Universidade Federal do Rio Grande do Sul

Universidade Federal do Pará

Programa de Pós-Graduação em Medicina: Ciências Médicas

DINTER UFRGS/UFPA

AVALIAÇÃO DE MODELO DE MENOPAUSA EM RATAS: PARÂMETROS FISIOLÓGICOS, COMPORTAMENTAIS, BIOQUÍMICOS E NOVAS ESTRATÉGIAS TERAPÊUTICAS

Aluna: Sônia Fátima da Silva Moreira

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**Se alguém julga saber alguma coisa, com efeito,
não aprendeu ainda como convém saber.**

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RESUMO

Introdução: Os sintomas psíquicos e vasomotores são altamente prevalentes na transição menopáusica e na pós-menopausa, e estão relacionados ao hipoestrogenismo decorrente da falência ovariana que ocorre na mulher na meia-idade. Sua exata fisiopatogenia é desconhecida, porém alterações de neurotransmissores, como a serotonina e a noradrenalina, parecem estar relacionados ao aparecimento dessa sintomatologia. A terapia estrogênica geralmente é efetiva em aliviar esses sintomas, no entanto muitas mulheres não podem ou não desejam este tipo de tratamento, por isso, diversas alternativas têm sido estudadas. Objetivos: esta tese teve como objetivo avaliar, em um modelo experimental de climatério em ratas, parâmetros fisiológicos, comportamentais e bioquímicos, visando testar duas novas terapias: a cetamina, um antagonista não competitivo do receptor N-metil-D-aspartato (NMDA) e a eletroestimulação transcraniana de corrente contínua (ETCC) respectivamente, para o comportamento do tipo depressivo e a disfunção termorregulatória. **Métodos**: Ratos Wistar fêmeas adultas (200 a 250 g) foram randomizadas pelo peso e submetidas a modelo de menopausa por meio de ovariectomia bilateral ou a procedimento sham (falsa cirurgia). No primeiro experimento, os animais foram submetidos ao teste do nado forçado para avaliar comportamento do tipo depressivo e posteriormente receberam uma dose de cetamina 10mg/kg de peso intraperitoneal. No segundo experimento, os animais foram avaliados quanto à disfunção termorregulatória e tratados com ETCC catódica. **Resultados:** No primeiro experimento, as ratas em estado hipoestrogênico apresentaram comportamento do tipo depressivo que foi revertido pela cetamina. As ratas sham apresentaram um quadro de menopausa precoce indexado por citologia vaginal, provavelmente decorrente da manipulação de anexos. Além disso, as ratas ovariectomizadas apresentaram comportamento tipo ansioso. No entanto, não houve alteração da atividade locomotora e exploratória entre os grupos. No segundo experimento, as ratas ovariectomizadas apresentaram aumento da temperatura retal que foi parcialmente revertido pela eletroestimulação transcraniana de corrente contínua; as ratas ovariectomizadas apresentaram níveis elevados de interleucina 8 no soro, em relação às não ovariectomizadas, sem diferença nos níveis hipotalâmicos; houve aumento dos níveis séricos e diminuição dos níveis hipotalâmicos de BDNF nas ratas ovariectomizadas e interação do

modelo com a ETCC em relação aos níveis corticais de BDNF. Nos testes nociceptivos, as ratas ovariectomizadas apresentaram diminuição do tempo de latência de resposta no teste da placa quente e alodinia mecânica no teste Von Frey, parcialmente revertida pela ETCC, no entanto, não houve diferença entre os grupos no teste tail flick. **Conclusão**: Nossos estudos demonstram que o modelo de ovariectomia utilizado foi eficaz em reproduzir os sintomas apresentados no período perimenopausa tendo, portanto, potencial translacional. Adicionalmente, sugerem que, além dos sistemas serotoninérgico e noradrenérgico, outros sistemas parecem estar associados ao aparecimento de sintomas na transição menopáusica como, por exemplo, o sistema glutamatérgico. Demonstram ainda que a cetamina e a ETCC podem ser eficazes como adjuvantes no tratamento dos sintomas do climatério, respectivamente, farmacológico (nos sintomas depressivos) e não farmacológico (nos “fogachos”).

Palavras-chave: menopausa, ETCC, cetamina, estradiol, BDNF, depressão, citocinas, fogachos.

ABSTRACT

Introduction: Psychological and vasomotor symptoms have a high prevalence in women during menopause transition and post menopausal years. Though these symptoms are associated to decline of estrogen levels due to ovarian failure, their exact pathophysiology is unknown. Variations on neurotransmitters such as serotonin and norepinephrine seem to be responsible by great amount of these symptoms. Estrogen therapy is usually effective in relieving these symptoms. However, many women have contraindications or do not wish to use this kind of treatment, thus several therapeutic alternatives have been studied. **Objectives**: This thesis was designed to evaluate, in an experimental model of menopause in rats, physiological, behavioral and biochemical parameters, aiming to test two new therapies: ketamine, a noncompetitive antagonist of the N-methyl-D-aspartate (NMDA) receptor and transcranial direct current stimulation (tDCS) respectively for the depressive-like behavior and thermoregulatory dysfunction. **Methods**: Female adult Wistar rats (200-250g) were randomized by weight and subjected to a menopause model through bilateral ovariectomy or sham (false surgery) procedure. In the first experiment, the animals were subjected to the forced swimming test to assess depressive-like behavior and subsequently received a dose of 10mg/kg of weight of intraperitoneal ketamine. In the second experiment, the animals were evaluated for thermoregulatory dysfunction and treated with cathodal tDCS. **Results**: In the first experiment, the rats in hypoestrogenic state showed depressive-like behavior that was reversed by ketamine. The sham rats presented a precocious menopause indexed by vaginal cytology, probably due to the surgical handling of the tubes and ovaries. Moreover, the ovariectomized rats showed anxiety-like behavior. However, there was no change in locomotor activity between groups. In the second experiment, the ovariectomized rats showed an increase in rectal temperature that was partially reversed by tDCS. Moreover, the ovariectomized rats showed elevated serum levels of interleukin-8, compared to non-ovariectomized rats, with no difference in hypothalamic levels; there was an increase in serum levels and decreased hypothalamic BDNF levels in ovariectomized rats, and there was interaction of ovariectomy and ETCC in relation to cortical BDNF levels. In the nociceptive tests, the ovariectomized rats presented decreased response latency in the hot plate test and mechanical alodinia in the von Frey test; however, there was

no difference between groups in the tail flick test. **Conclusion**: Our studies demonstrate that the ovariectomy model used was effective in reproducing the symptoms that women present during perimenopause and therefore has translational potential. Additionally, these data suggest that, beyond the serotonergic and noradrenergic systems, other systems seem to be associated with the onset of symptoms in menopausal transition such as the glutamatergic system. Our data also demonstrate that ketamine and ETCC can be effective adjuvant therapeutic tools to the relief of climacteric symptoms, respectively, pharmacological (on depressive symptoms) and non pharmacological (on "hot flashes").

Keywords: menopause, tDCS, ketamine, estradiol, BDNF, cytokines, hot flushes.

LISTA DE FIGURAS DA TESE

LISTA DE FIGURAS E TABELAS DOS ARTIGOS

Artigo 1.

OVX-V: rats under ovariectomy and vehicle (i.p.); OVX-K: rats under ovariectomy and ketamine (10 mg/kg i.p.).

*SHAM-K and OVX-K different from SHAM-V and OVX-V groups (one-way ANOVA/SNK, P=0.03).

Artigo 2.

Figure 1. TDCS electrode placement. The cathodal stimulus (negative electrode) was positioned over the neck and shoulder areas, and the anodal electrode (positive) was positioned at the midpoint of the lateral angle of the eyes (adapted from Adachi et al., 2012).

Figure 2. A. Metestrus: a combination of round ‘‘pavement cells,’’ some needle-like cells, and a few smaller leukocytes can be present during a transitional period during the early portion of the first day of diestrus. **B.** Diestrus: only leukocytes are present.

Figure 3. Female rats weight at baseline and at the end of tDCS treatment. At baseline the weight was not different between groups (one-way ANOVA, P>0.05).

* At the end of tDCS treatment, OT and OS were different from CT, SS and ST (one-way ANOVA/SNK, P<0.05).

CT: control group; SS: sham ovariectomy+sham tDCS; ST: sham ovariectomy+tDCS; OS: ovariectomy+sham tDCS; OT: ovariectomy+tDCS.

Figure 4. Mean rectal temperature at Celsius grade.

* significant difference from CT, SS and ST (Wald Chi square, P<0.001).

** significant difference from SS and ST (Wald Chi square, P<0.001).

CT: control group; SS: sham ovariectomy+sham tDCS; ST: sham ovariectomy+tDCS; OS: ovariectomy+sham tDCS; OT: ovariectomy+tDCS.

Figure 5. Locomotor activity assessed during ten minutes. Each column represents the mean ± SEM. (n = 5/6 per group). **Panel A:** locomotor activity in the first five minutes. There was no difference between groups (two-way ANOVA, P>0.05). **Panel B:** locomotor activity in the last five minutes. There was no difference between groups (two-way ANOVA, P>0.05). CT: control group; SS: sham ovariectomy+sham tDCS; ST: sham ovariectomy+tDCS; OS: ovariectomy+sham tDCS; OT: ovariectomy+tDCS.

Figure 6. Interleukin 8 serum levels (pg/ml). Each column represents the mean±SEM (n=8 per group). b different from a (two-way ANOVA, P<0.05). CT: control group; SS: sham ovariectomy+sham tDCS; ST: sham ovariectomy+tDCS; OS: ovariectomy+sham tDCS; OT: ovariectomy+tDCS.

Table 1. Interleukin 10 levels in the structures of the central nervous system. Data are expressed as mean±S.E.M (pg/mg of protein). CT: control group; SS: sham ovariectomy+sham tDCS; ST: sham ovariectomy+tDCS; OS: ovariectomy+sham tDCS; OT: ovariectomy+tDCS.

Artigo 3.

Figure 1. A: Metestrus - a combination of round ‘‘pavement cells,’’ some needle-like cells, and a few smaller leukocytes can be present during a transitional period during the early portion of the first day of diestrus. **B:** Diestrus – only leukocytes.

Figure 2. Tail flick test. Each column represents mean±SEM. There was no difference between groups (n=5/6 per group, one-way ANOVA, P>0.05). CT: control group; SS: sham ovariectomy+sham tDCS; ST: sham ovariectomy+tDCS; OS: ovariectomy+sham tDCS; OT: ovariectomy+tDCS.

Figure 3. Hot plate test. Each column represents mean±SEM. * OS and OT are different from CT, SS and ST (n=8/9 per group, one-way ANOVA P=0.01). CT: control group; SS: sham ovariectomy+sham tDCS; ST: sham ovariectomy+tDCS; OS: ovariectomy+sham tDCS; OT: ovariectomy+tDCS.

Figure 4. Von Frey test. Each column represents mean±SEM of right hind paw of each group. There was significant difference between groups * OS is different from CT, SS, ST and OT (n=5 per group, one-way ANOVA, P=0.03). CT: control group; SS: sham ovariectomy + sham tDCS; ST: sham ovariectomy + tDCS; OS: ovariectomy + sham tDCS; OT: ovariectomy+tDCS.

Figure 5. BDNF serum levels. Each column represents mean±SEM. There was no interaction between ovariectomy and tDCS treatment (n=8 per group, two-way ANOVA, P>0.05). There was significant effect of ovariectomy. * OS and OT are increased in comparison to CT, SS and ST (n=8 per group, two-way ANOVA, P=0.02). CT: control group; SS: sham ovariectomy+sham tDCS; ST: sham ovariectomy+tDCS; OS: ovariectomy+sham tDCS; OT: ovariectomy+tDCS.

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P=0.002, n=7-8/group). CT: control group; SS: sham ovariectomy+sham tDCS; ST: sham ovariectomy+tDCS; OS: ovariectomy+sham tDCS; OT: ovariectomy+tDCS.

Figure 8. BDNF hippocampal levels. Each column represents mean±SEM. * SS, ST, OS and OT decreased as compared to CT (one-way ANOVA, P<0.001, n=8/9 per group). CT: control group; SS: sham ovariectomy+sham tDCS; ST: sham ovariectomy+tDCS; OS: ovariectomy+sham tDCS; OT: ovariectomy+tDCS.

Figure 9. BDNF spinal cord levels. Each column represents mean±SEM. There was no difference between groups (two-way ANOVA, P>0.05). CT: control group; SS: sham ovariectomy+sham tDCS; ST: sham ovariectomy+tDCS; OS: ovariectomy+sham tDCS; OT: ovariectomy+tDCS.

LISTA DE ABREVIATURAS E SIGLAS

AMPA: ácido α-amino-3-hidroxi-5-metil-4-isoxazolepropiônico;

AVC: acidente vascular cerebral;

AVE: acidente vascular encefálico;

BDNF: fator neurotrófico derivado do cérebro;

CEUA: comissão de ética no uso de animais;

CNS: central nervous system;

CONCEA: Conselho Nacional de Controle de Experimentação Animal;

COX2: cicloxigenase 2;

CR2: enzima cinina redutase 2;

CREAL: Centro de Reprodução e Experimentação de Animais de Laboratório;

CSF: fator estimulador de colônias;

DBCA: Diretriz Brasileira para o Cuidado e a Utilização de Animais para fins Científicos e Didáticos;

ECRC: ensaio clínico randomizado controlado;

EMT: eletroestimulação magnética transcraniana;

EPM: erro padrão da média;

ETCC: estimulação transcraniana por corrente contínua;

EUA: Estados Unidos da América;

E2: estradiol;

FDA: Federal Drug Admnistration;

GABA: ácido gama-aminobutírico;

HCPA: Hospital de Clínicas de Porto Alegre;

5 HT: 5 hidroxitriptamina;

IL: interleucina;

IL-1: interleucina 1 ;

IL-1β: interleucina 1β;

IL- 8 : interleucina 8 ;

IL-10: interleucina 10 ;

IFN: interferon;

IMAO: inibidor da monoamino-oxidase;

IMS: International Menopause Society (Sociedade Internacional da Menopausa);

ISRS: inibidor seletivo da recaptação de serotonina;

ISRN:inibidor seletivo da recaptação de noradrenalina;

LTD: depressão de longo prazo;

LTP: potenciação de longo prazo;

NEs: neurônios nociceptivos específicos;

NF-kB: fator nuclear kapa beta;

NGF: neurotrophin growth factor;

NMDA: N-metil D-aspartato;

NT: neurotrofinas;

OVX: ovariectomizada(s);

PRGC: peptídeo relacionado ao gene da calcitonina;

REα: receptor de estradiol alfa;

REß: receptor de estradiol beta;

REPG: receptor de estradiol acoplado à proteína G;

SEM: Standard error of mean;

SERMs: moduladores seletivos dos receptores estrogênicos;

SNC: sistema nervoso central;

STRAW: Stages of Reprodutive Aging of Women;

SWAN: Study of Women's Health Across the Nation;

Tc: temperatura central;

tDCS: transcranial direct current stimulation;

TFL: tail flick latency - teste de latência de retirada da cauda ;

TNF: fator de necrose tumoral;

Trk: tirosina-quinase;

UAMP: Unidade de Análise Molecular e Proteínas;

UEA: Unidade de Experimentação Animal;

UFRGS: Universidade Federal do Rio Grande do Sul;

UPM: último período menstrual;

WHI: Women’s Health Initiative.

Sumário

1. Introdução

O envelhecimento da população é um fenômeno mundial que vem suscitando interesse em diversas áreas. Atualmente, a expectativa de vida das mulheres, na América latina e na Europa é de 73,6 e 80,9 anos, respectivamente (1). A idade da menopausa, no entanto, permanece em torno dos 48,7 anos, logo, durante cerca de um terço da vida, essas mulheres viverão sob os efeitos do hipoestrogenismo decorrente da falência ovariana (2).

Embora se trate de um fenômeno fisiológico, a diminuição da produção de estrogênios devido ao esgotamento dos folículos ovarianos pode causar diversas alterações que impactam negativamente na qualidade de vida das mulheres, tais como: alterações do humor, distúrbios do sono, sintomas vasomotores, ressecamento das mucosas e da pele, alterações cognitivas, diminuição da libido, e, mais tardiamente, alterações uroginecológicas, diminuição da densidade óssea e aumento do risco cardiovascular (2–5). A idade da mulher na menopausa, a concomitância de doenças crônicas e as características sociodemográficas e culturais são preditores da severidade e da frequência desses sintomas (6–8).

A estrogenioterapia é considerada o tratamento de escolha para o alívio dos sintomas relacionados ao hipoestrogenismo da mulher climatérica. Entretanto, desde a publicação dos resultados do estudo Women's Health Initiative (WHI), que encontrou associação entre terapia estrogênica ou estroprogestativa e aumento no risco de câncer de mama e tromboembolismo, seu uso tem sido limitado (9,10). Por essa razão, existe uma crescente demanda por alternativas terapêuticas para o alívio dos sintomas (11).

Os sintomas depressivos e ansiosos podem reduzir de maneira significativa a qualidade de vida das mulheres no climatério (12). Os mecanismos que levam ao aparecimento desses sintomas na transição menopáusica ainda não estão bem esclarecidos e, até mesmo a fisiopatogenia dos transtornos depressivos, vem sendo amplamente debatida. A teoria monoaminérgica postula que a depressão é causada

por uma diminuição da função das monoaminas no cérebro e, os fármacos antidepressivos são desenhados para aumentar a oferta dessas substâncias, inibindo sua recaptação (inibidores da recaptação de serotonina e inibidores da recaptação de serotonina e noradrenalina) ou de sua degradação (inibidores da monoaminooxidase)(13). Porém, o longo tempo para o início da ação terapêutica e as baixas taxas de remissão dos sintomas tem encorajado a procura por fármacos mais efetivos. A observação de que doses mínimas de cetamina produzem um rápido e transitório efeito antidepressivo aumentou o interesse em sistemas neurobiológicos que não eram explorados na depressão (14). A cetamina é um antagonista não competitivo do receptor de glutamato N-metil-D-aspartato (NMDA) utilizado como anestésico e seu efeito antidepressivo revelou o papel do glutamato na fisiopatogenia dos transtornos depressivos (15).

Na transição menopáusica, teoriza-se que as mudanças nos níveis hormonais afetam sistemas de neurotransmissores aumentando a vulnerabilidade da mulher à depressão (16). As ondas de calor, conhecidas como fogachos, são os sintomas mais frequentes e angustiantes associados à menopausa, no entanto, sua exata fisiopatogenia ainda não está bem esclarecida, o que dificulta o estabelecimento de um tratamento eficaz para mulheres que não podem ou não querem fazer uso de estrogênios (4,17).

As isoflavonas, compostos não esteroides estruturalmente similares ao estrogênio natural, habitualmente encontradas na soja e em vários outros tipos de vegetais, apresentaram efeitos benéficos em estudos experimentais, entretanto, os resultados de ensaios clínicos controlados, avaliando o efeito da isoflavona nos sintomas vasomotores, são contraditórios (18–21).

Diversos fármacos não hormonais têm sido utilizados para o tratamento dos fogachos, entre eles, a clonidina, um agonista alfa adrenérgico, cujo mecanismo proposto seria a diminuição da liberação de noradrenalina, e a veraliprida, um derivado benzidamida com efeitos antidopaminérgicos. Todavia, os efeitos colaterais destas substâncias levaram ao abandono de seu uso na terapêutica do climatério (22).

A terapia antidepressiva levou à observação, na década de 90, de que o uso de inibidores seletivos da recaptação de serotonina (ISRS) diminuía a frequência das ondas de calor nessas mulheres (23). Desde então, vários ensaios clínicos têm testado o uso de diferentes antidepressivos, tais como a venlafaxina, a desvenlafaxina, a paroxetina e a fluoxetina para o tratamento dos fogachos, com resultados promissores (24–26). No entanto, custo e efeitos colaterais são relacionados a baixa adesão dos pacientes (27).

Tendo em conta que a maioria das mulheres na transição menopáusica e na pós- menopausa apresentam transtornos que diminuem significativamente sua qualidade de vida e que, embora a terapêutica de reposição hormonal seja eficaz, evidências consistentes indicam maior incidência de potenciais complicações com seu uso, opções farmacológicas e não farmacológicas precisam ser investigadas para aliviar os sintomas dessas pacientes.

Técnicas de neuromodulação central têm galgado espaço no tratamento da depressão (28), dores crônicas (29) e epilepsia (30), e hipotetiza-se que possam vir a ser uma alternativa terapêutica no tratamento dos sintomas psicoafetivos e vasomotores relacionados ao climatério. Esta hipótese se fundamenta no efeito difuso desta intervenção sobre o sistema nervoso central, o qual pode ter ação nos sistemas noradrenérgicos e serotoninérgicos que estão associados ao aparecimento destes sintomas no hipoestrogenismo.

Estudos em modelos animais contribuem para o conhecimento da fisiopatogenia e investigação de novas terapias com potencial translacional. Nesta tese buscamos o melhor entendimento da fisiopatogenia de transtornos da transição menopáusica. E, avaliamos duas novas terapias: a cetamina na reversão do comportamento do tipo depressivo e a estimulação transcraniana de corrente contínua (ETCC) para o tratamento da disfunção termorregulatória, induzidos pelo hipoestrogenismo, em ratas ovariectomizadas.

A estrutura da apresentação desta tese segue as normas do Programa de Pós Graduação em Medicina: Ciências Médicas da Faculdade de Medicina do Rio Grande do Sul.

2. Revisão da literatura

2.1. Estratégia para localização de informações

Na revisão da literatura enfocamos os aspectos relacionados aos sintomas associados à menopausa e seu tratamento, com ênfase nos sintomas vasomotores e depressivos e sua fisiopatogenia. Também foram investigados a cetamina, o fator neurotrófico derivado do cérebro (BDNF), citocinas e a eletroestimulação transcraniana de corrente contínua. A estratégia de busca envolveu as seguintes bases de dados: LILACS, SciELO, PubMed/Medline e banco de teses da CAPES. Foram realizadas buscas através dos termos “menopause”, “menopause symptoms”, “menopause therapy”, “depressive symptoms”, “hot flushes”, “hot flushes therapy”, “ketamine”, “cytokine”, “BDNF” e “tDCS”. Na base de dados Pubmed/Medline, para os termos “menopause”, “menopause symptoms”, “menopause therapy”, “depressive symptoms”, “hot flushes”, “hot flushes therapy” e “cytokine” foram pesquisados apenas artigos dos últimos 5 anos. Em relação ao principal termo: “menopause”- 11.022 artigos foram encontrados no PUBMED nos últimos 5 anos, 6.622 no LILACS e 486 no SciELO e 221 teses no Banco da CAPES. Em relação ao termo ¨depressive symptoms” - 75.217 artigos foram encontrados no PUBMED nos últimos 5 anos, 26.262 no LILACS e 761 no SciELO e 179 teses no Banco da CAPES. Em relação ao termo “hot flushes” – 1.282 artigos foram encontrados no PUBMED nos últimos 5 anos, 1.206 no LILACS e 23 no SciELO e 03 teses no Banco da CAPES. Em relação ao termo “ketamine” – 13.964 artigos foram encontrados no PUBMED, 3.723 no LILACS e 226 no SciELO e 51 teses no Banco da CAPES. Em relação ao termo “cytokine” – 144.695 artigos foram encontrados no PUBMED nos últimos 5 anos, 1.547 no LILACS e 519 no SciELO e 03 teses no Banco da CAPES. Em relação ao termo “BDNF” – 14.017 artigos foram encontrados no PUBMED, 45 no LILACS e 28 no SciELO e 90 teses no Banco da CAPES. Em relação ao termo “tDCS” – 1.200 artigos foram encontrados no PUBMED, 437 no LILACS e 7 no SciELO e 13 teses no Banco da CAPES.

As referências dos artigos encontrados foram revisadas para localizar outros estudos não contemplados nesta busca. A busca de artigos com o cruzamento das palavras-chave está demonstrada no esquema abaixo (figura 1).

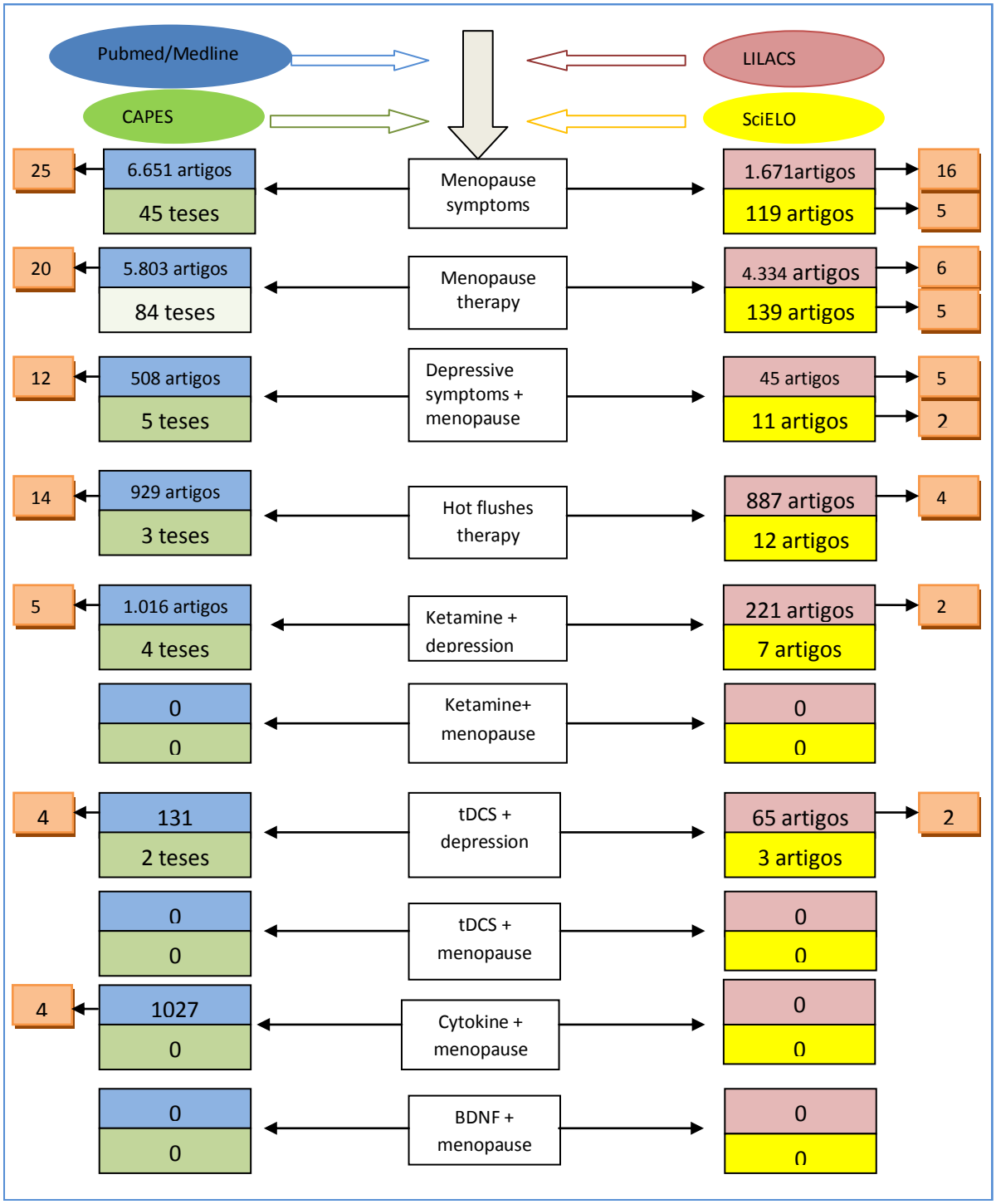


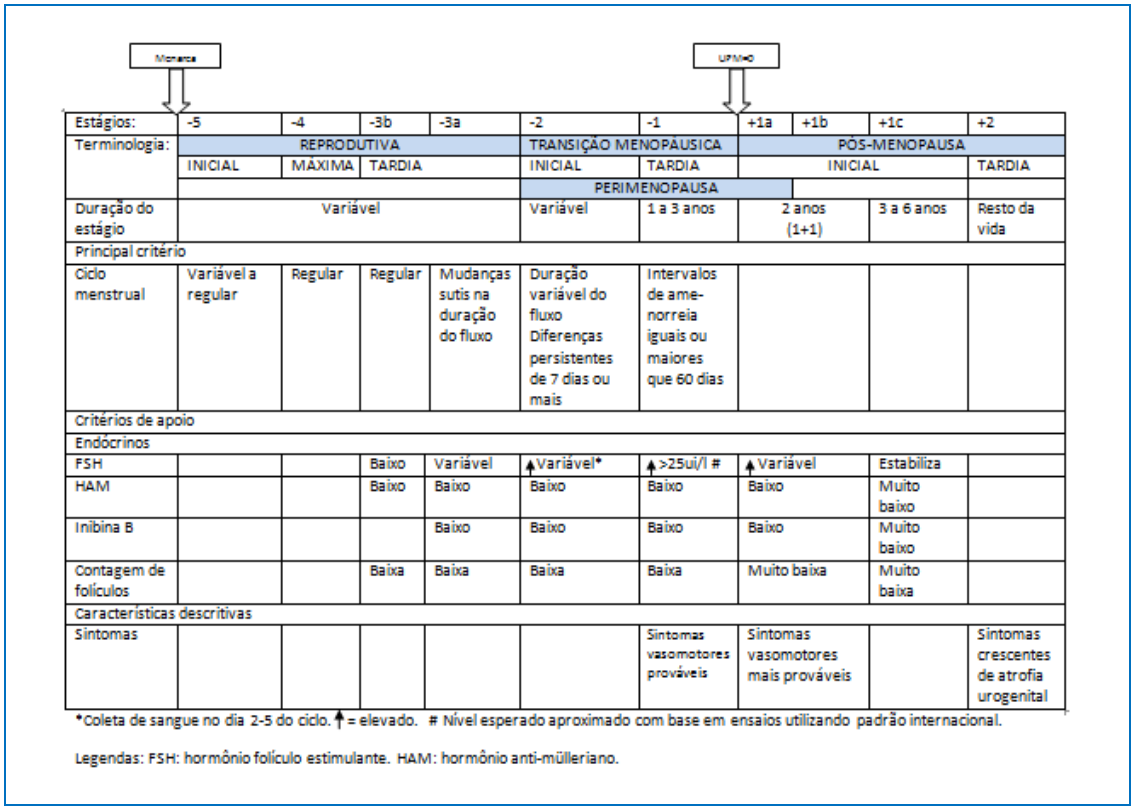
Figura 1.Estratégia de busca de referências bibliográficas sobre as bases que fundamentam este estudo com a combinação das palavras-chave. Obs: Na base de dados LILACS foram computados apenas os que não constavam na base de dados Medline/PubMed. As caixas externas indicam os artigos que foram incluídos na revisão ou nos artigos da tese.

2.2. Aspectos conceituais da menopausa

A diversidade de nomenclatura torna confusa a comparação de resultados entre os estudos. Por essa razão, esforços para definir formalmente e promover o uso de terminologia apropriada para o final do período reprodutivo da mulher ganhou impulso em um grupo de trabalho do Primeiro Congresso da Sociedade Internacional de Menopausa (IMS), em 1976. O climatério foi definido como a fase da vida da mulher que marca a transição da vida reprodutiva para a não reprodutiva, podendo se estender por vários anos, e a menopausa como o último período menstrual (UPM), determinado após amenorreia de 12 meses consecutivos e dividindo o climatério em dois períodos: o período pré-menopausa e o período pós menopausa. A "Síndrome Climatérica" foi definida como a presença de sintomas deletérios durante o climatério (31,32). Posteriormente, um Grupo Científico em Pesquisa na Menopausa foi organizado pela Organização Mundial de Saúde (OMS) e publicou recomendações iniciais, em 1981. Em 1996, essas recomendações foram atualizadas (33) e o termo "climatério" foi desencorajado devido seu uso inconsistente e ambíguo, além de ser recomendado o uso da terminologia "menopausa natural", definida como a cessação permanente da menstruação resultante da perda da atividade folicular ovariana e reconhecida após 12 meses consecutivos de amenorreia, para a qual não há causa fisiológica ou patológica óbvia. Essas recomendações foram endossadas pelo grupo de trabalho da IMS, no entanto, esse grupo depois incorporou os termos "climatério" e "síndrome climatérica" devido à difundida popularidade internacional desses descritores fora dos Estados Unidos. Nova terminologia foi proposta, em 2001, no *Stages of Reproductive Aging Workshop* (STRAW), considerando mais adequado utilizar o termo "transição menopáusica" em trabalhos científicos e restringindo a nomenclatura anterior para a comunicação com as pacientes e imprensa leiga (34). O objetivo da oficina STRAW foi desenvolver um sistema de estadiamento prático e padronizado para o envelhecimento reprodutivo feminino, que pudesse ser utilizado de maneira confiável, tanto no meio científico quanto no contexto clínico, além de produzir definições mais precisas para termos ambíguos, tais como perimenopausa e transição menopáusica.

O relatório STRAW divide a vida reprodutiva e pós-reprodutiva da mulher em estágios, iniciando na menarca e tendo como marco a menopausa natural, ou último período menstrual (UPM). Esses estágios variam em duração, com cinco deles anteriores e dois posteriores ao UPM. Dez anos depois, esses estágios foram revistos e redivididos com novos critérios para seu diagnóstico (35).

O quadro abaixo resume os estágios e sua terminologia.



Estágios:	-5	-4	-3b	-3a	-2	-1	+1a	+1b	+1c	+2
Terminologia:	REPRODUTIVA				TRANSIÇÃO MENOPÁUSICA		PÓS-MENOPAUSA			
	INICIAL	MÁXIMA	TARDIA		INICIAL	TARDIA	INICIAL			TARDIA
					PERIMENOPAUSA					
Duração do estágio	Variável				Variável	1 a 3 anos	2 anos (1+1)		3 a 6 anos	Resto da vida
Principal critério										
Ciclo menstrual	Variável a regular	Regular	Regular	Mudanças sutis na duração do fluxo	Duração variável do fluxo Diferenças persistentes de 7 dias ou mais	Intervalos de ame-norreia iguais ou maiores que 60 dias				
Critérios de apoio										
Endócrinos										
FSH			Baixo	Variável	↑Variável*	↑>25ui/l #	↑Variável		Estabiliza	
HAM			Baixo	Baixo	Baixo	Baixo	Baixo		Muito baixo	
Inibina B				Baixo	Baixo	Baixo	Baixo		Muito baixo	
Contagem de folículos			Baixa	Baixa	Baixa	Baixa	Muito baixa		Muito baixa	
Características descritivas										
Sintomas						Sintomas vasomotores prováveis	Sintomas vasomotores mais prováveis			Sintomas crescentes de atrofia urogenital

*Coleta de sangue no dia 2-5 do ciclo. ↑= elevado. # Nível esperado aproximado com base em ensaios utilizando padrão internacional.

Legendas: FSH: hormônio folículo estimulante. HAM: hormônio anti-mülleriano.

Figura 2. Estágios reprodutivos da vida reprodutiva da mulher. Fonte: Adaptado de Harlow et al. Menopause, 2012.

A menopausa natural geralmente ocorre entre os 40 e os 55 anos de idade, já a transição menopáusica inicia alguns anos antes e se caracteriza pela queda progressiva da secreção de estradiol, decorrente do esgotamento dos folículos ovarianos, culminando com a interrupção definitiva dos ciclos menstruais e o surgimento de sintomas como: ondas de calor, suores noturnos, alterações do humor, ressecamento de mucosas, insônia e diminuição da libido (17,32). A quimioterapia, a radioterapia pélvica, cirurgias ovarianas e histerectomia, além de

inúmeros fatores genéticos e ambientais, podem acelerar o envelhecimento ovariano, e, consequentemente, antecipar a menopausa natural.

2.3. Sintomatologia

Durante a transição menopáusica, oscilações mais irregulares dos níveis de hormônios ovarianos podem levar a uma variedade de sintomas físicos e psicológicos. Embora não sejam universais, estudos demonstram que 58 a 80% das mulheres relatam algum sintoma desagradável nessa fase da vida, sendo mais comuns os sintomas vasomotores e os decorrentes da atrofia genital (36–39). Alterações neuropsíquicas, tais como transtornos depressivos, ansiosos, de sono e nos processos cognitivos também têm sido associados à transição menopáusica e à pós-menopausa(40–44).

Os dados encontrados no estudo de corte, *Study of Women's Health Across the Nation* (SWAN), apoiam a hipótese de que a transição menopáusica esteja associada a risco aumentado de surgimento de sintomas depressivos (41). Antes deste, outro estudo de corte, o *Harvard Study of Moods and Cycles*, já sugeria que, dentro da mesma faixa etária, mulheres sem história prévia de transtorno depressivo, apresentavam risco significativo de ter o primeiro episódio depressivo quando iniciavam precocemente a transição menopáusica (45). Quanto aos transtornos ansiosos, geralmente precedem ou acompanham os sintomas depressivos, e os resultados de estudos experimentais sugerem que os hormônios ovarianos modulem os níveis de ansiedade (46–48).

A etiologia dos transtornos depressivos é multifatorial e complexa, com interação de fatores individuais, psicossociais e ambientais (49,50). A crescente evidência de que os estrogênios – por meio da modulação de neurotransmissores - desempenham papel crítico na patogenia destes transtornos em mulheres abre novos caminhos para pesquisas, tanto na área da fisiopatogenia quanto na área do tratamento do transtorno depressivo. As teorias existentes sobre as causas da depressão têm seu foco em neurotransmissores monoaminérgicos: dopamina, adrenalina, noradrenalina e serotonina; e, especialmente a serotonina tem sido alvo de um grande número de pesquisas sobre os efeitos alterações no sistema serotonérgico em transtornos do humor (51). Recentemente, estudos clínicos e experimentais têm demonstrado que a cetamina, antagonista não competitivo do

receptor NMDA, tem efeito antidepressivo, sugerindo que o glutamato tem papel na fisiopatogenia da depressão (13,14,52). Pesquisas apontam que os estrogênios influenciam a depressão e o comportamento do tipo depressivo por meio da interação com fatores neurotróficos e influenciando o sistema serotoninérgico. Particularmente, o estradiol aumenta os níveis de BDNF no cérebro e altera a expressão da serotonina em uma maneira subtipo de receptor específico (53).

Em relação às alterações nos processos cognitivos, o nível hormonal pode influenciar os processos neurais ligados às funções cognitivas, porém os achados clínicos ainda são inconsistentes (54,55). Em um estudo de corte de base populacional, o *The French Three-City Study*, tanto a menopausa cirúrgica quanto a menopausa natural precoce foram associadas com efeitos negativos, em longo prazo, sobre a função cognitiva, que não foram inteiramente compensados por terapia de reposição estrogênica (56).

Os sintomas decorrentes da atrofia genital, como o ressecamento vaginal, a dispareunia e a urgência miccional, podem estar associados à diminuição da libido, prejudicando sobremaneira a vida sexual de algumas mulheres (57,58).

Dentre os sintomas deletérios da transição menopáusica, os sintomas vasomotores, representados pelas ondas de calor ou fogachos e pela sudorese noturna, são os que costumam aparecer mais cedo, sendo, frequentemente, os primeiros sinais associados ao hipoestrogenismo. Os fogachos consistem em sensação repentina e passageira de calor moderado a intenso na região anterior do tórax, pescoço e face, podendo ser acompanhados de sudorese profusa e palpitação, que pioram no período noturno, estando muitas vezes relacionados a distúrbios do sono (4,33,59). Os episódios de ondas de calor variam em frequência, duração e intensidade, geralmente duram menos que cinco minutos e podem ser desencadeados por aumento da temperatura ambiente, comidas e bebidas quentes e estresse.

2.4. Epidemiologia dos fogachos

Estudos mundiais mostram que a prevalência de fogachos varia de acordo com a população estudada, em uma ampla faixa de 18%, em uma população rural

da China, a 74% em países ocidentais (36,60). Estudos realizados no Brasil mostraram prevalência de fogachos variando entre 53,2% a 70% (38,61,62).

Uma gama de fatores influencia a prevalência, a frequência e a intensidade dos sintomas vasomotores, entre eles destacam-se o clima, o tipo de dieta, o estilo de vida, os sentimentos frente ao fim da vida reprodutiva e ao envelhecimento e outros fatores socioculturais (36).

Em geral, os sintomas vasomotores aparecem de 2 a 3 anos antes do último período menstrual (UPM) e perduram por mais 2 a 3 anos na pós-menopausa, porém os resultados do estudo de corte *The Penn Ovarian Aging* mostraram uma duração média de ondas de calor intensas a moderadas por 10,2 anos, fortemente associada à idade da mulher na menopausa e mais longa em afroamericanas (63).

2.5. Fisiopatogenia dos fogachos

Embora a associação entre o declínio dos níveis de estradiol e o início dos sintomas vasomotores da transição menopáusica esteja bem estabelecida, o mecanismo exato que leva ao aparecimento dessa sintomatologia permanece obscuro. Sabe-se que não há relação somente com os níveis plasmáticos absolutos do estradiol, mas também com suas oscilações. Além do que, já foi comprovado que, em mulheres que viveram sempre em hipoestrogenismo, como as portadoras da síndrome de Turner, não há o aparecimento de fogachos, a não ser que essas pacientes sejam medicadas com estrogênio e, após algum tempo, o hormônio venha a ser retirado (64).

Uma hipótese sobre a patogenia dos fogachos é que as flutuações dos níveis estrogênicos afetariam diretamente a reatividade vascular. Jowsig e colaboradores teorizam que os baixos níveis de estradiol durante a pós-menopausa possam contribuir para reduzir a elasticidade dos vasos sanguíneos, resultando em atraso nas respostas às mudanças de temperatura interna (65). Yasui e colaboradores (2006) realizaram um estudo medindo os níveis séricos de 17 citocinas em 129 mulheres pré, peri e pós-menopausa e 50 mulheres ooforectomizadas e encontraram níveis séricos significativamente elevados de interleucina 8 (IL-8)

naquelas que apresentavam fogachos, sugerindo que a IL-8 está associada com a vasodilatação periférica em mulheres com sintomas vasomotores (66).

Vários autores caracterizam os fogachos como distúrbios da regulação da temperatura corporal e diversos mecanismos por meio dos quais a termorregulação estaria alterada na mulher perimenopáusica já foram relatados, entre eles, o estreitamento da chamada zona termoneutra, que é a faixa de temperatura central dentro da qual o organismo não necessita lançar mão de mecanismos reguladores para dissipar ou conservar calor (figura 3). Freedman, em estudos clínicos, demonstrou que a amplitude da zona termoneutra é de 0,4ºC em mulheres assintomáticas e praticamente nula naquelas que apresentam ondas de calor. Logo, nas mulheres com fogachos, bastam pequenos aumentos da temperatura interna para deflagrar vasodilatação e sudorese e assim diminuir a temperatura corporal, e por isso, frequentemente, os fogachos são sucedidos por sensação de frio e tremores (67,68).

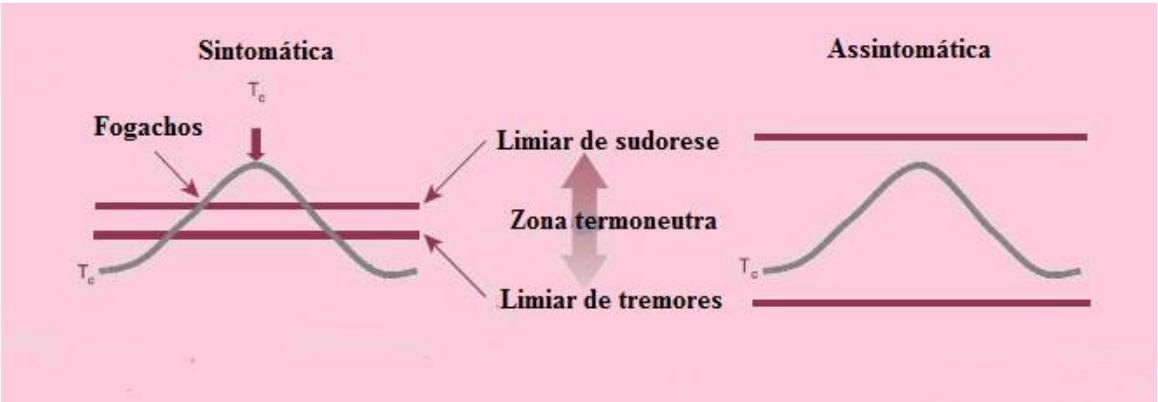


Figura 3. Zona termoneutra estreitada em mulheres com queixas de fogachos e normal em mulheres assintomáticas. Adaptado de imagem acessada em http://www.menopausemgmt.com/current-best-treatments-for-hot-flashes. Tc: temperatura central.

Outros estudos deste mesmo grupo demonstraram que, antes do início do episódio de fogacho, ocorre um pequeno aumento da temperatura central (69), porém estas elevações não são motivadas por vasoconstrição periférica ou aumento da taxa metabólica, mas por um mecanismo central noradrenérgico, apoiando a hipótese de que os fogachos são desencadeados por ativação noradrenérgica central, de origem provavelmente hipotalâmica (68).

O núcleo termorregulatório, localizado na área pré-ótica medial do hipotálamo, é responsável pela vasodilatação e transpiração, mecanismos que promovem a perda de calor. Estudos tem demonstrado que receptores estrogênicos estão localizados em regiões associadas ao sistema termorregulador, indicando que aquelas áreas podem ser influenciadas, em sua estrutura ou função, pelos níveis estrogênicos (70–72).

Os neurotransmissores serotonina e noradrenalina desempenham importante papel na modulação da temperatura central, de mensageiros neuroquímicos e na vascularização periférica e postula-se que os estrogênios podem regular a atividade de sistemas neurotransmissores dentro da área pré-ótica medial do hipotálamo. Um estudo clínico demonstrou que a ioimbina, um antagonista α2-adrenérgico de ação central, provoca ondas de calor, e que a clonidina, um agonista α2-adrenérgico, as reduz (73); corroborando o envolvimento da adrenalina na patogenia dos fogachos. Complementando, resultados de um estudo experimental indicaram que a ativação central de receptores 5-HT2a restaura a regulação da temperatura em dois modelos de disfunção termorregulatória induzida por ovariectomia em roedores (74).

Nos últimos anos, vários estudos têm buscado desvendar o papel da serotonina ou 5-hidroxitriptamina (5-HT) no mecanismo das ondas de calor do climatério. Depois da menopausa os níveis sanguíneos de 5-HT diminuem em torno de 50% e a terapia estrogênica restaura estes níveis. A interação entre os estrogênios, a serotonina e outros neurotransmissores pode ser explicada pela seguinte cadeia de eventos: (1) os estrogênios aumentam a síntese de 5-HT e de endorfinas, (2) as endorfinas e a 5-HT inibem a produção de noradrenalina; (3) a diminuição de estrogênios no climatério está associada a níveis decrescentes de endorfinas e 5-HT e a aumento dos receptores de 5-HT. Esta interação de efeitos resulta na perda do mecanismo de *feedback* negativo da produção de noradrenalina causando aumento da noradrenalina, o que pode reduzir a zona termoneutral e assim aumentar a propensão aos fogachos (75).

Em suma, esses estudos sugerem que a diminuição e variações expressivas dos níveis de estradiol levam a uma redução na densidade de receptores pré-sinápticos α2 adrenérgicos inibidores e a um aumento na liberação hipotalâmica de serotonina e noradrenalina. Então, essas aminas diminuem o ponto de ajuste do

núcleo termorregulatório que passa a desencadear mecanismos inapropriados de perda de calor, diante de elevações ínfimas da temperatura interna (67,70,71,76–78).

2.6. Tratamento dos sintomas vasomotores

2.6.1. Fármacos hormonais

Terapia estro/progestogênica

Os estrogênios têm sido usados há várias décadas para o alívio dos sintomas vasomotores e compreendem vários hormônios, incluindo a estrona, o estradiol e o estriol. O mais potente deles, o estradiol (E2), predomina durante o período reprodutivo da vida da mulher, o estriol predomina durante a gravidez e os níveis de estrona são maiores durante a pós-menopausa (79). O estradiol tem sido, classicamente, o hormônio de escolha para reposição hormonal na menopausa ou após ooforectomia. Uma variedade de medicamentos contendo estrogênios e progesteronas ou somente estrogênios têm sido utilizados com eficácia comparável. Existem várias formulações disponíveis por via oral, transdérmica ou vaginal. Para mulheres com útero intacto é recomendado uso combinado de estrogênios com progestagênios, para evitar o desenvolvimento de hiperplasia ou câncer endometrial (4,80).

Uma metanálise, avaliando 24 ensaios clínicos randomizados duplo-cegos controlados por placebo, concluiu que mulheres sintomáticas tratadas com várias formas de estrogênio ou estrogênio combinado com progesterona, administrados por via oral, tinham uma diminuição de duas a seis vezes no número de ondas de calor por dia comparadas com as que utilizaram placebo. Esse efeito era equivalente a 75% de redução na frequência. Além disso, usuárias de estrogênios apresentaram diminuição significativa da intensidade dos fogachos (81).

No entanto, embora os estrogênios sejam efetivos para o alívio dos sintomas associados ao hipoestrogenismo, preocupações com relação ao risco aumentado de tromboembolismo e câncer de mama em usuárias de terapia hormonal, levantadas

pelo estudo WHI, publicado em 2002, levaram ao aumento do interesse em terapias não hormonais para o tratamento dos sintomas vasomotores (82).

Tibolona

A tibolona [(7α,17α)-17-hydroxi-7-metil-19-norpregn-5(10)-en-20-in-3-ona] é um esteroide sintético cujos metabólitos têm ação estrogênica, progestagênica e androgênica que foi primeiramente testado em 1984, em macacas, com bom efeito na regulação das alterações de temperatura provocadas pela ovariectomia (83). Desde então, vários ensaios clínicos compararam o efeito da tibolona com placebo, estrogênios e terapia estro-progestagênica (84,85). Uma revisão, publicada em 2012, evidenciou que a tibolona, utilizada via oral na dose de 2,5 mg/dia, foi mais efetiva que o placebo, porém menos efetiva que a terapia estrogênica ou estro-progestagênica, no alívio dos sintomas vasomotores; e levantou preocupações quanto à segurança, a longo prazo, do uso da tibolona, pois houve aumento na recidiva de câncer de mama e aumento do risco de acidente vascular encefálico (AVE) em mulheres com média de idade de 60 anos (85). Efeitos adversos comuns da tibolona incluem sangramento uterino, ganho de peso, dor no corpo e cefaleia (86).

Moduladores Seletivos de Receptores Estrogênicos (SERMs)

Nova terapêutica combinando moduladores seletivos de receptores estrogênicos (SERMs) com estrogênios está sendo desenvolvida nos Estados Unidos da América (EUA). Os SERMs atuam como agonistas ou antagonistas em diferentes tecidos. Os efeitos negativos dos estrogênios nas mamas e endométrio são bloqueados pelo SERM apropriado permitindo apenas seus efeitos positivos na redução dos sintomas vasomotores, epitélio vaginal e nos ossos. Atualmente o SERM, Bazedoxifeno, agonista nos ossos e antagonista nas mamas e endométrio, tem sido estudado em combinação com estrogênios para o tratamento dos sintomas da menopausa (87).

2.6.2. Fármacos não hormonais

Fitoestrogênios / Isoflavonas

Algumas substâncias, presentes em vegetais, possuem ação estrogênica e antiestrogênica conhecida, por isso são denominadas de fitoestrogênios.

Os três maiores grupos de fitoestrogênios são as flavonas, as isoflavonas e os cumestranos. O poder estrogênico destas substâncias é variável. O grupo das isoflavonas (malonilgenistina, malonildaidzina, genistina, daidzina, genisteína, daidzeína, acetildaidzina, gliciteína, acetilgenistina e equol) tem maior atividade estrogênica e maior afinidade pelos receptores. As propriedades estrogênicas e antiestrogênicas dos fitoestrogênios decorrem da sua interação com os receptores de estrogênios (88,89).

As isoflavonas, compostos não esteroides estruturalmente similares ao estrogênio natural, apresentam um anel fenólico com um radical hidroxila no carbono 3, estrutura que lhe confere a capacidade de ligação seletiva, de alta afinidade, aos receptores estrogênicos (90). Estas substâncias são habitualmente encontradas na soja e em vários outros tipos de vegetais (91). Estudos experimentais encontraram efeitos benéficos de diversas plantas (18,20,89,92,93), entretanto, os ensaios clínicos controlados que avaliam o efeito da isoflavona nos sintomas vasomotores são contraditórios (21,94).

Alguns estudos sugerem que o extrato de soja, na dose de 50 a 120 mg/dia, seja seguro e uma alternativa terapêutica eficaz para a mulher na pós-menopausa com sintomas climatéricos moderados (95,96) e não parecem alterar a espessura endometrial (97,98). No entanto, a grande variedade de compostos contendo isoflavonas, em diferentes doses, nos inúmeros estudos publicados, tem dificultado a interpretação dos resultados e as revisões sistemáticas.

Em 2013, uma abrangente revisão foi publicada avaliando ensaios clínicos randomizados controlados (ECRC) utilizando fitoestrogênios para o tratamento de sintomas vasomotores. Foram encontrados 43 ECRCs, conduzidos até julho de 2013, incluindo 4084 participantes com ondas de calor, que estavam próximas à menopausa ou eram menopausadas. Muito poucos ECRCs apresentavam dados adequados para inclusão em uma metanálise. Quatro ensaios, que não puderam ser combinados em uma metanálise, sugeriam que altos níveis de genisteína (>30mg/d) reduziam consistentemente a frequência dos fogachos. No entanto, um

forte efeito placebo foi notado na maioria dos estudos. Muitos ECRCs nessa revisão eram pequenos, de curta duração e pobres em qualidade, e os tipos de fitoestrogênio eram muito variados. Os autores concluíram que não havia evidência irrefutável mostrando que suplementos de fitoestrogênios possam efetivamente reduzir a frequência e intensidade dos sintomas vasomotores, embora os benefícios dos concentrados de genisteína mereçam investigação posterior(19).

Outro fator que vem sendo apontado como causa das discrepâncias entre os achados experimentais e os achados clínicos nos benefícios da soja é a diferença na produção de equol. O equol é produto exclusivo do metabolismo bacteriano intestinal da isoflavona, daidzeína, presente na dieta e possui atividade estrogênica, com afinidade para ambos os receptores de estrogênio, REα e REβ. O equol é superior a todas as outras isoflavonas na sua atividade antioxidante. No entanto, ele não é produzido em todos os adultos saudáveis. Vários estudos de intervenção dietética sugerem que as respostas clínicas máximas para as dietas de proteína de soja são observadas em pessoas que são “boas produtoras de equol”. A eficácia clínica da proteína de soja na menopausa pode ser uma função da capacidade de biotransformar isoflavonas de soja nessa isoflavona estrogênica mais potente, o equol. A incapacidade de distinguir mulheres que são "equol-produtores" das "não equol-produtores", nos estudos clínicos, poderia explicar a variação nos dados relatados sobre os benefícios da soja (99,100).

Inibidores Seletivos da Recaptação de Serotonina / Inibidores Seletivos da Recaptação de Serotonina e Noradrenalina (ISRS/ISRSN)

Na década de 1990 foi observado que mulheres em uso de ISRS apresentavam diminuição na frequência de fogachos. Sendo, então, conjeturado que tais antidepressivos poderiam ter efeito terapêutico nos sintomas vasomotores. Desde então, vários ensaios clínicos e estudos experimentais têm sido realizados para testar essa hipótese, com diferentes agentes farmacológicos (18,92,101–104). Em geral, os resultados destas pesquisas têm sido promissores e apoiam a continuação de estudos sobre sua utilização, não só para os sintomas depressivos, mas para o tratamento de um espectro de sintomas associados à transição menopáusica e à menopausa (103–105).

Salientando que estudos sobre o papel da serotonina e da noradrenalina na fisiopatogenia dos fogachos corroboram o uso de antidepressivos na terapêutica dos fogachos. Contudo, estes fármacos podem ser contraindicados para algumas pacientes com câncer de mama, pois o Tamoxifeno, um SERM amplamente utilizado na prevenção e tratamento do câncer mamário, é metabolizado em sua forma ativa, o endoxifeno, pela enzima CYP2D6 no citocromo P450 e alguns destes antidepressivos, notadamente a paroxetina e a fluoxetina, podem inibir essa enzima resultando em níveis mais baixos de endoxifeno (106). Dados *in vitro* indicam que a desvenlafaxina tem pouco efeito no citocromo P450 e probabilidade mínima de interação com outros fármacos (107).

Apesar de relativamente seguros, os ISRS/ISRSN são contraindicados para usuários de inibidores da monoaminooxidase (IMAO), warfarina e tioridazina, e apresentam efeitos colaterais que levam alguns pacientes a abandonar o tratamento, como: boca seca, diminuição do apetite, náusea, obstipação intestinal, diminuição da libido, cefaleia, sedação e tremores (108).

Recentemente, um medicamento cuja base é a Paroxetina foi aprovado nos EUA pela *Food and Drug Administration* (FDA) para o tratamento dos fogachos, apesar do parecer contrário do FDA *Reproductive Health Drugs Advisory Committee*, seu próprio Comitê Consultivo para Fármacos em Saúde Reprodutiva. Este fármaco passou a ser o único tratamento não hormonal aprovado pelo FDA para o tratamento dos sintomas vasomotores. A pequena dose do ISRS, apenas 7,5mg V.O./dia, no período noturno e a necessidade de uma alternativa terapêutica para o tratamento das ondas de calor em mulheres portadoras de câncer de mama e outras patologias que contraindicam o uso de terapia estrogênica devem ter pesado a favor dessa decisão (109).

Clonidina

A Clonidina é um agonista α-adrenérgico que foi primeiramente sugerido para o tratamento das ondas de calor da síndrome climatérica na década de 1970 (110). O mecanismo de ação proposto é que a redução da liberação de noradrenalina, causada pela Clonidina, aumente o limiar de ativação de sudorese, aumentando, assim a zona termoneutra (111). Estudos experimentais demonstram que a clonidina

reduz alterações da termorregulação, em modelos animais de deficiência estrogênica (83,112). Contudo, as pacientes relataram efeitos adversos significantes em diversos ensaios clínicos, incluindo hipotensão, boca seca, obstipação intestinal, prurido corporal e sonolência (113).

Boekhout e colaboradores (2011) realizaram um ECRC, com dois braços, um utilizando venlafaxina e outro clonidina. Esse estudo iniciou com 102 pacientes, com história de câncer de mama, alocadas aleatoriamente (2:2:1) para receber venlafaxina 75 mg, clonidina 0.1 mg ou placebo diariamente por 12 semanas. Ao final do estudo, restaram 80 pacientes e os efeitos adversos mais frequentes (náusea, obstipação intestinal e severa perda do apetite) foram mais graves no grupo Venlafaxina. Os resultados desse estudo sugerem que a venlafaxina e a clonidina são efetivos para tratar os fogachos em mulheres com câncer de mama, sendo que os escores de ondas de calor com 12 semanas de tratamento foram mais baixos no grupo clonidina (24).

Gabapentina

A Gabapentina é estruturalmente relacionada ao neurotransmissor ácido gama-aminobutírico (GABA) e atualmente é utilizada como anticonvulsivante ou no tratamento de dor neuropática. Em 2000, Guttuso descreveu seis casos em que houve redução da frequência dos fogachos com o uso de gabapentina (114). Depois disto, muitos ensaios clínicos relataram a eficácia da gabapentina em reduzir a frequência dos fogachos comparada a placebo. Nestes ensaios clínicos, as doses de Gabapentina variaram de 300 a 2700 mg ao dia (115). Estes estudos mostraram que doses até 900 mg ao dia são suficientes para o tratamento dos fogachos, pois doses maiores aumentam os efeitos adversos, sem melhora significativa dos sintomas vasomotores (116). Sonolência, tonturas, ataxia, fadiga, nistagmo e edema periférico são efeitos adversos comumente relacionados ao uso da Gabapentina para o tratamento de epilepsia e dor neuropática, porém nas doses recomendadas para o controle das ondas de calor a sonolência foi o efeito colateral mais relatado (107).

Pregabalina

A Pregabalina é um fármaco análogo do GABA, de uma nova geração de compostos com mecanismo semelhante ao da Gabapentina, com ação

anticonvulsivante e também utilizada no tratamento da dor neuropática e da ansiedade. Devido à sua ação semelhante à da Gabapentina, foi levantada a hipótese de que também seria efetiva no tratamento dos fogachos. Foi realizado um ECRC com 207 mulheres alocadas aleatoriamente para receber placebo ou pregabalina 75 mg duas vezes ao dia ou 150 mg duas vezes ao dia, por seis semanas. Ao final do estudo restaram 163 participantes. Houve redução dos fogachos de 50% no grupo placebo e 65% e 71% nos grupos pregabalina 75mg duas vezes ao dia e pregabalina 150 mg duas vezes ao dia, respectivamente. Sonolência, tonturas, problemas cognitivos, visão borrada, visão dupla e ganho de peso foram os efeitos adversos mais frequentes com doses maiores de pregabalina.

Veraliprida

A Veraliprida é um derivado da benzidamida com efeitos dopaminérgicos, que tem sido estudada no tratamento dos fogachos (113). A Veraliprida foi comparada a placebo em 3 ensaios clínicos conduzidos na década de 1980, com número reduzido de participantes e registro de dados limitado. Dois ensaios clínicos com dose de 100 mg ao dia registraram redução dos escores de fogachos e em outro ensaio houve melhora subjetiva de 85% com Veralipride contra 50% de melhora com placebo (119). Mastodínia, galactorréia e queixas gastrointestinais foram os sintomas adversos mais frequentes. O uso de Veraliprida está associado a quadros de distúrbio extrapiramidal, incluindo discinesia aguda e parkinsonismo e, devido à sua toxicidade, não foi aprovado para uso nos EUA (119). Alguns autores defendem sua utilização pois os efeitos adversos estariam relacionados ao uso inadequado do fármaco (22,120,121).

2.6.3. Terapias não farmacológicas

Medidas comportamentais

A Sociedade Norte Americana de Menopausa recomenda, como primeira medida nos casos leves, mudanças de comportamento, como o uso de roupas leves, bebidas geladas, evitar comidas e ambientes quentes, uso de ventilador, etc (122).

Técnicas de relaxamento, respiração compassada, meditação e ioga têm sido testadas em diversos ensaios clínicos. Alguns desses estudos demonstraram redução dos sintomas vasomotores, provavelmente por reduzir o tono simpático (11,113,123). A hipnose foi testada em um pequeno ensaio clínico e houve redução de 68% nos escores de fogachos, nas mulheres tratadas com estas técnicas, além de melhora nos níveis de ansiedade e depressão autorrelatados e nos distúrbios do sono (124).

Exercícios físicos também têm sido testados para o alívio dos sintomas da transição menopáusica, com resultados conflitantes (116,125). De fato, exercícios físicos podem piorar os sintomas vasomotores por desencadear ondas de calor em mulheres com uma zona termoneutra diminuída (11).

Acupuntura

A Acupuntura envolve o uso de agulhas de calibre extremamente fino inseridas em pontos específicos do corpo e tem sido usada por milhares de anos na Ásia. Mais recentemente esta técnica tem ganhado popularidade no ocidente para o tratamento de múltiplas condições. Na última década, vários ensaios clínicos têm testado a acupuntura no tratamento das ondas de calor, porém ainda não há evidência de que seja efetiva nesse tipo de sintomatologia (113,126,127).

Bloqueio do gânglio estrelado

Outra terapia não farmacológica promissora, atualmente sob investigação, é o bloqueio do gânglio estrelado (113). Lipov e colaboradores teorizam que o bloqueio desse gânglio simpático, localizado anteriormente e abaixo das apófises transversas de C6 e C7, provoca o alívio dos sintomas vasomotores por interrupção do sistema nervoso simpático, talvez por permitir que os mecanismos termorreguladores que estão alterados se restabeleçam (128).

2.7. Alternativas terapêuticas testadas nesta tese

2.7.1. Cetamina

A cetamina é um antagonista de alta afinidade, não competitivo, do NMDA, receptor ionotrópico do glutamato, que é amplamente utilizada como agente anestésico (129). O glutamato é o principal mediador da transmissão sináptica excitatória no cérebro de mamíferos e tem um papel importante na plasticidade sináptica, aprendizagem e memória. Um crescente corpo de pesquisa pré-clínica implica o sistema glutamatérgico na fisiopatologia da depressão e no mecanismo de ação dos tratamentos antidepressivos (15,130). Berman et al. (2000), em um estudo clínico, publicou o primeiro relato de efeitos terapêuticos da cetamina em transtorno depressivo maior (14). Após este ECRC, muitos estudos forneceram evidências de que uma única dose intravenosa, subanestésica, de cetamina pode aliviar os sintomas depressivos em poucas horas. As propriedades antidepressivas da cetamina têm sido testadas em modelos animais de comportamento do tipo depressivo. Em particular os receptores NMDA parecem estar envolvidos, embora outros tipos de receptores também pareçam relacionados à depressão e aos efeitos dos antidepressivos. No nado forçado, a cetamina diminuiu o tempo de imobilidade, requerendo, para isso, a sinalização por meio de receptores de glutamato subtipo ácido α-amino-3-hidroxi-5-metil-4-isoxazolepropiônico (AMPA) (131). Em outro estudo experimental, esse efeito foi associado ao aumento da concentração do BDNF no hipocampo (52). Fraga e cols. sugeriram que os efeitos da cetamina no comportamento tipo depressivo e os níveis de BDNF no cérebro de ratos estão relacionados com o tempo em que eles foram avaliados após a administração da droga (132).

2.7.2. Técnicas de estimulação cerebral não invasiva

O controle da temperatura corporal é complexo e envolve integração de informações da periferia que são transmitidas para o cérebro. Vários estudos indicam que o córtex somatossensorial, o córtex da ínsula, o giro cingulado anterior, o tálamo e o hipotálamo são as regiões cerebrais que respondem às mudanças na temperatura cutânea, para regular as respostas homeostáticas às mudanças de temperatura ambiental. A temperatura e a dor estão intimamente associadas

funcional e anatomicamente no SNC, consistente com sua importância para a manutenção da integridade do organismo.

Nos últimos anos as técnicas de neuromodulação têm galgado espaço na terapêutica de patologias como depressão e dor crônica. Têm sido investigados, principalmente, os métodos relacionados à estimulação não invasiva do sistema nervoso central, em duas modalidades: a estimulação magnética transcraniana (EMT) e a ETCC. A ETCC tem demonstrado bons resultados em pacientes com depressão, AVC, alterações da excitabilidade cortical, por exemplo, distonia focal, cefaleia, dor crônica, depressão e epilepsia (28,30,133). A EMT é uma técnica segura de estimulação cerebral que apresenta efeitos positivos em pacientes com dor neuropática (134), porém ainda é uma ferramenta de alto custo. Já a ETCC é mais acessível, pois apesar de oferecer um método menos focal da estimulação do cérebro, a aplicação é simples e oferece pouco ou nenhum risco na aplicação (135).

A ETCC consiste na aplicação de corrente elétrica contínua sobre o couro cabeludo de forma a produzir alterações da excitabilidade cerebral. Tem sido proposto que o efeito modulador desse tipo de estímulo sobre o córtex cerebral ocorre em decorrência da hiperpolarização ou despolarização e, consequentemente, alteração da atividade e excitabilidade cortical. Esta mudança na excitabilidade pode ser explicada em função da estimulação catódica reduzir o disparo espontâneo de neurônios corticais, devido a uma hiperpolarização do corpo celular, enquanto que a estimulação anódica tem um efeito inverso (136).

Trabalhos abordando técnicas de captação de imagem encefálica como a tomografia por emissão de pósitron (PET) demonstram que a estimulação anódica aumenta o fluxo sanguíneo em algumas áreas corticais e subcorticais (137). Esse método também pode modular a excitabilidade cortical visual e motora (138,139). A aplicação de estimulação anódica no córtex motor resulta em melhor desempenho motor (140), aumento do rendimento do aprendizado motor implícito (137) e memória operacional em sujeitos saudáveis (141,142) e em pacientes com Doença de Parkinson (143).

É plausível teorizar que a ETCC possa vir a ser uma alternativa terapêutica no tratamento dos sintomas psicoafetivos e vasomotores relacionados ao climatério e

experimentos pré-clínicos podem trazer informações sobre os efeitos físicos e neuroquímicos dessa técnica. Esta hipótese se fundamenta no efeito difuso dessa intervenção sobre o sistema nervoso central, o qual pode ter ação nos sistemas noradrenérgico e serotoninérgico que estão associados ao aparecimento desses sintomas no hipoestrogenismo.

2.8. Marcadores avaliados nesta tese

2.8.1. Citocinas

As citocinas são conhecidas como proteínas de baixo peso molecular, produzidas por células do sistema imune, que atuam em outras células do organismo pertencentes ao sistema imune ou não (144). Nathan & Sporn definem citocina como uma (glico) proteína solúvel, de natureza não-imunoglobulina, liberada por células do hospedeiro vivo, que atuam não enzimaticamente, em concentrações nanomolares a picomolares, para regular a função da célula hospedeira (145).

As citocinas podem ser classificadas como interleucinas (IL), produzidas pelos leucócitos; interferons (IFN),que atuam na intermediação entre as células do sistema de defesa e certos vírus; os fatores de necrose tumoral (TNF),de crescimento (GF) e estimuladores de colônias (CSF)(146).

As citocinas podem ser divididas em dois grupos principais: citocinas pró-inflamatórias e citocinas anti-inflamatórias. As citocinas pró-inflamatórias atuam estimulando as células de defesa produtoras da inflamação, seja diretamente, seja pelo estímulo à produção de outras citocinas e, as mais conhecidas são: as interleucinas (IL-1alfa e beta, IL-6, IL-8) e TNF. As citocinas anti-inflamatórias atuam no sentido oposto, ou seja, inibem as células de defesa produtoras da reação inflamatória ou inibem a produção das citocinas que estimulam a inflamação e as mais conhecidas são: a interleucina-10 (IL-10) e a interleucina-13 (IL-13)(146).

As interleucinas têm diversas ações no organismo. Estudos mostraram que pessoas submetidas a stress mental agudo apresentam concentrações sanguíneas mais elevadas de IL-6, de TNF-α e de antagonista do receptor IL-1 (IL-1Ra) (147).

Vários pesquisadores têm demonstrado a relação entre o estradiol e as citocinas frente a diversas condições clínicas (148–152). Iwasa e cols.(2014) estudaram as mudanças nas respostas inflamatórias centrais e periféricas a lipopolissacarídeo, em ratas ovariectomizadas (OVX) e encontraram aumento do RNA mensageiro de todas as citocinas examinadas (IL-1β, TNF-α e IL-6) no hipotálamo das ratas OVX em relação às ratas não OVX (153).

2.8.2. Fator neurotrófico derivado do cérebro (BDNF)

As neurotrofinas (NT) são peptídeos encontrados no sistema nervoso central (SNC) que têm importância nos processos de crescimento, diferenciação e sobrevivência das células do tecido nervoso. O BDNF é membro da família das neurotrofinas, está envolvido no desenvolvimento do sistema nervoso dos vertebrados e regula a plasticidade sináptica do cérebro adulto, influenciando a migração de axônios e ajustando o número e tamanho de espinhas dendríticas em neurônios (154). O BDNF facilita o mecanismo fisiológico de potenciação de longo prazo (LTP) em hipocampo, por isso, tem sido associado com os processos de aprendizagem e memória (155). O BDNF é a neurotrofina mais abundante no SNC, tanto com relação à quantidade, quanto à distribuição, sendo seus mais altos níveis encontrados no hipocampo, cerebelo e córtex (154,156). As neurotrofinas exercem seus efeitos por meio de três classes de receptores do tipo tirosina-quinase (Trks):TrkA, TrkB e TrkC. Cada neurotrofina tem maior afinidade por determinado receptor, o BDNF liga preferencialmente ao TrkB (157).

A investigação sobre a regulação da expressão gênica do BDNF e os mecanismos de sinalização por meio do qual esta neurotrofina exerce os seus efeitos biológicos revelou interações entre a sinalização hormonal e o BDNF. Esta interação inclui a regulação da expressão do BDNF por hormônios esteróides gonadais, que podem influenciar o papel do BDNF no desenvolvimento sexualmente dimórfico do cérebro ou no dimorfismo sexual de comportamentos e funções cerebrais (158,159).

Alterações nos níveis de BDNF estão correlacionadas com uma série de doenças neurológicas, tais como depressão, epilepsia, síndrome de Parkinson,

doença de Alzheimer e doença de Huntington (160). Estudo de Fritsch (2010) sugere que a estimulação por corrente contínua promove plasticidade sináptica dependente do BDNF(161).

2.9. Modelos animais de fogachos

Os modelos animais podem prover dados extremamente úteis para ajudar a compreender uma gama de problemas de saúde e condições associadas à menopausa, inclusive sobre as ondas de calor.

Diversos modelos animais têm sido utilizados para o estudo das ondas de calor relacionadas ao hipoestrogenismo(162). A maioria dos pesquisadores utiliza fêmeas de roedores ovariectomizadas, principalmente de ratos e camundongos, mas também de cobaias (porquinhos- da- índia)(74,102,163). Kobayashi et al. (2000) utilizaram um termístor atado à cauda e outro introduzido no reto de ratas Sprague-Dawley ovariectomizadas, parcialmente contidas em caixas, e verificaram a temperatura a cada cinco minutos durante seis horas por dia, detectando aumento em ambos locais, após 2 e 8 semanas da cirurgia, respectivamente(164). Ovelhas e primatas não humanos são outros mamíferos que também têm sido utilizados com essa finalidade (83,165). Primatas não humanos são excelentes modelos para o estudo da disfunção termorregulatória associada à queda dos níveis estrogênicos, pois utilizam os mesmos mecanismos fisiológicos dos humanos para manter a temperatura corporal: sudorese e vasodilatação para diminuí-la e vasoconstrição e tremores para aumentá-la(166). No entanto, a complexidade e heterogeneidade dos sintomas vasomotores, que dificulta os estudos clínicos dos fogachos, também cria desafios na modelagem de animais para este distúrbio.

Para provocar o aumento da temperatura e testar drogas, alguns autores, além de realizar a ovariectomia, administram ioimbina, um antagonista seletivo dos receptores alfa-2 adrenérgicos(167), ou o peptídeo relacionado ao gene da calcitonina (PRGC)(168,169). Simpkins et al. (1983) desenvolveram um modelo em que induziam adição à morfina em ratas e depois administravam naloxona (antagonista de opioides) para provocar hipertermia por abstinência à droga(170). Alguns investigadores preconizam que a vasodilatação observada na cauda de ratas ovariectomizadas pode ser considerada um modelo de fogachos em humanos(171,172).

Detectar mudanças de temperatura e vasodilatação em animais conscientes e sem restrição de movimentos é outro desafio para estudos experimentais dos fogachos. Equipamentos dispendiosos, como termístores implantados nos animais, que necessitam ser colocados em caixas apropriadas para transmitir os dados para computadores e registradores de dados de temperatura com memória interna (dataloggers) são os mais utilizados, encarecendo as pesquisas (74,102,112,173,174).

3. Mapa conceitual/marco teórico

A figura resume o conhecimento atual da fisiopatogenia dos sintomas associados à menopausa e ação da terapia.

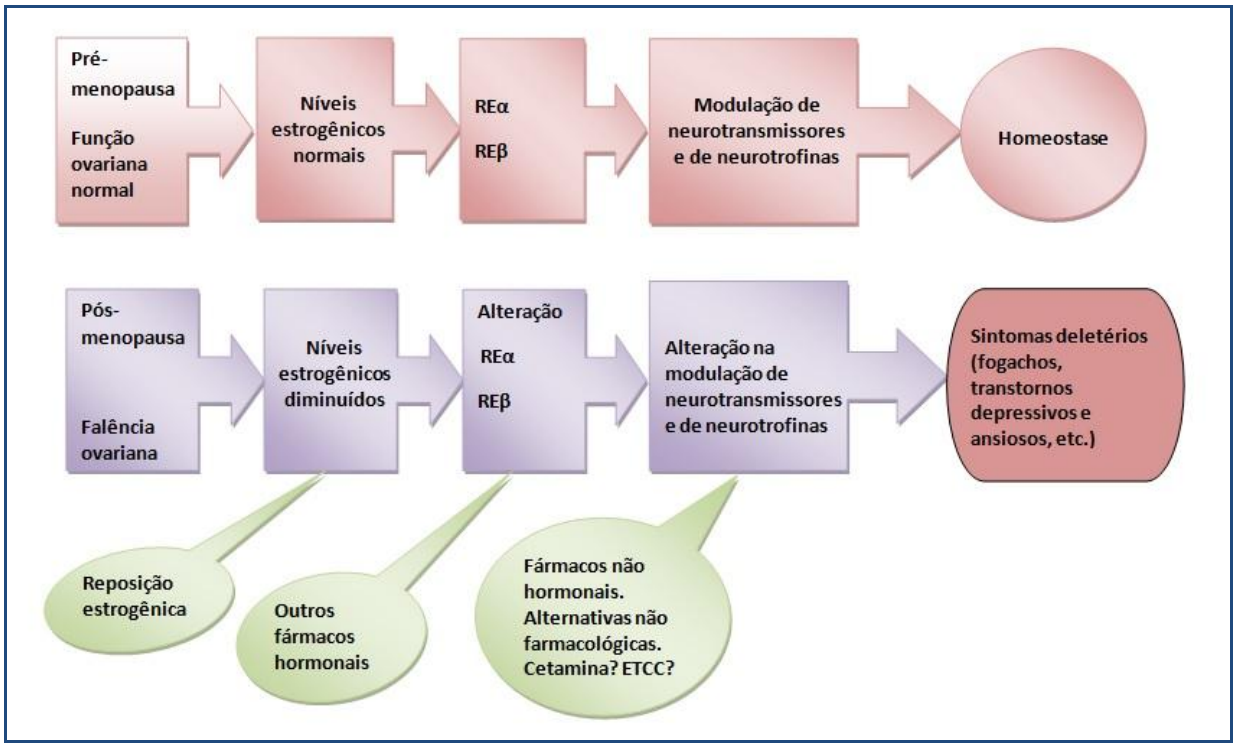


Figura 4. Mapa conceitual da tese. Acima: níveis estrogênicos normais →homeostase. Ao centro: níveis estrogênicos diminuídos na menopausa levando a alteração na modulação de neurotransmissores →sintomas. Abaixo: Alternativas terapêuticas. REα: receptor de estrogênio alfa. REβ: receptor de estrogênio beta.

4. Objetivos

4.1. Objetivo Geral

Avaliar, em um modelo animal de menopausa, parâmetros fisiológicos, comportamentais e bioquímicos objetivando testar duas abordagens terapêuticas: a cetamina, um antagonista não competitivo do receptor N-metil-D-aspartato (NMDA) e a eletroestimulação transcraniana de corrente contínua (ETCC) respectivamente, para comportamento do tipo depressivo e disfunção termorregulatória em ratas.

4.2. Objetivos específicos

Artigo 1: Ketamine reduced depressive-like behavior induced by ovariectomy in rats

Avaliar o efeito da ovariectomia sobre:

- o comportamento tipo ansioso;

- a atividade locomotora e exploratória;

- a citologia vaginal.

Avaliar o efeito da ovariectomia e da cetamina sobre:

- o comportamento tipo depressivo.

Artigo 2: Partial reversion of ovariectomy-induced thermoregulatory dysfunction by Cathodal Transcranial Direct Current Stimulation (tDCS)

Avaliar o efeito da ovariectomia sobre:

- os seguintes parâmetros fisiológicos: temperatura, peso corporal e citologia vaginal;

- os níveis de estradiol sérico;

Avaliar o efeito da ovariectomia e da ETCC sobre:

- a atividade locomotora e exploratória;

- a temperatura retal e cutânea;

- os níveis de interleucina 8 em soro e hipotálamo;

- os níveis de interleucina 10 no hipotálamo, hipocampo, córtex cerebral e medula espinhal.

Artigo 3: Neuromodulatory Effect of Estrogen and Transcranial Direct Current Stimulation (tDCS) on Nociception and BDNF in Ovariectomized Rats.

Avaliar o efeito da ovariectomia sobre:

- a citologia vaginal;

- os níveis de estradiol sérico;

Avaliar o efeito da ovariectomia e da ETCC sobre:

- a resposta nociceptiva e alodinia mecânica;

- os níveis de BDNF no soro, hipotálamo, hipocampo, córtex cerebral e medula espinhal.

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6. Artigos da tese

Artigo 1. Hypoestrogenism alters mood: ketamine reverses depressive-like behavior induced by ovariectomy in rats

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Hypoestrogenism alters mood: ketamine reverses depressive-like behavior induced by ovariectomy in rats

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Abstract: Menopause is a physiological process characterized by the loss of ovarian follicular activity that occurs in women between the ages of 40 and 55. As life expectancy for women has increased, they now spend a significant part of their lives in the postmenopausal state. Estrogen deficiency is associated with the onset of depressive and anxiety symptoms, cognitive impairment, and others. The use of animal models is very important because it makes it possible to study the pathophysiology of the depressive and anxiety disorders associated with a decrease in estrogen. This study investigates depressive-like and anxiety-like behaviors in ovariectomized rats and ketamine's effect on a forced swimming test. Twenty-eight female Wistar adult rats were initially divided into two groups: ovariectomized (OVX) and false surgery (SHAM). Hormonal status was verified by vaginal cytology, and the rats were subjected to a forced swimming (FS) test 18 days post-surgery, an open field (OF) test 28 days post-surgery, and an elevated plus maze (EPM) test 38 days post-surgery (Experiment 1). In addition, the effect of ketamine on depressive-like behavior of the female rats was evaluated (Experiment 2). The OVX group exhibited anxiety-like behavior on the EPM test (lower time spent and fewer entries in the open arms) without any difference in performance on the OF test. On the FS test, OVX rats showed depressive-like behavior (higher time of immobility) than SHAM rats. The SHAM group showed signs of hypoestrogenism (anestrus) at six months of age, probably induced by having their ovarian

and fallopian tubes exposed. Moreover, ketamine was able to reverse depressive-like behavior in the FS retest in both groups (OVX and SHAM). In conclusion, the OVX and the precociously menopausal SHAM rats showed depressive-like behavior most likely related to hypoestrogenism, which was reversed by ketamine. Our data are consistent with scientific evidence of the neuromodulatory effect of estrogen on mood, and the effective use of ketamine for depressive symptoms.

Key words: menopause; depression; ketamine; female rats; aging.

1. Introduction

Menopause is a physiological process due to the loss of ovarian follicular activity leading to a decrease in the production of estrogens. Hypoestrogenism can cause a variety of physiological and psychological disorders such as changes in the menstrual cycle, vasomotor and genital symptoms, sleep problems, mood swings, and impaired cognitive function [1,2]. Age, menopausal status, chronic diseases and socio- demographic characteristics (income, ethnicity and educational level) have been identified as predictors of the frequency and severity of menopausal symptoms [2, 3].

Data from two cohort studies in the United States showed increased risk of depression in women who enter menopausal transition [4, 5]. However, the mechanisms responsible for the development of depression in perimenopausal women remain unclear [6, 7]. On the other hand, Díaz-Véliz et al. (1997) suggest that ovarian hormones modulate anxiety levels and cognitive functions [8]. Anxiety may be a precursor for depression development [9] or may be accompanied by symptoms of depression [10],thus, it is important to consider equally anxiety and depression symptoms when investigating factors that may affect mood, such as hormonal status [11,12].

Depressive and anxious symptoms can significantly reduce the quality of life of postmenopausal women [13]. The mechanisms that lead to the emergence of these symptoms in the menopausal transition are not well understood, and even the pathophysiology of depressive disorders has been widely debated. The monoamine theory posits that depression is caused by a decreased function of monoamines in the brain, and antidepressant drugs are designed to increase the supply of these substances, inhibiting its reuptake (serotonin and norepinephrine reuptake inhibitors) or its degradation (monoamine oxidase inhibitors) [14]. However, the long time to the onset of the therapeutic action and the low rates of remission has encouraged the search for more effective drugs. The observation that small doses of ketamine produce a rapid and transient antidepressant effect increased

the interest in neurobiological systems that were not explored in depression [15]. Ketamine is a non-competitive N-methyl-D-aspartate (NMDA) receptor used in human and veterinary medicine, primarily for the induction and maintenance of general anesthesia. Berman et al. (2000) published the first report of ketamine's therapeutic effects on major depressive disorder [15]. After this randomized controlled trial (RCT), many studies provided evidence that a single, intravenous (IV), subanesthetic dose of ketamine may relieve depressive symptoms within hours [16]. Garcia et al. (2008) suggest that the increase of hippocampal BDNF protein levels induced by ketamine might be necessary to produce a rapid onset of antidepressant action [17]. Another study from the same laboratory demonstrated that Ketamine alters behavior and decreases BDNF levels in the rat brain as a function of time after drug administration, suggesting that the effects of ketamine on behavior and BDNF levels are related to the time at which they were evaluated after administration of the drug [18].

To understand the pathophysiology of anxiety and depression disorders associated with the decline of endogenous estrogen levels and devise interventions aimed at attenuating these symptoms, it is very important to establish and study animal models of menopause. This study investigated depressive-like and anxiety-like behaviors and cognitive performance in ovariectomized rats and ketamine's effect on performance on a forced swimming test.

2. Materials and methods

2.1. Animals

Twenty-eight female Wistar rats (90 days old, 200-300g) were randomized by weight and housed in cages of polypropylene material (49x34x16cm). They were housed four per cage and maintained with food and water available *ad libitum* on a 12h light/dark cycle (lights on at 7:00 AM, and lights off at 7.00 p.m.) in a temperature-controlled environment (22±2°C). The animals were initially distributed into two groups: ovariectomized (OVX) and false

surgery (SHAM) and subjected to the forced swimming test (FS) at 18th day post surgery, open field test (OF) at 28th day post surgery, and elevated plus maze test (EPM) at 38th day post-surgery. When the animals reached 180 days old, each group was subdivided into two more groups, which received ketamine or vehicle and were again subjected to the forced swimming test. The rats were handled for seven days prior to the experiments and remained in the laboratory for at least 30 min before being submitted to each test. All experiments and procedures were approved by the Institutional Animal Care and Use Committee (GPPG-HCPA protocol No. 110586) and were compliant with Brazilian guidelines involving the use of animals in research (Law N^{o}. 11.794). Additionally, all efforts were made to minimize suffering, pain and discomfort of the animals, as well as to reduce the number of animals.

2.2. Surgical procedures

One set of Wistar female rats underwent ovariectomy (surgical removal of the ovaries) and the other set underwent sham surgery (opening of the abdominal cavity and sewing it back). At 90 days of age, the rats were anesthetized with ketamine (80 mg/kg, i.p.; Syntec, Brazil) and Xylazine (20 mg/kg, i.p.; Sespo, Brazil) and underwent bilateral ovariectomy. The surgery consisted of a transversal dorsolateral incision of skin, between the last rib and pelvis and muscle dissection in order to expose the abdominal cavity. The ovary is located in a fat pad beneath the muscles. The periovarian fat was grasped to lift and exteriorize the ovary. The fallopian tube was crushed and ligated, and the ovary was removed by cutting above the clamped area. The muscle and the skin incision were closed with poligalactin and nylon suture. This procedure was repeated at the other side for bilateral ovariectomy. In sham surgery, rats underwent the same incisions, the ovaries and fallopian tubes were exposed and then put back in the abdominal cavity and the muscle and skin were closed. To reduce pain, all rats received dipyrone (25 mg/kg i.p.) after surgery, and recovered for ten days.

2.3. Vaginal smear

Ten days after surgery, vaginal smear was daily obtained in both groups to verify hormonal status. Samples were obtained and analyzed as described by Goldman et al. (2007) [19].

2.4. Behavioral tests

2.4.1. Locomotor activity assessed by Open Field (OF) Test

The behavioral assessment was performed in a varnished wood cage, measuring 60 cm x 40 cm x 50 cm with a glass front wall. The floor was covered with linoleum and divided up with dark lines: 12 squares of 13 cm x 13 cm each. The rats were gently placed in the left back corner and allowed to explore the surroundings for five minutes. The number of line crossings was taken as a measure of locomotor activity [20]. Rearing was defined as the moment the rat rose up on its hind legs, ending when one or both front paws touched the floor again [21], being evaluated as exploratory activity [22]. Grooming was defined as licking/washing of the head and body; it was assessed as a biological function of caring for the surface of the body [23]. The start of a trial occurred immediately after the rat was placed in the environment for scoring purposes. In this test, the animal was recorded as entering a new area when all four paws crossed the boundary into a different, marked-out area. Five measures were taken during the five-min test sessions: latency to leave the first quadrant (time in seconds); number of line crossings (i.e. horizontal activity), outer and inner crossings; number of rearing behaviors (i.e. vertical activity); grooming (time in seconds); and number of fecal boluses. The box was thoroughly cleaned between each trial.

2.4.2. Anxiety-like behavior assessed by Elevated plus-maze (EPM) test

The elevated plus-maze test was used to evaluate anxiety-like behavioral state. The maze was made of black PVC and elevated to a height of 50 cm above floor level. The apparatus included two open arms and two closed arms (50 cm x 40 cm x 10 cm), which extended from a common central platform (10 cm x 10 cm). The animal was placed in the central area of the EPM, facing one of the closed arms. Next, the following behavioral measures were recorded during the five-min test sessions: number of protected head-dipping movements (PHD); number of non-protected head-dipping movements (NPHD); number of entries in the open arms (EOA); number of entries in the closed arms (ECA); time spent on the open arms (TOA); time spent on the closed arms (TCA); time of grooming and number of rearing. Protected head dips involved dipping the head over the sides of the maze from within the central platform or a closed arm, whereas unprotected head dips were considered to occur when the animal dipped its head over the sides of the maze while on an open arm. In the EPM, entering a new area was recorded when all four paws crossed onto a new arm or into the central area [24]. After each test, the apparatus was cleaned thoroughly to remove any animal scent.

2.4.3. Depressive-like behavior assessed by Forced swimming (FS) test

The forced swimming test involved three expositions: training, testing and retesting under drug influence. A glass square tank (dimensions 40 cm x 40 cm x 52 cm, divided into four squares of 20 x 20cm to fit four rats) was filled with water (22-25°C) to a depth of 35 cm, on such a way that the rats' tail could not touch the bottom of the tank. For the first exposure, the rats were placed into water for 15 minutes (training session). 24 hours later, the animals were again placed in the water for a 5-min session (test session). The immobility time of rats was recorded in seconds, considering total immobility and/or movements to keep the head out of the water with no intention of escaping [25]. After the trial, rats were dried using soft towels and hair dryer, if necessary.

2.5. Experimental design

Experiment 1: Eighteen days after surgery (P108), groups OVX and SHAM were subjected to the sequence of FS, OF, and EPM tests. The tests were separate at least for one week between them.

Experiment 2: At P180, both groups (OVX and SHAM) were subdivided into two more groups that received ketamine (10 mg/kg, i.p., [17, 26]; Cetamin®, Syntec, Brazil) (SHAM-K and OVX-K) or vehicle (SHAM-V and OVX -V, saline, i.p.), and were subjected to FST (retest session).

2.6. Statistical Analysis

All data are presented as mean ±S.E.M. All analyses were performed using the Statistical Package for the Social Science (SPSS) software version 18.0. Student's t test or one-way ANOVA followed by Student-Newman-Keuls (SNK) were used to evaluate differences between groups. Critical significance level used for all comparisons was 5%.

3. Results

In this study, acyclic vaginal smears [void of nucleated (proestrus) cells] occurred in all ovariectomized rats (OVX), as verified since the beginning of vaginal smears procedures, ten days after surgery, and continuing up to the end of the experiments. Sham group presented normal estrous cycling in the first 60 days after surgery. Interestingly, after only three months, the SHAM group presented the cessation of normal estrous cycling, exhibiting aberrant cycling patterns at 180 days old, including an increase in the number of days per

cycle and a decrease in number of vaginal cells, on vaginal smears, followed by persistent diestrus (figure 1).

In experiment 1, OVX group showed increased immobility time in the FS test as compared to SHAM group (Student's t test, *P*=0.03; Figure 2, Panel B; n=14 per group). In the OF test, there was no significant difference between SHAM and OVX groups in all behaviors analyzed (Student's t test, *P*>0.05, data not shown, n=10-11 per group). In the EPM test, OVX group showed decreased time spent on open arms (*P*=0.01), lower number of entries on open arms (*P*=0.01) and lower number of NPHD (*P*=0.003) as compared to SHAM group, suggesting an anxiety-like behavior in the OVX female rats (Student's t test, Figure 3, Panel A, B and C, n=10-11 per group).

In experiment 2, at P180, the SHAM group presented a precocious menopause, showing signs of hypoestrogenism as indexed by vaginal smears, and depressive-like behavior linked to increased immobility time on FS retest (paired Student's t test, P=0.03, n=5-6 per group, Figure 4) and a tendency to be different in the swim time (paired Student's t test, P=0.051, n=5-6 per group, Figure 4). At P180, both groups that received ketamine (SHAM-K and OVX-K groups) improved its performance and decreased the immobility time, showing that ketamine reversed the depression-like behaviors in the FS retest (one-way ANOVA/SNK, P=0.03, n=5-6 per group, Figure 5).

4. Discussion

Our findings showed that female ovariectomized rats presented depressive-like and anxiety-like behaviors. In addition, at P180 the SHAM ovariectomized rats showed a precocious menopause with aberrant cycling patterns, and also presented a depressive-like behavior. Moreover, the NMDA receptor antagonist (ketamine) was able to reverse this behavior in both groups assessed (OVX and SHAM).

It is interesting to note that, the OVX female rats showed anxiety-like behavior in the EPM test, without any impairment in the locomotion assessed in the OF test. Our findings corroborate previous studies showing the modulatory effects of ovarian hormones upon behavioral indices of anxiety [8, 27]. Díaz-Véliz found different effects of diazepam on conditioning avoidance and motor activity in female rats, according to hormonal status [28]. Moreover, the anxiety-like behavior of OVX group on EPM test corroborates Kessler's et al. studies (2005, 2008) that showed association between depressive and anxiety disorders [29,10]. The estradiol injection, compared to vehicle, subcutaneously or into the hippocampus or amygdala of ovariectomized rats decreases anxiety-like and depression-like behaviors, as reported by Walf and Frye (2006)[27]. These authors also showed that chronic estradiol replacement to aged female rats reduces anxiety-like and depression-like behavior and enhances cognitive performance [30].

Female rats with surgical (OVX) and precocious menopause (SHAM) showed depressive-like behavior probably related to hypoestrogenism. These results are consistent with the scientific evidence about neuromodulatory effect of estrogen on mood [6,7,8,13]. The amygdala and hippocampus are brain regions known to be involved in mood regulation and animal studies have reported that the amygdala has one of the highest densities of estrogen receptors in the brain [31]. Estrada-Camarena et al. studied ovariectomized female Wistar rats, using the FS test, and found that estrogens have antidepressant-like effect characterized by a reduced immobility and increased swimming time and facilitate the action of fluoxetine and desipramine [32,33]. However, these effects were dependent on the type of estrogen used, and all combinations of estrogens and antidepressants decreased rats' locomotor activity when evaluated in the open field test [32,33].

In our study, the depressive-like behavior was reversed in both groups (OVX and SHAM) by ketamine, a non-competitive antagonist of the N-methyl D-aspartate receptor (NMDAR) that has been studied as an antidepressant drug in the sub-anesthetic dose. Most of the experimental studies on antidepressant effects of ketamine use male rodents [34].

Carrier and Kabbaj (2013) demonstrated that intact female rats are more sensitive than male rats to the antidepressant effects of ketamine [35]. In our study, we used a dose of 10 mg/Kg, as described by Garcia et al (2008)[17] and Yang et al (2012)[26], and the ovariectomized rats presented a significant reduction in the immobility time on the forced swimming test. Our finding complements those of Carrier and Kabbaj study, showing a dose-dependent action of ketamine in ovariectomized female rats.

In general, rodent models use stress-induced impairment of hippocampal function to produce the depressive-like behavior and test new antidepressants [36]. Estrogens act through ERα and ERß-receptor subtypes to modulate the transcription of genes, which, in turn, encode a variety of proteins. These proteins include many of the enzymes that play a key role in the synthesis and function of neurotransmitters including serotonin. Estrogens also have multiple effects on dopamine systems, including upregulation of dopamine D1A receptors [37] and increase of dopamine transporters' density [38]. Serotonin hetero-receptors are present in dopamine neurons creating multiple interaction points between estrogens, serotonin and dopamine neurotransmission that may be the link between depression and perimenopause in women with increased vulnerability. Yet, Ketamine does not work through the "conventional" antidepressant mono-aminergic targets of serotonin and noradrenaline [39] and then, its action on a model of menopause depressive-like behavior launches new pathways into the study of the pathophysiology of depression in this period of the women's life. It is important to note that the action of estradiol on glutamatergic system, in relation to the lordosis behavior of female rats, has been well established in the works of McCarthy [40]. Thus, we can suggest that the reversion of depressive-like behavior in ovariectomized rats makes a point of the role of glutamate in the depressive symptoms of menopause. The role of progesterone also has to be considered, since studies demonstrated proestrus increases in anxiolytic-like behavior in female rats that were coincident with elevated circulating and hippocampal progestin concentrations [41].

The non-ovariectomized group (SHAM group) showed signs of hypo-estrogenism (anestrous) at six months of age, as verified by vaginal smears. The precocity of the ovarian failure was an unexpected finding, for, in general, female rats only show aciclicity when they reach about twelve months [42]. Reproductive senescence in rodents is similar to menopause in several critical aspects, including similar alterations in pulsatile LH release and the LH surge, variability of cycle length prior to acyclicity, and ultimate cessation of hormone cycling. We hypothesized that ovarian and fallopian tubes exposition through the small dorsolateral opening may have accelerated ovaries aging by some mechanism such as trauma or impairment of blood circulation, which possibly elicits irreversible degenerative responses or necrosis in the ovaries cells.

Depressive-like behavior on aged SHAM group was less evident than on OVX group. This finding indicates that depressive-like behavior is present on both, natural or artificial ovarian depletion in female rats, the latter being more evident, probably because of gradual lowering of hormone levels on aging, and this mimics natural menopause in humans. There are several transgenic mice models of ovarian senescence. However, in such cases, fetal development of the reproductive system has been affected and normal reproductive function has never been achieved [43]. The depressive-like behavior in aged SHAM group was also reversed by ketamine, but less than the OVX group (figure 5). These data propose a new animal model of menopause transition, in which there is a gradual ovarian failure, which can be used in researches on menopause symptoms, more closely mimicking the biology of natural menopause in humans.

In conclusion, this study corroborates scientific evidences about neuromodulatory effect of estrogen on mood, and shows the ketamine's acute action on depressive-like behavior on a model of menopause. In addition, we suggest a new animal model of menopause, similar to natural evolution, induced by ovarian and fallopian tubes exposition. Further researches are needed to clarify the mechanisms by which ovarian and fallopian

tubes exposition through dorsolateral incisions may have caused precocious ovarian failure in female Wistar rats.

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Figure Legends

Figure 1 – Diestrus = only few leukocytes.

Figure 2 –Forced swimming test in the experiment 1. Data expressed as mean ± S.E.M., n=14 animals/group. Time was expressed in seconds. **Panel A:** Time of swimming. **Panel B:** Time of immobility.

*OVX was significantly different from SHAM (Student's *t* test, *P*=0.03).

Figure 3 – Elevated Plus Maze test in the experiment 1. Data expressed as mean ± S.E.M. **Panel A:** time spent in the arms was expressed in seconds. **Panel B:** number of entries in the arms. **Panel C:** number of PDH and NPHD behaviors. PHD: number of protected head dipping. NPHD: number of non-protected head dipping.

*OVX different from SHAM group (Student's *t* test; Panel A, *P* =0.01; Panel B, *P* = 0.01; Panel C, *P*=0.003).

Figure 4 – Forced swimming test at P108 and P180 in OVX and SHAM groups. Time was expressed in seconds. Data expressed as mean ±S.E.M. (n=5-6 animals/group). **Panel A:** Time of immobility. **Panel B:** Time of swimming.

*SHAM group different at P180 from P108 in the immobility time (paired Student's paired *t* test, *P* =0.03).

Figure 5 – Forced swimming test at 6 months in OVX and SHAM groups under use of ketamine or vehicle. Time was expressed in seconds. Data expressed as mean±S.E.M. (n=5-6 animals/group). SHAM-V: rats under sham ovariectomy and vehicle (i.p.); SHAM-K:

rats under sham ovariectomy and ketamine (10 mg/kg i.p.); OVX-V: rats under ovariectomy and vehicle (i.p.); OVX-K: rats under ovariectomy and ketamine (10 mg/kg i.p.).

*SHAM-K and OVX-K different from SHAM-V and OVX-V groups (one-way ANOVA/SNK, P=0.03).

Figure 1

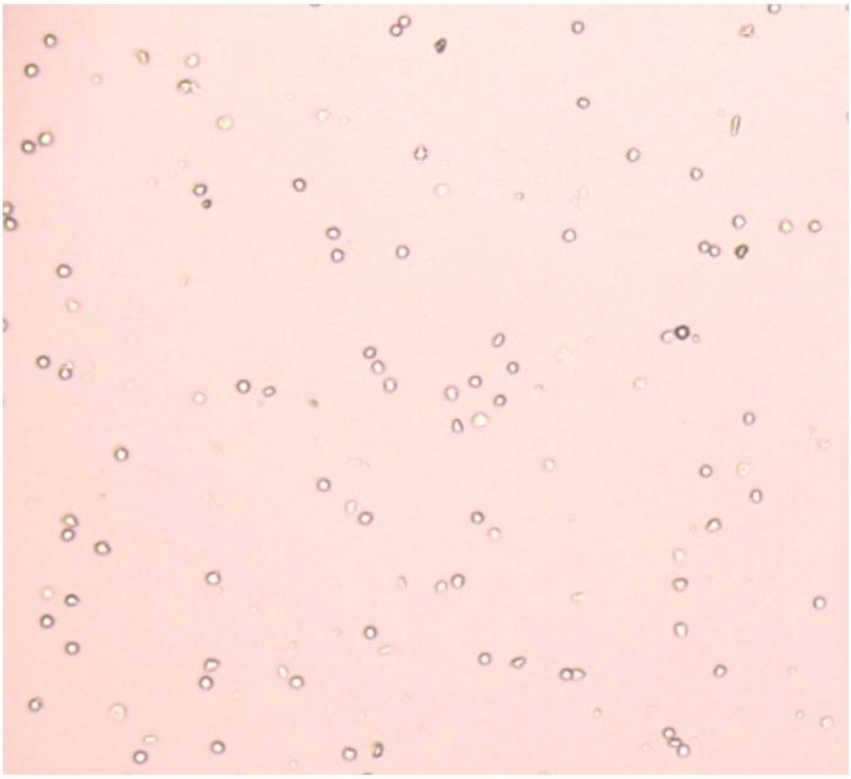
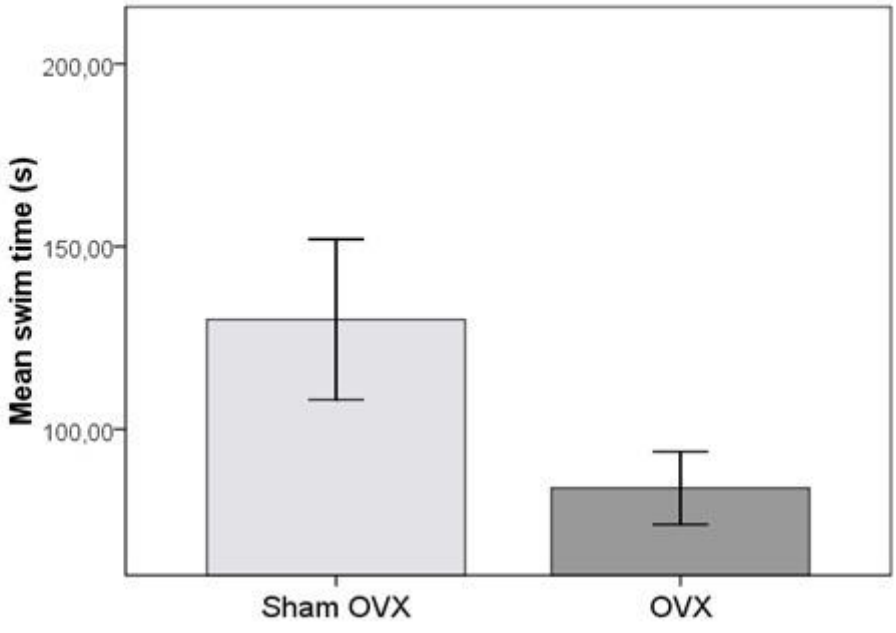


Figure 2

Panel A



Panel B

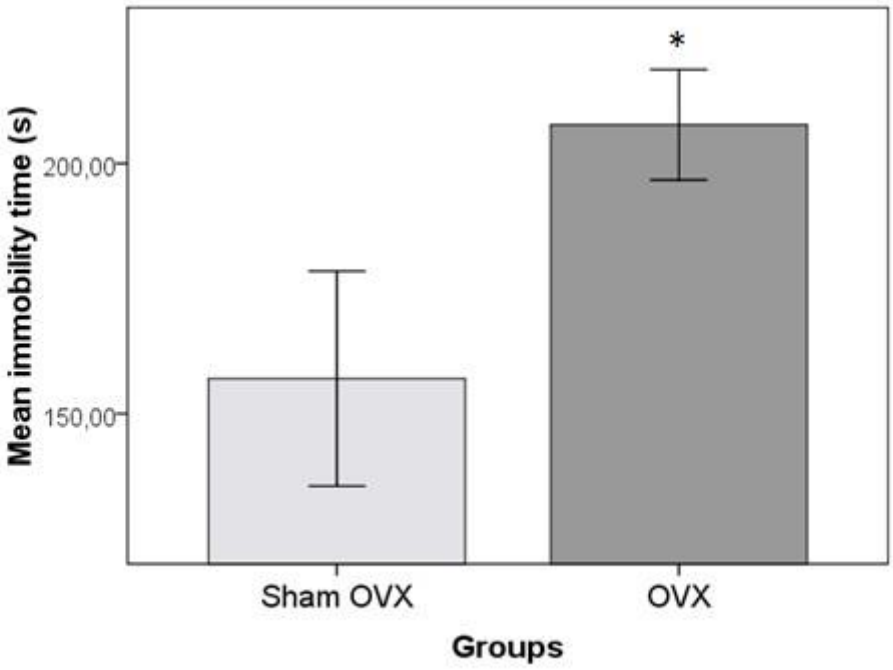
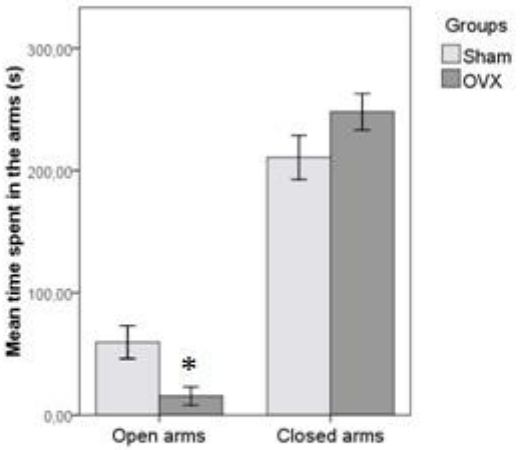
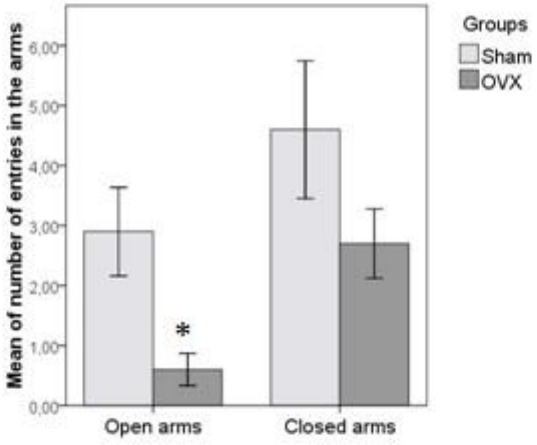


Figure 3

Panel A



Panel B



Panel C

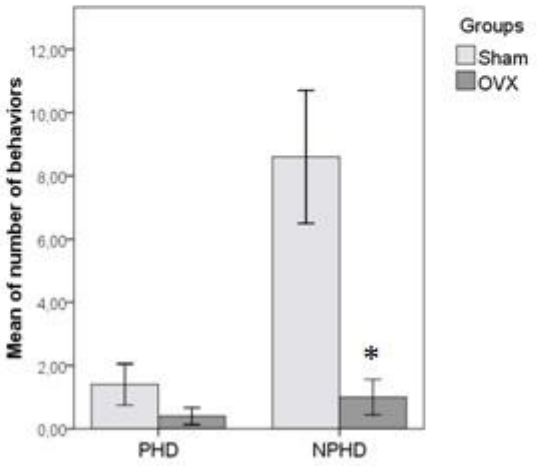
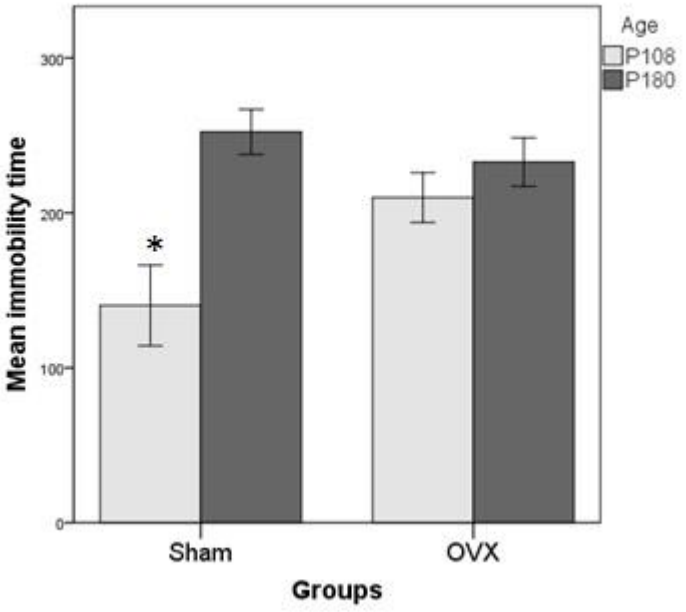


Figure 4

Panel A



Panel B

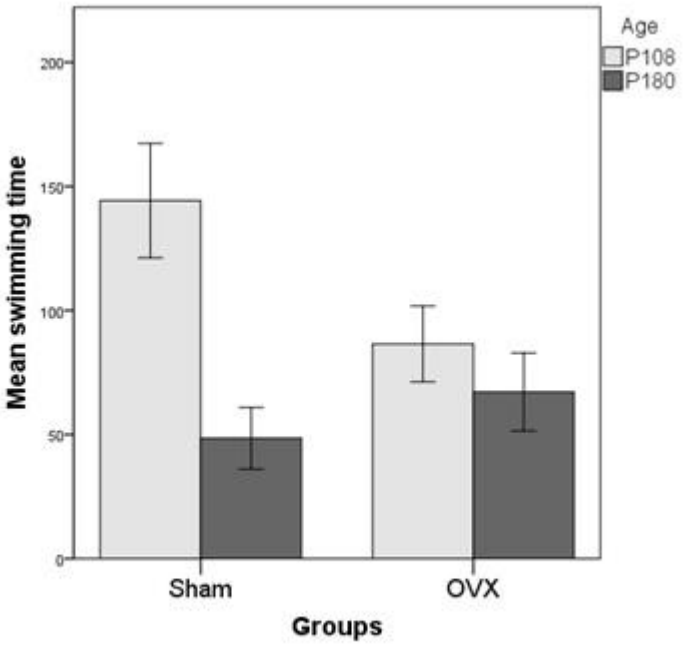
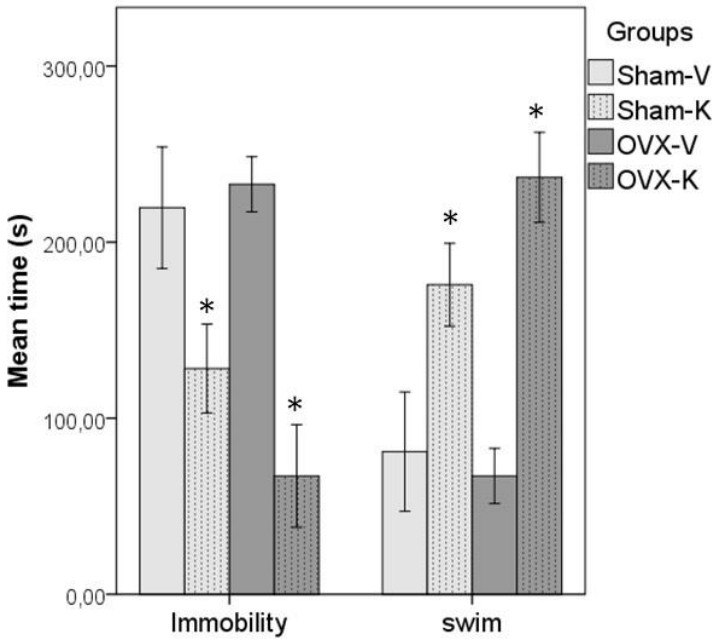


Figure 5


Groups
Sham-V
Sham-K
OVX-V
OVX-K
Mean time (s)
300,00
200,00
100,00
0,00
Immobility
swim
*
*
*
*

Artigo2. Partial reversion of ovariectomy-induced thermoregulatory dysfunction by Cathodal Transcranial Direct Current Stimulation (tDCS)

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Abstract

Hot flushes are the most common and characteristic complaints of women in the menopausal transition. The exact pathophysiology of hot flushes remains unknown; this challenges therapy for women who cannot receive estrogens. Hot flushes result from a dysfunction of the thermoregulatory system. Data from several studies suggest that transcranial direct current stimulation (tDCS) induces a cascade of events associated with glutamatergic, GABAergic, dopaminergic, serotonergic, and cholinergic activity modulation. It has also been suggested that tDCS is effective in treating pain and some neuropsychiatric disorders. In this study we test the hypothesis that cathodal tDCS may have a beneficial effect on the thermoregulatory dysfunction in ovariectomized rats. In addition, we evaluated locomotor activity, interleukin-8 (IL-8) and interleukin-10 (IL-10) levels. Forty-five female adult Wistar rats (200-250g) were randomized by weight into five groups: total control (CT), ovariectomized + tDCS (OT), ovariectomized + sham tDCS (OS), sham ovariectomized + tDCS (ST) and sham ovariectomized + sham tDCS (SS). The assessments for control menopause rat model efficacy were vaginal smears, estradiol level and weight measurement. Additionally we evaluated: rectal temperature, locomotor activity and IL-8 and IL-10 levels. Results: Ovariectomized rats showed low estradiol levels, anestrous at vaginal smears and increased weight in the end of the experiments confirming menopausal status. Ovariectomized rats showed increase in rectal temperature that was partially reversed by tDCS (P=0.01). The increased serum levels of interleukin-8 (IL-8) observed in rats corroborates data of study in women with severe hot flushes complaints. In conclusion, the animal model of ovariectomy can help us to understand the pathophysiology of menopause symptoms linked to peripheral and central biomarkers. In addition, our data suggest that tDCS may be an interesting therapeutic alternative to relieve the vasomotor symptoms. Further preclinical studies are needed to evaluate the montage of the electrodes, the period of treatment and neurochemical responses to tDCS on hot flushes.

Keywords: menopause, tDCS, female rats, cytokines, hot flushes.

1. **Introduction**

Currently, life expectancy of women in Latin America and in Europe is 73.6 and 80.9 years respectively (1). Age at menopause, however, remains around 48.7 years, therefore women live about a third part of their lifespan under the effects of menopausal hypoestrogenic status (2). Although depletion of ovarian follicles is a physiological phenomenon, decline in estrogen concentration can cause several changes which impact the women's quality of life, such as: mood changes, sleep disturbances, vasomotor symptoms, cognitive dysfunction, decreased libido, and, later, urogynecological problems, decreased bone density and increased cardiovascular risk (3,4).

Estrogen therapy is the gold standard treatment for the relief of symptoms associated to hypoestrogenism. However, since the Women's Health Initiative (WHI) study report, which found an association between estroprogestin therapy and increased risk of breast cancer and thromboembolism, its use has been limited (5,6). For this reason, there is a growing demand for alternative therapies to relieve these symptoms (7,8). Vasomotor symptoms (VMS), hot flushes and night sweats, are the most frequent and distressing symptoms of menopause transition and post menopause and leads many women to seek for medical assistance.

The exact pathophysiology of VMS is still unclear; this hinders the establishment of an effective treatment for women who may not use estrogens. It is theorized that hot flushes are a thermoregulatory dysfunction (9). Preoptical medial area in hypothalamus is responsible for body thermoregulation (10) and it is theorized that estrogen decline increases neurotransmitters serotonin and norepinephrine concentrations in this area, leading to reduction of the thermoneutral zone (11). Consequently, minimal increases on core body temperature trigger inadequate responses of vasodilation and sweating. Other brain regions were found to be involved on thermoregulation. Freedman and cols., using functional magnetic resonance imaging (fMRI), found activation of insula and anterior cingulate cortex (ACC) during hot flushes in post menopausal women and concluded that thermoregulation in humans appears to be represented in a distributed cortico-subcortical network rather than in a single

localized structure (12). Additionally, cytokines have been studied in the pathophysiology of hot flushes and increased serum levels of interleukin-8 (IL-8) were observed in women with severe hot flushes complaints (13).

New advances for the treatment of VMS are needed. Neuropsychiatric symptoms may benefit of neuromodulation techniques. Transcranial direct current stimulation (tDCS) is a relatively simple and low cost technique that has been studied to treat depression (14), pain (15,16) and epilepsy (17) and it can be useful to treat symptoms of menopause, such as VMS. Clinical studies have demonstrated that motor cortex stimulation shifts motor cortex excitability, according to stimulation polarity: anodal stimulation increases cortical excitability, while cathodal stimulation decreases excitability (18). The current model of tDCS effects is based on cortico-cortical interactions, with some subcortical components such as the ACC in these circuits (Fregni & Pascual-Leone, 2007)(19). Considering that cathodal tDCS is able to decrease the neuronal excitability, and data from the literature suggest a hyperexcitability of neurons associated to thermoregulation, the aim of this study was to evaluate the effect of cathodal tDCS on VMS, linked to temperature changes in female ovariectomized rats. In addition, it was evaluated locomotor activity and IL-8 and IL-10 serum levels. The assessments for control of efficacy of menopause model in rat were: estradiol levels, vaginal smears and weight measurement.

2. Materials and methods

2.1 Animals

Forty-five female Wistar rats (60 days old, 200-250g) were randomized by the weight and housed in cages of polypropylene material (49x34x16cm), with the floor covered by wood shaving. Four or five were housed per cage with food and water available ad libitum and maintained on a 12-h light/dark cycle (lights on at 7:00 AM, and lights off at 7.00 p.m.) in a humidity and temperature-controlled environment (22±2°C). The rats were randomized by the weight and distributed into five groups: control (CT), ovariectomy and tDCS (OT), ovariectomy and sham-tDCS (OS), sham-ovariectomy and tDCS (ST) and sham-ovariectomy and

sham-tDCS (SS). Animals were handled for fourteen days prior to the experiments. All experiments and procedures were approved by the Institutional Animal Care and Use Committee (GPPG-HCPA protocol No. 11-0586) and were compliant with Brazilian guidelines involving use of animals in research (Law No. 11.794). Additionally, all efforts were made to minimize the suffering, the pain and discomfort of the animals, as well as to reduce the number of animals.

2.2. Body weight

The animals were weighed using a semi-analytical balance 24 hours before surgery and 24 hours after the last session of tDCS. The data were expressed as grams (g) of body weight.

2.3 Surgical procedure

One set of Wistar female rats underwent ovariectomy (surgical removal of the ovaries) another one set underwent sham surgery (opening of the abdominal cavity and sewing it back) and the third set was not subjected to any surgical procedure at all. At 60 days of age, the rats were anesthetized with Isoflurane 5% for induction and 2% for maintenance and underwent bilateral ovariectomy as described by Park et al. (2010)(20). In sham surgery, rats underwent the same incisions, the ovaries and fallopian tubes were exposed and then put back in the abdominal cavity and the muscle and skin were closed. All rats received tramadol chloridrate (5mg/kg i.p.) to relieve pain after surgery. The animals recovered for ten days before experiments.

2.4. Vaginal smear

Ten days after surgery, vaginal smear was obtained daily to verify hormonal status. Samples were obtained and analyzed as described by Goldman et al. (2007)(21).

2.5. Measurement of Rectal Temperature: rectal temperature was measured with an Animal Digital Thermometer (Speedster Animal 6 seconds thermometer, precision 0.1ºC) which was calibrated to small animals and took four to six seconds to register rectal temperature. The speed measurement allowed the animal to stay only a few seconds under manual restraint, gently wrapped in a towel. The display exhibited an error message if there was some inaccuracy in the measurement.

2.6. Transcranial Direct Current Stimulation (tDCS)

Fourteen days after ovariectomy, the rats were subjected to a 20-min session of cathodal tDCS every afternoon for 8 days. This period was established because repetitive tDCS application has demonstrated better and longer-lasting effects, and in recent studies from our group antihyperalgesic response was achieved with this treatment period (15). The direct current was delivered from a battery-driven, constant current stimulator using ECG electrodes with conductive adhesive hydrogel. Rats' heads were shaved for better adherence and the electrodes were trimmed to 1.5cm^2 for better fit. After placement, electrodes were fixed onto the head with adhesive tape (MicroporeTM) and rats were involved in a towel to prevent them from removing the equipment (Figure 1). Three days before the beginning of the treatment, rats were daily wrapped in a towel for 20 minutes in order to minimize the stress for restraint. The cathodal electrode was positioned between the ears (parietal cortex)(Figure 1) (22) with modifications). The anodal electrode was positioned at the midpoint of the lateral angle of the eyes (supraorbital area). The electrodes were placed on the skin in a similar manner to that used in human studies of tDCS (23,24). A constant current of 0.5mA intensity was applied for 20min (25–27). This intensity has been used in other studies from our group and we have not observed any lesions on the animals' skin (15,28). This montage was chosen because we aimed to decrease cortical excitability and it is known that cathodal stimulation reduces spontaneous firing of cortical neurons due to hyperpolarization of the cell body (29). For sham stimulation, the

electrodes were placed in the same positions as for real stimulation; however, the battery was not plugged to the electrodes.

2.7. Locomotor activity

The method used for evaluation of spontaneous locomotor activity was adapted from Creese et al. (30). The animals were placed individually in locomotor activity cages (50 x 48 x 50 cm) equipped with six bars, each with 16 infrared light sensors that detect the relative position of the animal on the box (Insight Equipment Ltd., São Paulo, Brazil). The total distance traveled by the animal was evaluated for 10 min (5 initial minutes considered exploratory activity and the 5 final minutes the test session). The test was performed after 7 days of treatment.

2.8. Blood sampling and tissue collection

The animals were killed by decapitation and blood and tissue samples were collected 48 h after the last session of tDCS. A trained practitioner performed the euthanasia. The hypothalamus, hippocampus, cerebral cortex and spinal cord were immediately removed. In brief, full-thickness tissue samples were homogenized and centrifuged at 3,000 rpm for 10 minutes, and the supernatants were stored at -80°C until further analysis. Trunk blood was drawn and blood samples were centrifuged in plastic tubes for 5 min at 5,000 rpm, at room temperature. This method was used to enable the collection of large volumes of blood serum for analysis. This model also enables the determination of biochemical effects, including hormonal effects. Serum was obtained and frozen at −80°C until assays were performed.

2.9. Determination of Interleukin-8 (IL-8) and Interleukin-10 (IL-10) levels

Before the structures analyses, the total amount of proteins was measured by the Coomassie Blue method using bovine serum albumin as standard (31). Serum and structures levels were evaluated using DuoSet enzyme-linked immunosorbent assay (ELISA) kit (R&D Systems, Minneapolis, MN) according to the manufacturer's instructions, and the results were expressed as pg/mL and pg/mg tissue protein concentration, respectively.

2.10. Estradiol measurement

Estradiol levels were measured with an automated, monoclonal, competitive, chemiluminescent immunoassay (Enhanced Estradiol E2; Siemens Advia Centaur). The lower limit of sensitivity of this assay is 10.7 pg/mL.

2.11. Statistical analysis

Between-group differences were evaluated using one-way ANOVA/Student–Newman–Keuls (SNK) or two-way ANOVA/Bonferroni. Rectal temperatures repeated measures between groups were evaluated using the Generalized Estimating Equations (GEE)/Bonferroni. For the analysis of serum estradiol levels, statistical significance was certified by the Student's t test. In all statistical analyses, we regarded results as statistically significant if $P< 0.05$. Data are expressed as mean ± standard error of the mean (SEM).

3. Results

3.1. Assessment of menopause model

3.1.1. Body weight

At baseline, there was no difference in the mean body weight between the groups analyzed (CT: 209.11±7.4, SS: 204.56±8.6, ST: 203.78±8.63, OS: 205.78±7.99, OT: 206.44±7.855, n= 9 per group, one-way ANOVA, $P>0.05$). At

the end of the study, mean body weight in the ovariectomy groups (OS and OT) was found significantly higher than those in non-ovariectomy groups (CT, SS and ST) (CT: 244.78±6.7, SS: 238.78±5.33, ST: 244.25±4.23, OS: 267.11±5.93 and OT: 270.44±4.59, n=8-9 per group, one-way ANOVA/SNK, P<0.05., Figure 2).

3.1.2. Vaginal smears

Vaginal smears of the ovariectomized rats (OS and OT) presented acyclical pattern exhibiting only diestrus and methestrus samples, confirming the animal model of menopause (n=8-9 per group, Figure 3).

3.1.3. Estradiol serum levels

Significant difference was observed in the Estradiol serum levels between ovariectomy and sham-ovariectomy groups, confirming the efficacy of animal model of menopause (24.61±1.94 and 40.41±3.82 pg/mL, respectively; Students't test, P=0.001, n=14 per group).

3.2. Locomotor activity

Twenty-four hours after the 7th day of treatment, distance traveled in the initial five minutes was not different between groups (CT, SS, ST, OS and OT) that relates to the exploratory activity (two-way ANOVA, P>=0.05; n=8-9 per group, Figure 5 Panel A). In the final five minutes (test session) the distance traveled also was not different between groups (CT, SS, ST, OS and OT) that relates to the locomotor activity (two-way ANOVA, P>=0.05; n=8-9 per group, Figure 5 Panel B).

3.3. Rectal temperature assessment

It was observed group effect, time effect and interaction group *vs* time (Wald Chi-square, P=0.01, P<0.001 and P<0.001, respectively; n=8-9 per group). The both sham groups (SS and ST) were not different from the control group (CT), showing that the sham procedure did not affect the temperature of female rats. The ovariectomy plus sham tDCS was different from control group (CT), and both sham ovariectomized groups (SS, ST). The ovariectomy plus active tDCS group (OT) was not different from the control group (CT) and the ovariectomy plus sham tDCS group (OS), showing a partial reversion of the increased levels of rectal temperature; however, the OT group was different from both sham-ovariectomy groups (SS, ST). In relation to time, it was observed that temperature in the 10th and 14th day after ovariectomy were different from the baseline, 7th day after surgery and 24hs after the end of treatment (Figure 4).

3.4. Interleukin-8 levels

There was no difference between groups on IL-8 hypothalamic levels (CT: 1.73± 0.10, SS: 1.57±0.17, ST:2.14±0.26, OS:1.52±0.18 and OT:1.62±0.13, n=8-9/group, two-way ANOVA, P>0.05). Two-way ANOVA showed interaction between ovariectomy and tDCS, suggesting different effects of tDCS in ovariectomized and non-ovariectomized rats (p<0.05). Interleukin-8 serum levels were significantly higher in ovariectomized groups (OS and OT) (p<0.05, Figure 6).

3.5. Interleukin-10 levels

There was no difference between groups on IL-10 central levels (hypothalamus, hippocampus, cerebral cortex and spinal cord) (n=8-9/group, two-way ANOVA, P>0.05 for all)(Table 1).

4. Discussion

In this study, the ovariectomized rats showed low estradiol serum levels, vaginal smears acyclical patterns, and increased body weight, confirming a translational model of menopause. Also, the ovariectomized rats (OS) presented increased rectal temperature measurements in relation to ST and SS groups, which was more evident fourteen days after ovariectomy. Highlighting that this is the first time it is shown that the cathodal cortical tDCS treatment partially reversed the rise in rectal temperature in the ovariectomized rats. In addition, IL-8 serum levels were increased in ovariectomized rats without difference in the central levels (hypothalamus), suggesting that the IL-8 is related only to peripheral effects. This result corroborates previous human studies that found association between increased IL-8 serum levels and postmenopausal hot flushes (13,32). However, it is more likely that IL-8 serum is associated to vasodilation since the levels of this cytokine were also found increased in patients with hemodynamic instability after coronary artery bypass (33).

Moreover, our data showed no differences between groups regarding IL-10 levels in the structures of the central nervous system analyzed (hypothalamus, hippocampus, cerebral cortex and spinal cord). As IL-10 serum levels were not evaluated, this can be a limitation of our study since IL-8 serum levels were increased. Yet, in the study of Yasui and cols (2006), 17 cytokines were measured, including IL-10, and only IL-8 and macrophage inflammatory protein (MIP)-1β were correlated to hot flushes(13). It is interesting to note that Yasui proposes that the increase in IL-8 during hot flashes may be induced by the production and secretion from the hypothalamus and anterior pituitary gland through a decrease in estradiol level. However, in our study, we did not observe increase in the hypothalamic levels of IL-8, suggesting that another site contributes to the peripheral increase of this interleukin in women with vasomotor symptoms.

The rise in rectal temperature in the present study corroborates our previous findings in the pilot study (data not shown). The rectal temperature began to increase ten days after ovariectomy and continued higher than in non-ovariectomized rats up to the end of five weeks, showing that this method to measure the body temperature is reproducible. This finding corroborates another study that suggests that measurement of temperature in the rat by rectal probe

and telemetry yields compatible results (34). And, even though a rise in temperature produced by handling could be expected as described in the literature (35), this effect did not affect our experiment since all animals were handled similarly. Emphasizing, Kobayashi and cols., observed rise in tail skin and rectal temperatures two and eight weeks after ovariectomy, respectively, in female Sprague-Dawley rats (36). However, in our study we observed rise in rectal temperature earlier, ten days after ovariectomy and more evident two weeks after ovariectomy in female Wistar rats.

Interesting to note that, the hot flushes were partially relieved after 8 days of cathodal tDCS treatment. Previous data showed that cathodal tDCS, in general, is involved to decrease neuronal excitability (18). This central neuromodulation technique has been studied in the therapy of neuropsychiatric disorders in which neuronal excitability is imbalanced (23,37,38). The hyperexcitability of the hypothalamic neurons are reflected by VMS in the menopausal status (39–41). Estrogen decline down-regulates post synaptic receptors of serotonin and norepinephrine increasing the availability of these neurotransmitters in hypothalamus and narrowing thermoneutral zone, triggering an hypersensible response to changes in core body temperature (11,41,42). The mechanism of action of tDCS remains unclear; however studies suggested a hyperpolarization of neurons by cathodal tDCS. In addition, this effect over neurons involved on thermoregulation would decrease their set point to trigger mechanisms of heat dissipation. Considering that, the thermoregulation is a complex function involving several brain regions, it is plausible to speculate that tDCS could achieve insula and anterior cingulate cortex, which are regions known to be involved in hot flushes symptoms (12).

The locomotor activity was not different between groups, neither in rats that were subjected to surgery nor tDCS treatment in relation to the total control rats. The behavior assessment was performed seven days after the beginning of tDCS treatment and corroborates studies showing the safety of this technique (26,43). In relation to the surgical procedure, the locomotor test was performed in the 21th day post operatory (or 7th day of tDCS treatment) and showed that the animals were fully recovered from surgery. Also, there was no difference between

ovariectomized and non-ovariectomized groups showing that hypoestrogenic status did not affect locomotor activity, at this time point.

5. Conclusion

In summary, the animal model of ovariectomy proved to be a translational model of menopause, and it can help us to understand the pathophysiology of menopause status, linked to peripheral and central structures biomarkers. In addition, our data suggest that tDCS may be an interesting therapeutic alternative to relieve the vasomotor symptoms, mainly related to the mechanisms of action implicit in this technique. Further preclinical studies are needed to evaluate the montage of the electrodes, the period of treatment and the neurochemical responses to tDCS on hot flushes.

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Figures

Figure 1.

Figure 2.

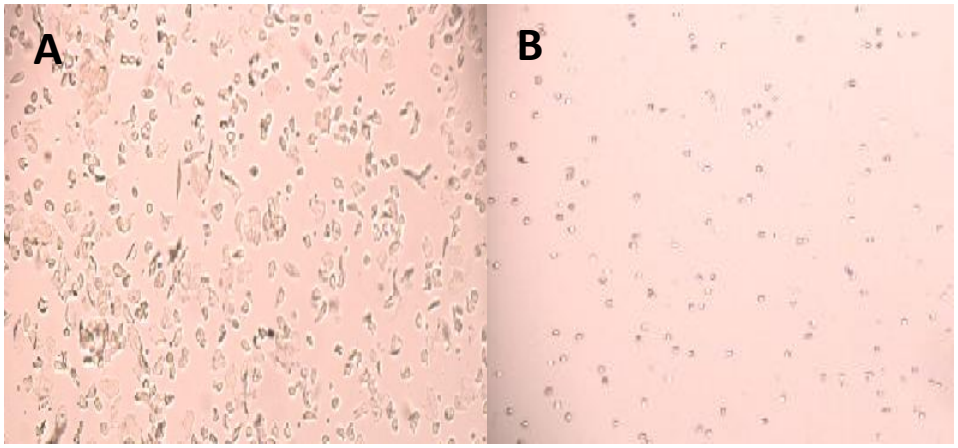
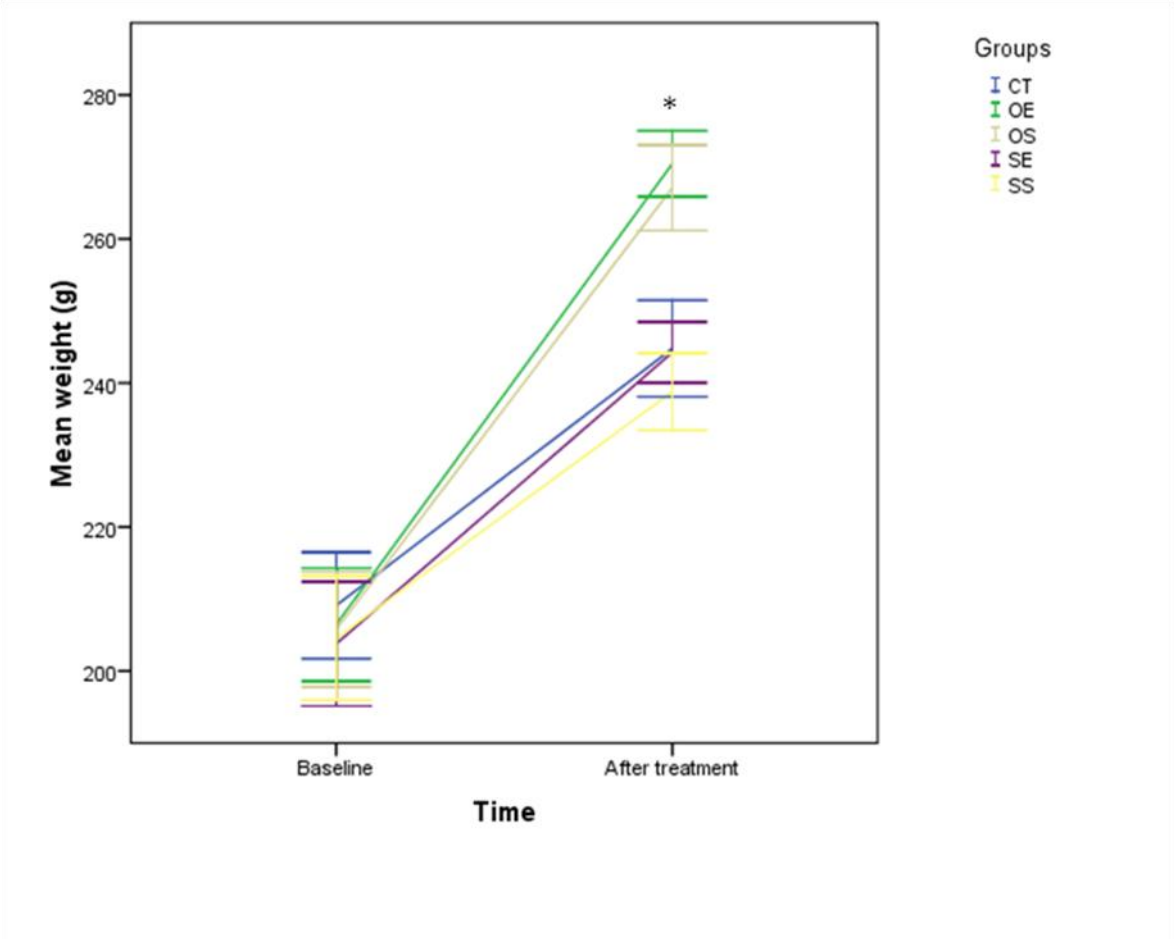


Figure 3.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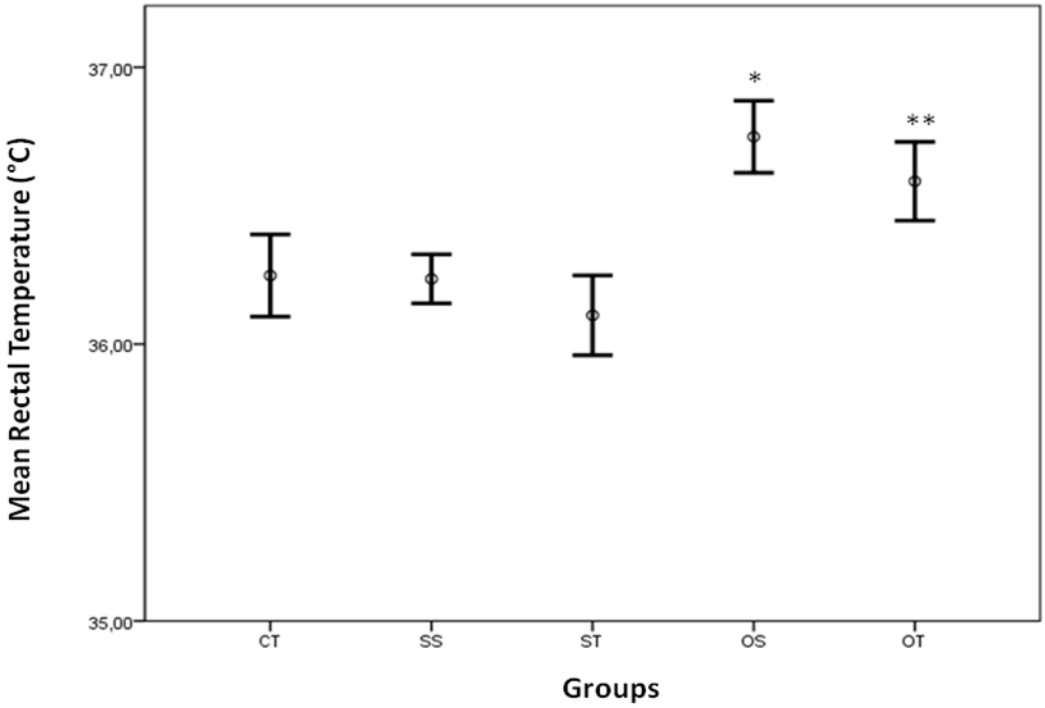
OE
OS
SE
SS
Mean weight (g)
280
260
240
220
200
*
Baseline
After treatment
Time

Figure 4.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Mean Rectal Temperature (°C)
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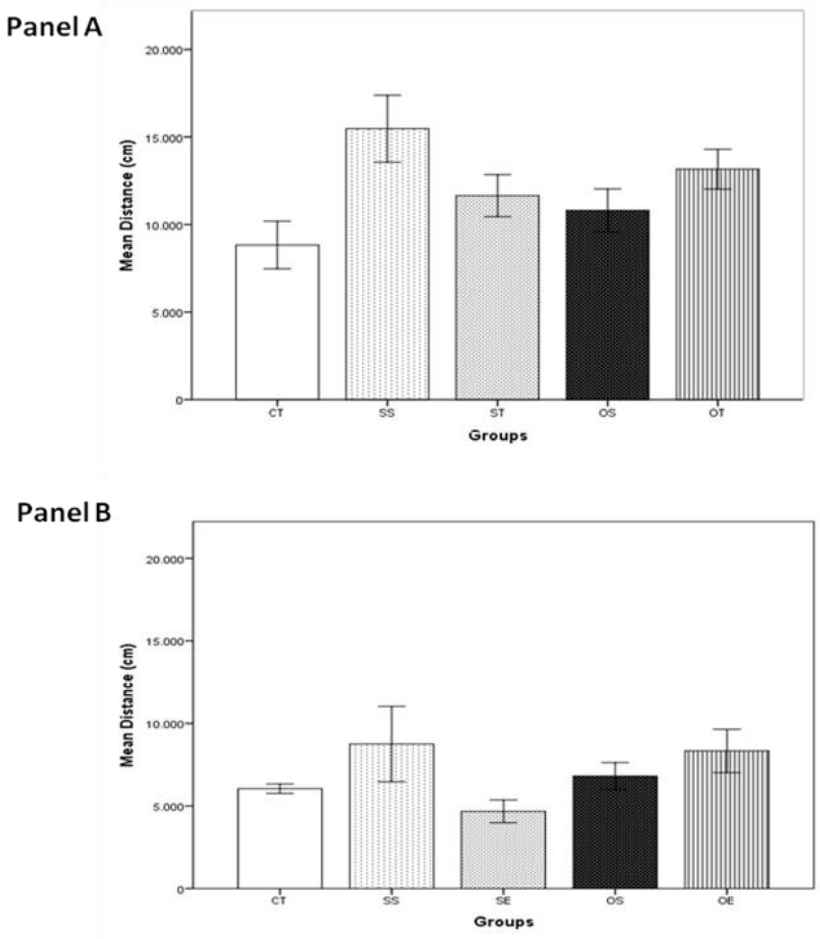
CT
SS
ST
OS
OT
Groups

Figure 5.


Panel A
Mean Distance (cm)
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5.000
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CT
SS
ST
OS
OT
Groups
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Mean Distance (cm)
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0
CT
SS
SE
OS
OE
Groups

Figure 6.

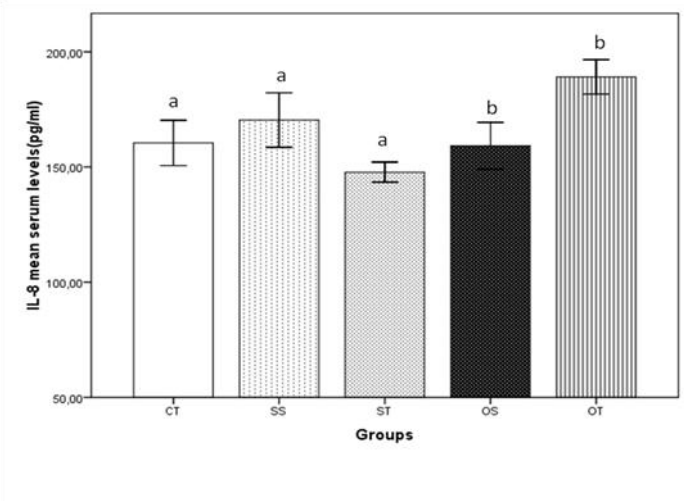

Table 1.

	Groups					
CNS structures	**CT**	**SS**	**ST**	**OS**	**OT**	
Hypothalamus	32.84 ± 9.7	87.39 ± 18.9	77.47 ± 24.1	69.37 ± 18.1	58.80 ± 12.2	P=0.21
Hippocampus	2.57±0.9	2.46 ± 0.7	3.69 ± 0.4	2.10 ± 0.3	3.15 ± 0.9	P=0.51
Cerebral cortex	7.43±1.7	8.04±2.3	10.25 ± 3.3	4.11 ± 1.5	10.0 ± 2.7	P=0.45
Spinal cord	20.88±6.3	29.55±10.8	26.60±7.9	16.82±8.2	11.97±4.4	P=0.49

Legends

Figure 1. TDCS electrode placement. The cathodal stimulus (negative electrode) was positioned over the neck and shoulder areas, and the anodal electrode (positive) was positioned at the midpoint of the lateral angle of the eyes (adapted from Adachi et al., 2012).

Figure 2. A. Metestrus: a combination of round ‘‘pavement cells,’’ some needle-like cells, and a few smaller leukocytes can be present during a transitional period during the early portion of the first day of diestrus. **B.** Diestrus: only leukocytes are present.

Figure 3. Female rats weight at baseline and at the end of tDCS treatment. At baseline the weight was not different between groups (one-way ANOVA, P>0.05).

* At the end of tDCS treatment, OT and OS were different from CT, SS and ST (one-way ANOVA/SNK, P<0.05). CT: control group; SS: sham ovariectomy+sham tDCS; ST: sham ovariectomy+tDCS; OS: ovariectomy+sham tDCS; OT: ovariectomy+tDCS.

Figure 4. Mean rectal temperature at Celsius grade. * significant difference from CT, SS and ST (Wald Chi square, P<0.001). ** significant difference from SS and ST (Wald Chi square, P<0.001). CT: control group; SS: sham ovariectomy+sham tDCS; ST: sham ovariectomy+tDCS; OS: ovariectomy+sham tDCS; OT: ovariectomy+tDCS.

Figure 5. Locomotor activity assessed during ten minutes. Each column represents the mean ± SEM. (n = 5/6 per group). **Panel A:** locomotor activity in the first five minutes. There was no difference between groups (two-way ANOVA, P>0.05). **Panel B:** locomotor activity in the last five minutes. There was no difference between groups (two-way ANOVA, P>0.05). CT: control group; SS: sham ovariectomy+sham tDCS; ST: sham ovariectomy+tDCS; OS: ovariectomy+sham tDCS; OT: ovariectomy+tDCS.

Figure 6. Interleukin 8 serum levels (pg/ml). Each column represents the mean±SEM (n=8 per group). b different from a (two-way ANOVA, P<0.05).CT: control group; SS: sham ovariectomy+sham tDCS; ST: sham ovariectomy+tDCS; OS: ovariectomy+sham tDCS; OT: ovariectomy+tDCS.

Table 1. Interleukin 10 levels in the structures of the central nervous system. Data are expressed as mean±S.E.M (pg/mg of protein). There were no differences (two-way ANOVA). CT: control group; SS: sham ovariectomy+sham tDCS; ST: sham ovariectomy+tDCS; OS: ovariectomy+sham tDCS; OT: ovariectomy+tDCS.

Artigo 3. Neuromodulatory Effect of Estrogen and Transcranial Direct Current Stimulation (tDCS) on Nociception and BDNF in Ovariectomized Rats.

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Abstract: Menopause is a physiological process characterized by the loss of ovarian follicular activity and consequent decline on sex steroids levels. As currently women have an increase on life expectancy, they spend a significant part of their lives in hypoestrogenic state of the postmenopause. Estrogen is known to interact with neurotransmitters and neurotrophins such as brain derived neurotrophic factor (BDNF). The transcranial direct current stimulation (tDCS) is a relatively safe and low cost brain stimulation technique that has been tested in the treatment of chronic pain. The present investigation aims to explore the effect of tDCS on pain behavior and peripheral and central BDNF levels in ovariectomized rats. Female Wistar adult rats were randomized by weight and distributed into five groups: control (CT), ovariectomy + tDCS (OT), ovariectomy + sham tDCS (OS), sham ovariectomy + tDCS (ST) and sham ovariectomy + sham tDCS (SS). Rats were subjected to cathodal tDCS. Hormonal status was verified by vaginal cytology and estradiol levels. Additionally we evaluated: tail flick test, electronic von Frey test, hot plate test and hypothalamic, hippocampal, spinal cord, cortical and serum BDNF levels. It was observed significant effect of ovariectomy in hot plate and von Frey tests, and in serum and hypothalamic BDNF levels. Hippocampal BDNF levels were significantly decreased in OT, OS, ST and SS groups as compared to control group. There was interaction between the effects of tDCS and ovariectomy in cortical BDNF levels. Moreover, cathodal tDCS reversed partially nociceptive hypersensitivity induced by ovariectomy. These data are consistent with the scientific evidence about neuromodulatory effect of estrogen and tDCS on nociception and BDNF.

Keywords: tDCS, BDNF, ovariectomy, pain.

1. Introduction

Menopause is a physiological process characterized by the loss of ovarian follicular activity and consequent decline on sex steroids levels. As currently women have an increase on life expectancy, they spend a significant part of their lives in the

hypoestrogenic state of post menopause(1). Estrogens are known to have many other actions besides its importance on reproductive function. Among these actions, estrogens are known to modulate neurotrophins such as neurotrophin growth factor (NGF) and brain derived neurotrophic factor (BDNF), and to interact with neurotransmitters (2,3). The term “estrogens” comprises several hormones, the most potent of them, the estradiol, predominates during the reproductive period of women’s lifespan(4). Epidemiological studies showed that higher prevalence of painful disorders in women compared to men could be related to estradiol(5). Experimental studies have shown gender differences regarding functional and structural characteristics of the pain pathways (6). Li and colleagues (2014) showed that the ovariectomy animal model induced a robust nociceptive hypersensitivity (7).

Non-invasive brain stimulation techniques, like transcranial direct current stimulation (tDCS), have been used to treat chronic pain (8,9). It is considered a relatively safe, low cost and painless technique(10). The mechanisms by which tDCS induces changes across different levels of the nervous system may involve tissue polarization and, consequently, the modulation of neuronal activity (11,12). The current model of tDCS effects is based on cortico-cortical interactions, with some subcortical components, including anterior cingulate cortex (ACC), in these circuits(13) .Previous studies of our research group using rats confirmed immediate and long-lasting effects of tDCS treatment on chronic inflammation (14)and hyperalgesia induced by chronic restraint stress model (15). Interestingly, BDNF contributes to common mechanism that underlies pain syndromes(16), chronic stress(17), and DCS effects(18). BDNF regulates synaptic neuronal survival and peripheral and central neuroplasticity (19), and has been studied in pathological conditions such as depression and chronic pain(20,21). In healthy subjects, the modulating effect of the BDNF on the nervous system seems to be dependent on the gender(22). The BDNF has a facilitatory effect on pain thresholds in healthy females, but the opposite occurs in healthy males(23). However, the role of serum BNDF in pain, including its function in motor cortex excitability modulation, is poorly understood. In fact estradiol modulates the BDNF mRNA in areas associated with nociceptive sensory processing, such as the hippocampus, cerebral cortex and spinal cord (24). Therefore, it is reasonable to explore the possible relationship between BDNF, estradiol, pain behavior and tDCS effects.

Thus, the present investigation aims to evaluate the effect of tDCS on nociceptive response and peripheral and central BDNF levels in ovariectomized rats. The BDNF levels and pain threshold (in the hot plate, tail flick and von Frey tests) were used to indirectly assess neuronal activity and pain sensitivity, respectively.

2. Materials and methods

2.1 Animals

Forty-five female Wistar rats (60 days old, 200-250g) were randomized by weight and housed in cages of polypropylene material (49x34x16cm) with the floor covered by wood shaving. Four or five were housed per cage with food and water available *ad libitum* and maintained on a 12-h light/dark cycle (lights on at 7:00 AM, and lights off at 7.00 p.m.) in a humidity and temperature-controlled environment (22±2°C). The animals were randomized by the weight and distributed into five groups: control (CT), ovariectomized and treated with tDCS (OT), ovariectomized and treated with sham tDCS (OS), false ovariectomized and treated with tDCS (ST) and false ovariectomized and treated with sham tDCS (SS). They were handled for fourteen days prior to the experiments. All experiments and procedures were approved by the Institutional Animal Care and Use Committee (GPPG-HCPA protocol No. 11-0586) and were compliant with Brazilian guidelines involving use of animals in research (Law No. 11.794). Additionally, all efforts were made to minimize the suffering, the pain and discomfort of the animals, as well as to reduce the number of animals.

2.2 Surgical procedures

One set of Wistar female rats underwent ovariectomy (surgical removal of the ovaries) another one set underwent sham surgery (opening of the abdominal cavity and sewing it back) and the third set was not subjected to any surgical procedure at all. At 90 days of age, the rats were anesthetized with Isoflurane 4% for induction and 2% for maintenance and underwent bilateral ovariectomy as described by Park et al., 2010(25). In sham surgery, rats underwent the same incisions, the ovaries and

fallopian tubes were exposed (but not removed) and then put back in the abdominal cavity and the muscle and skin were closed. All rats received tramadol chloridrate (5mg/kg i.p.) to relieve pain after surgery. The animals recovered for ten days before experiments.

2.3. Vaginal smear

Ten days after surgery, vaginal smear was obtained daily to verify hormonal status. Samples were obtained and analyzed as described by Goldman et al. (2007)(26).

2.4. Transcranial Direct Current Stimulation (tDCS)

Fourteen days after ovariectomy, the rats were subjected to a 20-min session of cathodal tDCS every afternoon for 8 days. This period was established because repetitive tDCS application has demonstrated better and longer-lasting effects, and in recent studies from our group antihyperalgesic response was achieved with this treatment period (15). The direct current was delivered from a battery-driven, constant current stimulator using ECG electrodes with conductive adhesive hydrogel. Rats' heads were shaved for better adherence and the electrodes were trimmed to 1.5cm^2 for better fit. After placement, electrodes were fixed onto the head with adhesive tape (MicroporeTM) and rats were involved in a towel to prevent them from removing the equipment (Figure 1). Three days before the beginning of the treatment, rats were daily wrapped in a towel for 20 minutes in order to minimize the stress for restraint. The cathodal electrode was positioned between the ears (parietal cortex)(Figure 1) (27) with modifications). The anodal electrode was positioned at the midpoint of the lateral angle of the eyes (supraorbital area). The electrodes were placed on the skin in a similar manner to that used in human studies of tDCS (28,29). A constant current of 0.5mA intensity was applied for 20min(30–32). This intensity has been used in other studies from our group and we have not observed any lesions on the animals' skin(14,15). This montage was chosen because we aimed to decrease cortical excitability and it is known that cathodal stimulation reduces

spontaneous firing of cortical neurons due to hyperpolarization of the cell body(33). For sham stimulation, the electrodes were placed in the same positions as for real stimulation; however, the battery was not plugged to the electrodes.

2.5. Nociceptive behavioral tests

The experimental animals were previously exposed to all the apparatus to acclimate to the procedure 24 h prior to the test sessions except for the locomotor activity. This was done because the novelty of the apparatus can itself induce antinociception (34).

2.5.1. Tail Flick latency (TFL)

TFL is used to assess heat threshold (35). Each animal was placed on the apparatus and its tail was laid across a nichrome wire coil that was then heated using an electric current. The equipment was calibrated to obtain three consecutive baseline tail-flick latencies between 3 s and 5 s. If at any time the animal failed to flick its tail before the temperature reached 75 °C, the tail was removed from the coil to prevent skin damage. Three TFL baselines were taken at 3 min intervals.

2.5.2. Von Frey test

The von Frey test is used to assess mechanical allodynia (pain induced by non-noxious stimuli)(36). Paw withdrawal threshold in response to a mechanical stimulus was determined using an electronic von Frey device. Animals were placed in a cage with a metal mesh floor, allowing them to move freely. Von Frey device stimuli were applied to the mid-plantar surface of the right hind paw through the mesh floor. Application was only performed when the animal's paw was in contact with the floor, and was repeated three times with an interstimuli interval of at least 5 seconds. The withdrawal threshold of the hind paw was expressed in force: grams (g).

2.5.3. Hot Plate

Twenty-four hours prior to testing, all rats were given 5 minutes to acclimate to the hot plate. The temperature of the plate was kept at 52±0.1°C. The animals were placed in glass funnels onto the heated surface. With a stopwatch, the experimenter recorded, for the latency of response in seconds, the time between placement of the animals and the onset of paw licking or jumping behavior.

2.6. Blood sampling and tissue collection

The animals were killed by decapitation and blood and tissue samples were collected 48 h after the last session of tDCS. A trained practitioner performed the euthanasia. The hippocampus, cerebral cortex, hypothalamus and spinal cord were immediately removed. In brief, full-thickness tissue samples were homogenized and centrifuged at 3,000 rpm for 10 minutes, and the supernatants were stored at -80°C until further analysis. Trunk blood was drawn and blood samples were centrifuged in plastic tubes for 5 min at 5,000 rpm, at room temperature. This method was used to enable the collection of large volumes of blood serum for analysis. This model also enables the determination of biochemical effects, including hormonal effects. Serum was obtained and frozen at −80°C until assays were performed.

2.7. Estradiol measurement

Estradiol levels were measured with an automated, monoclonal, competitive, chemiluminescent immunoassay (Enhanced Estradiol E2; Siemens Advia Centaur). The lower limit of sensitivity of this assay is 10.7 pg/mL.

2.8. BDNF measurement

BDNF levels in the serum, hippocampus, cerebral cortex, hypothalamus and spinal cord were determined by sandwich-ELISA using monoclonal antibodies specific for BDNF (R&D Systems, Minneapolis, United States). Total protein was

measured by Bradford's method (1976) using bovine serum albumin as standard(37).

2.9. Statistical analyses

Nociceptive behavioral tests were evaluated using one-way ANOVA followed by Student Neumans Keuls and BDNF levels differences between groups were evaluated using two-way ANOVA followed by Bonferroni test. Estradiol levels between ovariectomized and non-ovariectomized groups were assessed using Student's t test. The factors for two-way ANOVA were: tDCS and ovariectomy. In all statistical analyses, we regarded results as statistically significant if $P< 0.05$. Data are expressed as mean ± standard error of the mean (S.E.M.).

3. Results

3.1. Assessment of hormonal status

3.1.1. Vaginal smears

Vaginal smears of the ovariectomized rats (OS and OT) presented acyclical pattern exhibiting only diestrus and methestrus samples, confirming the animal model of menopause (Figure 1; n=8-9 per group, data not shown).

3.1.2. Estradiol serum levels

Significant difference was observed in the Estradiol serum levels between ovariectomy and sham-ovariectomy groups, confirming the efficacy of animal model of menopause (24.61±1.94 and 40.41±3.82 pg/mL, respectively; Students't test, P=0.001, n=14 per group).

3.2. Nociceptive behavioral tests

3.2.1. Tail flick measurement

After 24hs of the end of treatment, we did not observe difference between groups in the tail flick latency (CT: 3.09±0.11; SS: 3.10±0.11; ST: 3.29±0.11; OS: 3.13±0.12; OT: 3.32±0.11, one-way ANOVA, P>0.05, n=8-9/group).

3.2.2. Hot Plate

After 24hs of the end of treatment, the ovariectomy groups (OS and OT) presented decrease in the paw withdrawal latency compared to CT, SS and ST groups (CT: 6.18±0.38; SS: 6.88±0.56; ST: 6.66±1.32; OS: 3.65±0.48; OT: 3.34±0.65, one-way ANOVA, P=0.005, n=8-9/ group).

3.2.3. Von Frey

After the 7th day of treatment, OS group presented a decrease in the mechanical threshold of the right hind paw compared to CT group; however the tDCS reversed partially this behavior in the ovariectomized rats (OT)(CT: 75.00±5.89; SS: 58.53±1.9; ST: 67.41±4.68; OS: 53.17±5.1; OT: 69.22±5,47; one-way ANOVA, P=0.03, n=6/ group).

3.4. BDNF levels

3.4.1. Serum levels

There was a significant difference in the BDNF serum level between the groups, related to ovariectomy effect. It was observed an increase in the serum BDNF levels of ovariectomized rats (OS and OT groups) as compared to non-ovariectomized rats (CT, SS and ST groups), with no significant effect of tDCS, nor interaction between tDCS and ovariectomy (CT: 6.11±0.31, SS: 6.35±0.22, ST: 5.86±0.20, OS: 6.44±0.29, OT: 6.70±0.17; two-way ANOVA, P=0.02, n=8-9/group).

3.4.2. Cortical levels

There was interaction effect between tDCS and ovariectomy (CT: 12.84±1.09, SS: 11.42±0.79, ST: 9.19±0.72, OS: 8.47±0.95, OT: 12.16±0.96; two-way ANOVA, P=0.001, n=8-9/group).

3.4.3. Hypothalamic levels

There was a significant difference in the hypothalamic levels between the groups. It was observed a significant effect of ovariectomy in the hypothalamic BDNF levels, with no effect of tDCS, nor interaction between the two factors (CT: 119.55±11.99, SS: 144.17±17.91, ST: 146.78±7.29, OS: 108.69±7.46, OT: 85.45±10.34; two-way ANOVA, P=0.002, n=7-8/group).

3.4.4. Hippocampal levels

There was a significant decrease in the hippocampal BDNF levels in the OS, OT, SS and ST groups as compared to CT group (CT: 24.21±2.34, SS:9.72±1.42, ST:12.54±3.10, OS: 12.27±1.73, OT:10.86±1.36; one-way ANOVA, P<0.001, n=8-9/group). To assess the effect of ovariectomy, tDCS and their interaction we performed the two-way ANOVA; however we did not find effect, since this test uses only two independent variables (ovariectomy and tDCS).

3.4.5. Spinal cord levels

There was no difference between groups in the spinal cord BDNF levels (CT: 27.70±1.97, SS: 26.51±2.36, ST: 28.66±1.79, OS: 26.46±2.9, OT: 25.16±1.68; two-way ANOVA, P>0.05, n=8-9/group).

4. Discussion

In this study we showed that ovariectomized rats presented increased nociceptive response in hot plate and von Frey test without effect on TFL test. This finding corroborates study of Stoffel and cols that demonstrated sex differences in latency to respond in the hot plate test, suggesting a relationship with estrogen levels (38). This raises the possibility of gender differences in a variety of components associated with the regulation of nociception. Evidence indicates that estrogen is involved in the regulation of analgesia and nociception (39–41). In addition, pain thresholds may vary in females in different phases of the estrous cycle (42). It is important to note that, in our previous study we showed that ovariectomized rats presented depressive symptoms (Moreira SS personal communication). Pain and depression share neuroanatomical pathways and neurobiological substrates that might explain the increased vulnerability to pain in depressive patients, and vice versa (43).

Our results also demonstrated the importance of BDNF in pain response. In fact, BDNF has a facilitatory effect on pain threshold in females, but has an opposite effect in males (22); supporting the notion that BDNF is a modifier of pain threshold according to the hormonal state. A clinical study of Begliuomine et al. showed that plasma BDNF levels was positively correlated with estradiol (E2) and progesterone and negatively correlated with menopausal age (2). In the present study, we found the opposite, corroborating previous human study of our research group that suggests that experimental heat and pressure pain threshold is gender-related and BDNF dependent (22). In this study we showed that ovariectomized rats showed decreased pain threshold associated with increased serum BDNF levels. Possibly, it is due to the BDNF action as a regulator of neuronal excitability and modulator of synaptic plasticity in the central nervous system (CNS) (19). Changes in the frequency or potency of activation across synapses can result in long-term increases or decreases in synaptic strength, referred to as long-term potentiation (LTP) or long-term depression (LTD), respectively (44). BDNF is an important marker and modulator of neural activity in the NMDA receptor in the ascending and descending pain transmission pathways (45) and in the regulation of GABAergic interneurons in the cerebral cortex (46), binding to TrkB receptors. The TrkB receptors are strongly recruited during the activation of nociceptive pathways and mediate the polysynaptic C-fiber–evoked discharge by BDNF. Additionally, TrkB receptors can enhance an

NMDA response by increasing the open time of NMDA receptors(47–49). Furthermore consistent evidence supports the central role of BDNF in the initiation of central sensitization (50).

Other previous clinical study of our research group showed that the association between BDNF and pain is modulated by estradiol serum level (Medeiros FM personal communication). Each one increases the pressure pain threshold (PPT); however, when it was analyzed their interaction, the effect of BDNF on PPT is reversed. Although the underlie mechanism of this association is unknown. It led us to hypothesize a potential mechanism: it is possible that increased estradiol levels would increase baseline excitability and thus counteract the effects of decreased sensory afferent down-regulating pain. On the other hand, the lack of estradiol would lead to compensatory mechanisms that result in pain and thus increased serum BDNF levels would index mal-adaptive plasticity. Corroborating this hypothesis, in the present study, the peripheral BDNF levels were opposite to central BDNF levels. Since the ovariectomized rats presented an increase in BDNF serum levels associated to decrease levels in central structures. Our findings support the hypothesis that BDNF is a key mediator of synaptic plasticity, neuronal connectivity and dendritic arborization (51,52). In addition we showed that hippocampal BDNF levels were significantly decreased in all groups as compared to control group, we can suggest that this effect is induced by restraint needed to apply the tDCS. It is known that restraint stress induced hippocampal BDNF decrease (53) corroborating our findings.

In our study tDCS had a different action in ovariectomized and sham ovariectomized rats. The tDCS treatment increased cortical BDNF levels in ovariectomized rats and decreased cortical BDNF levels in the sham-ovariectomized rats. This datum is very interesting and shows the importance of determining hormonal status of women before tDCS treatment. Moreover, cathodal tDCS reversed partially nociceptive hypersensitivity induced by ovariectomy. It is important to highlight that the tDCS montage is cathodal, thus induced a tissue hyperpolarization. Since, tDCS may increase or decrease neuronal activity, depending on the type of stimulation or the circuits involved. In previous studies of our research group, using anodal stimulation in the male rats, we showed immediate and long-lasting effects of tDCS treatment on chronic inflammation (14) and

hyperalgesia induced by chronic restraint stress models (15). Thus, we can suggest that the gonadal hormone has an important role in the action mechanism of tDCS. However it is important to note that ovariectomized rats did not present a pain disorder, but an alteration in pain threshold. It suggests that the animal state is very important in the action tDCS.

5. Conclusion

In summary, we found that ovariectomized rats showed nociceptive hypersensitivity that was partially reversed by the cathodal tDCS. In addition, BDNF serum level can be correlated to pain and that this relationship seems to be modulated by estradiol level. This result supports the hypothesis that this gonadal hormone has an important regulatory effect on neuroplasticity as indexed by BDNF levels. Thus, further studies are required to: (1) elucidate the mechanism implicated in this relationship; (2) assess additional variables that may help to explain the variability in pain and other mediators among ovariectomized rats. These data are consistent with the scientific evidence about neuromodulatory effect of estrogen and tDCS on nociception and BDNF.

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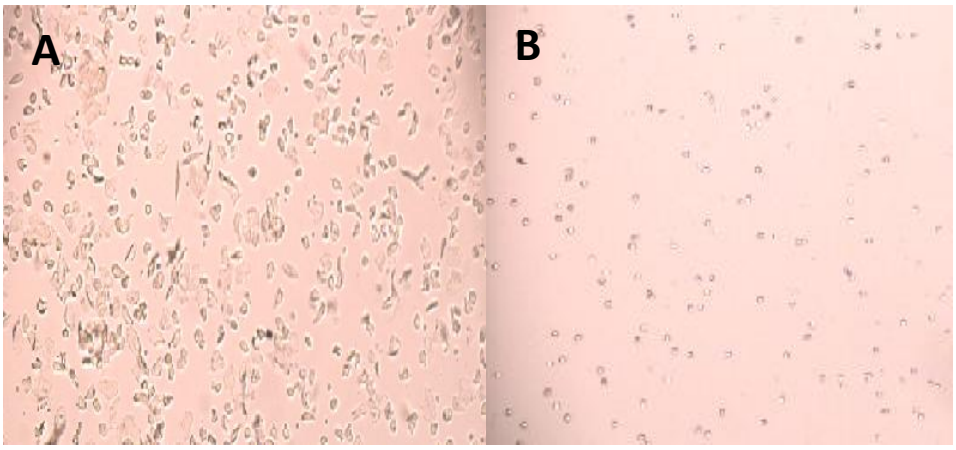
Figure 1.



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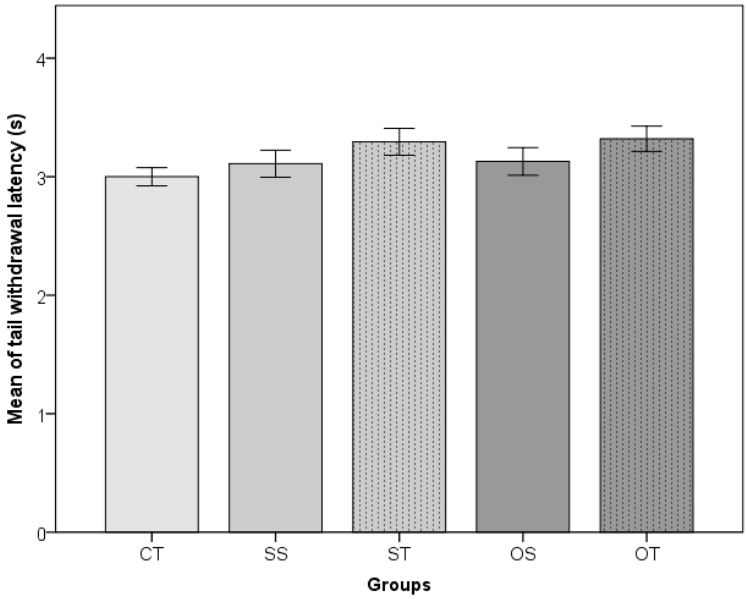

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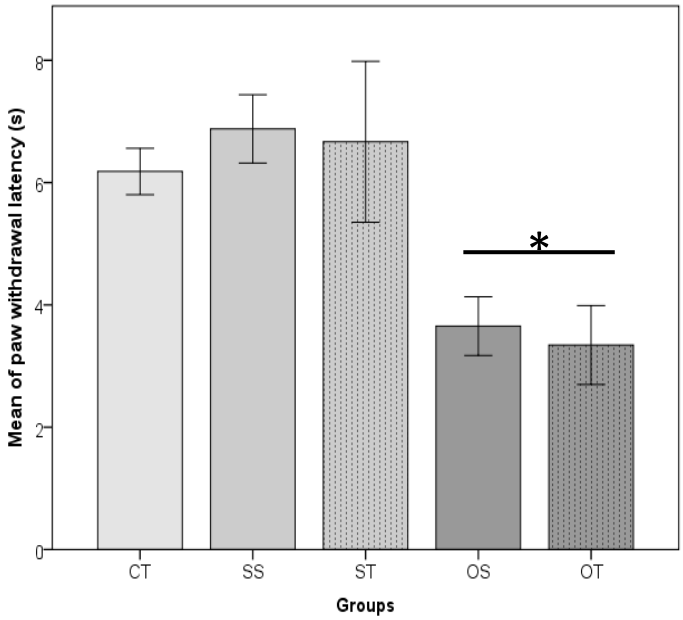

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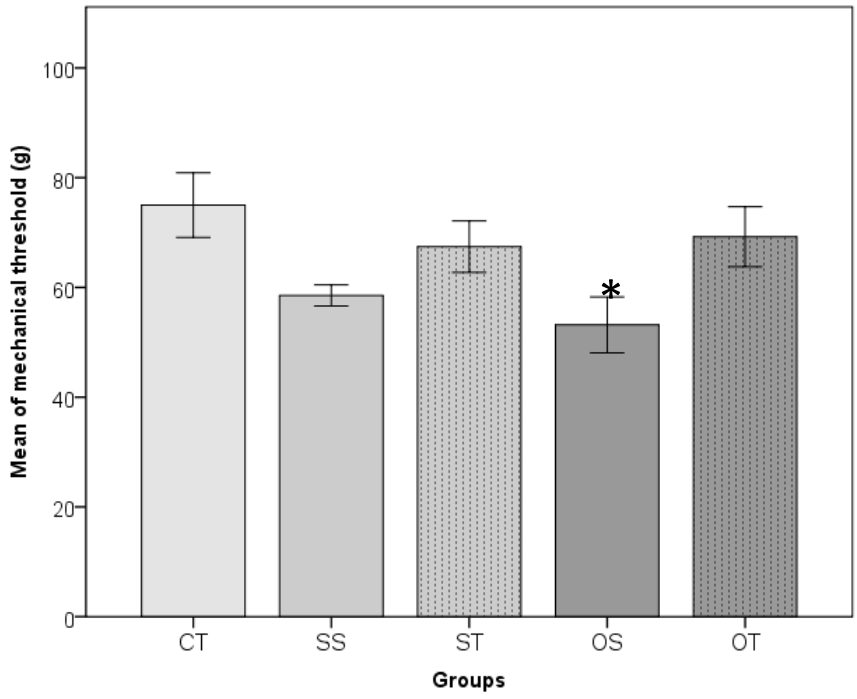

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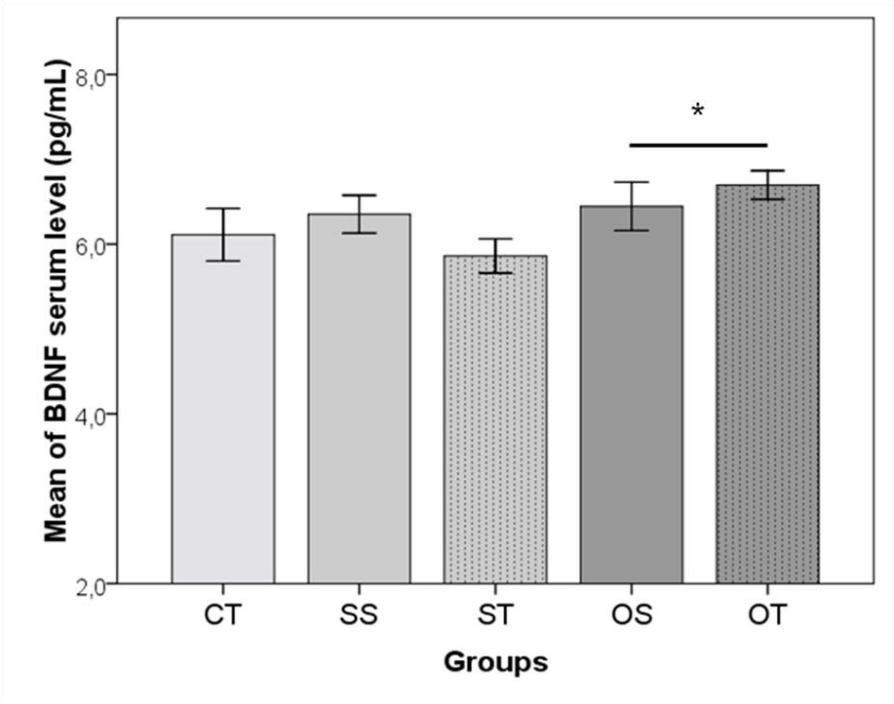

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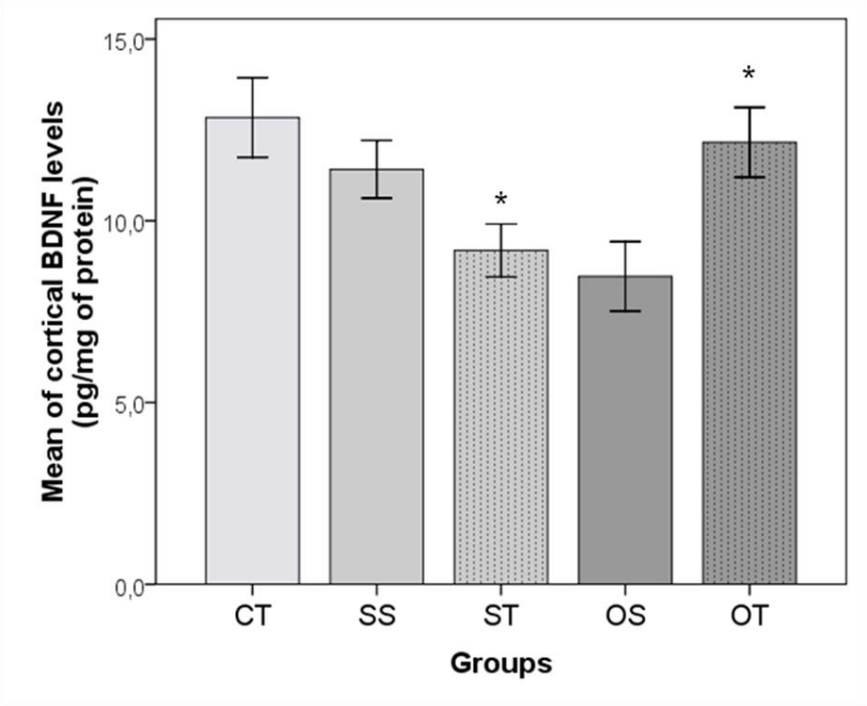

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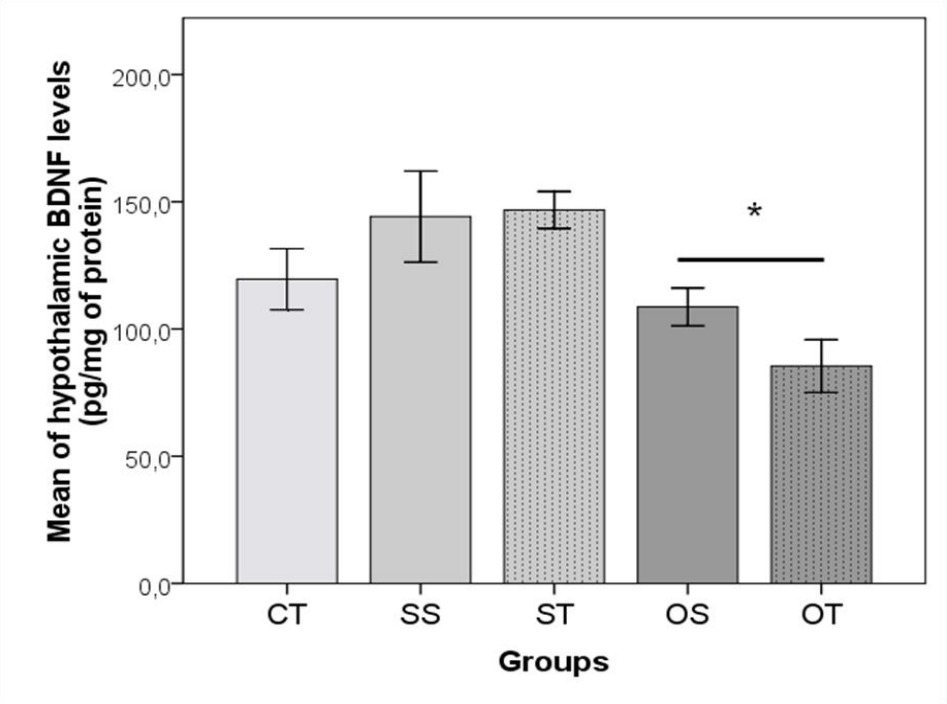

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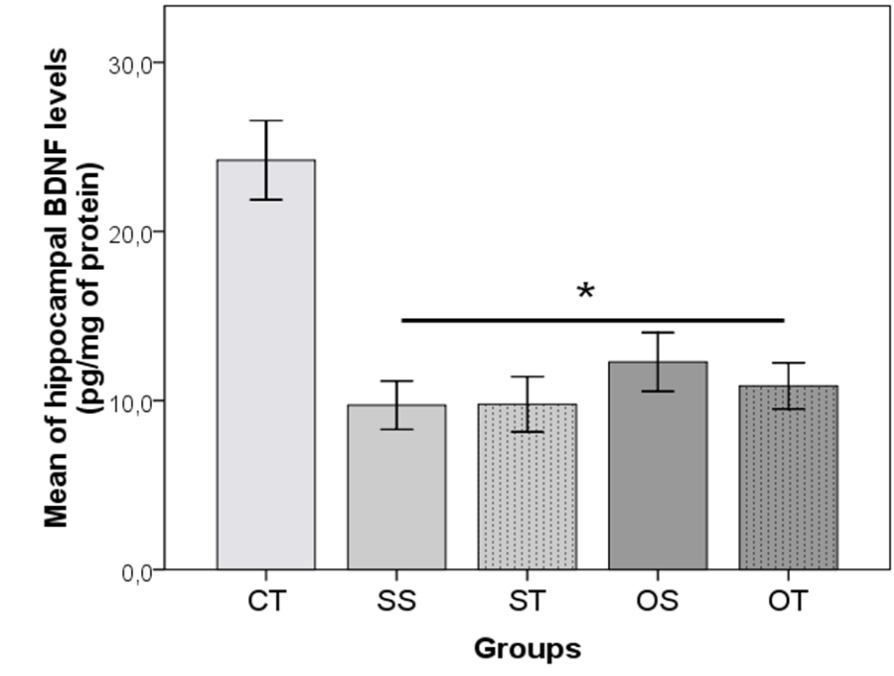

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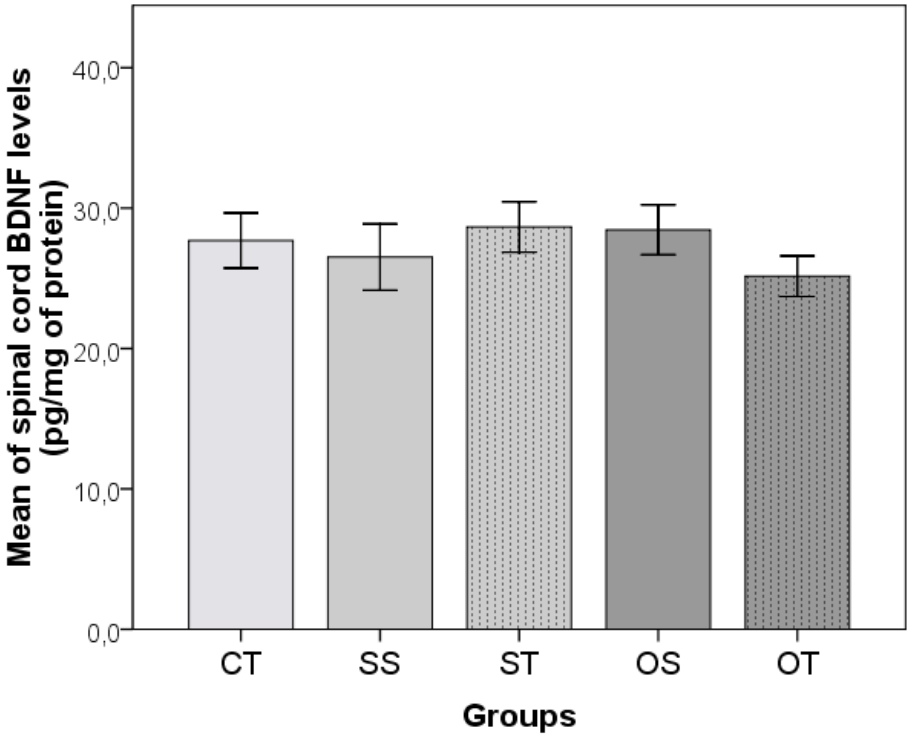
Mean of spinal cord BDNF levels (pg/mg of protein)
40,0
30,0
20,0
10,0
0,0
CT
SS
ST
OS
OT
Groups

Legends

Figure 1. A: Metestrus - a combination of round ‘‘pavement cells,’’ some needle-like cells, and a few smaller leukocytes can be present during a transitional period during the early portion of the first day of diestrus. **B**: Diestrus – only leukocytes.

Figure 2. Tail flick test. Each column represents mean±SEM. There was no difference between groups (n=5/6 per group, one-way ANOVA, P>0.05)**.** CT: control group; SS: sham ovariectomy+sham tDCS; ST: sham ovariectomy+tDCS; OS: ovariectomy+sham tDCS; OT: ovariectomy+tDCS.

Figure 3. Hot plate test. Each column represents mean±SEM. There was significant difference between groups. * OS and OT are different from CT, SS and ST (n=8/9 per group, one-way ANOVA=0.01). CT: control group; SS: sham ovariectomy+sham tDCS; ST: sham ovariectomy+tDCS; OS: ovariectomy+sham tDCS; OT: ovariectomy+tDCS.

Figure 4. Von Frey test. Each column represents mean±SEM of right hind paw of each group. There was significant difference between groups * OS is different from CT, SS, ST and OT (n=5 per group, one-way ANOVA, P=0.03)**.** CT: control group; SS: sham ovariectomy + sham tDCS; ST: sham ovariectomy + tDCS; OS: ovariectomy + sham tDCS; OT: ovariectomy+tDCS.

Figure 5. BDNF serum levels**.** Each column represents mean±SEM. There was no interaction between ovariectomy and tDCS treatment (n=8 per group, two-way ANOVA, P>0.05). There was significant effect of ovariectomy. * OS and OT are increased in comparison to CT, SS and ST (n=8 per group, two-way ANOVA,

P=0.02). CT: control group; SS: sham ovariectomy+sham tDCS; ST: sham ovariectomy+tDCS; OS: ovariectomy+sham tDCS; OT: ovariectomy+tDCS.

Figure 6. BDNF cortical levels**.** Each column represents mean±SEM. There was interaction between tDCS and ovariectomy (n=8/9 per group, two-way ANOVA, P=0.001). CT: control group; SS: sham ovariectomy+sham tDCS; ST: sham ovariectomy+tDCS; OS: ovariectomy+sham tDCS; OT: ovariectomy+tDCS.

Figure 7. BDNF hypothalamic levels**.** Each column represents mean±SEM. There was no interaction between ovariectomy and tDCS treatment (n=7/8 per group, two-way ANOVA, P>0.05). There was significant effect of ovariectomy (two-way ANOVA, P=0.002, n=7-8/group). CT: control group; SS: sham ovariectomy+sham tDCS; ST: sham ovariectomy+tDCS; OS: ovariectomy+sham tDCS; OT: ovariectomy+tDCS.

Figure 8. BDNF hippocampal levels**.** Each column represents mean±SEM. * SS, ST, OS and OT decreased as compared to CT (one-way ANOVA, P<0.001, n=8/9 per group). CT: control group; SS: sham ovariectomy+sham tDCS; ST: sham ovariectomy+tDCS; OS: ovariectomy+sham tDCS; OT: ovariectomy+tDCS.

Figure 9. BDNF spinal cord levels**.** Each column represents mean±SEM. There was no difference between groups (two-way ANOVA, P>0.05). CT: control group; SS: sham ovariectomy+sham tDCS; ST: sham ovariectomy+tDCS; OS: ovariectomy+sham tDCS; OT: ovariectomy+tDCS.

7. Considerações finais

A partir dos resultados desta tese podemos concluir:

1. O modelo de ovariectomia mostrou-se eficaz para mimetizar as alterações associadas ao hipoestrogenismo em mulheres, caracterizando uma importância translacional;

2. A manipulação de útero e anexos induziu um estado menopáusico precocemente, podendo se tornar outra opção como modelo animal translacional de transição menopáusica, necessitando estudos mais aprofundados dos mecanismos envolvidos;

3. Ambos os modelos induziram estado depressivo, que foi revertido pela cetamina;

4. O aumento de temperatura retal induzido pela ovariectomia foi revertido parcialmente pelo tratamento com ETCC;

5. Houve interação entre a ovariectomia e a ETCC nos níveis corticais de BDNF.

8. Perspectivas futuras

Testar o efeito da administração de melatonina em modelo de menopausa em ratas.

Testar novas montagens da aplicação de ETCC em modelo de menopausa em ratas.

Testar a cetamina em modelo animal de menopausa em ratas quanto à performance cognitiva e o comportamento tipo ansioso.

9. Anexos

HCPA - HOSPITAL DE CLÍNICAS DE PORTO ALEGRE
GRUPO DE PESQUISA E PÓS-GRADUAÇÃO

COMISSÃO DE ÉTICA NO USO DE ANIMAIS

A Comissão Científica e a Comissão de Ética no Uso de Animais (CEUA/HCPA) analisaram o projeto:

Projeto: 110586
Data da Versão do Projeto: 08/01/2012

Pesquisadores:
WOLNEI CAUMO
LICIANE FERNANDES MEDEIROS
IZABEL CRISTINA CUSTODIO DE SOUZA
SONIA FATIMA DA SILVA MOREIRA
STEFANIA GIOTTI CIOATO
IRACI LUCENA DA SILVA TORRES

Título: AVALIAÇÃO DO EFEITO DA ESTIMULAÇÃO ELÉTRICA TRANSCRANIANA EM RATAS SUBMETIDAS A UM MODELO DE SÍNDROME CLIMATÉRICA

Este projeto foi APROVADO em seus aspectos éticos e metodológicos de acordo com as Diretrizes e Normas Nacionais e Internacionais, especialmente a Lei 11.794 de 08/10/2008, que estabelece procedimentos para o uso científico de animais.

- Os membros da CEUA/HCPA não participaram do processo de avaliação de projetos onde constam como pesquisadores.
- Toda e qualquer alteração do Projeto deverá ser comunicada à CEUA/HCPA.
- O pesquisador deverá apresentar relatórios semestrais de acompanhamento e relatório final ao CEUA/HCPA.

Porto Alegre, 06 de março de 2012.

Dr. Alessandro Osvaldt
Coordenador CEUA/HCPA