, the AChE activity was determined according to the method described by Ellman et al. (1961) and modified by Fitzgerald

and Costa (1993). Briefly, proteins of all samples were adjusted to 0.1–0.2 mg/ml. 0.2 ml of intact cells were added to a solution containing 1.0 mM acetylthiocholine (ATC), 0.1 mM 5,5′-dithiobis (2-nitrobenzoic acid) (DTNB), and 0.1 M phosphate buffer (pH 8.0). Immediately before and after incubation for 30 min, at 27 °C, the absorbance was read on a spectrophotometer at 412 nm. AChE was calculated from the quotient between lymphocyte AChE activity and protein content, and the results were expressed as μmol/h/mg of protein.

Determination of AChE in whole blood and serum BuChE activities

The AChE enzymatic assay was determined by the method of Ellmann et al. (1961) modified by Worek et al. (1999). The specific activity of whole blood AChE was calculated from the quotient between the AChE activity and hemoglobin content and the results are expressed as mU/μmol of whole blood. The BuChE activity in the serum was determined by the method of Ellmann et al. (1961), except that the acetylcholine substrate was replaced by butyrylthiocholine and the results were expressed in the μmol BcSCh/h/mg of protein.

Histological studies

Three rats from each group were used for histological analysis of the lesion. These rats were perfused under deep anesthesia with 10% buffered formalin via the left ventricle at 7 (demyelination) and 21 (remyelination) days after injection of EB. Brain stem coronal slices of the lesion were embedded in paraffin for routine processing and 5 mm sections were produced and stained with hematoxilin and eosin (H&E).

Statistical analysis

For the biochemistry tests the statistical analysis used was one-way ANOVA, followed by Duncan's multiple range tests. All the data are expressed as the mean ± standard error. Differences were considered significant when the probability was $p < 0.05$ in all experiments.

Results

All animals in the sham group showed normal estrous cycle during the experiment. The estrogen levels were measured in all groups both in the demyelination and remyelination phases (Figure 1). In the demyelination phase, there was a significant reduction ($p<0.05$) of the estrogen levels of ovariectomized animals compared with the sham group. In the remyelination phase, groups supplemented with 17-β estradiol (OVX+E2 and OVX+E2+EB) were statistically similar to the sham group while the non-supplemented groups (OVX and OVX + EB) remained with a significant reduction ($p<0.05$) when compared with control.

The AChE activity was evaluated in the supernatant (S1) in all brain structures (Figure 2). In relation to CE, in the demyelination phase there was a significant increase ($p<0.05$) in the AChE activity in the OVX+EB group, and especially, in the OVX group. However, in the process of remyelination, only the animals from the OVX+EB group had a significant increased AChE activity ($p<0.05$), while the OVX + E2 group showed a significant decrease ($p<0.05$) in the enzyme activity. The groups OVX and OVX+E2+EB showed activity statistically similar to the sham and OVX and OVX+E2+EB groups.

In the CC, in the demyelination phase, there was a significant increase ($p<0.05$) in the AChE activity in the OVX+E2 group and mainly in the OVX+EB group. The OVX+E2+EB group showed activity statistically similar to the sham and OVX+E2. In

the remyelination phase, OVX and OVX+EB groups had a significantly increased AChE activity ($p<0.05$), while the OVX+E2+EB group significantly reduced ($p<0.05$) the enzyme activity.

The ST had a significant reduction ($p<0.05$) in the AChE activity in the OVX+E2 group in the demyelination phase. For the remyelination phase, the OVX+E2, and especially OVX and OVX+E2+EB groups showed a significant reduction ($p<0.05$) in the enzyme activity. The OVX+EB group showed a significant increase ($p<0.05$) in the AChE activity.

In the HP, during the demyelination, the OVX+E2, OVX+E2+EB and, mainly OVX+EB group had a significantly increased ($p<0.05$) AChE activity. However, the OVX group showed activity statistically similar to sham and OVX+E2 groups. In the remyelination phase, OVX+E2+EB and, especially, OVX and OVX+EB groups had significantly increased ($p<0.05$) AChE activity. The OVX+E2 group showed activity statistically similar to sham and OVX+E2+EB.

The results of serum BuChE and total blood and lymphocytes AChE are demonstrated in the Figure 3. In the demyelination phase, the serum BuChE activity was significantly increased ($p<0.05$) in OVX+E2 and especially in OVX+E2+EB group. However, OVX and OVX+EB groups showed activity statistically similar to sham and OVX+E2 groups. In the remyelination phase, there was a reduction in the enzyme activity ($p<0.05$) in the OVX+EB and OVX+E2+EB, and especially in the OVX. The OVX+E2 group showed a significant increase ($p<0.05$) of the serum BuChE activity at this stage.

The activity of AChE in total blood during the demyelination showed a significant gradual increase ($p<0.05$) in the OVX+E2, OVX+E2+EB and OVX+EB groups, respectively. In the remyelination phase, there was a significant reduction

($p<0.05$) in the enzyme activity in the OVX group. However, the OVX+E2+EB and, especially, OVX+EB and OVX+E2 groups remained with a significant increase ($p<0.05$) in the blood AChE activity.

In the demyelination phase, a significant decrease ($p<0.05$) was observed in the AChE activity of lymphocytes in OVX, OVX+E2 and OVX+EB+E2 groups, while the OVX+EB group showed a significantly increased ($p<0.05$) enzyme activity. In the process of remyelination, all groups showed physiological values of the enzyme activity close to the sham group. However, the OVX+E2+EB group showed an AChE activity of lymphocytes significantly higher ($p<0.05$) when compared with OVX and OVX+EB groups.

The histological analysis of the ventral area of the cisterna pontis revealed alterations caused by the EB injection (Figure 4). After 7 days, loss of myelin sheaths was seen as status spongiosus of the tissue in the OVX+EB and OVX+EB+E2 groups. After 21 days of EB procedure, an extensive area of demyelination with cystic cavities was observed in the OVX-EB group, while lesions were seen where spongiosis subsided through remyelination in the OVX+EB+E2 group.

Discussion

In the present study we verified the effect of 17-β estradiol on the AChE activity of the brain (ST, CC, HP and CE), blood and lymphocyte, as well as on the serum BuChE of demyelinated ovariectomized female rats. We have chosen 17-β estradiol because this hormone has been shown to be neuroprotective in nerve cells, thereby increasing the process of remyelination *in vitro* (Zhu and Glaser, 2008) and assisting in the modulation of the cholinergic system (Feng et al., 2004). This is relevant since cholinergic deficits contribute to cognitive impairment present in several

neurodegenerative events (Das et al., 2002; Schliebs and Arendt, 2006). Furthermore, ovariectomy is a model considered to be the best tool to mimic human ovarian hormone loss (Sato et al., 2003; Baeza et al. 2010) while EB was used as a demyelinating agent in order to mimic the pathophysiologic process of demyelinating diseases such as MS (Nassar et al., 2009). The pathophysiological mechanisms involved in the development of neurodegenerative diseases remain poorly understood. Thus, there is a need to investigate new therapeutic strategies and estrogens appear as a great promise due to their immunomodulatory, neuroprotective and promyelination actions (El-Etr et al., 2011).

In relation to the AChE activity in the CNS, our results revealed that the CE, in the demyelination phase, presented an increase in the activity of this enzyme in the OVX and OVX+EB groups; this increase was greater in the ovariectomized animals without EB. In the process of remyelination, there was an inhibition of enzyme activity in the OVX+E2 group, whereas the enzyme activity of the OVX and OVX+EB+E2 groups were similar in the values to the sham and OVX + E2 groups. Although the EB is a major inhibitor of the AChE activity of the CE *in vitro* (Mazzanti et al. 2006a), in our study the isolated effect of EB associated to ovariectomy caused stimulation of the enzyme in both phases which induces an AChE activation and leads to a fast ACh degradation and the subsequent downstimulation of ACh receptors (Grisaru et al., 1999). The different behaviors of the AChE activity in the OVX group in each phase may be related to the time of estrogen deprivation (Daniel et al., 2006), since this decrease was not as strong in the OVX + E2 group. In addition, the duration of estrogen therapy should be considered important for an influence on the brain AChE activity (Pereira et al., 2008).

In relation to CC, despite the OVX had no influence on the activity of AChE in the process of demyelination, there was an increase in the enzyme activity in the remyelination phase. Stimulation of the AChE activity was also observed by Monteiro et al. (2005), in 3 month-old female rats with 30 days of ovariectomy. In both phases, demyelination or remyelination, OVX+EB showed an activation of the activity of AChE, which could be related to a high degree of toxicity and inflammation promoted by the EB experimental model of demyelination (Levine and Reynols, 1999) in females with no estrogen supplementation. Among the groups who received estrogen supplementation, in the demyelination phase, the OVX+E2 group presented an increase in the AChE activity, although less intense when compared with the OVX+EB group. The OVX+E2+EB group showed similar activity to the sham and OVX+E2 groups. In the remyelination phase, the OVX and OVX+E2 groups showed a decrease in the AChE activity when compared with the sham group. The CC is characterized by the presence of estrogen receptor β (ER-β) (Shughrue and Merchenthaler, 2000), which may have facilitated the action of 17-β estradiol.

In ST, a structure rich in cholinergic pathways, only the OVX+E2 group showed a decrease in the activity of AChE in the demyelination phase. However, in the remyelination phase, the OVX and OVX+E2+EB groups showed a more intense inhibition of the AChE activity when compare with the OVX+ E2 group. On the other hand, the OVX+EB group showed an increase in the enzyme activity. Our study agrees with the results found in ST by Mazzanti et al. (2006b) where no changes were found *in vivo* in the AChE activity 7 days after demyelination by EB. In addition, OVX contributes to a heterogeneous change in the AChE activity in each phase (Das et al., 2002). Thus, in the remyelination phase, we can see that the estrogen supplementation could partially reverse the action of OVX. However, the combination of OVX with EB

impeded a better action of the estrogen hormone, which may have influenced its action on estrogen receptors α and β mRNA expressed by ST (Kuppers and Beyer, 1999).

Estrogens have been shown to have numerous effects on the functions of HP, a brain structure related to cognition, memory and learning (McEwen, 2002; Feng et al., 2004). In the demyelination phase, there was an increase in the AChE activity in the OVX+EB+E2, OVX+E2 OVX+EB groups, being that OVX+EB presented the highest increase. The OVX group showed enzyme activity similar to the sham and OVX+E2 groups. In the remyelination phase, the increase in the enzyme activity remained high; being more intense in the OVX and OVX+EB groups, while in the OVX+EB+E2 group the increase of AChE activity was less intense. The OVX+E2 group behaved similarly to the OVX+EB+E2 and sham groups. Although EB is an inhibitor of the AChE activity in HP *in vitro* (Mazzanti et al., 2006a), in our *in vivo* study we detected activation of the enzyme activity in the groups in which the EB was used, in both phases, mainly in the group where there was no estrogen supplementation (OVX + EB). AChE can increase its activity in inflammatory conditions (Das, 2007). The inflammation induced by EB (Levine and Reynolds, 1999) is potentiated by the disruption of the blood-brain barrier that occurs after the injection of the drug in the brainstem of animals (Bondan et al., 2002), since this barrier acts in the exclusion of inflammatory cells in the CNS. Thus, inflammation helps in the elimination of the inducing agent of demyelination and is also able to assist in the repair process (Das, 2006). The high AChE activity of the OVX group was more exacerbated in the remyelination phase, a period where estrogen levels were lower in the bloodstream, which may contribute to possible damage to the CNS (Schumacher et al., 2003). However, it is possible to see that the supplementation with 17-β estradiol in the OVX+EB+E2 group partially reversed the damage in HP induced by EB and ovariectomy (OVX+EB) in both demyelination and remyelination phases.

Estrogen supplementation for 21 days (remyelination phase) in the OVX+E2 group allowed the enzyme activity to be equivalent to OVX+E2+EB and the control groups. Interestingly, an increased activity of AChE can decrease the amount of ACh available in the synaptic cleft thus reducing the cholinergic activity in this brain region.

Our study also investigated the role of non-neuronal cholinergic system regarding the experimental model of demyelination with EB and ovariectomized rats with or without supplementation with 17-β estradiol since the evaluation of the non-neuronal cholinergic system is a relevant parameter that should be related to menopausal status, estrogenic therapy, as well as brain cholinergic system (Martins et al., 2012).

In the demyelination phase, the serum BuChE showed an increase in its activity in the OVX+E2 group, and mainly the OVX+EB+E2 group, whereas the OVX and OVX+EB groups were similar to the control and the OVX+E2 groups. On the other hand, in the same phase, the whole blood AChE also showed an increased activity in OVX+E2, OVX+EB+E2, and mainly in the OVX+EB group. However, in the remyelination phase, the OVX+EB, OVX+E2+EB, and especially OVX group showed a decrease in activity of serum BuChE, while the OVX+E2 group showed an increase in the enzyme activity. Our result is in accordance with Monteiro et al. (2005) who also found a decrease in the serum BuChE activity in young rats one month after ovariectomy. Even in this phase, the activity of the AChE in the whole blood remained increased in the OVX+EB, OVX+E2 and OVX+E2+EB group, but a decrease in its activity was observed in the OVX group.

Das (2007) also mentions that high activities of these enzymes are present in diseases that are important constituents of the X metabolic syndrome, such as Alzheimer's disease (AD). Thus, it is relevant to observe the increased serum BuChE

and blood AChE activities of the OVX+E2 group in both phases. A previous study from our laboratory (Martins et al., 2012) also found similar activity in blood AChE of 5 month-old ovariectomized rats with estrogen supplementation. On the other hand, groups that showed inhibition of BuChE activity and / or blood AChE in the phase of demyelination may also have impairment in the non-neuronal cholinergic system. An unexpected decline of serum BuChE as occurred in the OVX and OVX+EB groups in the remyelination phase can possibly be interpreted as a compensatory mechanism to decrease the hydrolysis of ACh, since these groups have increased AChE activities in the brain (Monteiro et al., 2005).

It is important to emphasize that a lower activity of these enzymes can occur in patients with terminal stages of AD, which may indicate a serious risk of death (Das, 2007; Rao et al., 2007). Thus, AChE and BuChE enzymes are reliable and robust markers of inflammation, and the measure of their activities could possibly be used as a guide to predict their development as well as in the prognosis and response to the treatment of neurodegenerative diseases (Das, 2007). ACh is synthesized, amongst others, by immune system (lymphocytes, dendritic cells, neutrophils) and endothelial cells (Kawashima and Fujii, 2000). It is possible that ACh derived from these sources is involved in the modulation of local inflammatory processes and regulation of immune functions, suggesting that a close interaction exists between immune response and cholinergic transmission (Rao et al., 2007).

In spite of the fact that estrogens modulate the onset and progress of inflammation (Nilsson, 2007), we could observe that in the demyelination phase our results in the AChE activity of lymphocytes showed an increase of the enzyme activity only in the OVX+EB group, while the other groups showed an inhibition of the activity when compared with the sham group. Non-neuronal ACh acts continuously as a local

signaling molecule involved in the regulation of basic cell functions (Wessler et al., 2003). Thus, it is possible that the changes observed in the cholinergic activity of lymphocytes may be related to immune disfunctions (Kawashima and Fujii, 2003). We can note that OVX+EB was able to stimulate a more intense inflammatory response of lymphocytes due to an increase in the AChE activity, which could reduce indirectly the levels of ACh, demonstrating that the absence of ovarian hormones in demyelinated females can be injurious in this aspect. ACh is considered an anti-inflammatory molecule, and alterations in its concentration may be related to some neurodegenerative diseases (Rao et al., 2007).

In the remyelination phase, all groups behaved similarly to the sham group. However, the OVX+E2+EB group showed a higher enzyme activity compared with the OVX and OVX+EB groups. It is possible that lymphocytic cholinergic system be involved in the regulation of lymphocyte function, acting via mAChRs coupled to phospholipase C or adenylyl cyclase and nAChRs forming ligand-gated ion channels (Kawashima and Fujii, 2004). Moreover, the inhibitory effect on non-neuronal acetylcholine may contribute to the clinical effectiveness of steroids (Wessler et al., 2003).

In our study, the AChE activity showed great variability among the different brain structures analyzed, as well as in different phases of the same structure. Thus, we suggest that the behavior of the enzyme can vary not only by the influence of the presence or absence of the hormone estrogen (Martins et al., 2012), and the heterogeneous functionality of the central cholinergic system (Das et al., 2001), but also by the processes of demyelination and remyelination induced experimentally by the EB. Histological analysis performed in our study showed that 7 days after the injection of EB there was a demyelinating lesion characterized by the loss of myelin sheaths within

the area of injury and cell accumulation suggestive of inflammatory infiltrate in the OVX+EB and OVX+EB+E2 groups. On the other hand, 21 days after the injection of EB it was possible to observe several areas of remyelination, with small areas of demyelination in resolution which is in agreement with previous studies (Levine and Reynolds, 1999; Woodruff and Franklin, 1999; Bondan et al. 2000; Graça et al. 2001; Mazzanti et al. 2006b, 2009; Ramos et al. 2009). The process of remyelination was more intense in the OVX+EB+E2 group compared with the OVX+EB group and this might be due to the presence of estrogen supplementation (Zhu and Glaser, 2008) for a period of 21 days after demyelination. Several brain cells express *in vivo* estrogen receptors, being important to note that the ER-β is co-localized in oligodendrocytes and in myelin sheaths (Zhang et al., 2004) while ER-α has been observed in microglia (Vegeto et al., 2003; Sierra et al., 2008;) and promoting the reactivity of brain macrophages (Vegeto et al., 2003). In this aspect, the OVX+E2+EB group, unlike what occurred in the OVX+EB group showed numerous foamy macrophages at the site of lesions caused by the EB.

Summarizing, the present study demonstrates that the experimental model of demyelination by EB in ovariectomized female rats with no estrogen supplementation increased the activity of AChE in different structures of the brain studied, both in the demyelination and remyelination phases. This behavior of the enzyme could reduce the amount of neutransmissor available in the synaptic cleft, impairing cholinergic neurotransmission. Estrogen supplementation normalized the AChE activity in the demyelination phases and inhibited in the CC, ST and CE at the remyelination phase, and this would make AChE and 17-β estradiol important tools for neurodegenerative diseases, such as MS. Non-neuronal AChE has an increase in its activity in demyelinating ovariectomized rats. The responses provided by non-neuronal cholinergic

system are dependent on the phase analyzed, demyelination or remyelination as well as the presence or absence of estrogen supplementation. Thus, 17-β estradiol modulates the neuronal and non-neuronal AChE activity in experimental model of demyelination by EB. Considering the possibility that these phenomena occur in humans, our data provide a good alternative for a new therapeutic strategy in neurodegenerative diseases in women as in MS.

Conflicts of interest

The authors declare that there are no conflicts of interest.

Acknowledgments

We would like to thank the Programa de Pós-graduação em Medicina Veterinária – UFSM (Universidade Federal de Santa Maria) and CAPES (Coordenação de Pessoal de Nível Superior) for the support. CMM and STAL are research fellows from the Brazilian agency CNPq (Conselho Nacional de Desenvolvimento Científico e Tecnológico). The data presented are part of the Doctorate thesis of DBM.

References

Acs, P., 2009. 17β-estradiol and progesterone prevent cupriozone provoked demyelination of corpus callosum in male mice. Glia 57, 807-814.

Aguiar, R.B., Dickel, O.E., Cunha, R.W., Monserrat, J.M., Barros, D.M., Martinez, P.E., 2008. Estradiol valerate and tibolone: effects upon brain oxidative stress and blood biochemistry during aging in female rats. Biogerontology 9, 285-298.

Baeza, I., De Castro, N.M., Giménez-Llort, L., De La Fuente, M., 2010. Ovariectomy, a model of menopause in rodents, causes a premature aging of the nervous and immune systems. J Neuroimmunol 219, 90-99.

Baumann, N., Pham-Dinh, D., 2001. Biology of oligodendrocyte and myelin in the mammalian central nervous system. Physiol. Rev. 81, 871-927.

Bergmeyer, H.U., 1983. Methods of Enzymatic Analysis. Vol. 3. London: Academic Press, 118-133.

Bondan, E.F., Lallo, M.A., Dagli, M.L.Z., Pereira, L.A.V.D., Graça, D.L., 2002. Ruptura da barreira hematoencefálica após injeção de droga gliotóxica no tronco encefálico de ratos wistar. Arq. Neuropsiquiatr. 60, 582-589.

Bondan, E.F., Lallo, M.A., Sinhorini, I.L., Pereira, L.A., Graça, D.L., 2000. The effect of cyclophosphamide on brainstem remyelination following local ethidium bromide injection in Wistar rats. J. Submicrosc. Cytol. Pathol. 32, 603-612.

Borovikova, L.V., Ivanova, S., Zhang, M., Yang, H., Botchkina, G.I., Watkins, L.R., Wang, H., Abumrad, N., Eaton, J.W., Tracey, K.J., 2000. Vagus nerve stimulation attenuates the systemic inflammatory response to endotoxin. Nature 405, 458–462.

Bowman, R.E, Ferguson, D., Luine,V.N., 2002. Effects of chronic restraint stress and estradiol on open field activity, spatial memory, and monoaminergic neurotransmitters in ovariectomized rats. Neuroscience 113, 401-410.

Böyum, A., 1968. Isolation of mononuclear cells and granulocytes from human blood. Isolation of mononuclear cells by one centrifugation, and of granulocytes by combining centrifugation and sedimentation at 1 g. Scan. J. Clin. Lab. Invest. Suppl. 97, 77–89.

Bradford, M., 1976. A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein–dye binding. Anal. Biochem. 72, 248–54.

Cokugras, A.N., 2003. Butyrylcholinesterase: structure and physiological importance. Turk. J. Biochem. 28, 54-61.

Confavreux, C., Vukusic, S., Adeleine, P., 2003. Early clinical predictors and progression of irreversible disability in multiple sclerosis: an amnesic process. Brain 126, 770-782.

Daniel, J.M., Hulst, J.L., Berbling, J.L., 2006. Estradiol replacement enhances working memory in middle-aged rats when initiated immediately after ovariectomy but not after a long-term period of ovarian hormone deprivation. Endocrinology 147, 607-614.

Das, A., Dikshit, M., Nath, C., 2001. Profile of acetylcholinesterase in brain areas of male and female rats of adult and old age. Life Sciences 68, 1545-1555.

Das, A., Dikshit, M., Srivastava, S.R., Srivastava U.K., Nath, C., 2002. Effect of ovariectomy and estrogen supplementation on brain acetylcholinesterase activity and passive-avoidance learning in rats. Can. J. Physiol. Pharmacol. 80, 907–914.

Das, A., Dikshit, M., Srivastava, S.R., Srivastava, U.K., Nath, C., 2002. Effect of ovariectomy and estrogen supplementation on brain acetylcholinesterase activity and passive-avoidance learning in rats. Can. J. Physiol. Pharmacol. 80, 907-914.

Das, U.N., 2006. Clinical laboratory tools to diagnose inflammation. Adv. Clin. Chem. 41, 189–229.

Das, U.N., 2007. Acetylcholinesterase and butyrylcholinesteraseas possible markers of low-grade systemic inflammation. Med. Sci. Monit. 13, 214-221.

El-Etr, M., Ghoumari, A., Sitruk-Ware, R., Schumacher, M., 2011. Hormonal influences in multiple sclerosis: New therapeutic benefits for steroids. Maturitas 68, 47-51.

Ellman, G.L., Courtney, D.K., Andres, V., Featherstone, R.M., 1961. A new and rapid colorimetric determination of acetylcholinesterase activity. Biochem. Pharmacol. 7, 88–95.

Feng, Z., Cheng, Y., Zhang, J., 2004. Long-term effects of melatonin or 17 beta-estradiol on improving spatial memory performance in cognitively impaired, ovariectomized adult rats. J. Pineal Res. 37, 198-206.

Fitzgerald, B.B., Costa, L.G., 1993. Modulation of muscarinic receptors an acetylcholinesterase activity in lymphocytes and brain areas following repeated organophosphate exposure in rats. Fundam. Appl. Toxicol. **20**, 210–216.

Frye, C.A., Rhodes, M.E., Dudek, B., 2005. Estradiol to aged female or male mice improves learning in inhibitory avoidance and water maze tasks. Brain Res. 1036, 101-108.

Gibbs, R.B., 2010. Estrogen therapy and cognition: a review of the cholinergic hypothesis. Endocr. Rev. 31, 224-253.

Graça, D.L., Bondan, E.F., Pereira, L.A.V.D., Fernandes, C.G., Maiorka, P.C., 2001. Behaviour of oligodendrocytes and schwann cells in na experimental modelo of toxic demyelination of the central nervous system. Arq. Neuropsiquiatr. 59, 358-361.

Grisaru, D., Sternfeld, M., Eldor, A., Glick, D., Soreq, H., 1999. Structural roles of acetylcholinesterase variants in biology and pathology. Eur. J. Biochem. 264, 272-286.

Kaaja, R.J., Greer, I.A., 2005. Manifestations of chronic disease during pregnancy. J. Am. Med. Assoc. 294, 2751-2757.

Kawashima, K., Fujii, T., 2000. Extraneuronal cholinergic system in lymphocytes. Pharmacol. Ther. 86, 29-48.

Kipp, M., Clarner, T., Dang, J., Copray, S., Beyer, C., 2009. The cuprizone animal model: new insights into an old story. Acta Neuropathol. 118, 723-736.

Kuppers, E., Beyer, C., 1999. Expression of estrogen receptor α and β mRNA in the developing and adult mouse striatum. Neurosci. Lett. 276, 95–98.

Levine, J.M., Reynolds, R. 1999. Activation and proliferation of endogenous oligodendrocyte precursor cells during ethidium bromide-induced demyelination. Exp. Neurol. 160, 333-347.

Marcondes, F.K., Bianchi, F.J., Tanno, A.P., 2002. Determination o the estrous cycle phases of rats: some helpful considerations. Braz. J. Biol. 62, 609-614.

Marcondes, F.K., Miguel, K.J., Melo, L.L., Spadari-Bratfisch, R.C., 2001. Estrous cycle influences the rsponse of female rats in the elevated plus-maze test. Physiol. Behav. 74, 435-440.

Martins, D.B., Mazzanti, C.M., França, R.T., Pagnoncelli, M., Costa, M.M., Souza, E.M., Gonçalves, J., Spanevello, R., Schmatz, R., Costa, P., Mazzanti, A., Beckmann, D.V., Cecim, M.S., Schetinger, M.R., Lopes, S.T.A., 2012. 17-β estradiol in the acetylcholinesterase activity and lipid peroxidation in the brain and blood of ovariectomized adult and middle-aged rats. Life Sciences, in press.

Mazzanti, C.M., Spanevello, R., Ahmed, M., Pereira, L.B., Gonçalves, J.F., Correa, M., Schmatz, R., Stefanello, N., Leal, D.B.R., Mazzanti, A., Ramos, A.T., Martins, T.B., Danesi, C.C., Graça, D.L., Morsch, V.M., Schetinger, M.R.C., 2009. Pre-

treatment with ebselen and vitamin E modulate acetylcholinesterase activity: interaction with demyelinating agents. Int. J. Dev. Neurosci. 27, 73-80.

Mazzanti, C.M., Spanevello, R., Ahmed, M., Schmatz, R., Mazzanti, A., Salbego, F.Z., Graça, D.L., Sallis, E.S.V., Morsch, V.M., Schetinger, M.R.C., 2007. Cyclosporine A inhibits acetylcholinesterase activity in rats experimentally demyelinated with ethidium bromide. Int. J. Dev. Neurosci. 25, 259-264.

Mazzanti, C.M., Spanevello, R., Obregon, A., Pereira, L.B., Streher, C.A., Ahmed, M., Mazzanti, A., Graça, D.L., Morsch, V.M., Schetinger, M.R.C., 2006a. Ethidium bromide inhibits rat brain acetylcholinestearse activity in vitro. Chem. Biol. Interact. 162, 121-127.

Mazzanti, C.M., Spanevello, R.M., Pereira, L.B., Gonçalves, J.F., Kaizer, R., Correa, M., Ahmed, M., Mazzanti, A., Festugatto, R., Graça, D.L., Morsch, V.M., Schetinger, M.R.C., 2006b. Acetylcholinesterase activity in rats experimentally demyelinated with ethidium bromide and treated with interferon beta. Neurochem. Res. 31, 1027-1034.

McEwen, B., 2002. Estrogen actions throughout the brain. Rec. Prog. Horm. Res. 57, 357–384.

Mesulam, M.M., Guillozet, A., Shaw, P., Levey, A., Duysen, E.G., Lockridge, O., 2002. Acetylcholinesterase knockouts establish central cholinergic pathways and can use butyrylcholinesterase to hydrolyse acetylcholine. Neuroscience 110, 627-39.

Monteiro, S.C., Stefanello, F.M., Vianna, L.P., Matté, C., Barp, J., Belló-Klein, A., Trindade, V.M.T., Wyse, A.T.S., 2005. Ovariectomy enhances acetylcholinesterase activity but does not alter ganglioside content in cerebral cortex of female adult rats. Metab. Brain Dis. 20, 35-44.

Moorthy, K., Yadav, U.C.S., Siddiqui, M.R., Mantha, A.K., Basir, S.F., Sharma, D., Cowsik, S.M., Baquer, N.Z., 2005. Effect of hormone replacement therapy in normalizing age related neuronal markers in different age groups of naturally menopausal rats. Biogerontology 6, 345-356.

Nassar, C.C.S., Bondan, E.F., Alouche, S.R., 2009. Effects of aquatic exercises in a rat model of brainstem demyelina tion with ethiduim bromide on the beam walking test. Arq. Neuropsiquiatr. 67, 652-656.

Nilsson, B.O., 2007. Modulation of the inflammatory response by estrogens with focus on the endothelium and its interactions with leukocytes. Inflamm. Res. 56, 269-273.

Pereira, R.T.S., Porto, C.S., Godinho, R.O., Abdalla, F.M.F., 2008. Effects of estrogens on intracellular signaling pathways linked to activation of muscarinic acetylcholine receptors and on acetylcholinesterase activity in rat hippocampus. Biochem. Pharmacol. 75, 1827-1834.

Ramos, A.T.; Maiorka, P.C.; Dagli, M.L.Z., Hosomi, F.Y.M., Violin, K.B., Latorre, A., Viott, A.M., Masuda, E.K., Trost, M.E., Martins, T.B., Graça, D.L.Remyelination in experimentally demyelinated connexin 32 knockout mice. Arq. Neuropsiquiatr. 67, 488-493.

Rao, A.A., Gumpeny, R.S., Das, U.N., 2007. Elevated butyrylcholinesterase and acetylcholinesterase may predict the development of type 2 diabetes mellitus and Alzheimer's disease. Med. Hypotheses 69, 1272-1276.

Rocha JBT, Emanuelli T, Pereira ME., 1993. Effects of early undernutrition on kinetic parameters of brain acetylcholinesterase from adult rats. Acta Neurobiol. Exp. 53, 431-437.

Rosas-Ballina, M., Tracey, K.J., 2009. Cholinergic control of inflammation. J. Intern. Med. 265, 663-679.

Sallis, E.S.V., Mazzanti, C.M., Mazzanti, A., Pereira, L.A.V., Arroteia, K.F., Fustigatto, R., Pelizzari, C., Rodrigues, A., Graça, D.L., 2006. OSP-immunofluorescent remyelinating oligodendrocytes in the brainstem of toxically-demyelinated wistar rats. Arq. Neuropsiquiatr. 64, 240-244.

Sato, T., Teramoto, T., Tanaka, K., Ohnishi, Y., Irifune, M., Nishikawa, T., 2003. Effects of ovariectomy and calcium deficiency on learning and memory of eight-arm radial maze in middle-aged female rats. Behav. Brain Res. 142, 207-216.

Schetinger, M.R.C., Porto, N.M., Moretto, M.B., Morsch, V.M., Rocha, J.B.T., Vieira, V., Moro, F., Neis, R.T., Bittencourt, S., Bonacorso, H.G., Zanatta, N. (2000) New benzodiazepines alter acetylcholinesterase and ATPase activities. Neurochem. Res. 25, 949–955.

Schliebs, R., Arendt, T., 2006. The significance of the cholinergic system in the brain during aging and in Alzheimer's disease. J. Neural. Transm. 113, 1625–1644.

Schumacher, M., Weill-Engerer, S., Liere, P., Robert, F., Franklin, R.J.M., Garcia-Segura, L.M., Lambert, J.J., Mayo, W., Melcangi, R.C., Parducz, A., Suter, U., Carelli, C., Baulieu, E.E., Akwa, Y., 2003. Steroid hormones and neurosteroids in normal and pathological aging of the nervous system. Prog. Neurobiol. 71, 3-29.

Shughrue, P.J., Merchenthaler, I., 2000. Estrogen is more than a "sex hormone": Novel sites for estrogen action in the hippocampus and cerebral cortex. Front. Neuroendocrinol. 21, 95-101.

Sicotte, N.L., Liva, S.M., Klutch, R., Pfeiffer, P., Bouvier, S., Odesa, S., Wu, T.C.J., Voskuhl, R.R., 2002. Treatment of multiple sclerosis with the pregnancy hormone estriol. Ann. Neurol. 52, 421-428.

Siegel, G., 1999. Basic Neurochemistry: molecular, cellular and medical aspects. 6 ed. Philadelphia: Lippincott Willians & Wilkins,1183p.

Sierra, A., Gottfried-Blackmore, A., Milner, T.A., McEwen, B.S., Bulloch, K., 2008. Steroid hormone receptor expression and function in microglia. Glia 56, 659–674.

Straub, R.H., 2007. The complex role of estrogens in inflammation. Endocr. Rev. 28, 521-574.

Taylor, L.C., Puranam, K., Gilmore, W., Ting, J.P.Y., Matsushima, G.K., 2010. 17β-estradiol protects male mice from cuprizone-induced demyelination and oligodendrocyte loss. Neurobiol. Dis. 39, 127-137.

Thakur, M.K., Sharma, P.K., 2006. Aging of brain: role of estrogen. Neurochem. Res. 31, 1389-1398.

Tougu, V., Kesvatera, T., 1996. Role of ionic interactions in cholinesterase catalysis. Biochim. Biophys. Acta 1298, 12-30.

Vegeto, E., Belcredito, S., Etteri, S., Ghisletti, S., Brusadelli, A., Meda, C., Krust, A., Dupont, S., Ciana, P., Chambon, P., Maggi, A., 2003. Estrogen receptor-α mediates the brain antiinflammatory activity of estradiol. PNAS 100, 9614-9619.

Woodruff, R.H., Franklin, R.J.M., 1999. Demyelination and remyelination of the caudal cerebellar peduncle of adult rats following stereotaxic injections of lysolecithin, ethidium bromide, and complement/anti-galactocerebroside: a comparative study. Glia 25, 216-228.

Worek, F., Mast, U., Kiderlen, D., Diepold, C., Eyer, P., 1999. Improved determination of acetylcholinesterase activity in human whole blood. Clin. Chim. Acta 288, 73–90.

Yener, T., Tunc, A.T., Aslan, H., Aytan, H., Caliskan, A.C., 2007. Determination of oestrous cycle of the rats by direct examination: how reliable? Anat. Histol. Embryol. 36, 75-7.

Zhang, Z., Cerghet, M., Mullins, C., Williamson, M., Bessert, D., Skoff, R., 2004. Comparison of in vivo and in vitro subcellular localization of estrogen receptors alpha and beta in oligodendrocytes. J. Neurochem. 89, 674–684.

Zhu, T.S., Glaser, M., 2008. Neuroprotection and enhancement of remyelination by estradiol and dexamethasone in cocultures of rat DRG neurons and Schwann cells. Brain Res. 1206, 20-32.

Zhu, W.H., Lu, C.Z., Huang, Y.M., Link, H., Xiao, B.G., 2007. A putative mechanism on remission of multiple sclerosis during pregnancy: estrogen-induced indoleamine 2,3-dioxygenase by dendritic cells. Mult Scler 13, 33-40.

Figure Legends

Figure 1. Serum 17-β estradiol levels evaluated by immuno-chemiluminescence of female ovariectomized rats submitted to the model of demyelination with ethidium bromide (EB). Sham-A (adult female rat in proestrus), OVX (ovariectomized rats, not demyelinated, treated just with vehicle), OVX+EB (demyelinated ovariectomized rats treated just with vehicle), OVX+E2 (ovariectomized rats, not demyelinated, treated with 0.1µg/g of 17-β estradiol), and OVX+EB+E2 (demyelinated ovariectomized rats treated with 0.1µg/g of 17-β estradiol) at demyelination (7 days after EB injection) and remyelination (21 days after EB injection) phases. Each column represents mean±S.E. Duncan's multiple range test (n=6): groups that show different letters are statistically different ($p<0.05$).

Figure 2. Acetylcholinesterase (AChE) activity of cerebellum, cerebral cortex, striatum and hippocampus of female ovariectomized rats submitted to the model of demyelination with ethidium bromide (EB). Sham-A (adult female rat in proestrus), OVX (ovariectomized rats, not demyelinated, treated just with vehicle), OVX+EB (demyelinated ovariectomized rats treated just with vehicle), OVX+E2 (ovariectomized rats, not demyelinated, treated with 0.1µg/g of 17-β estradiol), and OVX+EB+E2 (demyelinated ovariectomized rats treated with 0.1µg/g of 17-β estradiol) at demyelination (7 days after EB injection) and remyelination (21 days after EB injection) phases. Each column represents mean±S.E. Duncan's multiple range test (n=6): groups that show different letters are statistically different ($p<0.05$).

Figure 3. Butyrylcholinesterase (BuChE) activity of serum and acetylcholinesterase (AChE) of blood and lymphocytes of female ovariectomized rats submitted to the model of demyelination with ethidium bromide (EB). Sham-A (adult female rat in proestrus), OVX (ovariectomized rats, not demyelinated, treated just with vehicle), OVX+EB (demyelinated ovariectomized rats treated just with vehicle), OVX+E2 (ovariectomized rats, not demyelinated, treated with 0.1µg/g of 17-β estradiol), and OVX+EB+E2 (demyelinated ovariectomized rats treated with 0.1µg/g of 17-β estradiol) at demyelination (7 days after EB injection) and remyelination (21 days after EB injection) phases. Each column represents mean±S.E. Duncan's multiple range test (n=6): groups that show different letters are statistically different ($p<0.05$).

Figure 4. Histopathological analysis of the pons of female ovariectomized rats submitted to the model of demyelination with ethidium bromide (EB). Sham-A (adult

female rat in proestrus), OVX (ovariectomized rats, not demyelinated, treated just with vehicle), OVX+EB (demyelinated ovariectomized rats treated just with vehicle), OVX+E2 (ovariectomized rats, not demyelinated, treated with 0.1µg/g of 17-β estradiol), and OVX+EB+E2 (demyelinated ovariectomized rats treated with 0.1µg/g of 17-β estradiol) at demyelination (D) (7 days after EB injection) and remyelination (R) (21 days after EB injection) phases. Sham D - Round area of edema and central cell accumulation. Sham R - Resolution in the swollen area. OVX D - Swollen area with the presence of foamy macrophages. OVX R - Elongated area of mild edema and moderate cellular infiltration. OVX+EB D – It can be observed along the needle path areas of demyelination and cellular accumulation suggestive of inflammatory infiltration. OVX+EB R - An extensive area of demyelination with cystic cavities. OVX+E2 D - Winding area of edema probably infiltrated by inflammatory cells. OVX+E2 R - Restricted area of edema with mild cellular infiltration. OVX+EB+E2 D - An extensive area of demyelination with inflammatory infiltrate consisting of foamy macrophages (gitter cells). OVX+EB+E2 R -Small area of demyelination in the presence of numerous foamy macrophages. Hematoxilin and eosin (HE), 10x.

Figure 1

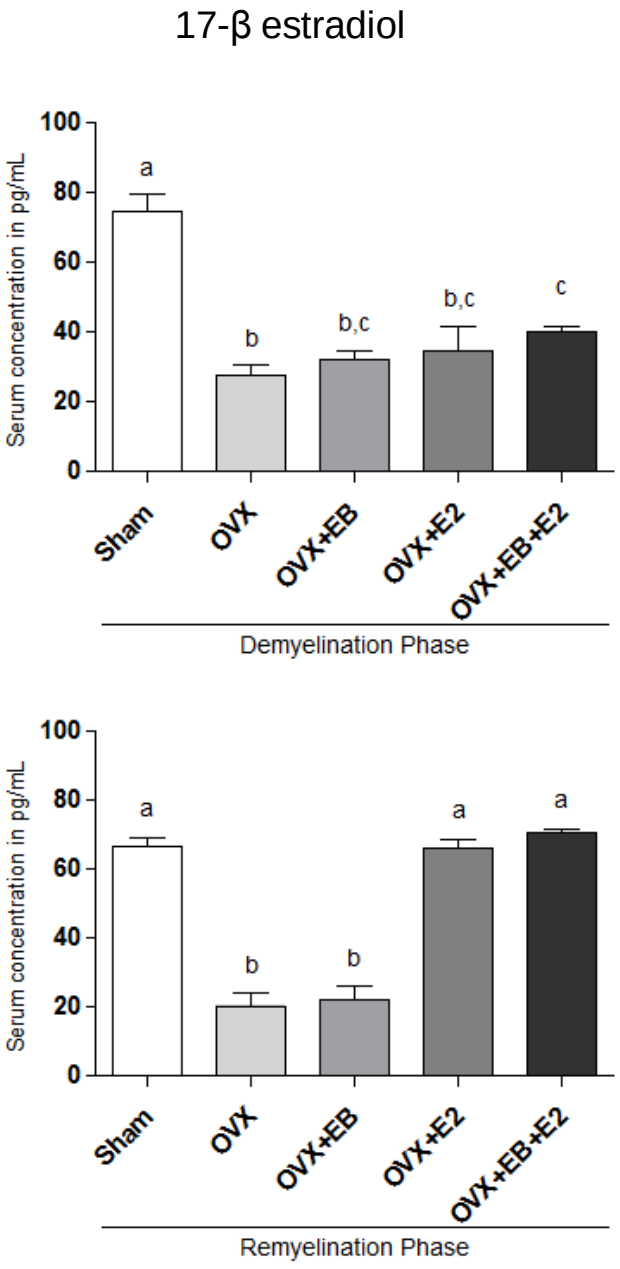

17-β estradiol
Serum concentration in pg/mL
100
80
60
40
20
0
a
b
b,c
b,c
c
Sham
OVX
OVX+EB
OVX+E2
OVX+EB+E2
Demyelination Phase
Serum concentration in pg/mL
100
80
60
40
20
0
a
b
b
a
a
Sham
OVX
OVX+EB
OVX+E2
OVX+EB+E2
Remyelination Phase

Figure 2

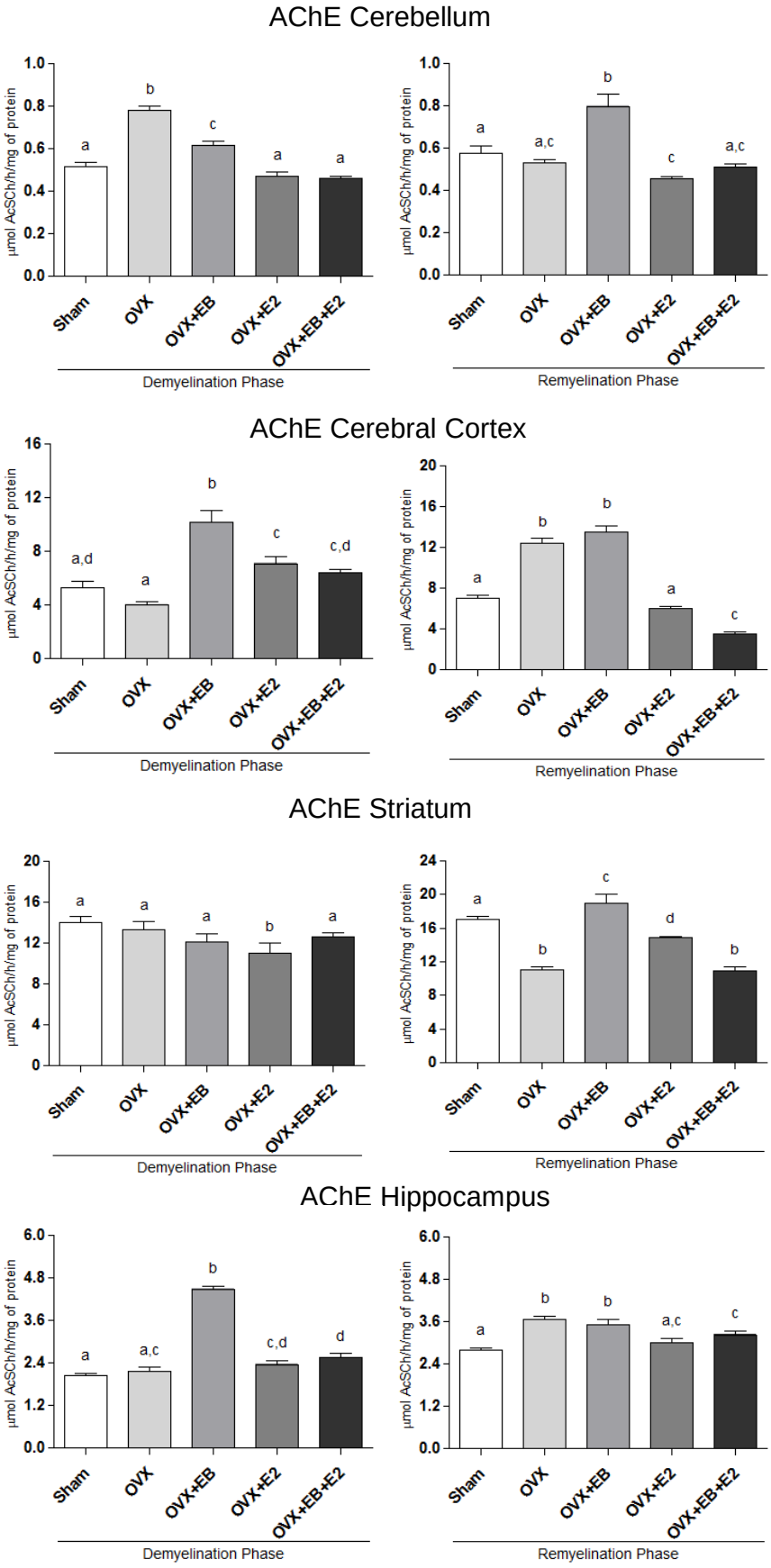
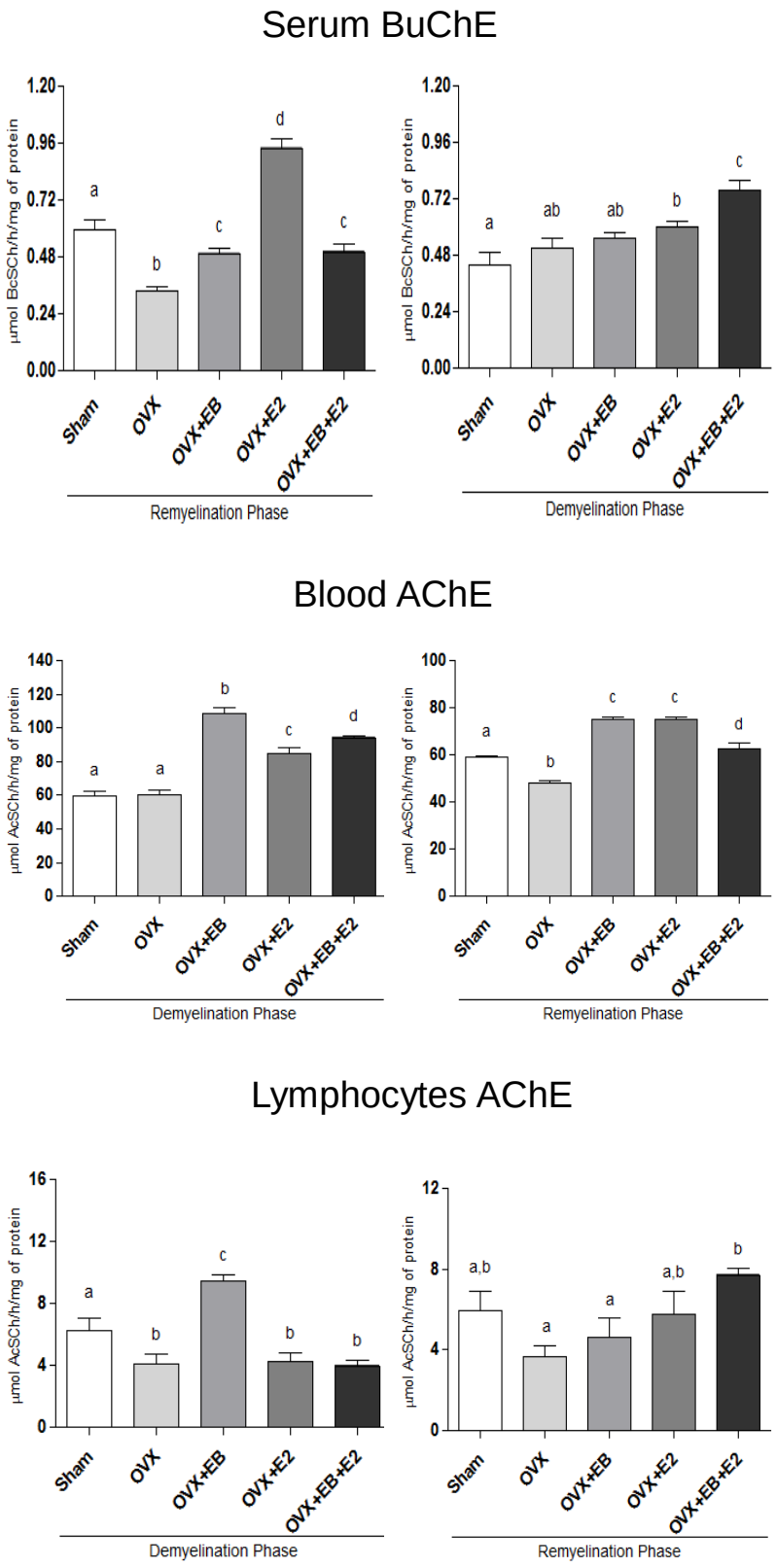
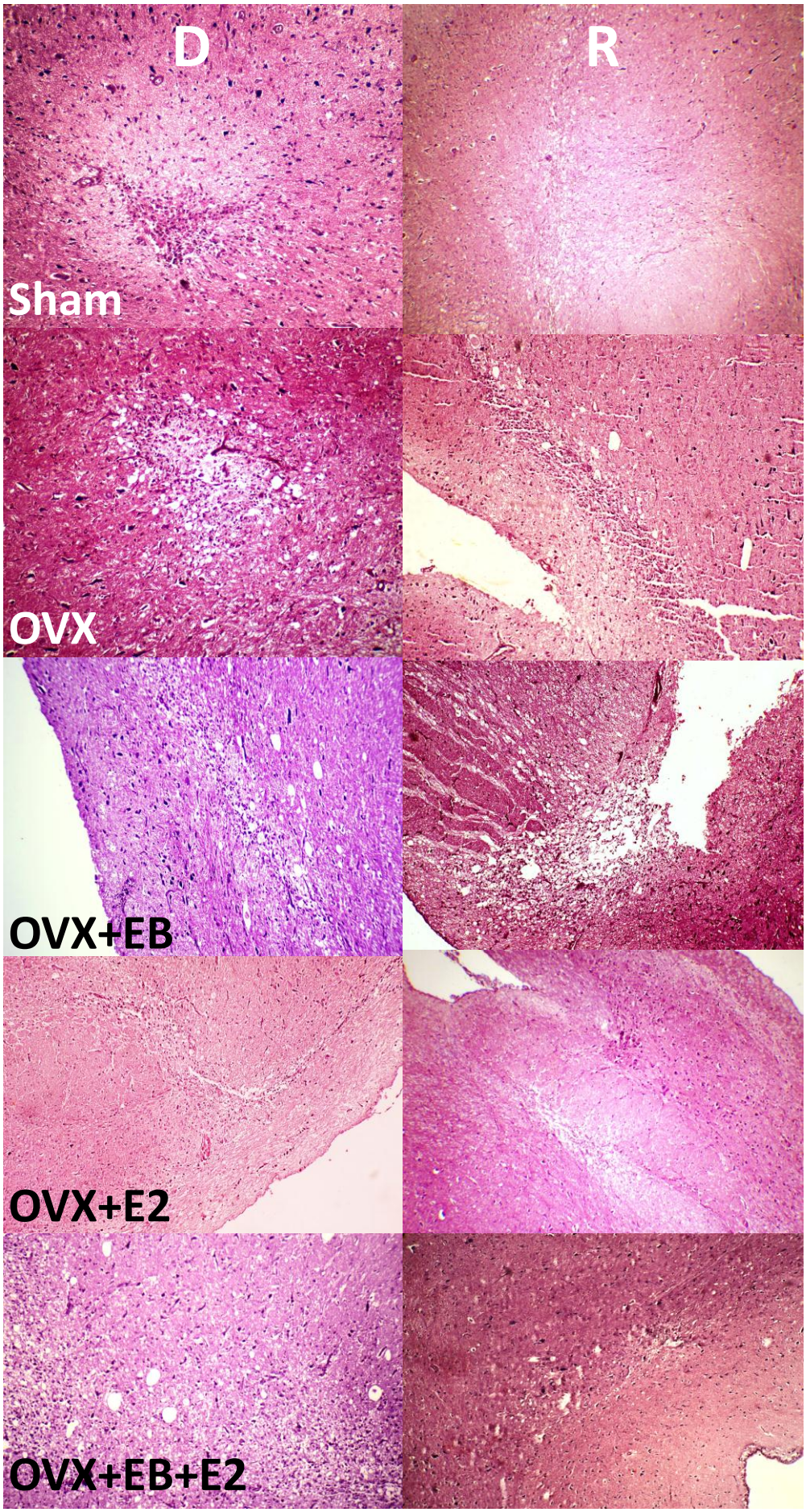

AChE Cerebellum
µmol AcSCh/h/mg of protein
Sham
OVX
OVX+EB
OVX+E2
OVX+EB+E2
Demyelination Phase
Remyelination Phase
AChE Cerebral Cortex
AChE Striatum
AChE Hippocampus

Figure 3


Serum BuChE
µmol BcSCh/h/mg of protein
1.20
0.96
0.72
0.48
0.24
0.00
a
b
c
d
c
Sham
OVX
OVX+EB
OVX+E2
OVX+EB+E2
Remyelination Phase
a
ab
ab
b
c
Demyelination Phase
Blood AChE
µmol AcSCh/h/mg of protein
140
120
100
80
60
40
20
0
a
a
b
c
d
Demyelination Phase
a
b
c
c
d
Remyelination Phase
Lymphocytes AChE
16
12
8
4
0
a
b
c
b
b
Demyelination Phase
a,b
a
a
a,b
b
Remyelination Phase


D
R
Sham
OVX
OVX+EB
OVX+E2
OVX+EB+E2

3.3 MANUSCRITO 2:

Complete blood count and acetylcholinesterase activity of lymphocytes of demyelinated and ovariectomized rats treated with resveratrol

Danieli B. Martins, Cinthia M. Mazzanti, Márcio M. Costa, Raqueli França, Marcielen Pagnoncelli, Roberto M. Maciel, Roberta Schmatz, Lizielle Oliveira, Vera Morsch, Grasiela Facco, Diandra Visentini, Thais Mann, Alexandre Mazzanti, Sonia T. A. Lopes

Manuscrito aceito pela Immunopharmacology And Immunotoxicology

Complete blood count and acetylcholinesterase activity of lymphocytes of demyelinated and ovariectomized rats treated with resveratrol

Danieli B. Martins[a*] Cinthia M. Mazzanti[a] Márcio M. Costa[a] Raqueli França[a] Marcielen Pagnoncelli[a] Roberto M. Maciel[a] Roberta Schmatz [b] Lizielle Oliveira[b] Vera Morsch[b] Grasiela Facco[b] Diandra Visentini[a] Thais Mann[a] Alexandre Mazzanti [a] Sonia T. A. Lopes[a]

[a]Departamento de Clínica de Pequenos Animais and [b]Departamento de Química, Universidade Federal de Santa Maria, Santa Maria –RS, Brazil

Keywords: leukocytes, brain, cholinergic system, ethidium bromide, phytoestrogen.

*Corresponding author:

Danieli Brolo Martins

Hospital Veterinário Universitário, Centro de Ciências Rurais, Universidade Federal de Santa Maria, Av. Roraima, 97105-900, Santa Maria, RS, Brazil.

Telephone number: + 55 -55 -3220-8814

Fax number: +55 -55 -3220 -8978

Email: vetdanielimartins@yahoo.com.br

Abstract

Resveratrol is a phytoestrogen that has many beneficial actions. This study aimed to evaluate the effect of resveratrol on the complete blood count (CBC) and the acetylcholinesterase (AChE) activity of lymphocytes of ovariectomized rats experimentally demyelinated by ethidium bromide (EB). Forty adult female Wistar rats (60 days, 200-220 g) were divided randomly into 5 groups (n=4) to evaluate the demyelination phase and 5 groups (n=4) to evaluate the remyelination phase. In each phase, the groups consisted of sham rats - G1; ovariectomized rats, not demyelinated, treated only with vehicle (ethanol 25%) - G2; demyelinated ovariectomized rats treated only with vehicle – G3; ovariectomized rats, not demyelinated, treated with resveratrol – G4; and demyelinated ovariectomized rats treated with resveratrol – G5. Only during the remyelination phase, CBC showed a significant difference ($p < 0.05$) in the number of monocytes between G2 and G5 groups. In the demyelination phase there was a significant decrease ($p < 0.05$) in the AChE activity in the G4 group, while the G5 group was statistically similar to the G1, G2 and G4 groups. In the remyelination phase, there were no significant differences in the AChE activity among the groups. The treatment for 7 days with resveratrol with or without the experimental demyelization with EB appears to influence the AChE activity of lymphocytes, without changing the number of these cells in the circulation. However, in the remyelination phase, there seems to be stabilization in its effect on the lymphocyte AChE activity.

Introduction

Phytoestrogens are a class of natural plant components that have molecular structures and actions similar to estrogen (1). Among the various types of phytoestrogens, resveratrol (3,5,4 '-trihydroxy-trans-stilbene) is a phytoalexin that has beneficial properties to the organism (2), such as the enhancement of cerebral blood flow (3) and neuroprotection against degenerative diseases (4) due to in part its potential anti-inflammatory and antioxidant properties (5,6).

The mechanisms by which resveratrol exerts its neuroprotective effects are not well established (7,8). One possible factor that may explain the mechanism of neuroprotection against some diseases of the nervous system is by increasing or preserving cholinergic neurotransmission (9). A key element of the cholinergic functioning is performed by the enzyme acetylcholinesterase (AChE) (AChE; E.C. 3.1.1.7) (10). However, apart from the classical role in the brain cholinergic transmission, there is evidence that AChE participates in non-neuronal cholinergic system of blood cells (11,12).

AChE has been identified in lymphocytes which probably play an important role in the regulation of immune functions (13). However, the function of this system in blood cells is not clear yet, but it seems to be involved in the activity of various biological functions such as cell-cell contact as well as increase of cell activation and immune regulation (14,15,16). The non-neuronal cholinergic system, widely expressed in human cells independent of the nervous function, represents a local regulatory system contributing to cell and organ homeostasis (17).

Demyelinating diseases are a serious consequence of the destruction of myelin sheaths present in the central nervous system (CNS) and peripheral nervous system

(PNS) (18). Moreover, experimental demyelination helps in the analysis of potential cellular changes that occur in demyelinating neurodegenerative diseases (19). Toxic demyelination is one of the commonly used models used to explore the reparative capacity of the central nervous system (CNS) against toxins, such as ethidium bromide (EB), which can be injected into the white matter of experimental animals leading to selective myelin loss (20). In addition, the nervous system communicates with the immune system in a bi-directional way. Nervous tissues synthesize neuropeptides and cytokines and immune cells serve as the molecular basis of neuro-immune interactions (21). The EB demyelinating model consists in a important tool to understand the pathological mechanisms of many demyelinating diseases that are still unclear (22,23,24), such as the relation between the non-neuronal cholinergic system and the immune response. Additionally, the rat is a good animal model to experimental studies about demyelination (25).

Several types of leukocytes, as granulocytes and mononuclear cells, are involved on the pathophysiology of neurodegenerative diseases, such as multiple sclerosis in humans (26), canine distemper (27) and in some cases inflammation may increase the total leucocyte count (28). However, literature data show that resveratrol is able to reduce the exaggerated action of white blood cells such as polymorphonuclear neutrophils (PMN) (29), as well as modulate the release of proinflammatory substances by these cells (30).

Considering that resveratrol has demonstrated anti-inflammatory properties against neurodegenerative diseases and that the cholinergic extra-neural system has played important roles in the body, we aimed to evaluate and correlate the total blood count (CBC) and the AChE activity of lymphocytes from ovariectomized rats experimentally demyelinated with EB and treated with resveratrol.

Methods

Chemicals and reagents

Resveratrol (3,5,4 '-trihydroxy-trans-stilbene, with approximately 99% purity) and other enzymes, co-enzymes, substrates and buffers were obtained from Sigma Chemical Co. (St. Louis, MO, USA). All other reagents used in the experiment were of analytical grade and high purity.

Animals

Forty adult female Wistar rats (60 days, 200-220 g) from the Central Animal House of the Universidade Federal de Santa Maria (UFSM) were used in this experiment (demyelination or remyelination). The animals were kept in a constant temperature (21 ± 2 ° C) with light cycle 12h light / 12h dark with free access to food and water. The study was conducted in accordance with the UFSM Ethics Committee Guidelines for Experiments with Animals (process 86/2009).

After a week of adaptation, the animals were submitted to vaginal cytology for 2 weeks to verify the presence of normal estrous cycle (31,32). Only rats with normal estrous cycle entered the experiment.

Surgical Procedures

Anesthesic procedure

The animals were anesthetized initially with 4% halothane in a 40% O_2/60% N_2O mixture and the anesthesia was maintained during both surgeries with 1.5–2.5% halothane breathing spontaneously via facemask.

Ovariectomy

The control group (G1) underwent a sham surgery, with an incision in the Alba line, but without gonad removal, while the in other groups the gonads were completely removed. After a recovery period of 10 days, all animals were submitted again to vaginal smear to confirm the efficacy of the surgical procedures (4 days). On day 15 after ovariectomy the animals were submitted to the demyelination procedure.

Experimental demyelination

With the aid of a roof motor of orthodontic use and a drill number 2, a hole was made 0.85 cm to the right of the bregma until exposing the duramater. With the use of a Hamilton syringe with a removable needle of caliber 26 s, the solutions were injected 2 mm deep into the subcortical white water. Ten microliters of EB (0.1%) was injected in the animals of the G3 and G5 groups, and the same volume of 0.9% saline solution was injected in the animals of the G1, G2 and G4 groups. The duramater was left open and the skin, along with the remainder of the subcutaneous tissue, was sutured with a 4.0 nylon thread.

Experimental groups and treatments

The animals were divided randomly into 5 groups (n= 4) to evaluate the demyelination phase (seven days after EB surgery) and 5 groups (n= 4) to evaluate the remyelination phase (21 days after EB surgery). In each phase, the groups consisted of

sham rats – not ovariectomized, not demyelinated (only physiologic solution instead EB), and treated only with vehicle (ethanol 25%) instead resveratrol - G1 (G1-D for demyelination and G1-R for remyelination); ovariectomized rats, not demyelinated, treated only with vehicle (ethanol 25%) - G2 (G2-D for demyelination and G2-R for remyelination); demyelinated ovariectomized rats treated only with vehicle (ethanol 25%) – G3 (G3-D for demyelination and G3-R for remyelination); ovariectomized rats, not demyelinated, treated with resveratrol – G4 (G4-D for demyelination and G4-R for remyelination) and demyelinated ovariectomized rats treated with resveratrol – G5 (G5-D for demyelination and G5-R for remyelination).

On the same day of the surgical procedure of demyelination 10 mg/kg resveratrol by gavage was administered to the animals of the G4 and G5 groups. Resveratrol was freshly prepared in 25% ethanol and was administered at 3 to 4 PM, once a day, for 7 days (animals to evaluate the demyelination phase) or 21 days (animals to evaluate the remyelination phase), at a volume not exceeding 0.1 ml/100 g rat weight (33) (Figure 1). G1, G2 and G3 groups were submitted to gavage only with 25% ethanol for the same periods.

Laboratorial analysis

Blood collection

The animals were anesthetized with halothane and after that submitted to euthanasia. The blood was immediately collected by intracardiac puncture and placed in tubes using EDTA 10% as anticoagulant. For the AChE activity of lymphocytes, peripheral blood was isolated and AChE activity was measured immediately. For the CBC, 1 ml of blood from each sample was separated and analyzed within 2 h after collection.

AChE activity of lymphocytes

The peripheral lymphocytes were isolated using Ficoll Hypaque density gradient (34). After separation, only samples with at least 95% of lymphocytes, as verified in the coulter STKS (Miami—USA), were used. Lymphocyte viability and integrity were confirmed by determining the percentage of cells, excluding 0.1% trypan blue and measuring lactate dehydrogenase (LDH) activity (35). After the isolation of the lymphocytes, the AChE activity was determined according to the method described by (36) modified by (37). Briefly, proteins of all samples were adjusted to 0.1–0.2 mg/mL. The amount of 0.2 mL of intact cells was added to a solution containing 1.0 mM acetylthiocholine (ATC), 0.1 mM 5,5′-dithiobis (2-nitrobenzoic acid) (DTNB), and 0.1 M phosphate buffer (pH 8.0). Immediately before and after incubation for 30 min at 27 °C, the absorbance was read on a spectrophotometer at 412 nm. AChE was calculated from the ratio between lymphocyte AChE activity and protein content and the results are expressed as μmol/h/mg of protein.

CBC

The red blood cells and total leukocyte counts and hemoglobin quantification were performed with an automatic counter Mindray BC 2800 Vet®. The determination of hematocrit was obtained in a micro-hematocrit centrifuge after 5 min in rotation of 19,720 G. The measurement of total plasma proteins (TPP) was performed by refractometry and the result obtained in milligrams per deciliter (mg/dL). The mean corpuscular volume (MCV) and mean corpuscular hemoglobin concentration (MCHC) were determined by indirect calculations. The leukocyte differential count was performed on blood smears stained with Diff Quick.

Statistical analysis

A normality test was performed (Kolmogorov Smirnov test) in all data for comparison of groups of a same phase, verifying that they had a normal distribution. Thus, data were subjected to analysis of variance (ANOVA one way) followed by Tukey test. For comparison of groups at different phases, data were submitted to the Student's t test for independent samples. In both cases data were considered significantly different when probability (p) was less than 5%. The SPSS ® version 15.0 was used to perform all statistical tests.

Since the variables (AChE activity of lymphocytes and lymphocyte absolute value) presented a normal distribution (Kolmogorov Smirnov test) in both times, they were tested with Pearson correlation to verify the linear relationship between them. Data were considered significantly different when a probability (p) was less than 5%.

Results

During the demyelination phase there were no significant changes in any of the parameters of the CBC. However, during the remyelination phase, the blood count showed a significant difference in the number of monocytes ($p < 0.05$) between the G2 and G5 groups (Table 1).

The AChE activity of lymphocytes is shown in Figure 2. There was a significant difference ($p < 0.05$) between the control groups (G1-D and G2-D) and the group G4-D. The G5-D group was statistically similar to G1-D, G2-D and G4-D groups. The AChE activity in the remyelination phase had no significant differences among the groups. On the other hand, when comparing the same groups taking into account their different

phases, demyelination or remyelination, the AChE activity of lymphocytes showed a significant difference ($p < 0.05$) between the G4 and G5 groups.

There was no correlation between the AChE activity and the number of lymphocytes in the bloodstream at the demyelination phase as well as in the remyelination phase.

Discussion

Several benefits have been attributed to resveratrol (4,7). However, few studies have evaluated the effect of this phytoestrogen on blood profile (38,39) or AChE activity (33). In our study, we evaluated the effects of resveratrol in the CBC and in the AChE activity of lymphocytes. Moreover, we investigated the correlation of the AChE activity of lymphocytes with the number of lymphocytes in the bloodstream of ovariectomized rats submitted to the experimental demyelinating/ remyelinating brain lesion.

The oral form for resveratrol administration was chosen because this form mimics the primary use for the consumption of this substance through the ingestion of red wine (2). Furthermore, previous studies (33,38) have showed no toxic effects in different organs or structures examined. We used 10mg/kg of resveratrol because this dose has demonstrated beneficial effects on different organs as brain, liver and kidneys (33,40).

In CBC, the erythrogram showed no significant difference between groups with both 7 days and 21 days of daily use of 10 mg/kg of resveratrol. Thus, it can be observed that ovariectomy (G2) did not influence the number of red blood cells when compared with the sham rat group (G1). In addition, the dosage of resveratrol used in our study was safe to maintain the hematocrit. This result was similar to that found by

(38) that used resveratrol 20 mg/kg for 28 days orally in rats. However, long term *in vitro* of resveratrol exposure was found to inhibit the clonal growth of normal hematopoietic progenitor cells, although this inhibitory effect is partially reversible. On the other hand, this phytoestrogen does not induce or enhance spontaneously occurring apoptotic death in normal hematopoietic progenitor cells (41). Moreover, resveratrol has antioxidant effects on red blood cells, what is an important tool to preserve the cellular membrane integrity (42).

In the leucogram, the total leukocytes remained similar among the groups, regardless of the phase observed. This fact is relevant because the EB demyelination model, used in this research, does not induce the presence of a systemic inflammatory condition as can be observed in CBC of G3-D and G3-R groups, both at the peak of demyelination and remyelination, respectively. The use of this phytoestrogen seems to reduce PMN activation, a potential mechanism involved in many diseases, such as acute vascular ischaemic disease. The down-regulation of PMN function by resveratrol might also contribute to the recently observed anti-inflammatory and cancer preventive activities of this compound (43). However, in our study, resveratrol does not appear to influence a change in the number of white blood cells, with the exception of monocytes in the process of remyelination. In this aspect, we can observe a difference in the number of monocytes between G2-R and G5-R groups.

A decreased monocyte count is rarely observed in animals, and no specific significance is associated with monocytopenia (44), as we can see in the remyelineted group treated with resveratrol for 21 days (G5-R). Monocytes participate in inflammatory responses and are considered intermediated cells in a continuing maturation process. They migrate to tissues where they continue to develop, reaching the form of macrophages. These mononuclear cells phagocyte various structures, such

as damaged cells, cell debris and foreign particles (45). In this context, histological analysis has shown that EB intracisternal injection in the brain of rats causes degenerative changes in oligodendrocytes and astrocytes after 48-72 h of induction (46,47,48), presenting later macrophage infiltration (23). Although the G3, G4 and G5 groups have shown a lower number of monocytes compared with G1 and G2 groups, this difference was significant only between G2 and G5 groups. It is possible to see a downward trend in this cell line in blood during the process of remyelination. However, resveratrol is able to modulate the activity of mononuclear cells, such as reducing the production of monocyte tissue factor, which in humans is related to situations of chronic inflammation (49).

We can observe a significant decrease in AChE activity of lymphocytes in group G4-D. Additionally, group G5-D showed an intermediate AChE activity, it was statistically similar to G4-D and the other G1-D, G2-D and G3-D groups. It is interesting to note that both groups, G4-D and G5-D had 7 days of treatment with resveratrol and only G5-D was affected by the the demyelination process. Thus, it is possible that resveratrol behaves as an anti-inflammatory substance, especially when administered for short periods, since G4-R and G5-R groups did not show such inhibition of AChE activity of lymphocytes after 21 days of treatment with resveratrol. A decrease in the AChE activity of lymphocytes may indirectly reflect increased levels of ACh that may reduce local and systemic inflammatory events, due to the presence of negative feedback control exerted by ACh (21,50). It is possible that ACh derived from this source be involved in the modulation of local inflammatory processes and regulation of immune functions (21).

Studies have shown that due to its high lipid solubility, resveratrol is able to cross the blood brain barrier and enter into the brain tissue (51,52) which contributes to

its neuroprotective effect. Thus, resveratrol could promote an alternative and an early intervention would help in preventing further damage to the nervous tissue (7).

AChE inhibitors possess anti-inflammatory properties that promote cholinergic up-regulation by reducing lymphocyte proliferation and the secretion of pro-inflammatory cytokines (53). However, in our study we did not observe a decrease in the number of lymphocytes in the groups that showed a decrease in the enzyme activity. Additionally, our study showed no correlation between the number of lymphocytes of the different groups and AChE activity of these kinds of cells. In this context, it may be that ACh in T cells is synthesized when necessary and then directly released without storage. That said, further study will be needed to confirm the role of ACh storage mechanism in lymphocytes (54).

In the demyelization lesions induced by EB in the brain of rats there is the presence of inflammatory cells such as lymphocytes, infiltrating meningeal cells and Schwann cells (55,56). Although EB is able to inhibit the AChE activity in rat brain *in vitro* (57) as well as in various parts of the brain in the demyelination process (23), such inhibition was not observed in the AChE activity of lymphocytes of the demyelinated group without treatment with resveratrol (G3-R). However, it is possible that the G5-D group has been influenced in part by the process of demyelination caused by EB (55) despite the use of resveratrol, since this group did not show exactly the same intensity of response of the group treated with resveratrol without demyelination (G4-D). Thus, it is possible that non-neuronal ACh released from T cells modulates local macrophage activity via interaction through cell surface molecules (13,17).

Comparing AChE activity of lymphocytes between the different stages of demyelination and remyelination, the G4 and G5 groups showed again significant differences. Unlike the process of demyelination where there was change in the enzyme

activity in the G4-D and G5-D groups, the remyelination phase showed no change in the AChE activity of lymphocytes between the G4-R and G5-R groups. It is known that 21 days after EB injection, the remyelination is in an advanced stage (56), which may have favored the stabilization of the enzyme. However, further studies are still needed to clarify the behavior of this enzyme compared to resveratrol treatment in cases of demyelination and remyelination.

Conclusions

The treatment for 7 days with resveratrol is able to modulate the AChE activity of lymphocytes with or without the experimental demyelization with EB. The decrease of AChE activity may increase the levels of acetylcholine (ACh), an anti-inflammatory molecule, leading to a better cholinergic transmission. However, this modulation doesn't change the number of white cells in the circulation, especially lymphocytes. After 21 days of treatment with resveratrol, in the remyelination phase, there seems to be stabilization in its effect on the AChE activity of lymphocytes, suggesting a possible adaptation of the phytoestrogen to the extraneural cholinergic system. Taken together, these findings contribute to this phytoestrogen be considered a promising supplementary therapeutic substance for demyelinating diseases.

Acknowledgements

This study was supported by CAPES (Coordenação de Aperfeiçoamento de Pessoal de Nível Superior) and Programa de Pós-Graduação em Medicina Veterinária – UFSM. The data presented are part of the Doctorate thesis of DBM.

Conflicts of interest

The authors declare that there are no conflicts of interest.

References

(1) Whitten PL, Kudo S, Okubo KK. Isoflavonoids. In: D'Mello JPF, ed. Handbook of Plant and Fungal Toxicants. Boca Raton, FL: CRC Press, 1997: 117-37.

(2) Frémont L. Biological effects of resveratrol. Life Sci 2000; 66:663-73.

(3) Kennedy DO, Wightman EL, Reay JL, Lietz G, Okello EJ, Wilde A, Haskell CF. Effects of resveratrol on cerebral blood flow variables and cognitive performance in humans: a double-blind, placebo-controlled, crossover investigation. Am J Clin Nutr 2010; 91:1590-97.

(4) Rocha-Gonzalez HI, Ambriz-Tututi M, Granados-Soto V. Resveratrol: a natural compound with pharmacological potential in neurodegenerative diseases. CNS Neurosci Ther 2008; 14:234–47.

(5) Surh YJ, Hurh YJ, Kang JY, Lee E, Kong G, Lee SJ. Resveratrol, an antioxidant present in red wine, induces apoptosis in human promyelocytic leukemia (HL-60) cells. Canc Lett 1999; 140:1-10.

(6) Labinskyy N, Csiszar A, Veress G, Stef G, Pacher P, Oroszi G, Wu J, Ungvari Z. Vascular dysfunction in aging: potential effects of resveratrol, an anti-inflammatory phytoestrogen. Curr Med Chem 2006; 13:989-96.

(7) Saiko P, Szakmary A, Jaeger W, Szekeres T. Resveratrol and its analogs: defense against cancer, coronary disease and neurodegenerative maladies or just a fad? Mutat Res Rev 2008; 658:68-94.

(8) Farooqui T, Farooqui AA. Aging: an important factor for the pathogenesis of neurodegenerative diseases. Mech Ageing Dev 2009; 130:203-15.

(9) Norbury R, Cutter WJ, Compton J, Robertson DM, Craig M, Whitehead M, Murphy DG. The neuroprotective effects of estrogen on the aging brain. Exp Geront 2003; 38:109-17.

(10) Appleyard ME. Secreted acetylcholinesterase: non-classical aspects of a classical enzyme. Trends Neurosci 1992; 15:485–90.

(11) Tayebati SK, El-Assouad D, Ricci A, Amenta F. Immunochemical and immunocytochemical characterization of cholinergic markers in human peripheral blood lymphocytes. J Neuroimmunol 2002; 132:147–55.

(12) Kawashima K, Fujii T. The lymphocytic cholinergic system and its contribution to the regulation of immune activity. Life Sci 2003; 74: 675-96.

(13) Kawashima K, Fujii T. Extraneuronal cholinergic system in lymphocytes. Pharmacol Ther 2000; 86:29-48.

(14) Santos SCR, Vala I, Miguel C, Barata JT, Garção P, Agostinho P, Mendes M, Coelho AV, Calado A, Oliveira CR, Silva JM, Saldanha C. Expression and subcellular localization of a novel nuclear acetylcholinesterase protein. J Biol Chem 2007; 282:597–603.

(15) Paleari L, Grozio A, Cesario A, Russo P. The cholinergic system and cancer. Semin Cancer Biol 2008; 18:211–17.

(16) Battisti V, Schetinger MRC, Maders LDK, Santos KF, Bagatini MD, Correa MC, Spanevello RM, Araujo MC, Morsch VM. Changes in acetylcholinesterase (AChE) activity in lymphocytesand whole blood in acute lymphoblastic leukemia patients. Clin Chim Acta 2009; 402:114-18.

(17) Wessler I, Kilbinger H, Bittinger F, Unger R, Kirkpatrick CJ. The non-neuronal cholinergic system in humans: expression, function and pathophysiology. Life Sci 2003; 72:2055–61.

(18) Siegel G. Basic Neurochemistry: molecular, cellular and medical aspects. 6th ed. Philadelphia: Lippincott Willians & Wilkins, 1999; 1183p.

(19) Levine JM, Reynolds R. Activation and proliferation of endogenous oligodendrocyte precursor cells during ethidium bromide-induced demyelination. Exp Neurol 1999; 160:333-47.

(20) Stangel M, Hartung HP. Remyelinating strategies for treatment of multiple sclerosis. Prog Neurobiol 2002; 68:361-76.

(21) Das UN. Acetylcholinesterase and butyrylcholinesterase as possible markers of low-gradesystemic inflammation. Med Sci Monit 2007; 13:214-21.

(22) Mazzanti CM, Spanevello R, Ahmed M, Schmatz R, Mazzanti A, Salbego FZ, Graça DL, Sallis ESV, Morsch VM, Schetinger MRC. Cyclosporine A inhibits acetylcholinesterase activity in rats experimentally demyelinated with ethidium bromide. Int J Dev Neurosci 2007; 25: 259-64.

(23) Mazzanti CM, Spanevello R, Ahmed M, Pereira LB, Gonçalves JF, Correa M, Schmatz R, Stefanello N, Leal DBR, Mazzanti A, Ramos AT, Martins TB, Danesi CC, Graça DL, Morsch VM, Schetinger MRC. Pre-treatment with ebselen and vitamin E modulate acetylcholinesterase activity: interaction with demyelinating agents. Int J Dev Neurosci 2009; 27: 73-80.

(24) Nassar CCS, Bondan EF, Alouche SR. Effects of aquatic exercises in a rat model of brainstem demyelina tion with ethiduim bromide on the beam walking test. Arq Neuropsiquiatr 2009; 67: 652-6.

(25) Schreiner B, Heppner FL, Becher B. Modeling multiple sclerosis in laboratory animals. Semin Immunopathol 2009; 31:479-95.

(26) Mossberg N, Movitz C, Hellstrand K, Bergström T, Nilsson S, Andersen O. Oxygen radical production in leukocytes and disease severity in multiple sclerosis. J Neuroimmunol 2009; 231:131-34.

(27) Beineke A, Puff C, Seehusen F, Baumgärtner W. Pathogenesis and immunopathology of systemic and nervous canine distemper. Veterinary Immunology and Immunopathology 2009, 127:1-18.

(28) Crowell JA, Korytko PJ, Morrissey RL, Booth TD, Levine BS. Resveratrol-associated renal toxicity. Toxicol Sci 2004; 82:614-19.

(29) Rotondo S, Rajtar G, Manarini S, Celardo A, Rotilio D, De Gaetano G, Evangelista V, Cerletti C Effect of trans-resveratrol, a natural polyphenolic compound, on human polymorphonuclear leucocyte function. Braz J Pharmacol 1998; 123:1691-99.

(30) Richard N, Porath D, Radspielerand A, Schwager J. Effects of resveratrol, piceatannol, tri-acetoxystilbene, and genistein on the inflammatory response of human peripheral blood leukocytes. Mol Nutr Food Res 2005; 49:431–42.

(31) Marcondes FK, Bianchi FJ, Tanno AP. Determination of the estrous cycle phases of rats: some helpful considerations. Braz J Biol 2002; 62:609-14.

(32) Yener T, Turkkani TA, Aslan H, Aytan H, Cantug CA. Determination of oestrous cycle of the rats by direct examination: how reliable? Anat Histol Embryol 2007; 36:75–7.

(33) Schmatz R, Mazzanti CM, Spanevello R, Stefanello N, Gutierres J, Correa M, Rosa MM, Rubin MA, Schetinger MRC, Morsch VM. Resveratrol prevents memory deficits and the increase in acetylcholinesterase activity in streptozotocin-induced diabetic rats. Eur J Pharmacol 2009; 610:42-8.

(34) Boyum A. Isolation of mononuclear cells and granulocytes from human blood. Isolation of mononuclear cells by one centrifugation, and of granulocytes by combining centrifugation and sedimentation at 1g. Scand J Clin Lab Invest Suppl 1968; 97:77-89.

(35) Bergmeyer HU. Methods of Enzymatic Analysis. 3rd vol. London: Academic Press, 1983: 118-33.

(36) Ellman GL, Courtney DK, Andres V, Featherstone RM. A new and rapid colorimetric determination of acetylcholinesterase activity. Biochem Pharmacol 1961; 7: 88–95.

(37) Fitzgerald BB, Costa LG. Modulation of muscarinic receptors an acetylcholinesterase activity in lymphocytes and brain areas following repeated organophosphate exposure in rats. Toxicol Sci 1993; 20:210–16.

(38) Juan ME, Vinardell MP, Planas JM. The daily oral administration of high doses of trans-resveratrol to rats for 28 days is not harmful. J Nutr 2002; 132:257-60.

(39) Gao X, Deeb D, Media J, Divine G, Jiang H, Chapman RA, Gautam SC. Immunomodulatory activity of resveratrol: discrepant in vitro and in vivo immunological effects. Biochem Pharmacol 2003; 66:2427–35.

(40) Schmatz R, Perreira LB, Stefanello N, Mazzanti C, Spanevello R, Gutierres J, Bagatini M, Martins CC, Abdalla FH, Serres JDS, Zanini D, Vieira JM, Cardoso AM, Schetinger MR, Morsch VM. Effects of resveratrol on biomarkers of oxidative stress and on the activity of delta aminolevulinic acid dehydratase in liver and kidney of streptozotocin-induced diabetic rats. Biochimie 2012; 94: 374-83.

(41) Gautam SC, Xu YX, Dumaguin M, Janakiraman N, Chapman RA Resveratrol selectively inhibits leukemia cells: a prospective agent for ex vivo bone marrow purging. Bone Marrow Transplant 2000; 25:639-45.

(42) Tedesco I, Russo M, Russo P, Iacomino G, Russo GL, Carraturo A, Faruolo C, Moio L, Palumbo R. Antioxidant effect of red wine polyphenols on red blood cells. J. Nutr. Biochem. 2000, 11:114-9.

(43) Jang M, Cai L, Udeani GO, Slowing KV, Thomas CF, Beecher CWW, Fong HHS, Farnworth NR, King-Horn AD, Mehta RG, Moon RC, Pezzuto JM. Cancer chemopreventive activity of resveratrol, a natural product derived from grapes. Science 1997, 275:218-20.

(44) Bienzle D, Stanton JB, Embry JM, Bush SE, Mahaffey EA. Evaluation of an in-house centrifugal hematology analyzer for use in veterinary practice. J Am Vet Med Assoc 2000; 217:1195–200.

(45) Thrall MA. Hematologia e bioquímica clínica veterinária. São Paulo: Roca, 2006: 582.

(46) Yajima K, Suzuki K. Demyelination and remyelination in the rat central nervous system following ethidium bromide injection. Lab Invest 1979; 41:385-92.

(47) Bondan EF. Estudo morfológico do processo de remielinização no tronco encefálico de ratos wistar submetidos experimentalmente ao modelo gliotóxico do brometo de etídio e tratados com ciclofosfamida ou ciclosporina - Doctorate Thesis. São Paulo: Universidade de São Paulo, 1997: 190.

(48) Bondan EF, Lallo MA, Dagli MLZ, Pereira LAVD, Graça DL. Ruptura da barreira hematoencefálica após injeção de droga gliotóxica no tronco encefálico de ratos wistar. Arq Neuro-Psiquiatr 2002; 60:582-89.

(49) Kaur G, Roberti M, Raul F, Pendurthi UR. Suppression of human monocyte tissue factor induction by red wine phenolics and synthetic derivatives of resveratrol. Thromb Res 2007;119:247-56.

(50) Rao AA, Gumpeny RS, Das UN. Elevated butyrylcholinesterase and acetylcholinesterase may predict the development of type 2 diabetes mellitusand Alzheimer's disease. Med Hypotheses 2007; 69: 1272-6.

(51) Wang Q, Xu J, Rottinghaus GE. Resveratrol protects against global cerebral ischemic injury in gerbils. Brain Res 2002; 958:439-47.

(52) Jannin B, Menzel M, Berlot JP, Delmas D, Lançon A, Latruffe N. Transport of resveratrol, a cancer chemopreventive agent, to cellular targets: plasmatic protein binding and cell uptake. Biochem Pharmacol 2004; 68:1113–8.

(53) Nizri E, Hamra-Amitay Y, Sicsic C, Lavon I, Brenner T. Anti inflammatory properties of cholinergic up-regulation: a new role for acetylcholinesterase inhibitors. Neuropharmacology 2006; 50:540-7.

(54) Kawashima K, Fujii T. Basic and clinical aspects of non-neuronal acetylcholine: overview of non-neuronal cholinergic systems and their biological significance. J Pharmacol Sci 2008; 106:167-73.

(55) Bondan EF, Lallo MA, Orsini H, Bentubo HLD, Yazbek A, Macrini DJ, Bernardi MM, Graça DL. Avaliação da atividade locomotora após indução local de desmielinização tóxica no tronco encefálico de ratos Wistar. Arq Neuro-Psiquiatr 2006; 64:496-503.

(56) Sallis ESV, Mazzanti CM, Mazzanti A, Pereira LAV, Arroteia KF, Fustigatto R, Pelizzari C, Rodrigues A, Graça DL. OSP-immunofluorescent remyelinating

oligodendrocytes in the brainstem of toxically-demyelinated wistar rats. Arq Neuro-Psiquiatr 2006; 64:240-44.

(57) Mazzanti CM, Spanevello RM, Pereira LB, Gonçalves JF, Kaizer R, Corrêa M, Ahmed M, Mazzanti A, Festugatto R, Graça DL, Morsch VM, Schetinger MR. Acetylcholinesterase activity in rats experimentally demyelinated with ethidium bromide and treated with interferon beta. Neurol Res 2006; 31:1027-32.

Figure Legends

Figure 1 – Time line of the experimental protocol with ovariectomized rats during the resveratrol treatment in the different phases of demyelination and remyelination. EB: ethidium bromide.

Figure 2 – Lymphocyte AChE activity in the demyelination and remyelination phase in the different groups (n=4): sham rats - G1; ovariectomized rats, not demyelinated, treated just with vehicle (ethanol 25%) - G2; demyelinated ovariectomized rats treated just with vehicle (ethanol 25%) – G3; ovariectomized rats, not demyelinated, treated with resveratrol – G4 and demyelinated ovariectomized rats treated with resveratrol – G5. Each column represents mean± S.D. Tukey test: groups that show different letters are statistically different ($p<0.05$) when compared in a same phase. Independent samples T test: groups that show # are statistically different ($p<0.05$) when compared in a different phase.

Table Legend

Table 1 – Complete blood count in the demyelination and remyelination phase in the different groups (n=4). Each column represents mean ± S.D. Tukey test: groups that show different letters are statistically different ($p < 0.05$).

Figure 1

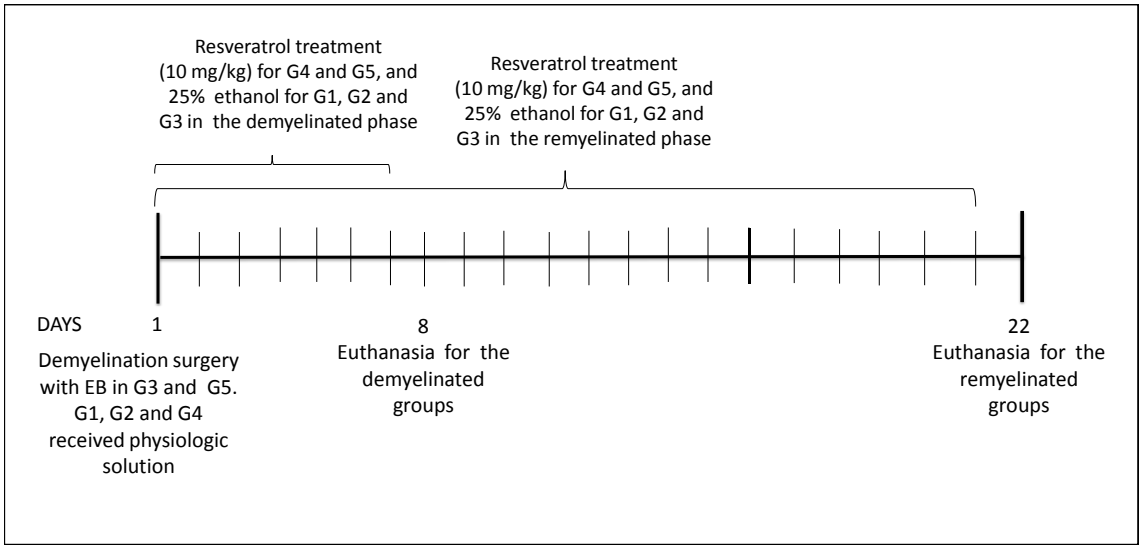
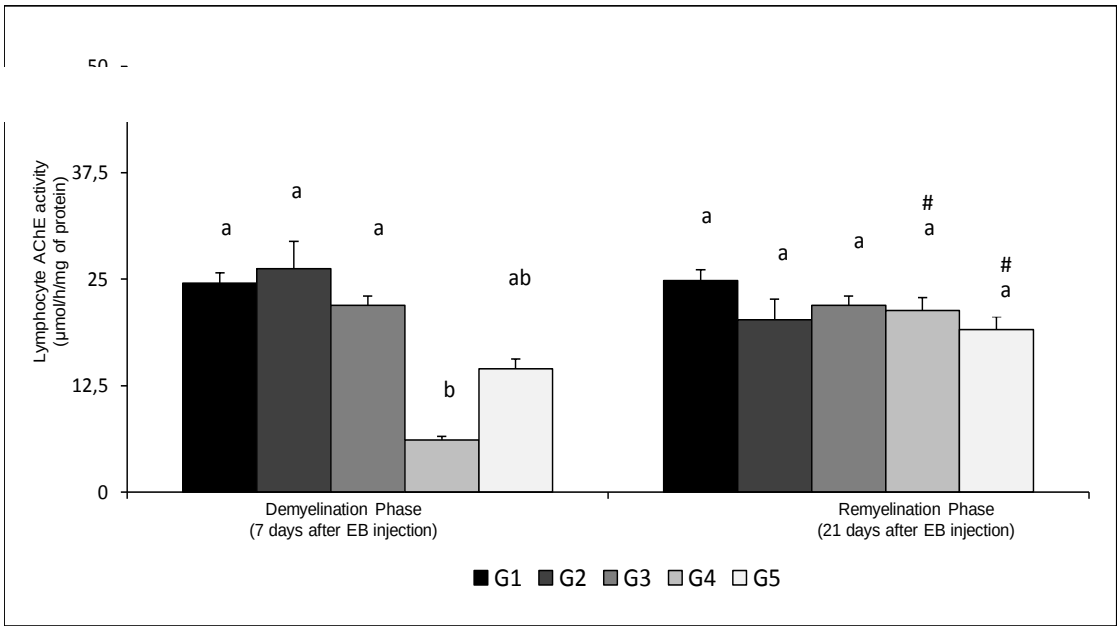


Figure 2



Table

Hematological Variables	Sham female rats, ethanol 25% (G1)	Ovariectomized rats, not demyelinated, ethanol 25% (G2)	Ovariectomized rats, demyelinated, ethanol 25% (G3)	Ovariectomized rats, not demyelinated, ethanol 25% + resveratrol (G4)	Ovariectomized rats, demyelinated, ethanol 25% + resveratrol (G5)
	Demyelination phase (7 days)				
Red blood cells					
Erythrocytes	6,97 ±0,12	6,28 ±0,40	7,21 ±0,29	6,94 ±0,45	7,03 ±0,19
Hemoglobin	11,96 ±0,15	11,25±0,91	12,60 ±0,87	10,36 ±3,37	12,12 ±1,07
Hematocrit	41 ±1,73	39 ±2,82	42 ±2,51	41 ±1,52	41 ±2,5
Mean corpuscular volume, fL	58,7 ±2,73	62,0 ±0,56	58,6 ±1,22	59,5 ±2,98	58,6 ±2,0
Mean corpuscular hemoglobin concentration, g/L	29,2 ±0,98	28,8 ±0,28	29,7 ±0,64	29,4±0,25	29,3 ±1,41
White blood cells					
Total leukocytes	5,07 ±3,07	5,8 ±0,99	6,4 ±0,98	5,2 ±2,6	5,08 ±1,32
Neutrophils	1013 ±447	2104 ±884	1825 ±508	1367 ±798	1535 ±674
Lymphocytes	3982 ±2522	3554 ±121	4555 ±598	3509 ±1511	3426 ±900
Eosinophils	56 ±96	116 ±19	17 ±30	192 ±124	64 ±48
Monocytes	14 ±25	25 ±36	35 ±61	164 ±216	49 ±49
Total plasmatic protein	7,6 ±0,20	7,2 ±0,00	7,8 ±0,30	7,7 ±0,30	7,3 ±0,30
	Remyelination phase (21days)				
Red blood cells					
Erythrocytes	7,62 ±0,57	7,9 ±0,57	7,4 ±0,20	7,3 ±0,17	7,6 ±0,19
Hemoglobin	13,57 ±0,89	14,10 ±0,56	13,20 ±0,26	12,23 ±0,65	12,9 ±0,64
Hematocrit	44 ±2,21	47 ±4,24	43 ±1,0	40 ±2,08	43 ±1,73
Mean corpuscular volume, fL	58,7 ±2,91	58,8 ±0,99	58,0 ±1,21	55,2 ±1,89	56,1 ±2,00
Mean corpuscular hemoglobin concentration, g/L	30,2 ±0,86	30,0 ±1,48	30,5 ±0,52	30,1 ±0,36	29,9 ±1,30
White blood cells					
Total leukocytes	3,5 ±1,71	4,25 ±1,71	4,77 ±2,05	3,93 ±0,56	4,16 ±1,57
Neutrophils	931 ±552	717 ±60	965 ±552	1100 ±467	1387 ±767
Lymphocytes	2537 ±1189	3402 ±342	3767 ±1566	2705 ±476	2647 ±1110
Eosinophils	70 ±71	87 ±67	26 ±26	112 ±131	124 ±97
Monocytes	35^{ab} ±17	42^{a} ±3,53	8^{ab} ±14	14^{ab} ±25	0^{b} ±0
Total plasmatic protein	8,0 ±0,48	7,5 ±0,42	7,6 ±0,11	7,8 ±0,50	7,8 ±0,23

4. DISCUSSÃO

Durante as últimas décadas, diversos trabalhos tem estudado as diferentes propriedades dos estrógenos, sendo a neuroproteção uma das mais comentadas. Além disso, estes hormônios também influenciam o crescimento, a diferenciação, a maturação e a homeostasia de vários tecidos-alvo. Contudo, com passar da idade, há uma queda gradativa na produção estrogênica. No entanto, atualmente a expectativa cada vez maior faz com que as mulheres vivam períodos maiores de carência estrogênica, visto que o início da menopausa tem se mantido fixo. Este fato contribui para uma maior incidência de patologias como disfunção cognitiva e doenças neurodegenerativas (WISE et al., 2001; WISE, 2002).

Várias são as doenças neurodegenerativas de alta importância, como a doença de Alzheimer e a Esclerose Múltipla (EM). Na EM, em especial, chama a atenção o fato de que as mulheres são mais acometidas que os homens (GRZESIUK, 2006). Todavia, é interessante ressaltar, que durante a gravidez, quando os níveis estrogênicos estão mais elevados, há uma melhora significativa na sintomatologia apresentada por estas pacientes (ZHU & GLASER, 2008). Sendo a EM uma enfermidade desmielinizante inflamatória, há a necessidade de se encontrar novas opções terapêuticas, as quais combinem propriedades neuroprotetoras com efeitos antiinflamatórios (GOLD & VOSKUHL, 2009). Desta forma, os estrógenos são candidatos primários como agentes terapêuticos na EM.

Neste trabalho, buscou-se estudar primariamente o 17-β estradiol, um dos tipos de estrógenos mais expressivos em humanos e roedores (McEWEN,

2001), e secundariamente o resveratrol, um fitoestrógeno com propriedades similares às apresentadas pelo hormônio. Para isso, no artigo investigou-se as ações do 17-β estradiol frente a ratas adultas e de meia-idade, visto que a EM acomete a faixa etária entre 19 e 57 anos (GRZESIUK, 2006). Além disso, apesar de mulheres de meia-idade serem vulneráveis a doenças neurodegenerativas (WISE et al., 2001), mulheres adultas que tem uma queda drástica em seus níveis estrogênicos, como nos casos de ooforectomia bilateral, são mais propensas a desenvolverem desordens neurológicas mais precocemente (GARCÍA-SEGURA et al., 2001; VEGETO et al., 2008). Assim, no primeiro experimento, buscou-se avaliar a atividade da AChE e relacioná-la com sistema colinérgico não-neuronal frente a terapia de reposição hormonal em fêmeas de diferentes idades. Em adição a isto, foi demonstrado se tais mudanças enzimáticas poderiam ser atribuídas à peroxidação lipídica.

Os resultados encontrados na primeira pesquisa, evidenciaram que a reposição com 17-β estradiol é capaz de influenciar a atividade da AChE nas diferentes estruturas encefálicas estudadas. O cerebelo, um local com poucas vias colinérgicas (ZIMMERMAN e SOREQ, 2006), foi a única estrutura onde a atividade da AChE manteve-se constante em todos os grupos nas diferentes idades. Já a inibição na atividade desta enzima esteve presente na presença de reposição hormonal no estriado dos animais de meia idade e no córtex cerebral dos animais adultos e de meia idade. Em determinadas condições a inibição na atividade da AChE poderia contribuir para o aprendizado e memória devido ao possível aumento dos níveis de ACh (DAS et al., 2005). O aumento na atividade da AChE foi mais freqüente nos grupos ovariectomizados sem suplementação estrogênica, como no hipocampo dos animais de meia-idade, e

no estriado nos animais adultos e de meia-idade. No entanto, o estriado dos animais adultos que tiveram reposição estrogênica foi o único caso onde a presença do 17-β estradiol elevou a atividade enzimática neuronal. GRISARU et al. (1999) comentaram que um aumento na atividade da AChE faz com que haja uma degradação mais rápida da ACh disponível na fenda sináptica, o que poderia ser prejudicial por diminuir a estimulação dos receptores deste neurotransmissor. Tal fato sugere que a baixa nos níveis hormonais podem ser prejudicial à homeostase do sistema colinérgico feminino.

Também é importante ressaltar que a atividade AChE no sangue total e nos linfócitos dos animais adultos apresentaram um alta atividade da enzima, refletindo uma ativação do sistema colinérgico nesta faixa etária com ou sem presença estrogênica. No entanto, os animais ovariectomizados de meia-idade tiveram inibição na atividade da AChE. As anormalidades do sistema colinérgico não-neuronal parece estar relacionado a certas doenças neurodegenerativas, e há evidências que sugerem que este sistema poderia ser usado como um alvo terapêutico em potencial (KAWASHIMA & FUJII, 2008)

Além disso, diversas pesquisas propõem que os estrógenos são importantes neuroprotetores devido a sua atividade antioxidante nos neurônios (VEDDER et al., 1999; AGUIAR et al., 2008). Nesta primeira pesquisa foi sugerido que a idade possa interferir na produção de radicais livres, que induzem ao aparecimento da peroxidação lipídica, na presença da ovariectomia e na reposição estrogênica. Neste sentido, a ovariectomia aumentou a peroxidação lipídica em todas as estruturas encefálicas estudadas dos animais adultos sem reposição hormonal. No entanto, a reposição estrogênica reverteu

este resultado. Apesar da ovariectomia não ter aumentado a peroxidação lipídica em todas as estruturas dos animais de meia idade, a reposição hormonal reverteu apenas parcialmente este efeito no cerebelo. Tais efeitos podem ser devido às diferentes concentrações de componentes anti-oxidantes presentes nas distintas partes estudadas do encéfalo (VATASSERY, 1992), bem como, pelas diferentes idades apresentadas pelos animais.

Desta forma, os dados obtidos neste período forneceram novas perspectivas no tratamento estrogênico direcionado às mulheres com enfermidades neurodegenerativas, visto que as ações do 17-β estradiol são variáveis conforme a idade e a região encefálica estudada. Neste aspecto, os achados deste trabalho corroboram com estudos de BROWNE et al. (2009) que demonstraram um efeito benéfico do estrógeno no número e no tamanho dos neurônios colinérgicos em macacas ovariectomizadas de diferentes idades.

No manuscrito 1, foi introduzido ao estudo do 17-β estradiol o modelo experimental de desmielinização pelo brometo de etídio (BE), uma importante ferramenta para a compreensão da patogênese de muitas doenças desmielinizantes que ainda necessitam de maiores esclarecimentos (MAZZANTI et al., 2007; MAZZANTI et al., 2009; NASSAR et al., 2009). Este modelo produz grandes lesões na ponte da cisterna basal, comprometendo de 1/3 a 1/2 desta estrutura (BONDAN, 1997). Em pacientes com EM, a ponte é o quarto maior local de incidência de lesões de desmielinização (28.6%) quando comparado com a substância branca periventricular, um local onde ocorre a maior prevalência de lesões (82.7%) (MINGUETTI, 2001; SKENDER-GAZIBARA et al., 2001).

As análises histológicas realizadas neste estudo foram fundamentais para validar o modelo experimental de desmielinização utilizado. Tais análises demonstraram que os grupos desmielinizados pela injeção de BE, decorridos sete dias do processo, apresentavam lesões caracterizadas por perda das bainhas de mielina em uma área com acúmulo celular compatível com infiltrado inflamatório. No entanto, na fase de remielinização, 21 dias após a injeção de BE, observou-se diversas áreas de remielinização, sendo outras pequenas áreas em processo de resolução, o que concorda com outros estudos anteriores (BONDAN, 1997; GRAÇA et al., 2001; MAZZANTI, 2007; RAMOS et al., 2009). É importante ressaltar que o processo de remielinização ocorreu de forma mais intensa no grupo de animais ovariectomizados e desmielinizados que receberam reposição estrogênica, fato que vem ao encontro com suas possíveis propriedades neuroprotetora e remielinizante (ZHU & GLASER, 2008).

Assim, foi a avaliado o efeito do 17-β estradiol na atividade da AChE de diferentes partes do encéfalo, sangue total e linfócitos, além da atividade da butirilcolinesterase (BuChE) sérica na fase de desmielinização e remielinização experimental pelo BE em ratas ovariectomizadas.

Nesta segunda investigação, foi demonstrado que a desmielinização experimental pelo BE em ratas sem suplementação estrogênica eleva a atividade da AChE nas diferentes estruturas encefálicas estudadas, tanto na desmielinização quanto na remielização. O aumento na atividade da AChE observado neste estudo, pode causar uma degradação muito rápida do neurotransmissor acetilcolina (ACh), diminuindo sua captação pelos receptores locais (ZHOU et al., 2002). Este fato deve ser considerado, uma vez que o

grupo que apresentou um maior aumento na atividade da AChE foi OVX+EB, ou seja, ratas ovariectomizadas, sem reposição por 17-β estradiol, e desmielinizadas. A ACh também é considerada uma molécula com efeitos anti-inflamatórios (DAS, 2007), e sua baixa disponibilidade na fenda sináptica poderia prejudicar diversos processos biológicos no encéfalo.

Ainda no mesmo estudo, a reposição estrogênica conseguiu normalizar a atividade da AChE na fase de desmielinização na maioria das estruturas encefálicas avaliadas. No entanto, houve uma inibição na atividade da enzima na fase de remielinização, no córtex cerebral, no estriado e no cerebelo. Apesar do não conhecimento se a EM tem associação com uma redução seletiva nos neurônios colinérgicos, parece que o processo de desmielinização e de dano aos axônios que caracterizam esta patologia poderiam interromper a sinalização neuronal por causarem um bloqueio na condutibilidade neuronal e prejuízo no transporte axonal (CHRISTODOULOU et al., 2006). Por isso, a AChE e o 17-β estradiol se tornam importantes ferramentas contra doenças neurodegenerativas.

Já no hipocampo, uma estrutura ligada a memória e a cognição, foi observado um aumento na atividade da AChE nas fêmeas com reposição estrogênica nas fases de desmielinização e remielinização. A teoria da neuroproteção estrogênica sobre a cognição tem sido bem aceita, todavia, seu mecanismo ainda não está claro (ZURKOVKY et al., 2007).

Também se verificou no manuscrito 1 que os resultados do sistema colinérgico não-neuronal, como atividade da AChE dos linfócitos e de sangue total, e atividade da BuChE sérica, foram dependentes da fase analisada (desmielinização ou remielinização), bem como, da presença ou não do 17-β

estradiol. As enzimas AChE e a BuChE são marcadores úteis da presença de inflamação, e é possível que a mensuração de suas atividades possa ser usada como um guia para predizer o desenvolvimento, o prognóstico e a resposta ao tratamento de diversas doenças ligadas ao sistema nervoso central e outras condições em que haja pequenas inflamações sistêmicas (DAS, 2007; RAO et al., 2007). Desta maneira, o conjunto de dados obtidos com o segundo experimento permite dizer que o 17-β estradiol é capaz de modular a atividade da AChE neuronal e não-neuronal no modelo experimental de desmielinização pelo BE.

A terceira e última parte deste experimento é apresentada no manuscrito 2. Neste trabalho, foi adicionado ao seguimento do estudo da desmielinização pelo BE, o fitoestrógeno resveratrol, um componente com propriedades similares ao estrógeno e encontrado em diversos derivados da uva (KALITA & MILLIGAN, 2010). O resveratrol tem demonstrado propriedades antiinflamatórias contra doenças neurodegenerativas (SCHMATZ et al., 2009; SHINDLER et al., 2010). Por sua vez, o sistema colinérgico não-neuronal tem desempenhado um importante papel na defesa imune do organismo (WESSLER et al., 2003; DAS, 2007; RAO et al. 2007). Por isso, nesta etapa se avaliou e correlacionou este fitoestrógeno à atividade da AChE nos linfócitos e ao hemograma de ratas ovariectomizadas e desmielinizadas pelo BE.

Os dados obtidos nesta etapa revelaram que no hemograma, com exceção dos monócitos no período de remielinização, não houve alteração no eritrograma e leucograma, independente da fase analisada. Não há uma significância clínica associada a diminuição dos monócitos circulantes (BIENZLE et al., 2000). No entanto, KAUR et al. (2007) verificaram que o

resveratrol é capaz de reduzir a produção exagerada de fatores inflamatórios produzidos por essas células em situações crônicas.

Em relação a atividade da AChE nos linfócitos houve uma diminuição da sua atividade nos grupos tratados com resveratrol durante o período de desmielinização, sendo a inibição mais intensa no grupo não desmielinizado. Um decréscimo na atividade da AChE pelo resveratrol pode contribuir para o aumento nos níveis de ACh (MESULAM et al., 2002; SCHMATZ et al., 2009). Contudo, é possível que esta ação dependa do tempo de exposição ao fitoestrógeno, visto que na fase de remielinização houve uma normalização na atividade da enzima. Neste trabalho, não foi observado uma diminuição no número de linfócitos nos grupos que apresentaram decréscimo na atividade da enzima. Soma-se a isso, o fato de não haver correlação entre o número de linfócitos nos diferentes grupos avaliados e a atividade da AChE nestas células.

O conjunto de dados analisados leva a observação de que o 17-β estradiol reverta alguns parâmetros bioquímicos e comportamentais, de forma parcial ou total, prejudicados pela queda estrogênica causada pela ovariectomia. Estes efeitos são dependentes de fatores como a idade e a estrutura encefálica analisada. Além disso, o modelo experimental de desmielinização pelo BE mimetize uma condição inflamatória como verificado pelas análises enzimáticas, sem, no entanto, influenciar os parâmetros hematológicos. A reposição estrogênica reverteu o efeito do BE na maioria dos fatores investigados. Neste aspecto, o resveratrol também demonstrou efeitos benéficos frente ao modelo de desmielinização utilizado. Contudo, maiores estudos são necessários para a elucidação completa dos mecanismos de ação do 17-β estradiol e do resveratrol no modelo experimental de desmielinização

pelo BE. Caso os presentes resultados sejam ratificados em humanos, o 17-β estradiol e resveratrol abrem uma nova perspectiva e opção terapêutica para diversas doenças neurodegenerativas, em especial aquelas onde as mulheres são o principal grupo de risco, como a EM.

5. CONCLUSÕES

Artigo 1:

- ✓ O 17-β estradiol modula a atividade da acetilcolinesterase (AChE) no estriado e no córtex cerebral de ratas adultas, e no estriado, córtex cerebral e hipocampo de ratas de meia-idade, sugerindo que este hormônio possa interagir com o sistema colinérgico neuronal.
- ✓ A ovariectomia induz a um aumento na peroxidação lipídica, principalmente nos animais adultos e o 17-β estradiol consegue reverter essa situação na maioria dos casos, indicando que a queda estrogênica é prejudicial ao sistema nervoso central e que a reposição estrogênica é fundamental para a reversibilidade do quadro nesta idade.
- ✓ A queda nos níveis hormonais causados pela ovariectomia prejudica de forma mais intensiva o sistema colinérgico não-neural dos animais de meia-idade, mas a reposição estrogênica pode reverter essa condição nesta faixa etária. Sugere-se que a avaliação do sistema colinérgico não-neuronal é uma ferramenta fundamental para a percepção precoce de possíveis processos inflamatórios.
- ✓ A ovariectomia induz a um ganho de peso mais intenso nos animais adultos quando comparado aos animais de meia-idade, sugerindo que uma queda abrupta de estrogênio em animais adultos seja prejudicial nesse aspecto.

- ✓ A união dos dados obtidos demonstra que os efeitos deste hormônio variam de acordo com a idade e as diferentes partes do encéfalo.

Manuscrito1:

- ✓ O modelo experimental de desmielinização pelo brometo de etídio (BE) aumenta a atividade da AChE em diferentes partes do encéfalo de ratas ovariectomizadas sem reposição estrogênica, tanto na fase de desmielinização quanto na fase de remielinização. Sugere-se, desta forma, que a associação do modelo utilizado e o declínio hormonal interfiram na neurotransmissão colinérgica, e consequentemente, nos níveis de acetilcolina disponíveis na fenda sináptica.
- ✓ O 17-β estradiol estabiliza a atividade da AChE na fase de desmielinização e inibe a atividade desta enzima no córtex cerebral, estriado e cerebelo na fase de remielinização. Tal fato instiga o 17-β estradiol como um possível candidato terapêutico colinérgico frente a doenças desmielinizantes em mulheres, pois é capaz de contribuir para a normalização na atividade da AChE no período de maior agressão às células neuronais.
- ✓ No hipocampo, a atividade da AChE permanece alta em ambas as fases nas ratas com suplementação estrogênica, sem haver, no entanto, prejuízo a cognição nestes animais quando comparado ao grupo ovariectomizado, como demonstrado pelo teste de esquiva inibitória, o que enfatiza a importância da reposição estrogênica no

tratamento de doenças desmielinizantes associadas com disfunção cognitiva.

- ✓ A desmielinização associada a ovariectomia aumenta a atividade da AChE no sangue total e nos linfócitos, sugerindo presença de inflamação.
- ✓ A BuChE sérica demonstrou aumento da atividade nos animais ovariectomizados e tratados com terapia estrogênica, tanto na fase de desmielinização quanto na fase de remielinização. No entanto, na fase de remielinização, a atividade da BuChE foi inibida nos animais ovariectomizados e desmielinizados tratados ou não com estrógeno.
- ✓ O 17-β estradiol modula a atividade da AChE neuronal e não-neuronal no modelo de desmielinização do BE, sugerindo que a terapia estrogênica seja uma ferramenta em potencial para o tratamento de doenças neurodegenerativas.

Manuscrito 2:

- ✓ O tratamento por sete dias com resveratrol inibe a atividade da AChE dos linfócitos, com ou sem a presença de desmielinização, sem alterar o número dessas células na circulação, o que sugere uma modulação do sistema colinérgico não-neuronal por este fitoestrógeno, podendo contribuir para o aumento da acetilcolina, uma molécula com propriedades antiinflamatórias.
- ✓ Após 21 dias de tratamento com resveratrol parece haver, na fase de remielinização, estabilização do efeito do resveratrol sobre a

atividade da AChE linfocitária, demonstrando uma possível adaptação da atividade da enzima a este fitoestrógeno, fazendo com que a atividade enzimática volte à normalidade.

- ✓ Tais achados contribuem para que esse fitoestrógeno seja considerado uma substância terapêutica complementar promissora para as doenças desmielinizantes.

6. IMPORTÂNCIA DO TRABALHO E PERSPECTIVAS

Este foi o primeiro trabalho que buscou analisar o comportamento do sistema colinérgico neural e não-neuronal no modelo de desmielinização pelo brometo de etídio (BE) e seus efeitos na terapia estrogênica. Buscou-se esta pesquisa, visto que, mulheres com declínio na taxa hormonal são mais propensas a doenças neurodegenerativas, e a esclerose múltipla, uma doença neurodegenerativa de caráter desmielinizante, que afeta significativamente mais mulheres do que homens.

No entanto, procurou-se saber antes como se comportava a atividade da acetilcolinesterase (AChE) e o nível da peroxidação lipídica de animais adultos e de meia-idade frente ao declínio hormonal e a reposição estrogênica, já que a EM acomete mulheres adultas jovens e de meia idade. Observou-se que o sistema colinérgico é bastante afetado pela queda estrogênica, especialmente nos animais de meia idade. De forma similar, a baixa hormonal contribui para o aumento da peroxidação lipídica. Mas, a reposição com o 17-β estradiol consegue reverter este quadro, especialmente nos animais adultos.

Logo após, o modelo de desmielinização pelo BE demonstrou potencializar o prejuízo ao sistema colinérgico já causado pela ovariectomia em diversas partes do encéfalo. Todavia, a reposição estrogênica conseguiu atenuar ou reverter tais efeitos na maioria dos casos. Na análise do comportamento, os animais desmielinizados e com terapia estrogênica tiveram melhor desenvoltura na esquiva inibitória do que os animais apenas ovariectomizados, evidenciando mais um fator que contribui para os efeitos benéficos deste hormônio no processo desmielinizante. Além disso, foi possível

observar através da análise do sistema colinérgico não-neuronal e de análises histopatológicas que o modelo de desmielinização utilizado sucinta uma pequena resposta inflamatória local e sistêmica, que pode envolver de forma indireta a acetilcolina (ACh), uma molécula com ações antiinflamatórias.

Por fim, optou-se por utilizar o mesmo modelo de desmielinização frente a uma molécula alternativa, ou seja, com propriedades similares ao 17-β estradiol e que fosse de fácil aquisição e uso. Nesta pesquisa, foi evidenciado que este composto modula a atividade da AChE provavelmente em condições inflamatórias como ocorre após uma queda brusca hormonal ou um processo cirúrgico de desmielinização. É importante ressaltar que apesar da atividade da AChE ser sensível a mudanças nesta fase, nenhuma alteração foi evidenciada pelas células de defesa do sangue. Já na fase de remielinização ou estabilização dos níveis hormonais, a AChE permaneceu com sua atividade normalizada. Tais achados enfatizam a importância da continuação de estudos utilizando o resveratrol, ou mesmo outras substâncias similares, para uso preventivo ou tratamento complementar de doenças neurodegenerativas.

Ainda há muito a ser estudado e descoberto nesta área, e neste aspecto pode-se aprofundar ainda mais os estudos sobre o assunto a partir dos dados obtidos com o presente trabalho:

a) Mensurar o estresse oxidativo utilizando o 17-β estradiol e o modelo de desmielinização pelo BE.
b) Realizar o estudo morfométrico comparativo da análise histológica dos encéfalos dos animais desmielinizados e tratados com 17-β estradiol e resveratrol.

c) Verificar o sistema colinérgico neuronal nas diferentes estruturas encefálicas utilizando o modelo de desmielinização e o resveratrol.

d) Utillizar tanto o resveratrol quanto o 17-β estradiol de forma preventiva no modelo de desmielinização pelo BE.

e) Avaliar a atividade da AChE e o estresse oxidativo em ratas ovariectomizadas jovens, adultas, meias-idades e senis tratadas com resveratrol.

f) Identificar por métodos imunoistoquímicos a expressão da AChE no sistema nervoso central.

g) Administrar doses mais baixas de 17-β estradiol por um período mais extenso, antes da lesão experimental.

h) Utilizar em futuras pesquisas produtos de fácil acesso aos portadores de esclerose múltipla como o suco de uva, um produto rico em resveratrol.

7. REFERÊNCIAS BIBLIOGRÁFICAS

ACS, P. et al. 17β-estradiol and progesterone prevent cupriozone provoked demyelination of corpus callosum in male mice. **Glia**, v.57, p.807-814, 2009.

AGUIAR, R.B. et al. Estradiol valerate and tibolone: effects upon brain oxidative stress and blood biochemistry during aging in female rats. **Biogerontology**, v.9, p.285-298, 2008.

ALBERTAZZI, P.; PURDIE, D. The nature and utility of the phytoestrogens: a review of the evidence. **Maturitas**, v.42, p.173-185, 2002.

ANDERSEN, J.K. Oxidative stress in neurodegeneration: cause or consequence? **Nature Medicine**, v.10S, p.S18-S25, 2004.

APPLEYARD, M.E. Non-cholinergic functions of acetylcholinesterase. **Biochemical Society Transactions**, v.22, p.749-755, 1994.

BAEZA, I. et al. Ovariectomy, a model of menopause in rodents, causes a premature aging of the nervous and immune systems. **Journal of Neuroimmunology**, v.219, p.90-99, 2010.

BAUMANN, N.; PHAM-DINH, D. Biology of oligodendrocyte and myelin in the mammalian central nervous system. **Physiological Reviews**, v.81, p.871-927, 2001.

BAUR, J.A.; SINCLAIR, D.A. Therapeutic potential of resveratrol: the in vivo evidence. **Nature Reviews - Drug Discovery**, v.5, p.493-506, 2006.

BEBO, B.F. et al. Low-dose estrogen therapy ameliorates experimental autoimmune encephalomyelitis in two different inbred mouse strains. **The Journal of Immunology**, v.166, p.2080-2089, 2001.

BEINEKE, A. et al. Pathogenesis and immunopathology of systemic and nervous canine distemper. **Veterinary Immunology and Immunopathology**, v.127, p.1-18, 2009.

BELCHER, S.M.; ZSARNOVSZKY, A. Estrogenic actions in the brain: estrogen, phytoestrogens, and rapid intracellular signaling mechanisms. **Journal of Pharmacology and Experimental Therapeutics**, v.299, p.408-414, 2001.

BIANCHI, M.L.P.; ANTUNES, L.M.G. Radicais livres e os principais antioxidantes da dieta. **Revista de Nutrição**, v.12, p.123-130, 1999.

BIENZLE, D. et al. Evaluation of an in-house centrifugal hematology analyzer for use in veterinary practice. **Journal of American Veterinary Medical Association**, v.217, p.1195–200, 2000.

BONDAN, E.F. **Estudo morfológico do processo de remielinização no tronco encefálico de ratos wistar submetidos experimentalmente ao modelo gliotóxico do brometo de etídio e tratados com ciclofosfamida ou ciclosporina**, 1997. 190f. Tese (Doutorado em Patologia Experimental e Comparada) – Universidade de São Paulo, São Paulo.

BONDAN, E.F. et al. Avaliação da atividade locomotora após indução local de desmielinização tóxica no tronco encefálico de ratos Wistar. **Arquivos de Neuro-Psiquiatria**, v.64, 496-503, 2006.

BONDAN, E.F. et al. Ruptura da barreira hematoencefálica após injeção de droga gliotóxica no tronco encefálico de ratos wistar. **Arquivos de Neuro-Psiquiatria**, v.60, p.582-589, 2002.

BOWERS, J.L. et al. Resveratrol acts as a mixed agonist/antagonist for estrogen receptors alpha and beta. **Endocrinology**, v.141, p.3657-3667, 2000.

BRANN, D.W. et al. Neurotrophic and neuroprotective actions of estrogen: basic mechanisms and clinical implications. **Steroids**, v.72, p.381-405, 2007.

BRAUGHLER, J.M. et al. Novel 21-amino steroids as potent inhibitors of iron-dependent lipid peroxidation. **The Journal of Biological Chemistry**, v.262, n.22, p.10438-10440, 1987.

BROWNE, C. et al. Effects of two years of conjugated equine estrogens on cholinergic neurons in young and middle-aged ovariectomized monkeys. **Brain Research**, v.1264, p.13-23, 2009.

CHEN, J.H.; HALES, C.N.; OZANNE, S.E. DNA damage, cellular senescence and organismal ageing: causal or correlative? **Nucleic Acids Research**, v.35, n.22, p.7417-7428, 2007.

CHRISTODOULOU, C. et al. Effects of donepezil on memory and cognition in multiple sclerosis. **Journal of the Neurological Sciences**, v.245, p.127-136, 2006.

COKUGRAS, A.N. Butyrylcholinesterase: structure and physiological importance. **Turkish Journal of Biochemistry**, v.v.28, p.54-61, 2003.

COMBARROS, O. et al. Interaction between CYP19 aromatase and butyrylcholinesterase genes increases Alzheimer's disease risk. **Dementia and Geriatric Cognitive Disorders**, v.20, p.153-157, 2005.

COMBARROS, O. et al. Interaction between estrogen receptor-α and butyrylcholinesterase genes modulates Alzheimer's disease risk. **Journal of Neurology**, v.254, p.1290-1292, 2007.

CRAFT, R.M. Modulation of pain by estrogens. **Pain**, v.132, p.S3-S12, 2007.

CRAIG, M.C. et al. The interactive effect of acute ovarian suppression and the cholinergic system on visuospatial working memory in young women. **Psychoneuroendocrinology**, v.35, p.987-1000, 2010.

DARREH-SHORI, T. et al. Differential CSF butyrylcholinesterase levels in Alzheimer's disease patients with the ApoE ε4 allele, in relation to cognitive function and cerebral glucose metabolism. **Neurobiology of Disease**, v.24, p.326-333, 2006.

DARVESH, S et al. Butyrylcholinesterase in normal human amygdale and hippocampal formation. **The Journal of Comparative Neurology,** v.393, p.374-390, 1998.

DARVESH, S. et al. Neurobiology of butyrylcholinesterase. **Nature Reviews – Neuroscience**, v.4, p.131-138, 2003.

DAS, A. et al. Effect of ovariectomy and estrogen supplementation on brain acetylcholinesterase activity and passive-avoidance learning in rats. Canadian **Journal of Physiology and Pharmacology**, v.80, p.907-914, 2002.

DAS, A.; DIKSHIT, M.; NATH, C. Profile of acetylcholinesterase in brain areas of male and female rats of adult and old age. **Life Sciences**, v.68, p.1545-1555, 2001.

DAS, U.N. Acetylcholinesterase and butyrylcholinesteraseas possible markers of low-grade systemic inflammation. **Medical Science Monitor**, v.13, p.214-221, 2007.

DAY, T.; GREENFIELD, S.A. A non-cholinergic, trophic action of acetylcholinesterase on hippocampal neurons in vitro: molecular mechanisms. **Neuroscience**, v. 111, p.649-656, 2002.

DESCARRIES, L. et al. Diffuse transmission by acetylcholine in the CNS. **Progress in Neurobiology**, v.53, p.603-325, 1997.

DWOSH, E. et al. The interaction of MS pregnancy: a critical review. **International Multiple Sclerosis Journal**, v.10, p.38-42, 2003.

ELLIS, M.J. et al. Lower-dose vs high-dose oral estradiol therapy of hormone receptor positive, aromatase inhibitor resistant advanced breast cancer: a phase 2 randomized study. **Journal of American Medical Association**, v.302, p.774-780, 2009.

EVANS, J.L. et al. Oxidative stress and stress-activated signaling pathways: a unifying hypothesis of type 2 diabetes. **Endocrinology Reviews**, v.23, p.599-622, 2002.

FAILACE, R. **Hemograma: manual de interpretação**. 4ed. Porto Alegre: Artmed, 298p., 2003.

FALKENSTEIN, E. et al. Multiple actions of steroid hormones – a focus on rapid, nongenomic effects. **Pharmacological Reviews**, v.52, p.513-555, 2000.

FAROOQUI, T.; FAROOQUI, A.A. Aging: an important factor for the pathogenesis of neurodegenerative diseases. **Mechanisms of Ageing and Development**, v.130, p.203-215, 2009.

FELDMAN, R.S.; QUENZER, L.F. **Fundamentals of Neuropsychofarmacology**. Sunderland: Sinauer Associates, 1984. 528p.

FENG, Z. et al. Long-term effects of melatonin or 17 beta-estradiol on improving spatial memory performance in cognitively impaired, ovariectomized adult rats. **Journal of Pineal Research**, v.37, p.198-206, 2004.

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

FREMONT, L. Biological effects of resveratrol. **Life Sciences**, v.66, p.663-673, 2000.

FUSHIMI, S.; SHIRABE, T. Expression of insulin-like growth factors in remyelination following ethidium bromide-induced demyelination in the mouse spinal cord. **Neuropathology**, v.24, p.208-218, 2004.

FUSHIMI, S.; SHIRABE, T. The reaction of glial progenitor cells in remyelination following ethidium bromide-induced demyelination in the mouse spinal cord. **Neuropathology**, v.22, p.233-242, 2002.

GAO, X. et al. Immunomodulatory activity of resveratrol: discrepant in vitro and in vivo immunological effects. **Biochemical Pharmacology**, v.66, p2427–2435, 2003.

GAO, Z.B. et al. Inhibition of excitatory synaptic transmission by trans-resveratrol in rat hippocampus. **Brain Research**, v. 1111, p. 41-47, 2006.

GARCÍA-SEGURA, L.M. et al. Neuroprotection by estradiol. **Progress in Neurobiology**, v.63, p. 29-60, 2001.

GILGUN-SHERKI, Y. et al. Oxidative stress induced-neurodegenerative diseases: the need for antioxidants that penetrate the blood brain barrier. **Neuropharmacology**, v.40, p.959-975, 2001.

GOLD, S.M.; VOSKUHL, R.R. Estrogen treatment in multiple sclerosis. **Journal of the Neurological Sciences**, v.286, p.99-103, 2009.

GRAÇA, D.L. et al. Behaviour of oligodendrocytes and Schwann cells in na experimental modelo f toxic demyelination of the central nervous system. **Arquivos de Neuro-Psiquiatria**, v.59, p.358-361, 2001.

GRISARU, D. et al. Structural roles of acetylcholinesterase variants in biology and pathology. **European Journal of Biochemistry**, v.264, p.272-286, 1999.

GRZESIUK, A.K. Características clínicas e epidemiológicas de 20 pacientes portadores de esclerose múltiplaacompanhados em Cuiabá – Mato Grosso. **Arquivos de Neuro-Psiquiatria**, v.64, p.635-638, 2006.

GUAZZO, E. A technique for producing demyelination of the rat optic nerves. **Journal of Clinical Neuroscience.** v.12, p.54-58, 2005.

HA, B.J. et al. The role of *Salicornia herbacea* in ovariectomy-induced oxidative stress. **Biological Pharmacy Bulletin**, v.29, p.1305-1309, 2006.

HALLIWELL, B.; GUTTERIDGE, J.C. **Free Radicals in Biology and Medicine**. 4th ed. New York: Oxford University, 2007. 851p.

HEINZ, H. et al. Oestrogen-A protective factor in schizophrenia? **Current Psychiatry Reviews**, v.2, p. 339-352, 2006.

HENRY, L.A.; WITT, D.M. Resveratrol: phyestrogen effects on reproductive physiology and behavior in female rats. **Hormones and Behavior**, v.41, p.220-228, 2002.

HUNTLEY, A.L. Grape flavonoids and menopausal health. **Menopause International**, v.13, p.165-169, 2007.

JOHNSON, G.; MOORE, S.W. The adhesion function on acetylcholinesterase is located at peripheral anionic site. **Biochemical and Biophysical Research Communications**, v.258, p. 758-762, 1999.

KAAJA, R.J.; GREER, I.A. Manifestations of chronic disease during pregnancy. **Journal of the American Medical Association**, v.294, p.2751-2757, 2005.

KALITA, J.C.; MILLIGAN, S.R. In vitro estrogenic potency of phytoestrogen-glycosides and some plant flavanoids. **Indian Journal of Science and Technology**, v.3, p.1142-1147, 2010.

KAUR, G. et al. Suppression of human monocyte tissue factor induction by red wine phenolics and synthetic derivatives of resveratrol. **Thrombosis Research**, v.119, p.247-256, 2007.

KAWASHIMA, K.; FUJII, T. Extraneuronal cholinergic system in lymphocytes. **Pharmacology & Therapeutics**, v.86, p.29-48, 2000.

KAWASHIMA, K.; FUJII, T. Basic and clinical aspects of non-neuronal acetylcholine: overview of non-neuronal cholinergic systems and their biological significance. **Journal of Pharmacological Sciences**, v.106, p.167-173, 2008.

KIPP, M. et al. The cuprizone animal model: new insights into an old story. **Acta Neuropathologica**, v.118, p.723-736, 2009.

KOSTOVIC, I.; GOLDMAN-RAKIC, P.S. Transient cholinesterase staining in the mediodorsal nucleus of the thalamus and its connections in the developing human and monkey brain. **Journal of Comparative Neurology**, v.219, p.431–447, 1983.

KUMAR, P. et al. Physiological and biochemical effectsof 17β estradiol in aging female rat brain. **Experimental Gerontology**, v.46, p.597-605, 2011.

LABINSKYY, N. et al. Vascular dysfunction in aging: potential effects of resveratrol, an anti-inflammatory phytoestrogen. **Current Medicinal Chemistry**, v.13, p.989-996, 2006.

LEVINE, J.M.; REYNOLDS, R. Activation and proliferation of endogenous oligodendrocyte precursor cells during ethidium bromide-induced demyelination. **Experimental Neurology**, v.160, p.333-347, 1999.

LI, X. et al. The action of resveratrol, a phytoestrogen found in grapes, on the intervertebral disc. **Spine**, v.33, p.2586-2595, 2008.

LOVE, S. Demyelinating diseases. **Journal of Clinical Pathology**, v.59, p.1151-1159, 2006.

MARDER, K.; SANO, M. Estrogen to treat Alzheimer's disease: too little, too late?: so what's a woman to do? **Neurology**, v.54, p.2035-2037, 2000.

MARITIM, A.C. et al. Diabetes, oxidative stress, and antioxidants. **Journal of Biochemistry and Molecular Toxicology**, v.17, p.24-38, 2003.

MARQUES, F.Z. et al. Resveratrol: cellular actions of a potent natural chemical that confers a diversity of health benefits. **The International Journal of Biochemistry & Cell Biology**, v.41, p.2125-2128, 2009.

MATTSON, M.P.; LIU, D. Energetics and oxidative stress in synaptic plasticity and neurodegenerative disorders. **Neuromolecular Medicine**, v.2, p.215-231, 2002.

MAZZANTI, C.M. et al. Pre-treatment with ebselen and vitamin E modulate acetylcholinesterase activity: interaction with demyelinating agents. **International Journal of Developmental Neuroscience**, v.27, p.73-80, 2009.

MAZZANTI, C.M.A. **Efeito do interferon beta, da ciclosporina A, do ebselen e da vitamina E no sistema colinérgico e purinérgico de ratos normais e submetidos à desmielinização pelo brometo de etídio**, 2007. 160f. Tese (Doutorado em Bioquímica) - Universidade Federal do Rio Grande do Sul, Porto Alegre.

McCABE, M.P. et al. Mood and quality of life among people with progressive neurological illness. **International Journal of Clinical and Health Psychology**, v.9, p.21-35, 2009.

McEWEN, B.S.; ALVES, S.E. Estrogen actions in the central nervous system. **Endrocrine Reviews**, v.20, p.279-307, 1999.

McEWEN, B.S. Estrogens effects on the brain: multiple sites and molecular mechanisms. **Journal of Applied Physiology**, v.91, p.2785-2801, 2001.

MESULAM, M.M. et al. Acetylcholinesterase knockouts establish central cholinergic pathways and can use butyrylcholinesterase to hydrolyse acetylcholine. **Neuroscience**, v.110, p.627-639, 2002.

MINGUETTI, G. Ressonância magnética na esclerose múltipla. **Arquivos de Neuropsiquiatria**, v.59, 563-569, 2001.

MOKNI, M. et al. Effect of resveratrol on antioxidant enzyme activities in the brain of healthy rat. **Neurochemical Research**, v.32, p.981-987, 2007.

MONTEIRO, S.C. **Alterações bioquímicas e comportamentais causadas pela ovariectomia em ratas adultas. Efeito da suplementação com antioxidantes e soja,** 2007. 188f. Tese (Doutorado em Bioquímica) - Universidade Federal do Rio Grande do Sul, Porto Alegre.

MONTEIRO, S.C. et al. Ovariectomy enhances acetylcholinesterase activity but does not alter ganglioside content in cerebral cortex of female adult rats. **Metabolic Brain Disease**, v.20, p.35-44, 2005.

MOORTHY, K. et al. Effect of hormone replacement therapy in normalizing age related neuronal markers in different age groups of naturally menopausal rats. **Biogerontology**, v.6, p.345-356, 2005.

MOSSBERG, N. et al. Oxygen radical production in leukocytes and disease severity in multiple sclerosis. **Journal of Neuroimmunology**, v.213, p.131-134, 2009.

MUELLER, S.O. et al. Phytoestrogens and their human metabolites show distinct agonistic and antagonistic properties on estrogen receptor α (ERα) and ERβ in humans cells. **Toxicological Sciences**, v.80, p.14-25, 2004.

NASSAR, C.C.S. et al. Effects of aquatic exercises in a rat modelo f brainstem demyelination with ethidium bromide on the beam walking test. **Arquivos de Neuro-Psiquiatria**, v.67, p.652-656, 2009.

ODA, Y. Choline acetyltransferase: the structure, distribution and pathologic changes in the central nervous system. **Pathology International**, v.49, p.921-937, 1999.

OSOSKI, A.L.; KENNELLY, E.J. Phytoestrogens: a review of the present state of research. **Phytoterapy Research**, v.17, p.845-869, 2003.

ORSINI, H. et al. Marcação imunoistoquímica da expressão astrocitária de proteína glial fibrilar ácida e de vimentina no sistema nervoso central de cães com cinomose. **Arquivos de Neuro-Psiquiatria**, v.65, p.1070-1077, 2007.

PAXINOS, G. **The rat nervous system. Forebrain and midbrain**. Sidney: Academic, 1985. p. 487-508.

PEREIRA JUNIOR, M. et al. Estrogen effects on pilocarpine-induced temporal lobe in rats. **Maturitas**, v.62, p.190-196, 2009.

PEREIRA, R.T.S. et al. Effects of estrogenson intracellular signalingathways linked to activation of muscarinic acetylcholine receptors and on acetylcholinesterase activity in rat hippocampus. **Biochemical Pharmacology**, v.75, p.1827-1834, 2008.

PERRY, E.; WALKER, M.; GRACE, J.; PERRY, R. Acetylcholine in mind: a neurotransmitter correlate of consciousness? **Trends in Neuroscience**, v.22, n.6, p.273-280, 1999.

RAMOS, A.T. Remyelination in experimentally demyelinated connexin 32 knockout mice. **Arquivos de Neuro-Psiquiatria**, v.67, p.488-493, 2009.

RAO, A.A. et al. Elevated butyrylcholinesterase and acetylcholinesterase may predict the development of type 2 diabetes mellitus and Alzheimer's disease. **Medical Hypotheses**, v.69, p.1272-1276, 2007.

RICHARD, N. et al. Effects of resveratrol, piceatannol, tri-acetoxystilbene, and genistein on the inflammatory response of human peripheral blood leukocytes. **Molecular Nutritional Food Research**, v.49, p.431–442, 2005.

RIET-CORREA, G. et al. Ethidium bromide-induced demyelination of the sciatic nerve of adult Wistar rats. **Brazilian Journal of Medical and Biological Research**, v.35, p.99-104, 2002.

ROTONDO, S. et al. Effect of trans-resveratrol, a natural polyphenolic compound, on human polymorphonuclear leucocyte function. **Brazilian Journal of Pharmacology**, v.123, p.1691-1699, 1998.

SAIKO, P. et al. Resveratrol and its analogs: defense against cancer, coronary disease and neurodegenerative maladies or just a fad? **Mutation Research Review**, v.658, p.68-94, 2008.

SALLIS, E.S.V. **Resposta astrocitária e oligodendroglial no tronco encefálico de ratos wistar imunossuprimidos e submetidos ao modelo desmielinizante do brometo de etídio**, 2005. 69f. Tese (Doutorado em Medicina Veterinária) – Universidade Federal de Santa Maria, Santa Maria.

SALLIS, E.S.V. et al. OSP-immunofluorescent remyelinating oligodendrocytes in the brainstem of toxically-demyelinated Wistar rats. **Arquivos de Neuro-Psiquiatria**, v.64, p.240-244, 2006.

SAIKO, P. et al. Resveratrol and its analogs: defense against cancer, coronary disease and neurodegenerative maladies or just a fad? **Mutation Research Review**, v.658, p.68-94, 2008.

SAKAMOTO, T. et al. Effects of diverse dietary phytoestrogens on cell growth, cell cycle and apoptosis in estrogen-receptor-positive breast cancer cells. **Journal of Nutritional Biochemisty**, v.21, p.856-864, 2010.

SATO, T. et al. Effects of ovariectomy and calcium deficiency on learning and memory of eight-arm radial maze im middle aged female rats. **Behavioural brain research**, v.142, p.207-216, 2003.

SAUTTER, C.K. et al. Determinação do resveratrol em sucos de uva no Brasil. **Ciência e Tecnologia dos Alimentos**, v.25, p.437-442, 2005.

SCHMATZ, R. et al. Resveratrol prevents memory deficits and the increase in acetylcholinesterase activity in streptozotocin-induced diabetic rats. **European Journal of Pharmacology**, v.610, p.42-48, 2009.

SCHMDIT, C. et al. Densidade mineral óssea em cadelas submetidas à ovarioisterectomia com e sem reposição estrogênica oral. **Arquivo Brasileiro de Medicina Veterinária e Zootecnia**, v.58, p.506-510, 2006.

SCHMDIT, C. et al. Perfil lipoprotéico de cadelas submetidas à ovarioisterectomia com e sem reposição estrogênica. **Arquivo Brasileiro de Medicina Veterinária e Zootecnia**, v.56, p.449-456, 2004.

SHINDLER, K.S. et al. Oral Resveratrol Reduces Neuronal Damage in a Model of Multiple Sclerosis. **Journal of Neuro-Ophthalmology**, v.30, p. 328-339, 2010.

SHUGHRUE, P. et al. Comparative distribution of estrogen receptor-α (ER-α) and β (ER-β) mRNA in the rat pituitary, gonad, and reproductive tract. **Steroids**, v.63, p.498-504, 1998.

SICOTTE, N.L. et al. Treatment of multiple sclerosis with the pregnancy hormone estriol. **Annals of Neurology**, v.52, p.421-428, 2002.

SIEGEL, G. **Basic Neurochemistry: molecular, cellular and medical aspects**. 6 ed. Philadelphia: Lippincott Willians & Wilkins, 1999, 1183p.

SIES, H. Strategies of antioxidant defense - review. **European Journal of Biochemistry**, v.215, p.213-219, 1993.

SKENDER-GAZIBARA, M. et al. Etiopathogenesis of multiple sclerosis. A**rchives of Oncology**, v.9, p.8-10, 2001.

SOREQ, H.; SEIDMAN, S. Acetylcholinesterase – new roles for an old actor. **Nature Reviews - Neuroscience**, v.2, p.8-17, 2001.

TAKUR, M.K.; SHARMA, P.K. Aging of brain: role of estrogen. **Neurochemical Research**, v.31, p.1389-1398, 2006.

TEDESCO, I. et al. Antioxidant effect of red wine polyphenols on red blood cells. **Journal of Nutritional Biochemistry**, v.11, p.114-119, 2000.

TEMPFER, C.B. et al. Side effects of phytoestrogens: a meta-analysis of randomized trials. **The American Journal of Medicine**, v.122, p.939-946, 2009.

TURKO, I.V. et al. Diabetes-associated nitration of tyrosine and inactivation of succynil-CoA: 3-oxoacid CoA-transferase. **American Journal of Physiology. Heart and Circulatory Physiology**, v.281, p.H2289-H2294, 2001.

VALENTE, S.G. et al. Castration in female rats modifies the development of the pilocarpine model of epilepsy. **Epilepsy Research**, v.49, p.181-188, 2002.

VATASSERY, G.T. Vitamin E: Neurochemistry and implication for Parkinson's disease. **Annals of the New York Academy of Sciences**, v.669, p.92-110, 1992.

VEDDER, H. et al. Estrogen hormones reduce lipid peroxidation in cells and tissues of the central nervous system. **Journal of Neurochemistry**, v.72, n.6, p.2531-2538, 1999.

VEGETO, E. et al. Estrogen anti-inflamatory activity in brain: a therapeutic opportunity for menopause and neurodegenerative diseases. **Frontiers in Neuroendocrinology**, v.29, p.507-519, 2008.

VEGETO, E. et al. Estrogen receptor-α mediates the brain antiinflammatory activity of estradiol. **PNAS**, v.100, p.9614-9619, 2003.

VITRAC, X. et al. Determination of stilbenes (δ-viniferin, trans-astringin, trans-piceid, cis and trans-resveratrol, β-viniferin) in Brazilian wines. **Journal of Agricultural and Food Chemistry**, v.53, p.5664-5669, 2005.

VOSKUHL, R.R. Hormone-based therapies in MS. **International MS Journal**, v.10, p.60-66, 2003.

YAJIMA, K. Demyelination in the rat central nervous system following ethidium bromide injection. **Laboratory Investigation**, v.41, p.385-392, 1979.

YASUI, T. et al. Biological effects of hormone replacement therapy in relation to serum estradiol levels. **Hormone Research**, v.56, p.38-44, 2001.

WANG, Q. et al. Resveratrol protects against neurotoxicity induced by kainic acid. **Neurochemical Research**, v.29, p.2105-2112, 2004.

WESSLER, I. et al. The non-neuronal cholinergic system in humans: expression, function and pathophisiology. **Life Sciences**, v.72, p.2055-2061, 2003.

WISE, P.M. Estrogens and neuroprotection. **Trends in Endocrinology and Metabolism**, v.13, p.229-230, 2002.

WISE, P.M. et al. Neuroprotective effects of estrogen – new insights into mechanisms of action. Endocrinology, v.142, p.969-973, 2001.

ZIMMERMAN, G.; SOREQ, H. Termination and beyond: acetylcholinesterase as a modulator of synaptic transmission. Cell Tissue Research, v.326, p.655-669, 2006.

ZHANG, J.Q. et al. Distribution and differences of estrogen receptor beta immunoreactivity in the brain of adult male and female rats. **Brain Research**, v.935, p.73-80, 2002.

ZHENG, P. Neuroactive steroid regulation of neurotransmitter release in the CNS: action, mechanism and possible significance. **Progress in Neurobiology**, v.89, p.134-152, 2009.

ZHOU, F.M. et al. Cholinergic interneuron characteristics and nicotinic properties in the striatum. **Journal of Neurobiology**, v.53, p.590-605, 2002.

ZHU, T.S.; GLASER, M. Neuroprotection and enhancement of remyelination by estradiol and dexamethasone in cocultures of rat DRG neurons and Schwann cells. **Brain Research**, v.1206, p.20-32. 2008.

8 ANEXOS

Preview

From: jirillo@midim.uniba.it

To: vetdanielimartins@yahoo.com.br

CC:

Subject: Immunopharmacology And Immunotoxicology - Decision on Manuscript ID LIPI-2011-0218.R2

Body: @@date to be populated upon sending@@

Dear Dr Martins:

Ref: Complete blood count and acetylcholinesterase activity of lymphocytes of demyelinated and ovariectomized rats treated with resveratrol

Our referees have now considered your paper and have recommended publication in Immunopharmacology And Immunotoxicology. We are pleased to accept your paper in its current form which will now be forwarded to the publisher for copy editing and typesetting. The reviewer comments are included at the bottom of this letter, along with those of the editor who coordinated the review of your paper.

You will receive proofs for checking, and instructions for transfer of copyright in due course.

The publisher also requests that proofs are checked and returned within 48 hours of receipt.

Thank you for your contribution to Immunopharmacology And Immunotoxicology and we look forward to receiving further submissions from you.

Sincerely,
Professor Jirillo
Editor in Chief, Immunopharmacology And Immunotoxicology
jirillo@midim.uniba.it

Referee(s)' Comments to Author:

Referee: 1
Comments to the Author
All modifications that was solicited were realized.

Editor's Comments to Author:

Date Sent: 02-Apr-2012