Faculdade de Medicina

Programa de Pós-Graduação em Medicina: Ciências Médicas

TESE DE DOUTORADO

Avaliação dos Efeitos da Acupuntura e da Eletroacupuntura em Modelo Animal de Dor Neuropática: Parâmetros Comportamentais e Bioquímicos

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Porto Alegre

2017

UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL

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Tese de Doutorado apresentada ao Programa de Pós-Graduação em Medicina: Ciências Médicas como requisito para obtenção do título de Doutor em Ciências Médicas, da Universidade Federal do Rio Grande do Sul.

Porto Alegre

2017

CIP - Catalogação na Publicação

Adachi, Lauren Naomi Spezia
Avaliação dos Efeitos da Acupuntura e da Eletroacupuntura em Modelo Animal de Dor Neuropática: Parâmetros Comportamentais e Bioquímicos / Lauren Naomi Spezia Adachi. -- 2017.
155 f.

Orientadora: Iraci Lucena da Silva Torres.
Coorientador: Rafael Vercelino.

Tese (Doutorado) -- Universidade Federal do Rio Grande do Sul, Faculdade de Medicina, Programa de Pós-Graduação em Medicina: Ciências Médicas, Porto Alegre, BR-RS, 2017.

1. Acupuntura. 2. Eletroacupuntura. 3. Isoflurano. 4. Dor crônica. 5. Fator de crescimento neural. I. Torres, Iraci Lucena da Silva, orient. II. Vercelino, Rafael, coorient. III. Título.

Elaborada pelo Sistema de Geração Automática de Ficha Catalográfica da UFRGS com os dados fornecidos pelo(a) autor(a).

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"Os que se encantam com a prática sem a ciência são como os timoneiros que entram no navio sem timão nem bússola, nunca tendo certeza do seu destino."

Leonardo da Vinci

Aos meus pais.

AGRADECIMENTOS

- Agradeço imensamente à minha Orientadora Dra. Iraci Lucena da Silva Torres, pela oportunidade concedida desde a iniciação científica até hoje. Obrigada pela confiança, por todos os ensinamentos durante estes 11 anos de orientação, pelo apoio e empenho. Obrigada por tudo.
- Pela amizade, confiança e ensinamentos agradeço ao meu co-orientador Dr. Rafael Vercelino. Muito obrigada por toda ajuda prestada durante este trabalho.
- Obrigada às amigas e colegas Dra. Carla de Oliveira e Dra. Vanesa Leal Scarabelot pelo apoio, companheirismo, amizade e incansável ajuda durante este trabalho.
- Também gostaria de agradecer às colegas Dra. Joanna Ripoll Rozisky e Dra. Liciane Medeiros que foram muito importantes para minha formação, desde o meu início no grupo de pesquisa. Obrigada por toda ajuda!
- Às alunas de Iniciação Científica Tizye de Lima Rizzo, Camila Muneretto e Natália de Paula Silveira, meu agradecimento por toda ajuda na execussão deste trabalho.
- Gostaria de agradecer às colegas Dra. Andressa de Souza, Joice Soares de Freitas e Roberta Ströher pela ajuda nos testes bioquímicos, muito obrigada.
- A todos os colegas do grupo de pesquisa em Farmacologia da Dor e Neuromodulação: Investigações Pré-Clínicas, pelo convívio e auxílio sempre que necessário.
- Ao Grupo de Pesquisa e Pós-Graduação do Hospital de Clínicas de Porto Alegre - GPPG-HCPA, pelo apoio financeiro para o desenvolvimento do projeto (13-0298), por dispor da Unidade de Experimentação Animal (UEA) e da Unidade de Análises Moleculares e Proteína (UAMP) onde o trabalho foi desenvolvido.

- Agradeço a ajuda e disposição das colegas Francele Valente Piazza, do Laboratório de Histofisiologia Comparada, e Bruna Bellaver, do Departamento de Bioquímica pela ajuda nos experimentos e disponibilidade.
- Pela assistência e atenção, gradeço à Marta Cioato, Tuane N Alves Garcez e à Daniela Campagnol da Unidade de Experimentação Animal. Também agradeço a Rosalina A Braga, Karen Schwambach, Sônia Rodrigues e Vera L Gonçalves por todo suporte para o desenvolvimento deste estudo.
- Pela disposição e generosidade, agradeço ao Everaldo B de Almeida (CPE) e Jeferson B da Silva (UAMP). Obrigada por toda ajuda prestada.
- Agradeço à UFRGS, pela oportunidade, a CAPES, por ter financiado meus estudos. Agradeço também aos órgãos de fomento CNPq e FIPE que contribuíram para realização deste trabalho.
- Ao Programa de Pós-Graduação em Medicina: Ciências Médicas pela oportunidade de desenvolver meu Doutorado nesta instituição.
- Ao coordenador do Programa de Pós-Graduação em Medicina: Ciências Médicas, Dr. Wolnei Caumo, obrigada pela oportunidade e confiança.
- A Vera Susana V. Ribeiro e aos demais funcionários da secretaria do Programa de Pós-Graduação em Medicina: Ciências Médicas, pelas informações e ajuda quando solicitadas.
- BIC/UFRGS, PIBIC CNPq/UFRGS e PROCAD, pelas bolsas dos alunos que participaram deste trabalho.
- Meu agradecimento especial ao meu amor Guilherme Mahlman Mülheres, que sempre esteve ao meu lado, incentivando e apoiando. Muito obrigada por abdicar de algumas coisas para que pudesse me dedicar ao meu trabalho. Te amo muito.

- Agradeço imensamente ao meu pai Yoshimasa Adachi e à minha mãe Vandira Spezia Adachi, por serem meu porto seguro, por apoiarem minhas escolhas, e acreditarem em mim. Enfim, por todo esforço e todo amor dedicados a mim, muito obrigada. Amo vocês.
- Agradeço o incentivo e apoio de minha amada irmã Tayanee Akemi Spezia Adachi e meu cunhado Cezar Dall Pizzol, obrigada pelo apoio sempre.
- Por fim, agradeço a todos que de alguma forma contribuiram para a relização deste trabalho. A realização deste trabalho não seria possível sem a ajuda de todos.

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LISTA DE ABREVIATURAS

5- HT = Serotonina

AC = Acupuntura

ACTH = Hormônio Adrenocorticotrófico

AMPA = Ácido α-Amino-3-Hidróxi-5-Metil-4-Isoxazolepropiônico

AMPc = Adenosina Monofosfato Cíclico

ANOVA = *Analysis of Variance* (Análise de Variância)

ATP = Adenosina Trifosfato

AVC = Acidente Vascular Cerebral

BDNF = B*rain Derived Neurotrophic Factor* (Fator Neurotrófico Derivado do Encéfalo)

CCI = *Chronic Contriction Injury*

CGRP = Peptídeo Relacionado ao Gene da Calcitonina

CSA = *Cros-Sectional Area*

DCFH = Diacetato de Diclorofluoresceína

DN = Dor Neuropática

EA = Eletroacupuntura

GABA = Ácido Gama-Aminobutírico

GDR = Gânglio da Raiz Dorsal

GSH = Glutationa

IASP = *International Association for Study of Pain* (Associação Internacional para o Estudo da Dor)

IL-1β= Interleucina 1 beta

IL-10 = Interleucina 10

IL-6 = Interleucina 6

HHA = Eixo Hipotálamo-Hipófise-Adrenal

LCR = Líquido Céfalo Raquidiano

LTP = *Long Term Potentiation* (potenciação de longa duração)

LSD = *Fisher's LSD statistic test*

NGF **=** *Neural Growth Factor* (fator de crescimento neural)

NK = Neurocinina

NMDA = N-metil-d-aspartato

NP= *Neuropathic pain*

NRs = neurotrofinas

PG-E2 = Prostaglandina E2

PMRs = Receptores Polimodais

RMIf = Ressonância Magnética Funcional

ROS = Espécies Reativas de Oxigênio

SC = Sensibilização Central

SN = Sistema Nervoso

SNC = Sistema Nervoso Central

SNP = Sistema Nervoso Periférico

SP = Sensibilização Periférica

SNK = Student-Newman-Keuls

SNL = *Spinal nerve ligation*

TNF-α = *Tumor Necrosis Factor Alpha* (Fator de Necrose Tumoral Alfa)

TrkB = Receptor Tirosina Quinase B

TRPV1 = Receptores Vaniloides sub-tipo 1

APRESENTAÇÃO

Esta Tese está estruturada em 3 capitulos e 2 anexos:

- Capitulo I - Introdução, Revisão da Literatura, Objetivos e Referências Bibliográficas;

- Capitulo II - Materiais e Métodos, Resultados e Discussão na forma de artigos científicos;

- Capitulo III – Considerações Finais

- Anexos I - Aprovação do comitê de ética

- Anexo II - Produção acadêmica durante o período de doutorado.

O item Referências Bibliográficas refere-se somente às referências contidas nos itens Introdução, Revisão da Literatura e Considerações Finais.

Detalhes técnicos mais precisos sobre a metodologia empregada em cada um dos trabalhos apresentados podem ser encontrados nos trabalhos científicos.

RESUMO

Dor neuropática (DN) é definida como "dor iniciada ou causada por lesão primária ou disfunção em sistema nervoso", porém sua prevalência depende do tipo de trauma e da disfunção relacionada. Apesar desta condição dolorosa ser considerada altamente prevalente e debilitante, os tratamentos disponíveis são relacionados a efeitos adversos dificultando a adesão. Devido a isso, buscam-se alternativas não farmacológicas para o tratamento deste tipo de dor, entre elas, as técnicas de neuromodulação periférica, como acupuntura (AC) e eletroacupuntura (EA). Estas técnicas podem ser combinadas com intervenções farmacológicas e não farmacológicas e têm apresentado resultados promissores no tratamento da dor neuropática. No entanto, seus mecanismos de ação não estão totalmente elucidados, desta forma a utilização de modelos animais é de grande valia para o estudo destes mecanismos no tratamento da dor neuropática e da patofisiologia deste tipo de dor crônica. É importante salientar que a aplicação de AC e EA em animais acordados é complexa, visto que gera desconforto e pode alterar a analgesia induzida pelo tratamento. Em muitos estudos a anestesia com isoflurano é utilizada durante a aplicação dos tratamentos, porém sua utilização pode gerar um viés no estudo, considerando a possível interferência do fármaco nos resultados comportamentais e neuroquímicos. Outro importante foco de estudo consiste em comparar as duas técnicas, AC e EA, buscando determinar qual destas é a mais eficaz no tratamento da dor neuropática. Considerando o exposto acima, os objetivos desta tese foram: 1) avaliar os parâmetros comportamentais e neuroquímicos dos efeitos da utilização de anestesia na aplicação de AC e EA em ratos submetidos ao modelo de DN; 2) comparar os efeitos da AC e EA em modelo animal de DN por meio de parâmentros comportamentais, neuroquímicos e histológicos. Considerando os resultados obtidos nesta tese, concluímos que o isoflurano aumenta a analgesia promovida por AC e EA, provavelmente diminuindo o efeito do estresse gerado pela aplicação dos tratamentos em animais acordados,

resultado que é corroborado pela diminuição do nível de S100β periférico (marcador de morte neuronal central); Por outro lado, o isoflurano diminuiu os níveis de fator de crescimento neuronal (NGF) no nervo periférico lesado, indicando diminuição do processo de regeneração neural, enquanto a EA aumentou. Ao mesmo tempo, o isoflurano alterou os efeitos dos tratamentos nos comportamentos exploratórios e nos níveis de N-metil D-aspartato em tronco encefáfio e medula espinhal. A AC apresentou-se mais eficaz no tratamento da DN em comparação à EA, porém nenhum dos tratamentos foi capaz de alterar os danos causados pela indução da DN no músculo gastrocnemio esquerdo dos animais demonstrado na histologia. Todavia, este resultado não alterou a analgesia gerada pelos tratamentos.

Palavras-Chave: dor neuropática, acupuntura, eletroacupuntura, isoflurano, NGF, S100b, NMDA.

ABSTRACT

Neuropathic pain (NP) is defined as "pain initiated or caused by primary injury or dysfunction in the nervous system," but its prevalence depends on the type of trauma and related dysfunction. Although this painful condition is considered to be highly prevalent and debilitating, the available treatments are related to adverse effects, making adherence difficult. Because of this, non-pharmacological alternatives for the treatment of this type of pain are sought, among them, the techniques of peripheral neuromodulation, such as acupuncture (AC) and electroacupuncture (EA). These techniques can be combined with pharmacological and non-pharmacological interventions and have shown promising results in the treatment of neuropathic pain. However, its mechanisms of action are not fully elucidated, so the use of animal models is of great value for the study of these mechanisms in the treatment of neuropathic pain and the pathophysiology of this type of chronic pain. It is important to emphasize that the application of AC and EA in awake animals is complex, since it generates discomfort and can alter the analgesia induced by the treatment. In many studies, anesthesia with isoflurane is used during the application of the treatments, but its use may generate a bias in the study, considering the possible interference of the drug in the behavioral and neurochemical results. Another important focus of the study is to compare the two techniques, AC and EA, seeking to determine which is the most effective in the treatment of neuropathic pain. Considering the above, the objectives of this thesis were: 1) to evaluate the behavioral and neurochemical parameters of the effects of the use of anesthesia in the application of AC and EA in rats submitted to the DN model; 2) to compare the effects of AC and EA on animal model of DN by means of behavioral, neurochemical and morphological parameters. Considering the results obtained in this thesis, we conclude that isoflurane increases the analgesia promoted by AC and EA, probably decreasing the effect of the stress generated by the application of the treatments in agreed animals, a result that is corroborated by the decrease

in the level of peripheral S100β (biomarker of central neuronal injury); On the other hand, isoflurane decreased the levels of neural grown factor (NGF) in the injured peripheral nerve, indicating a decrease in the neural regeneration process, while the EA increased. At the same time, isoflurane altered the effects of treatments on exploratory behaviors and N-metil-D-aspartato (NMDA) levels in the brainstem and spinal cord. AC was more effective in the treatment of DN compared to EA, but none of the treatments was able to alter the damage caused by DN induction in the left gastrocnemius muscle of the animals showed in histology. However, this result did not alter the analgesia generated by the treatments.

Keywords: neuropathic pain, acupuncture, electroacupuncture, isoflurane, NGF, S100b, NMDA.

I. INTRODUÇÃO

1. INTRODUÇÃO

Dor aguda é uma resposta protetora do organismo a estímulos nocivos que, quando não controlada, resulta em efeitos indesejáveis que podem desencadear um processo de doença crônica. Dentre as dores crônicas, a dor neuropática (DN) é um problema clínico importante e debilitante; pode ser decorrente de uma lesão nervosa central ou periférica, afetando o sistema somatossensorial. Apesar do grande comprometimento clínico causado por esta condição, os tratamentos farmacológicos disponíveis são relacionados a efeitos adversos, diminuindo a adesão e dificultando o tratamento (Li *et al.*, 2016). Por isso na busca por novas terapias que auxiliem a diminuição dos sintomas clínicos da DN, estudos em modelos animais são extremamente importantes. Nas últimas décadas, o modelo da lesão constritiva crônica do nervo ciático (CCI), descrita por Bennet e Xie,1988 tem sido o modelo animal mais utilizado para o estudo da patofisiologia e tratamento da DN (Bennett e Xie, 1988; Wang *et al.*, 2016)

Dentre as técnicas não farmacológicas que apresentam bons resultados no tratamento de quadros dolorosos crônicos, a Acupuntura (AC) e a Eletroacupuntura (EA) se destacam como técnicas promissoras complementarares ou mesmo substitutivas a terapias farmacológicas, quando apresentam resultados satisfatórios. Ambos os tratamentos têm sido amplamente utilizados clinicamente nos países asiáticos no tratamento de uma série de doenças como osteoartrite (Liu, Y. H. *et al.*, 2017), depressão (Dong *et al.*, 2017) e cefaléia (Liu e Yu, 2016); incluindo doenças que cursam com dor aguda e crônica.

Apesar da analgesia induzida pela AC e EA ser reconhecida, seu mecanismo de ação ainda não foi totalmente esclarecido (Wang *et al.*, 2016). Sabe-se que AC e EA apresentam mecanismos complexos, que alteram diversos sistemas endógenos, como por exemplo, o sistema opioide, que aumenta a liberação de encefalinas e dinorfinas nos sistemas descendente

inibitório da dor, assim como ativa o eixo hipotálamo-hipófise-adrenal (HHA) (Han, 1999); Alvarenga et al., 2014).

Com o aumento da utilização destas tecnologias nos países ocidentais, e com o objetivo de oferecer tratamentos eficazes e livres de efeitos contextuais (placebo), algumas dúvidas em relação ao real efeito destas terapias permanecem. Uma dúvida frequente é reconhecer se há diferença entre o tratamento de AC em relação a sua associação à corrente elétrica, a EA. Existem poucos estudos pré-clínicos que comparem os efeitos comportamentais e neuroquímicos da AC manual e da EA no tratamento da DN. Dentre os estudos existentes, as metodologias não são consistentes ao comparar ambas as técnicas diretamente (Langevin *et al.*, 2015). A escolha do melhor tratamento depende da doença a ser tratada, das especificidades dos pacientes e da experiência clínica dos profissionais. Em DN, por exemplo, tanto AC ou EA têm bons resultados, mas ainda é necessário pesquisar sobre as possíveis diferenças entre os seus efeitos sobre esta condição dolorosa e seus mecanismos de ação.

O uso de modelos animais de dor crônica, como a DN, aliados ao tratamento de AC e EA, é de extrema importância para elucidarmos estes questionamentos. No entanto, dentro da prerogativa translacional, existe grande dificuldade na aplicação destes tratamentos em modelos animais, o que consequentemente dificulta a interpretação das respostas obtidas em um ambiente de pesquisa de estudos pré-clínicas. As formas utilizadas para a aplicação de AC e EA descritas na literatura são: animais acordados e livres, o que tende a gerar estresse, já que a inserção da agulha pode gerar desconforto ao animal; animais restritos, além do desconforto da inserção da agulha, a restrição é conhecida por causar estresse (agudo ou crônico) que influencia diretamente a analgesia induzida pelos tratamentos; animais anestesiados com isoflurano, apesar de ser um anestésico inalatório de pouca metabolização, pouco se sabe sobre as possíveis interferências nas respostas comportamentais e neuroquímicas induzidas pelos tratamentos de AC ou EA.

Dentre os mecanismos neuroquímicos relacionados à DN e aos tratamentos com AC e EA, têm sido indentificados diversos biomarcadores. Dentre os mais estudados está: o fator de crescimento neural (NGF), uma neurotrofina atuante no processo nociceptivo, tanto em nível central quanto periférico (Bannwarth e Kostine, 2014); proteína S100β, relacionada a processos de dor crônica, e utilizada como biomarcador de transtorno ou dano cerebral (Martins *et al.*, 2006); citocinas inflamatórias, como o fator de necrose tumoral-α (TNF alfa) com importante papel na indução e manutenção da DN (Wang *et al.*, 2016), e alteradas por AC e EA em sistema nervoso central (SNC) e sistema nervoso periférico (SNP) (Geis *et al.*, 2017). Os receptores N-metil D-aspartato (NMDAr) também participam ativamente do processo de sensibilização central, envolvido na DN (Ji e Strichartz, 2004), e o tratamento com AC e EA parecem alterar a expressão destes receptores (Lu *et al.*, 2016). Existem evidências que estes tratamentos também causam alterações nos níveis de diclorofluoresceína diacetato (DCFH), importante marcador de estresse oxidativo que, em SNP, também está alterado em condições de DN (Choi *et al.*, 2012).

Considerando o exposto acima, os objetivos desta tese foram: avaliar a influência da anestesia inalatória na aplicação de AC e EA em parâmetros comportamentais e neuroquímicos em ratos com DN; e comparar os efeitos da AC e EA em um modelo animal de DN em parâmetros comportamentais, neuroquímicos e histológicos

II. REVISÃO DA LITERATURA

2.1 Estratégias para Localizar e Selecionar Informações

Nesta revisão de literatura buscou-se estudar os principais aspectos da dor neuropática, acupuntura, eletroacupuntura e a relação com biomarcadores. A estratégia de busca envolveu as seguintes bases de dados: MEDLINE (site PubMed), LILACS, SciELO. Foram selecionados artigos publicados entre 1997 e 2017.

Nos sites PubMed, LILACS, SciELO foram realizadas buscas utilizando os termos: *neuropathic pain, acupuncture, electroacupuncture, NGF, S100 β, TNF-α, NMDA* e *DCFH*. Em relação ao termo *neuropathic pain*, foram encontrados 26.070 artigos no PubMed e 260 artigos no LILACS, já no SciELO foram encontrados 267 artigos. Utilizando-se o termo *acupuncture* foram encontrados 18.935 artigos no PubMed, 596 artigos no LILACS e 318 no SciELO. Com o descritor *electroacupuncture* a busca no PubMed encontrou 3.249 artigos, 55 no LILACS e 36 no SciELO. Com o descritor *NGF*, foram encontrados 9.540 no PubMed, 85 no LILACS e 22 no SciELO. Para *S100 β*, 3.045 artigos foram encontrados no PubMed, 65 no LILACS e 229 no SciELO. Em relação ao *TNF-α* 123.917 artigos foram encontrados no PubMed, 838 no LILACS e 301 no SciELO. A busca por *NMDA* revelou 30.807 artigos no PubMed, 173 no LILACS e 103 no SciELO. Por último, a busca simples de *DCFH* revelou 1.165 artigos no PubMed, 9 no LILACS e 7 no SciELO.

Refinando-se a busca, com cruzamentos entre as palavras-chave foi encontrado um reduzido número de artigos como mostrado na Figura 1.

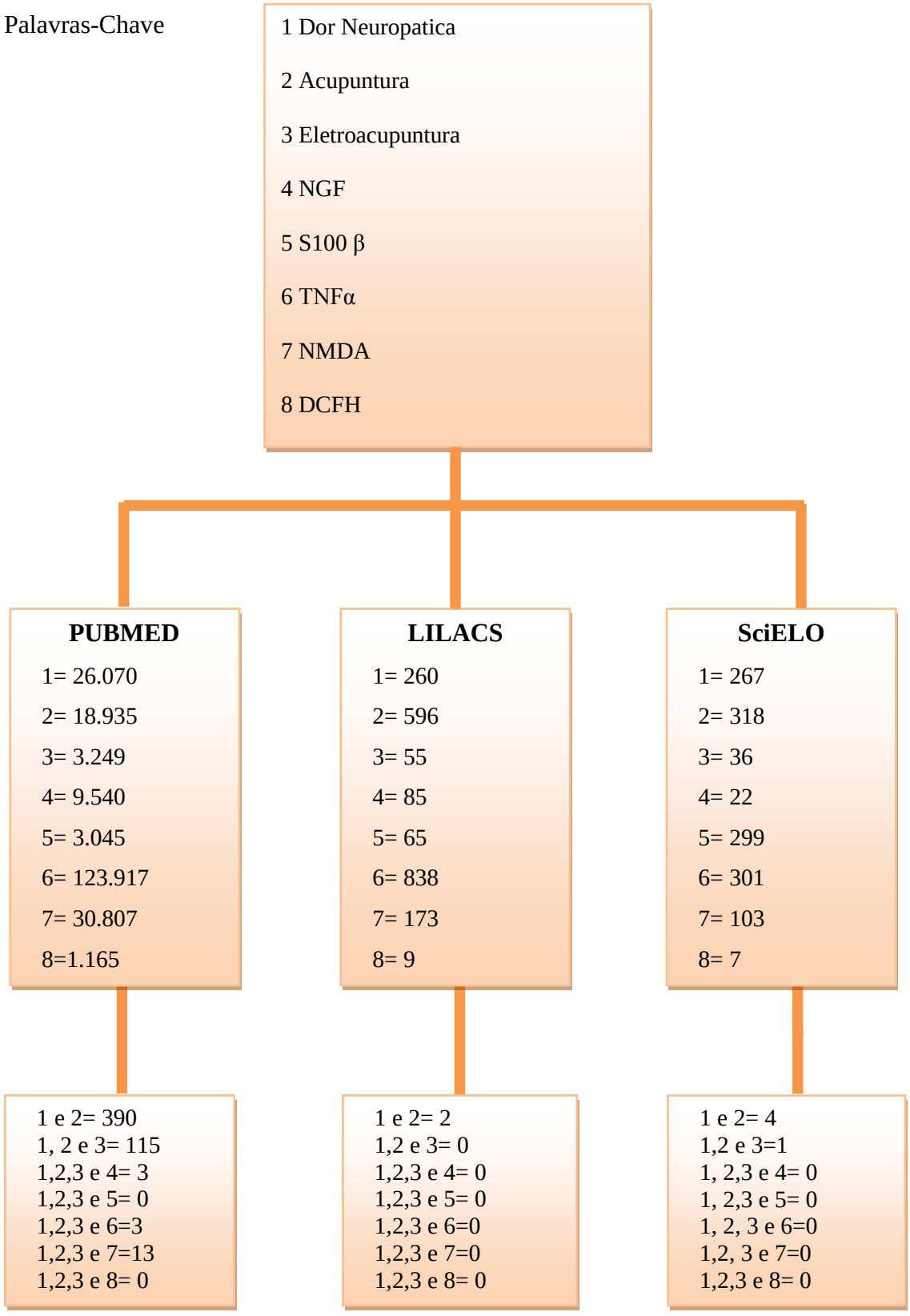

Figura 1. Fluxograma da pesquisa realizada com as palavras-chave

2.2 Dor

Dor é conceituada como "uma experiência sensorial e emocional desagradável associada a dano tecidual real ou potencial, ou descrita em termos de tal dano" pela Associação Internacional para o Estudo da Dor (IASP) desde 1979 (Merskey, 1994). Entretanto, Williams e Craig sugerem uma atualização do conceito, com maior abrangência, que beneficiaria o entendimento e o tratamento da dor (Williams e Craig, 2016). Assim, a nova definição considera dor "uma experiência angustiante associada a dano tecidual real ou potencial com componentes sensoriais, emocionais, cognitivos e sociais" (Williams e Craig, 2016).

Seguindo critério temporal, a dor pode ser classificada em aguda ou crônica. Devido à sua natureza angustiante e desagradável, a dor aguda exerce um papel protetor contra danos teciduais e geralmente está relacionada a causa recente. É causada por traumas, doenças subjacentes, alterações funcionais musculares ou viscerais e, na maioria dos casos, cessa em alguns dias ou semanas, sendo responsiva a analgésicos clássicos (Tajerian e Clark, 2017).

Por outro lado, a dor pode se tornar persistente, deixando de ser um sintoma, e passa a ser por si só a doença, caracterizada por um quadro de dor crônica. Estes casos são decorrentes da incapacidade do organismo de reverter a lesão relacionada à dor, como em casos inflamatórios crônicos, como a osteoartrite, ou então quando há o desequilíbrio entre os sistemas inibitórios e excitatórios nociceptivos, gerando, desta forma, uma resposta mal adaptativa a dor (D'mello e Dickenson, 2008). Em consequência disto há inúmeros prejuízos, mas pode-se salientar como principal, a perda da qualidade de vida destes pacientes.

Entre os critérios diagnósticos para classificação da dor crônica preconizada pela IASP, e que a dor deve ter duração de pelo menos três meses, porém alguns autores sugerem que dores com duração de pelo menos um mês já podem ser consideradas crônicas (Morgan e Whitney, 1996; Loeser, 2001). Dor crônica é uma condição de difícil tratamento e diagnóstico, está associada a sofrimento psicológico e prejuízo funcional (Wolfe *et al.*, 1990; Verhaak *et al.*,

1998). Sendo por estas razões que é considerada um problema de saúde pública que afeta 30% da população adulta nos EUA (Johannes *et al.*, 2010). No Brasil a prevalência da dor crônica difere de acordo com as regiões do país, variando de 28% a 54% da população (Dias *et al*., 2009; Mendonza-Sassi *et al*., 2006). Com a incidência e prevalência crescentes, esta doença gera custos de bilhões de dólares relacionados a seus tratamento e comorbidades, como alterações do humor (Mcwilliams *et al.*, 2004) e prejuizo cognitivo (Berryman *et al.*, 2013).

Um dos mecanismos sugeridos para a cronificação da dor e resistência às formas clássicas de tratamento envolve o conceito de sensibilização central, no qual eventos sensoriais desencadeados por trauma, gradualmente alteram o sistema nervoso central (SNC) amplificando a dor, mesmo sem lesão tecidual e sensibilização periférica (Tajerian e Clark, 2017). Dentre as causas mais comuns de dor crônica relacionada à sensibilização central estão fibromialgia, migrânea e dor neuropática (DN) (Naro *et al.*, 2016).

2.3 Dor Neuropática

DN é definida pela IASP como "dor que surge como consequência direta de uma lesão ou doença do sistema somatossensorial" (Jensen *et al.*, 2011). O sistema somatossensorial é responsável pela percepção do toque, pressão, temperatura, posição, movimento, vibrações e dor. Nervos somatossensoriais encontram-se em pele, músculos, articulações e fáscia; incluem termorreceptores, mecanorreceptores, quimiorreceptores, pruriceptores e nociceptores que enviam sinais para a medula espinhal e, finalmente, para o encéfalo para o seu processamento (Colloca *et al.*, 2017). A maioria dos processos sensoriais envolve núcleos talâmicos que recebem um sinal sensorial que é então direcionado ao córtex cerebral. Lesões ou doenças do sistema nervoso somatossensorial podem levar à transmissão alterada e desordenada de sinais sensoriais na medula espinhal e no encéfalo (Borsook, 2012). Condições comuns associadas à

DN incluem dor pós-herpética, neuralgia trigeminal, dor radicular, neuropatia diabética, amputação, lesão nervosa periférica e acidente vascular cerebral (AVC). De acordo com a origem e etiologia da DN, podemos classificá-la em central ou periférica, sendo que a de origem central é proveniente de lesões ou doenças que acometem o encéfalo ou a medula espinhal (por exemplo: AVC, lesão medular, afecções desmielinizantes, doenças inflamatórias, entre outras). Por outro lado, as dores periféricas são provenientes de alterações nervosas periféricas (por exemplo: diabetes, traumas, tumores, doenças, infecciosas, entre outras) (Baron *et al.*, 2010). No entanto, nem todos os pacientes com neuropatia periférica ou lesão nervosa central desenvolvem o quadro de DN. Um grande estudo com pacientes portadores de diabetes melitus demonstrou que apenas 21% deles desenvolveram sintomas de DN, sendo que todos apresentaram formas diferentes de neuropatias (Costigan *et al.*, 2009).

A prevalência global de sintomas de DN é de 21% em pacientes com neuropatia clínica (Colloca *et al.*, 2017) e depende do tipo de trauma e disfunção relacionados (Smith e Torrance, 2012). DN crônica é mais frequente em mulheres (8% versus 5,7% nos homens) e pessoas com mais de 50 anos (8,9% versus 5,6% naqueles <49 anos de idade). Além disto, afeta mais frequentemente a parte inferior das costas, membros inferiores, pescoço e membros superiores (Bouhassira, 2008). Os sintomas mais característicos da DN são: hiperalgesia, caracterizada pelo aumento da resposta aos estímulos nocivos mecânicos ou térmicos; e alodinia, que apresenta respostas dolorosas aos estímulos táteis inócuos. Estes sintomas são característicos de sensibilização periférica e central, decorrentes da lesão nervosa periférica (Baron *et al.*, 2010; Nickel *et al.*, 2012).

2.3.1 Sensibilização Periférica

Sensibilização periférica ocorre após inflamação periférica e é caracterizada por redução do limiar nociceptivo decorrente do aumento da resposta dos nociceptores periféricos a mediadores inflamatórios sensibilizantes. Perl, em 1976, descreveu primeiramente o fenômeno da sensibilização periférica decorrente da estimulação persistente de nociceptores promovendo redução do limiar de despolarização de terminações livres e aumento no tempo de respostas aos estímulos nociceptivos (Perl *et al.*, 1976).

Nociceptores são terminações de fibras nervosas livres específicas para responder a estímulos nocivos. Quando ativados por substâncias algogênicas transformam estímulos de natureza térmica, química ou mecânica em estímulo elétrico (potencial de ação) (transdução do sinal). Este potencial excitatório será transmitido pelas fibras nervosas periféricas (fibras Aδ e C) até SNC e interpretado no córtex cerebral como dor (Levine e Taiwo, 1990) (transmissão do sinal) (Fig.2). As fibras Aδ, em função da presença da bainha de mielina, transmitem o estímulo doloroso de forma rápida, enquanto as fibras C são responsáveis pela transmissão lenta da dor. Após o estímulo nociceptivo, no sítio da lesão a partir de axônios danificados e de células satélites, ocorre liberação de substâncias denominados algogênicas como íons, prostaglandina E2 (PG-E2), bradicinina, adenosina trifosfato (ATP), histamina, serotonina, interleucina-1β (IL-1β), interleucina 6 (IL-6), TNF-α, glutamato, endotelina1, NGF, e várias quimiocinas. Em sequência, ocorre migração de mastócitos e de leucócitos para o sítio da lesão (Piotrowski e Foreman, 1986; Campbell, 1989). Prostaglandinas e bradicinina causam alterações em receptores vanilóides subtipo 1 (TRPV1) acoplados a canais iônicos ligante-dependente via ativação da adenosina 3',5'-monofosfato cíclico (AMPc). Ocorre redução do tempo pós-hiperpolarização de membrana neural reduzindo o limiar de disparo da fibra nervosa.

As neurotrofinas, como o NGF, aumentam a síntese de substância P (SP) e do peptídeo relacionado com o gene da calcitonina (CGRP) nas fibras C, promovem redução da atividade do ácido gama-aminobutírico (GABA), em terminações nervosas periféricas e centrais, além de induzirem mudanças em receptores vanilóides (VR1) de fibras Aδ acoplados a canais iônicos ligante-dependente. Outra função relacionada às neurotrofinas, que esta relacionada ao mecanismo de sensibilização periférica é o recrutamento de proteínas quinases ativadas por mitógenos (MAPK) que fosforilam o AMPc e iniciam a transcrição gênica responsável por alterações fenotípicas que contribuem para amplificação da eficácia sináptica (Fletcher *et al.*, 1996; Aida *et al.*, 1999).

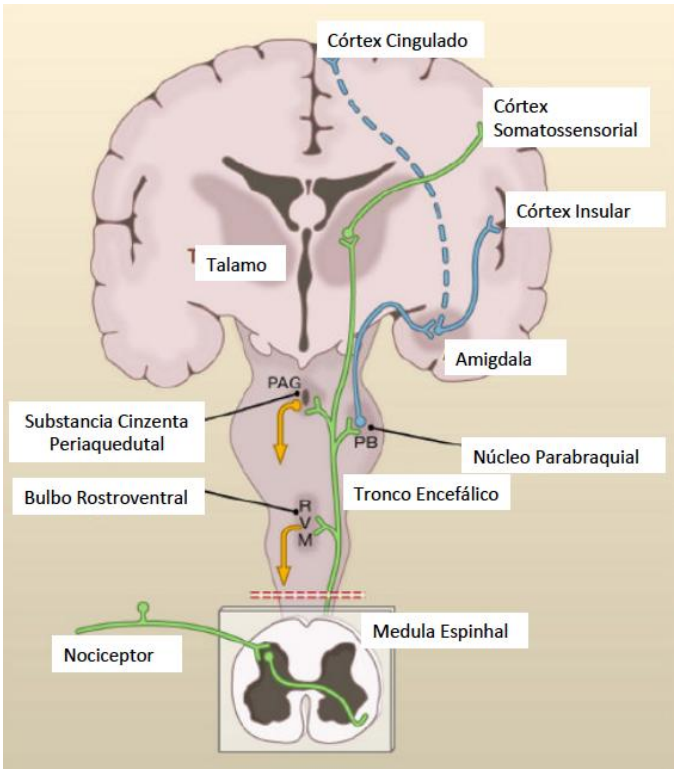

Figura 2. Transmissão do estímulo nociceptivo (Adaptado de Basbaum *et al.*, 2009).

A persistência do estímulo nociceptivo induz modificações no SNP e sensibilização de fibras nervosas periféricas e centrais, com consequente hiperalgesia primária e secundária e

aumento dos níveis de AMPc e cálcio nos nociceptores. Esta sensibilização resulta em alodinia e hiperalgesia (Fletcher *et al.*, 1996; Aida *et al.*, 1999). Além disto, a sensibilização periférica também ocorre após lesões nervosas na presença (neurite periférica) e ausência de inflamação tecidual e assim, pode contribuir para a hipersensibilidade da área de inervação do nervo afetado. A hiperalgesia secundária é caracterizada pela sensibilização da área adjacente à lesão, inervada pelo mesmo segmento da hiperalgesia primária. Isto ocorre devido a maior capacidade de resposta dos neurônios do corno dorsal que inervam o segmento da fonte primária da lesão (Latremoliere e Woolf, 2009).

A lesão do nervo periférico resulta em alterações orquestradas semelhantes à degeneração walleriana, levando a alterações estruturais e funcionais que afetam todo o SNP, incluindo terminações nervosas periféricas, fibras aferentes, gânglio da raiz dorsal (GDR) e terminais aferentes centrais na medula espinhal (Austin *et al.*, 2012; Nickel *et al.*, 2012). As alterações incluem edema do corpo celular, perda de corpúsculo de Nissl e deslocamento do núcleo do centro do neurônio para uma posição próxima à membrana celular. Após a lesão do nervo periférico, observa-se degeneração axonal, edema endoneurial e desmielinização maciça, associados à degeneração axonal e infiltração de células imunes em gânglio da raiz dorsal (GDR) e cornos dorsais de medula espinhal (Zochodne, 2012). Consequências da lesão nervosa incluem déficits funcionais e comportamentais que representam desafios para a identificação de novas estratégias terapêuticas para o tratamento de DN (Von Hehn *et al.*, 2012; Komirishetty *et al.*, 2016).

Outra consequência debilitante da lesão nervosa periférica é a dor espontânea, que ocorre na ausência de qualquer estímulo externo, podendo ser resultado da atividade espontânea gerada ao longo da via nociceptiva. Dor espontânea após lesões nervosas periféricas parece ser gerada pela hiperexcitabilidade no neurônio sensorial primário, levando a descarga de potenciais de ação ectópicos no local da lesão, mas também em locais axonais proximais, incluindo o soma

(Amir *et al.*, 2005; Amir *et al.*, 2015). Atividade ectópica é uma das principais sensações espontâneas que se manifestam após lesões nervosas, produzindo parestesia, disestesia e dor. DN pode ser episódica ou contínua, superficial ou profunda, muitas vezes apresenta-se como choque, em rajadas, fisgadas ou ardência. Enquanto muitas alterações ocorrem nos neurônios lesionados, fibras não lesionadas vizinhas passam a ser fonte de atividade ectópica neuropática, pois se comportam como uma entrada aferente produzindo sensações dolorosas (Wu *et al.*, 2002; Djouhri *et al.*, 2006). Alterações nestes neurônios podem induzir a liberação de mediadores pelos axônios lesados, células imunes e células de Schwann denervadas. A atividade espontânea é um componente importante no tratamento da DN por ter um papel fundamental nas alterações centrais nas vias nociceptivas, amplificando a sensibilização central e consequentemente, a dor (Von Hehn *et al.*, 2012).

2.3.2 Sensibilização Central

A Sensibilização Central (SC) é clinicamente caracterizada pela hiperalgesia, alodinia, dor irradiada ou dor persistente descrita como desagradável, latejante, em queimação ou dormência (Yunus, 2007). É definida como o aumento da capacidade de resposta de neurônios centrais à sinalização de nociceptores periféricos. Ocorre a partir de modificações em medula espinhal, entre o neurônio pré e pós sináptico, da via aferente nociceptiva no corno dorsal da medula. Estas alterações contribuem para redução do limiar de disparo dos nociceptores, aumento da eficácia sináptica e aumento do campo receptivo contribuindo para amplificação da dor. Estes fenômenos ocorrem em uma ordem temporal: alterações dos impulsos periféricos, aumento da excitabilidade de neurônios nociceptivos, descargas persistentes após estímulos repetidos e ampliação dos campos receptivos de neurônios do corno dorsal. O processo de SC tem como consequência a alteração no processamento sensorial central, mau funcionamento do

mecanismo antinociceptivo descendente, aumento da atividade da rota facilitatória da dor e aumento do período da segunda dor (somação temporal) (Nijs *et al.*, 2010).

SC é considerada o principal mecanismo fisiopatológico em condições de DN. Primeiramente ocorre sensibilização sináptica causada por uma sequência de estímulos periféricos nociceptivos repetidos aumentando as respostas das fibras Aδ, C e Aβ. Este fenômeno ocorre como consequência da liberação de aminoácidos excitatórios (ex. glutamato e aspartato), de peptídeos (ex. SP e CGRP) e de neurotrofinas [ex. NGF e fator neurotrófico derivado do encéfalo (BDNF)] no corno dorsal da medula espinhal (Woolf e Salter, 2000). Após a liberação destas substâncias e sua interação com receptores específicos, tais como os receptores N-metil-D-aspartato (NMDA), neurocinina 1 (NK1) e Tirosina Quinase B (TrkB), há ativação de cascatas de segundos mensageiros, promovendo a abertura de canais de cálcio, produção de prostaglandinas e óxido nítrico. Em sequencia, ocorre liberação de glutamato, aspartato, SP e CGRP, contribuindo para a ampliação do processo álgico (Ji e Strichartz, 2004).

Outro mecanismo fundamental na manutenção da SC é fenômeno *wind up, que* é o resultado da somação de potenciais sinápticos lentos após estimulação aferente repetida por tempo prolongado. As fibras C, amielínicas e de condução lenta, enviam impulsos nociceptivos para os neurônios de ampla faixa dinâmica (*wide-dinamic range* [WDR]) no corno dorsal da medula. Com impulsos intensos, ambos os neurônios são ativados de modo que até estímulos não dolorosos, como o toque, sejam percebidos como dolorosos (Yunus, 2007), estimulando a liberação dos neurotransmissores excitatórios como glutamato e aspartato no corno dorsal da medula espinhal induzindo despolarização neuronal pelos receptores NMDA. Ocorre, a partir desses eventos, o aumento da condutividade ao cálcio, e consequentemente maior resposta à dor, a cada estímulo repetido e de mesma intensidade (Li *et al.*, 1999). Os mecanismos que contribuem para o aumento da eficácia da transmissão sináptica são decorrentes da fosforilação de receptores de membrana e de alterações do tempo de abertura de canais iônicos ou do

aumento da síntese e liberação de mediadores excitatórios. O processo de facilitação sináptica envolve a ativação de fatores de transcrição, tais como a expressão de genes de formação imediata, como *c-fos* e *c-jun*, e de genes de resposta lenta que codificam a pró dinorfina, o receptor NK1 e trkB no corno dorsal da medula espinhal. Assim, ocorre regulação das vias ascendentes para síntese de citocinas, quimiocinas e moléculas de adesão. Deste modo, há mudança fenotípica no GRD (Hunt *et al.*, 1987) (Fig. 3).

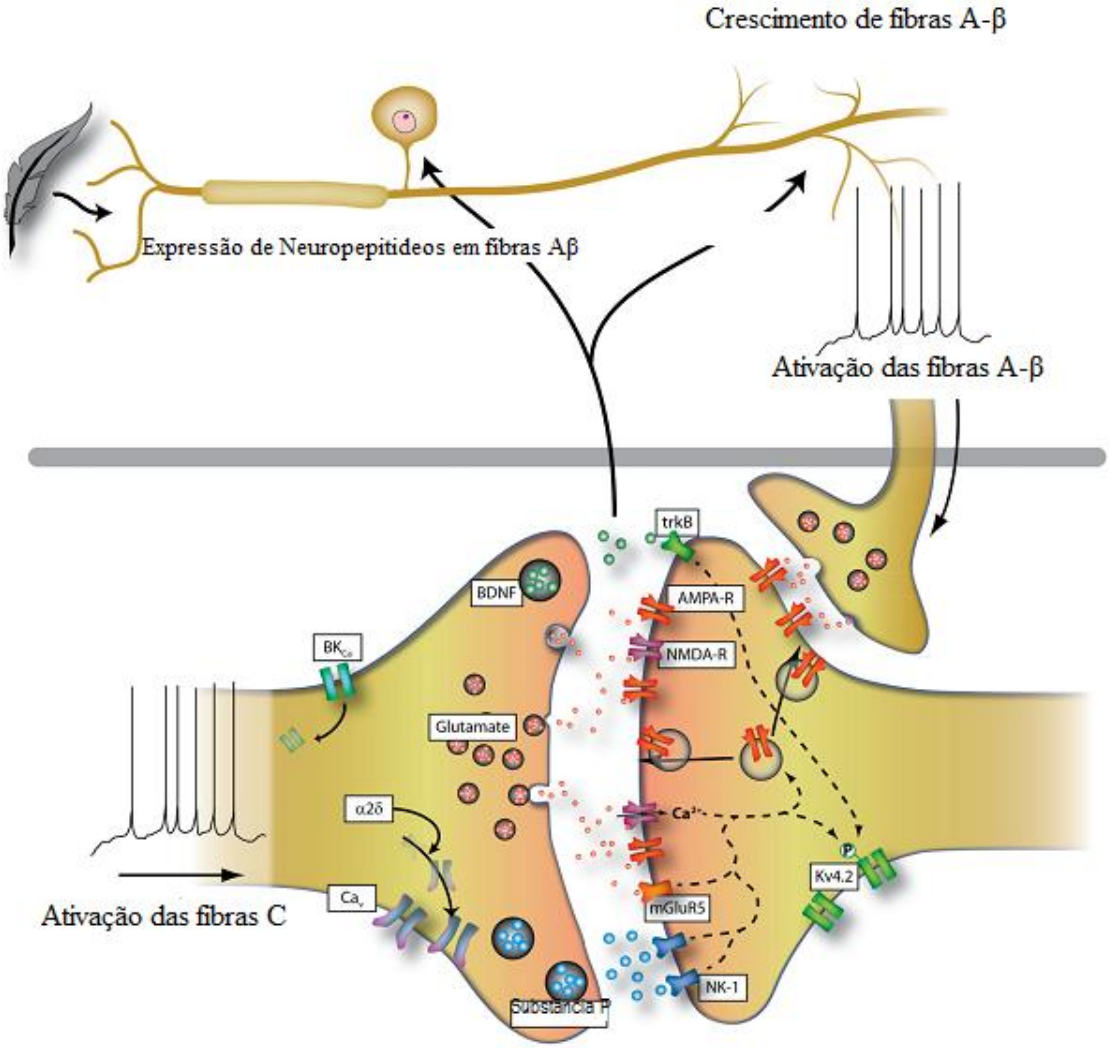

Figura 3. Sensibilização Central Adaptado de Von Hehn 2013.

Pode-se caracterizar a DN como uma combinação de sinais e sintomas que se manifestam com hiperalgesia e alodinia, intercalados ou em conjunto (Cruccu 2010; Baron 2012; Backonja 2013). Para avaliar perda (sinais sensoriais negativos) ou ganho (sinais sensoriais positivos) da função somatossensorial, as respostas são classificadas como normais, diminuídas ou aumentadas. O estímulo evocado positivo é classificado como hiperalgésico, (sensibilidade aumentada à um estímulo doloroso) ou alodínico (dor à um estímulo inócuo), e relacionado à dinâmica ou caráter estático do estímulo (Baron et al., 2017).

Atualmente existem muitos tratamentos farmacológicos para a DN, a maioria atua predominantemente na transdução de sinais neuronais incluindo bloqueadores de canais de cálcio e de sódio, antidepressivos e anticonvulsivantes. Contudo, como os demais tipos de dores crônicas, a DN não apresenta tratamentos farmacológicos satisfatórios. Os fármacos oferecem benefícios terapêuticos limitados e os pacientes podem apresentar muitos efeitos adversos (Finnerup *et al.*, 2010; Teasell *et al.*, 2010). Os opioides, considerados potentes agentes analgésicos, são menos eficazes para dores superficiais tipo pontada. Além de que os pacientes referem discreta redução na intensidade e melhora no desconforto gerado pela dor (Finnerup *et al.*, 2010; Teasell *et al.*, 2010). Sendo assim, a busca por terapias mais eficazes e com menos efeitos adversos se faz necessária.

Com objetivo de potencializar ou mesmo substituir terapias farmacológicas no tratamento de quadros dolorosos, técnicas alternativas como estimulação do SNC e SNP, utilizando diferentes abordagens, estão sendo estudadas (Spezia Adachi *et al.*, 2015; Liu, L. *et al.*, 2017). Em relação aos métodos de estimulação do SNP, considerados seguros e de fácil aplicação, a Acupuntura e a Eletroacupuntura são técnicas que apresentam potencial benefício no tratamento das dores crônicas (Zhao, 2008).

2.4 Acupuntura e Eletroacupuntura

Acupuntura, do latim *acus* = agulha e *pungere* = puncionar, utilizando a aplicação de agulhas em determinados pontos do corpo, chamados de acupontos, tem como objetivo tratar e aliviar os sintomas de doenças (Schoen *et al.*, 1986; Jaggar, 1992). Os acupontos pertencem aos 12 canais de energia divididos pelo corpo. Este tratamento também é considerado uma terapia reflexa, já que o estímulo realizado em determinado ponto tem ação sobre outras áreas do corpo (Hayashi, 2007).

Os meridianos e seus acupontos foram determinados de forma empirica durante milhares de anos de prática da Medicina Tradicional Chinesa (Ristol, 1997). Sugere-se que o acuponto é uma região em que há grande concentração de terminações nervosas sensoriais, podendo estar próximo à nervos, vasos sanguíneos, tendões, periósteo e cápsulas articulares (Wu, 1990). As propriedades elétricas dos acupontos são: condutância elevada, menor resistência, padrões de campo organizados e diferenças de potencial elétrico; geralmente possuem um diâmetro de 0,1 a 5 cm (Altman, 1992). Quando um destes pontos é puncionado, ocorre sensação de parestesia elétrica, calor podendo ocorrer contração muscular (Taffarel 2009, Rosted 1998, Scognamillo-Szabó 2001).

Acupuntura tem sido amplamente utilizada na China e em outros países asiáticos desde sua antiguidade para prevenção e tratamento de uma série de doenças, como depressão, estresse crônico e dor aguda e crônica (Dong *et al.*, 2017; Li *et al.*, 2017; Liu, L. *et al.*, 2017). Atualmente também é considerada um método alternativo para o tratamento de inúmeras doenças em diversos países ocidentais (Wang *et al.*, 2016). Sabe-se que a EA apresenta bons resultados clínicos no tratamento de dores agudas ou dores crônicas inflamatórias (Ceccherelli *et al.*, 1999; Liao *et al.*, 2017), assim como no tratamento da DN, com efeito analgésico potente (Kim, H. K. *et al.*, 2004; Wang *et al.*, 2016).

As agulhas utilizadas para puncionar os acupontos podem ser estimuladas por rotação manual ou estimulação elétrica, técnica conhecida como EA. O efeito antinociceptivo da AC e da EA é desencadeado pela ativação de vias opioides e não-opioides. Além disto, ocorre hiperestimulação das terminações nervosas de fibras mielínicas Aδ e consequente, ativação de vias modulatórias da dor. A modulação nociceptiva em estruturas do SNC, como no mesencéfalo ocorre por meio da liberação de serotonina e norepinefrina nos sistemas descendentes. Concomitante a isso, em medula espinhal, a modulação ocorre por inibição pré-sináptica, devido à ação de encefalinas e dinorfinas (Taffarel, 2009). AC ativa o eixo Hipotálamo Hipófise Adrenal (HHA) liberando hormônio liberador de corticotrofina (CRH) hormônio adrenocorticotrófico (ACTH), glicocorticoides (cortisol) e ß-endorfinas. Entretanto, existem opiniões controvérsas acerca da participação de hormônios glicocorticoides no efeito anti-inflamatório da AC (Alvarenga *et al*. 2014).

Prévios estudos demonstram alterações na ressonância magnética funcional (RMIf) de diversas áreas cerebrais após tratamento com AC sugerindo efeitos hipocampais da acupuntura. A manipulação de agulhas em ambas as mãos produziu diminuições proeminentes de sinais na RMIf no núcleo accumbens, amígdala, hipocampo, parahipocampo, hipotálamo, área tegmental ventral, giro cingulado anterior, caudado, putamen, pólo temporal e insula. Em contraste, os aumentos de sinal foram observados principalmente no córtex somatossensorial (Hui *et al.*, 2000; Yang *et al.*, 2012). Da mesma forma, EA parece modular a função de interneurônios no hipocampo, aumentando a LTP hipocampal em longo prazo no giro denteado em experimento relacionado à memória (He *et al.*, 2012).

Os efeitos da AC parecem estar relacionados, além da ativação de fibras do tipo Aδ e C (Zhao, 2008), também à ativação de receptores polimodais (PMRs) (Kawakita e Funakoshi, 1981; Kawakita e Gotoh, 1996; Kawakita *et al.*, 2006). PMRs são terminações nervosas livres capazes de responder a estímulos mecânicos e térmicos (não necessariamente nocivo), o que

pode explicar a sobreposição de efeitos de técnicas baseadas em estimulo mecânico (acupuntura) ou térmico (moxabustão) (Kawakita e Gotoh, 1996; Kawakita *et al.*, 2006).

Tambem é conhecido o envolvimento de opioides endógenos e de serotonina (5-HT) nos efeitos antinociceptivos da AC. A analgesia induzida por EA pode ser bloqueada pela naloxona, um antagonista competitivo opioide, tanto em humanos como em ratos, sugerindo a participação de opioides endógenos neste efeito (Pomeranz e Chiu, 1976; Mayer *et al.*, 1977). Adicionalmente, um estudo de Han (2004) demonstrou que naloxona bloqueia analgesia induzida por EA de baixa frequência (4 Hz), mas não de alta frequência (200 Hz) (Han, 2004). Isto pode ser devido à baixa frequência provocar liberação de opioides e a alta frequência atuar também em outros sistemas de neurotransmissão. Por outro lado, Hökfelt demonstrou que os neuropeptídeos só podem ser liberados por alta frequência de estimulação e não por baixa (Hökfelt, 1991).

Adicionalmente foi demonstrado o efeito neuroprotetor da EA por meio da ação de neurotrofinas (NRs) utilizando um modelo animal de lesão parcial de medula espinhal. Maiores níveis de NGF, BDNF e NT-3 foram observados após lesão parcial de medula espinhal e aplicação da EA de alta frequência indicando plasticidade espinhal induzida pelo aumento dos níveis de NTs promovida pela EA (Wang *et al.*, 2007). Usando o mesmo modelo experimental, também foi demonstrado que a EA de alta frequência aumenta os níveis de mRNA e NGF, BDNF, NT-3 em neurônios do GRD. Desta forma, estende-se aos neurônios sensoriais primários a hipótese de NTs mediarem o efeito da EA na plasticidade em neurônios com lesão (Chen *et al.*, 2007). As mesmas observações foram estendidas para NT-4, sugerindo um envolvimento da família NT inteira no efeito da EA induzindo o processo de neuroplasticidade em medula espinhal após lesão do nervo periférico (Liu et al., 2009). Estudo recente sugere que a analgesia promovida peto tratamento com EA é relacionada a IL-1β e TNF-α RNAm e BDNF, NGF, e NT3 / 4, mas não a IL-6. EA utilizada em ratos com dor crônica inibiu a expressão de citocinas e

a liberação de fatores neurotróficos por astrócitos podendo este ser um dos mecanismos da analgesia induzida por EA repetida (Wang *et al.*, 2016).

AC e EA demonstram bons resultados quando utilizados para aliviar a dor em humanos e em modelos animais de DN (Wang *et al.*, 2008; Norrbrink e Lundeberg, 2011). Porém, devido à dificuldade da aplicação desses tratamentos em animais acordados e em movimento, a maioria dos estudos utiliza alguma forma de imobilização (Tu *et al.*, 2012) ou anestesia (Cha *et al.*, 2012). No entanto, a administração de anestésicos e a imobilização dos animais durante a AC e EA pode ser uma fonte de viés na pesquisa pré-clínica, causando alterações fisiológicas que podem prejudicar a eficácia das técnicas. O efeito da AC e da EA pode ser influenciado pelo estresse e habituação de animais concientes (Park *et al.*, 2010) ou pelos anestésicos utilizados para sedação destes animais. A aplicação destes tratamentos em animais acordados atua como potencial estressor, já que a simples inserção da agulha e a estimulação manual ou elétrica podem favorecer o mecanismo de estresse. Sabe-se que imobilização, choque e até mesmo medo podem desencadear a analgesia induzida pelo estresse agudo interferindo desta forma nos resultados obtidos a partir de modelos animais de AC e EA (De Medeiros *et al.*, 2003). Desta forma, a analgesia por AC e EA pode ser severamente reduzida quando os estressores concomitantes não são adequadamente controlados.

Na maioria dos estudos em animais que utilizam AC ou EA, o anestésico utilizado é o isoflurano, que é de fácil administração e mantém as características comportamentais e fisiológicas da anestesia geral sem adjuvante (Purdon *et al.*, 2015). No entanto, não há estudos que comprovem que a utilização deste anestésico não altera a resposta analgésica da AC ou EA e/ou os níveis de alguns biomarcadores, como o NGF.

2.7 Biomarcadores

Biomarcadores são utilizados como parâmetros de avaliação de diagnóstico, relação causa-efeito e efetividade de tratamento. Biomarcadores podem ser encontrados periférica ou centralmente, sendo de fácil mensuração no caso de marcadores sanguineos. Considerando que processo nociceptivo ativa diversos sistemas como endócrino, autonômico e imune, estudos recentes tem buscado determinar marcadores que possam estar relacionados com processos de dor crônica (Nwagwu *et al.*, 2016).

O fator de crescimento neuronal (NGF) é uma neurotrofina essencial, amplamente expressa no SNC e SNP em desenvolvimento, envolvida na sobrevivência neuronal, no crescimento axonal e na diferenciação neural (Huang e Reichardt, 2001). Tem sido demonstrado que o NGF também atua no processo nociceptivo, tanto em nível central quanto periférico (Bannwarth e Kostine, 2014). NGF apresenta-se diminuído em neurônios sensoriais do GRD e do corno dorsal da medula espinhal em um modelo de neuropatia periférica (Aloe e Manni, 2009). Em contraste, Watson e colaboradores (2008), encontraram aumento nos níveis de NGF em um modelo de dor neuropática (Watson *et al.*, 2008). Sabe-se que após a lesão do nervo, muitas substâncias, incluindo o NGF, são liberadas principalmente pelos astrócitos do SNC. No corno dorsal da medula espinhal, um aumento do NGF está relacionado à sensibilização central provocando sintomas clínicos relacionados à DN (Campbell *et al.*, 1988). De forma semelhante, estudo prévio associa o aumento dos níveis de NGF no SNC ao aumento da dor (Chiang *et al.*, 2014). No entanto, quando se trata do SNP, há divergências em relação ao NGF e as condições dolorosas. Tem sido sugerido que o aumento dos níveis periféricos de NGF está relacionado à DN (Zhao *et al.*, 2016), no entanto, um estudo utilizando mobilização neural mostrou aumento de 52% nos níveis de NGF no nervo afetado comparado ao grupo submetido ao modelo de constrição do nervo ciático para indução da DN, indicando que o NGF contribui para a

regeneração neural do nervo afetado (Da Silva *et al.*, 2015). Em nível central o tratamento por AC ou EA diminui os níveis de NGF aumentados pela DN em ratos submetidos á lesão medular (Hains *et al.*, 2005).

A proteína S100β está relacionada ao processo de dor crônica, sendo altamente específica para SNC, encontrada em células gliais, astrócito, células de Schwann e algumas populações de neurônios (Portela *et al.*, 2002). Embora o papel fisiológico da proteína S100β ainda não seja totalmente conhecido, tem sido observado níveis aumentados no sangue e no líquido céfalo raquidiano (LCR) em lesões agudas e crônicas, e esta diretamente relacionado a intensidade e a extensão das lesões no SNC (Martins *et al.*, 2006), sugerindo um papel como biomarcador de transtorno ou dano encefálico. O aumento nos níveis centrais e em soro da S100β está relacionado à diminuição do limiar nociceptivo, induzido por modelos de dor inflamatória e neuropática (Tanga *et al.*, 2006; Zanette *et al.*, 2014). Estudo prévio demonstrou que o tratamento com AC e EA diminui os níveis periféricos de S100β após lesões cerebrais em humanos (Lu *et al.*, 2010), porém não foram encontrados estudos relacionados ao efeito analgésico de AC ou EA e níveis desta proteína.

As citocinas e outros mediadores inflamatórios são capazes de alterar correntes iônicas em nociceptores alterando o limiar nociceptivo (Binshtok *et al.*, 2008) na presença de sensibilização perférica. O sistema imune, quando ativado, promove a liberação de mediadores como óxido nítrico, IL-6, TNF-α, IL-1β, e NGF (Sommer e Kress, 2004; Coutaux *et al.*, 2005), contribuindo tanto para a sensibilização periférica como para a central (Julius e Basbaum, 2001; Coutaux *et al.*, 2005; Planells-Cases *et al.*, 2005). Células gliais medulares, como microglia e astrócitos, têm um papel importante na indução e manutenção da DN. A microglia aumenta a liberação de fatores neurotróficos, e os astrócitos liberam citocinas, incluindo TNF-α, interleucina-1 beta (IL-1β) e IL-6, mediadores essenciais para indução da DN (Guo *et al.*, 2007; Gao e Ji, 2010). Assim, está bem descrito que estas citocinas, principalmente TNF-α, estão

aumentadas tanto em SNC como em SNP (Wang *et al.*, 2016). Porém pouco é conhecido dos efeitos que da DN em estruturas musculares afetadas pela constrição neural e nos níveis de TNF-α. Por outro lado, está bem estabelecido que os tratamentos com AC ou EA diminuem os níveis de citocinas inflamatórias, principalmente o TNF-α, tanto em estruturas centrais quanto perifericamente (Wang *et al.*, 2016; Geis *et al.*, 2017). No entanto, não são conhecidos os efeitos da AC e EA em estruturas musculares.

Após o mecanismo de lesão, há a liberação de aminoácidos excitatórios, estes interagem com receptores específicos, como receptores N-metil D-aspartato (NMDAR), que estão diretamente relacionados ao processo de sensibilização central, NK1 e trkB, que ativam uma cascata de segundos mensageiros, promovendo abertura de canais de cálcio, contribuindo para a ampliação do processo álgico (Ji e Strichartz, 2004). Além do aumento da excitabilidade sináptica, a atividade aumentada do NMDA no corno dorsal espinhal desempenha um papel chave no desenvolvimento de dor neuropática (Chaplan *et al.*, 1997; Chen *et al.*, 2014). Muitos estudos demonstraram que o efeito antinociceptivo da AC pode estar relacionado à alteração na expressão de receptores ionotrópicos, incluindo NMDARs, canal iônico ácido sensível (ASIC) -3, receptor de potencial transiente vaniloide (TRPV) -1, TRPV4 e canais de sódio voltagem-dependentes (Lin *et al.*, 2015; Lu *et al.*, 2016).

Outro mecanismo envolvido na indução e manutenção da condição de DN é a presença de espécies reativas de oxigênio (ROS) (Geis *et al.*, 2017). Sabe-se que ROS atuam na fisiopatologia da DN (Kim, J. H. *et al.*, 2004; Yowtak *et al.*, 2011). Um modelo animal de lesão do nervo, a ligadura do nervo espinhal (SNL), causou aumento de estresse oxidativo evidenciado por alterações na peroxidação lipídica e na concentração de nitrito, GSH, SOD e catalase (Pottabathini *et al.*, 2015). O diacetato de diclorofluoresceína (DCFH) é um marcador direto de ROS e seu aumento é observado em condições de atrofia muscular (Powers *et al.*, 2007). Um dos possíveis mecanismos de ação da AC e EA está relacionado à diminuição de marcadores de ROS

em SNC e SNP. Choi e colaboradores sugerem que o tratamento com acupuntura diminuiu os níveis elevados de ROS em um modelo animal de DN (Choi *et al.*, 2012).

III. JUSTIFICATIVA

3. **JUSTIFICATIVA**

Dor neuropática é um quadro de dor crônica, altamente prevalente, debilitante, que afeta diretamente a qualidade de vida dos pacientes. Apesar de existirem diversos fármacos para seu tratamento, estes apresentam falta de eficácia e muitos efeitos adversos dificultando a adesão ao tratamento. Desta forma, a busca por alternativas terapêuticas de tratamento é de extrema importância. Dentre elas, a AC e EA apresentam-se como técnicas promissoras que tem demonstrado bons resultados no tratamento da dor neuropática. Apesar destas técnicas serem bastante utilizadas, permanecem dúvidas sobre seus mecanismos de ação, aumentando a necessidade de estudos em modelos animais que possam melhor elucidar estes mecanismos. As técnicas de AC e EA são de difícil aplicação em animais; a maioria dos estudos utiliza anestésicos que podem influenciar o efeito do tratamento. Considerando a importância do entendimento dos mecanismos envolvidos na dor neuropática e no seu tratamento com AC e EA, e acreditando-se que estes tratamentos podem reverter quadros de hiperalgesia e alodinia, a utilização de modelos animais para indução de dor neuropática podem elucidar possíveis alterações neuroquímicas e comportamentais provocadas pelo quadro de dor neuropática e o efeito dos tratamentos de AC e EA nestes parâmetros, permitirá melhor compreender estes parâmetros. Desta forma, espera-se que a melhor compreensão destas terapêuticas e dos mecanismos neuroquímicos envolvidos na DN, auxilie o tratamento de pacientes com quadros de dor, aumentando o espectro clínico por meio de abordagens translacionais. Resumidamente, a realização desta pesquisa contribuiu para um melhor entendimento dos mecanismos de ação da AC e EA na reversão da DN, aumentando seu entendimento e utilização para casos específicos de dores crônicas.

IV. OBJETIVOS

4.1 Objetivo Geral

Investigar o efeito do isoflurano na aplicação repetida de AC e EA, em parâmetros comportamentais e neuroquímicos de ratos submetidos a um modelo de dor neuropática; e comparar os efeitos da AC e EA em modelo animal de dor neuropática nas respostas comportamentais, bioquímicas e histológicas.

4.2 Objetivos Específicos

Avaliar o efeito de 8 sessões diárias de 20 minutos de AC ou EA em modelo animal de dor neuropática.

Artigo I

- ✓ Avaliar a resposta nociceptiva de alodinia mecânica;
- ✓ Avaliar dano neuronal periférico e central (NGF e S100β);
- ✓ Comparar a utilização ou não do anestésico durante o tratamento;

Artigo II

- ✓ Avaliar a resposta comportamental exploratória e exploratória;
- ✓ Avaliar NMDA em tronco encefálico e medula espinhal;
- ✓ Comparar a utilização ou não do anestésico durante o tratamento;

Artigo II

- ✓ Avaliar a resposta hiperalgésica térmica;
- ✓ Avaliar a resposta nociceptiva a estímulo mecânico;
- ✓ Avaliar histologia e imunohistoquímica de citocina inflamatória;
- ✓ Avaliar marcador de estresse oxidativo em tecido muscular;

V. REFERÊNCIAS DA REVISÃO DA LITERATURA

REFERÊNCIAS DA REVISÃO DA LITERATURA

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VI. ARTIGOS CIENTÍFICOS

6.1 Artigo 1

Isoflurane enhances the analgesic effect of acupuncture and electroacupuncture on neuropathic pain in rats

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Abstract

Introduction: Due to the difficulty of applying acupuncture (Ac) and electroacupuncture (EA) to the treatment of neuropathic pain (NP) in awake and freely moving rats, most studies use restraint or anesthesia. However, both conditions could be a potential source of bias. The purpose of the present study was to determine whether isoflurane interferes with the analgesic effects of Ac and EA using an NP rat model. We also investigated the effect of isoflurane on the levels of S100β protein in serum and nerve growth factor (NGF) in the left sciatic nerve of the animals. Methods: Using 140 male Wistar rats, we evaluated the nociceptive response induced by isoflurane using the von Frey test, serum levels of S100β, and NGFlevelsinthe left sciatic nerve. NP was induced by constriction of the left sciatic nerve. The treatments, with or without isoflurane anesthesia, started 14days after surgery (20min/day/8days). The von Frey test was performed at baseline, 14 days postoperatively, and immediately, 24h and 48h after the last treatment session. Animals were killed by decapitation. Serum and constricted nerves were collected and frozen at -80^{o}C. Generalized estimating equations/Bonferroni were used to analyze the results of the nociceptive test and three-way analysis of variance/SNK or Fisher's LSD test was used to analyze the results of the biochemical analysis. Results were considered significant if $P \leq 0.05$. Results: At baseline, there were no differences in the nociceptive response threshold among all groups. Fourteen days after surgery, the groups with NP had a decreased pain threshold compared to the other groups, showing that NP was established ($P<0.05$). Ac and EA enhanced the mechanical pain threshold immediately after the last session in the groups with NP and without anesthesia. In addition, when all groups received isoflurane, the nociceptive threshold significantly increased ($P<0.001$). These results were maintained for 48h after the last treatment session. There was an interaction between the independent variables: pain x treatments x isoflurane in serum S100β levels ($P<0.001$) and NGF levels in the left sciatic nerve ($P<0.001$).

Conclusion: Isoflurane enhanced the analgesic effects of Ac and EA and altered serum S100β levels and NGF levels in the left sciatic nerve in rats with NP.

Keywords: Neuropathic pain, Acupuncture, Electroacupuncture, Isoflurane.

1. Introduction

Acupuncture (Ac) and electroacupuncture (EA) treatments have yielded good results when used to alleviate pain in humans and experimental animals with neuropathic pain (NP)[1,2]. Although these techniques have been recognized as suitable for analgesia, the exact mechanisms of action are unknown. Moreover, due to the difficult application of Ac and EA treatments to awake and freely moving animals, most studies have used restraint[3] or anesthesia[4]. However, anesthetic administration and animal immobilization during treatment could be a potential source of bias as physiological changes may occur, thus having detrimental effects on the efficacy of the techniques.

The evaluation of Ac and EA may be biased by restraint stress or habituation in conscious animals[5] or by the anesthetics used in sedated animals. The application of Ac and EA treatments to awake animals may be viewed as a stressor, i.e., the simple insertion of a needle and the manual or electric stimulation of animals may be harmful. Restraint, shock and even fear are known to trigger stress-induced analgesia when animals are awake, as shown in models of Ac and EA analgesia[6]. Therefore, Ac and EA analgesia can be significantly reduced if concomitant stressors are not adequately controlled.

In animal studies utilizing Ac or EA, isoflurane is the most commonly used anesthetic[4].It offers an easy route of administration and is able to maintain the behavioral and physiological characteristics of general anesthesia without an adjunct[7]. However, whether it can alter the analgesic response to Ac or EA treatment is unknown. Interestingly, anesthesia can influence the interpretation of the experimental results of biomarkers. Mice exposed to isoflurane during postnatal brain development had increased serum levels of S100β, a protein used as a neurodegenerative biomarker, which could be associated with brain damage[8]. However, no study has demonstrated the same results in adult rats.

Neurotrophins also have an important role in neuronal survival, growth, and differentiation and may be affected by isoflurane as well. Nerve growth factor (NGF) is a pain-related neurotrophin that can exert pro- or antinociceptive effects, depending on concentration and site of administration[9]. Most importantly, Chen et al. demonstrated the neuroprotective effect of neurotrophins by EA in an animal model of spinal cord injury[10], indicating that a possible mechanism by which EA reduces pain is trough neurotrophic modulation.

Based on these findings, we believe that isoflurane is able to potentiate the analgesic effect of Ac and EA treatments and modify neuromodulation parameters. To test this hypothesis, we evaluated the nociceptive response induced by isoflurane, using the von Frey test, the serum levels of S100β and NGF levels in the left sciatic nerve of NP ratstreated with Ac or EA. Concurrently, we assessed locomotor behavior to demonstrate the extent to which the animals were affected by anesthesia.

2. Methods

2.1 Animals

Initially, 140 male Wistar rats (weight ≥250 g) aged 55–65 days were used in the experiment. Based on previous studies from our research group, 140 was the number of animals necessary to produce reliable scientific data[11; 12; 13].All animals were housed individually in polypropylene cages (49 × 34 × 16 cm) and maintained in a controlled environment (22 ± 2 °C) under a standard light–dark cycle (lights-on at 0700 h and lights-off at 1900 h), with free access to water and chow (Nuvital, Porto Alegre, Brazil). All experimental procedures were approved by the Institutional Committee for Animal Care and Use (GPPG-HCPA protocol no. 13-0298) and conformed to the Guide for the Care and Use of Laboratory Animals (8th ed., 2011). Animal maintenance followed the Brazilian Law 11794, which establishes the procedures for the use of

animals in scientific research. The experimental protocol complied with the ethical and methodological standards of the ARRIVE guidelines[14].

2.2 Neuropathic pain (NP) model: chronic constriction injury (CCI) of the sciatic nerve

The CCI of the sciatic nerve was performed as described by Bennett and Xie[15] to induce NP. The animals were anesthetized with isoflurane (5% for induction, 2.5% for maintenance), and the surgical site was shaved. The skin was antiseptically cleaned with 2% alcoholic iodine[15]. The left sciatic nerve was approached in the mid-thigh by removing part of the biceps femoris muscle. Three ligatures (4-0 Vicryl) were tied 1mm apart, close to the sciatic trifurcation. Ligation was tightened until muscle contraction of the leg could be observed, ensuring epineural blood flow. The same investigator performed the ligatures in all animals. In the sham-surgery groups, animals were anesthetized and the left sciatic nerve was exposed, but not constricted. The control groups did not undergo any surgical procedure.

2.3 Acupuncture (Ac)

Two stainless steel Ac needles with guide tubes (Suzhou Huanqiu Acupuncture Medical Appliance Co. Ltd., 218, China), measuring 0.18mm in length and 0.8 mm in diameter, were used. The needles were insertedapproximately 2–3 mm into the acupoint BL-24, which is located on the side depression of the lower edge of the L3 spinous process of the third lumbar vertebra (Fig.1)

----------*Insert Figure 1*----------

2.4 Electroacupuncture (EA)

The Ac needles were connected to an electrostimulation device (Model EL 608 NKL, Brusque, SC, Brazil) with an alternating frequency of 2 Hz and 100 Hz (2/10 Hz, 0.3 ms width) for 20 min. Procedures were performed in rats that were either freely moving or anesthetized with isoflurane (under oxygen flow; 2% for induction, 0.5% for maintenance).

2.5 von Frey test

Twenty-four hours prior to the test, the rats were acclimated to the apparatus for a period of 5 min. On the day of the test, the rats were placed on the analgesiometer, and the sensor containment box was positioned next to the paw, guided by the reflection in the mirror. The test was based on the maximum pressure (grams) required for the animal to show sensitivity to touch on the paw[16]. Three measurements were performedand the mean value was considered the pain threshold. Measurements were made at baseline, 14 days postoperatively, and immediately, 24h and 48h after the last treatment session. The same trained investigator, who was blinded to the treatment groups, performed all tests. All tests were performed in awake and freely moving animals, without any sedation.

2.6 Open field test

Behavioral assessment was performed 24h after the sixth session and before the seventh session to determine the chronic effect of six sessions of Ac and EA with and without anesthesia, avoiding the acute effect. It was performed in a 60 × 40 × 50 cmcage with the floor divided by lines into 12 squares of 13 × 13 cm each.Each test started immediately after the animals were placed in the back left corner and allowed to explore the surroundings for 5 min[17; 18].The number

ofline crossings (all paws crossing the boundary into an adjacentmarked-out area) was taken as a measure of locomotor activity[19].

2.7 Experimental design

The animals were acclimated to the study environment for 2 weeks before the beginning of the experiment, after which they were randomly allocated into one of the 14 following groups: Control (C), Sham surgery (Ss), Sham surgery+Ac (SsAc), Sham surgery+EA (SsEA), NP (Np), NP+Ac (NpAc), NP+EA (NpEA), Control+Anesthesia (CAn), Sham surgery+Anesthesia (SsAn), Sham surgery+Ac+Anesthesia (SsAcAn), Sham surgery+EA+Anesthesia (SsEAAn), NP+Anesthesia (NpAn), NP+Ac+Anesthesia (NpAcAn), and NP+EA+Anesthesia (NpEAAn)(Fig.2). Subsequently, the Np and Ss groups received their respective interventions (CCI or sham surgery). Fourteen days later, the von Frey test was performed to evaluate hyperalgesia in order to confirm the establishment of NP. Then, animals were treated for 8 days according to the specific protocol for each group (Ac, EA, or no treatment). The von Frey test was performed at baseline, 14 days postoperatively, and immediately, 24h and 48h after the last treatment session.

---------- Insert Figure 2 ----------

2.8 S100β measurement in blood serum

Animals were killed by decapitation 24 hours after the treatment ends. Blood serum was collected and frozen at -80°C until the time of testing. Serum levels of S100β were measured by a competitive enzyme-linked immunosorbent assay (ELISA) kit (MyBiosource, California, USA), according to manufacturer's instructions.

2.9 NGF measurement in the left sciatic nerve

The nerve was removed and frozen at -80°C until the time of testing. NGF levels were determined by a sandwich ELISA using monoclonal antibodies specific for each measurement (R&D Systems, Minneapolis, United States). Total protein was measured by Bradford's method using bovine serum albumin as a standard.

2.10 Statistical analysis

Data were expressed as mean ± standard error of the mean (SEM). Generalized estimating equations (GEE) followed by Bonferroni test were performed to analyze the results of nociception. The biochemical and open-field data of all groups were compared using a three-way analysis of variance (ANOVA) followed by Student-Newman-Keuls (SNK) test or Fisher's least significant difference (LSD) test. The results were reported as mean ± SEM and considered significant if $P \leq 0.05$. SPSS version 20.0 for Windows was used for all statistical analyses.

3. Results

3.1 von Frey test showed that isoflurane potentiates analgesia induced by Ac and EA

GEE showed a significant time×treatments×interaction (χ^2=1419.33; 52; P<0.001)(Fig. 3). At baseline, all groups had a pain threshold similar to that of the control group ($P \geq 0.05$). Fourteen days postoperatively, the pain threshold in pain groups was different from that in the control and sham groups, confirming the establishment of NP (P<0.001). Immediately after the last treatment session (21 days after surgery), both treatments (Ac and EA) enhanced the mechanical pain threshold of animals exposed to the NP model (NpAc and NpEA groups), but these results did not differ from those of animals exposed to the NP model without treatment (Np and NpAn groups) (P>0.05). Conversely, when all groups received isoflurane anesthesia, the increase in pain threshold was significantly different between the Np and NpAn groups

(P<0.001). This result was maintained 24h and 48h after the last treatment session (22 and 23 days after surgery).

---------- *Insert Figure 3* ----------

3.2 Isoflurane decreased locomotor activity

The analysis of the open field test showed no interactions between the independent variables: pain×treatments×anesthesia (three-way ANOVA/SNK, P>0.05). However, there was an anesthesia×treatments interaction (three-way ANOVA/SNK, $F_{(2.125)}$=3.03; P=0.052). Significant effects of anesthesia were observed (ANOVA/SNK, $F_{(1.125)}$=13.92; P<0.01). The results show that Ac and EA induced an increase in locomotor activity, but anesthesia was able to reverse this effect (Fig. 4).

---------- *Insert Figure 4* ----------

3.3 Isoflurane altered serum S100β levels

The analyses showed interactions between the independent variables: pain×treatments×anesthesia (three-way ANOVA/SNK, $F_{(2.68)}$=4.21; P<0.05), pain×treatments (three-way ANOVA/SNK, $F_{(2.68)}$=6.57; P<0.05), and pain×anesthesia (three-way ANOVA/SNK, $F_{(2.68)}$=8.05; P<0.05). These results indicate that animals exposed to both pain and treatment had an increase in S100β levels, while animals exposed to both pain and anesthesia had a decrease in S100β levels. Significant effects of anesthesia (three-way ANOVA/SNK, $F_{(1.68)}$=34.96; P<0.01) and treatments (three-way ANOVA/SNK, $F_{(2.68)}$=6.158; P<0.01) were also observed, with a decrease in serum S100β levels in animals exposed to anesthesia compared to those in groups

without anesthesia. Conversely, EA treatment induced an increase in serum S100β levels, while Ac treatment did not affect the levels of S100β (Fig. 5).

---------- *Insert Figure 5* ----------

3.3 Isoflurane altered NGF levels in the affected nerve

The analyses showed interactions between the independent variables: pain×treatments×anesthesia (three-way ANOVA/LSD, $F_{(2.116)}$=9.47; P<0.05). Significant independent effects of anesthesia (three-way ANOVA/LSD, $F_{(1.116)}$=30.33; P<0.05), pain (three-way ANOVA/LSD, $F_{(1.126)}$=101.15;P<0.05) and treatments (three-way ANOVA/LSD, $F_{(2.116)}$=6.54; P<0.05) were observed. Animals receiving isoflurane had decreased NGF levels, similar to those found in animals in the pain model. Conversely, animals that received EA treatment had increased NGF levels in the affected nerve (three-way ANOVA/LSD, P<0.05)(Fig. 6).

---------- *Insert Figure 6* ----------

4. Discussion

In the present study, we demonstrated that repeated exposure to isoflurane enhances the analgesic effect of treatment with Ac or EA. However, isoflurane alone does not have an analgesic effect. It is important to note that, although the use of needles in conscious rats is an important translational step, it can complicate the interpretation of the results. It is known that animals become agitated during Ac and, not rarely, the needle is inserted outside the acupoint. In addition, needle insertion and electric stimulation can be considered painful or uncomfortable stimuli[20], which could interfere with the analgesic response to Ac or EA. It is well established

that Ac has a segmental and extrasegmental response and even central actions[21]. Therefore, when applied once, this stimulus can act as an acute stressor leading to an analgesic response. However, when applied several times, it could act as a chronic stressor[22], thus altering the analgesic effect of Ac or EA.

Stress has a direct influence on pain sensitivity. Acute stress reduces pain sensitivity, which is known as stress-induced analgesia and is probably mediated by brainstem modulation. In contrast, chronic stress increases pain sensitivity, inducing hyperalgesia[12; 23]and allodynia[12; 23]. This relationship between stress and pain sensitivity probably occurs due to activation of the hypothalamic-pituitary-adrenal (HPA) axis, leading to glucocorticoid release, which can alter the serotonergic and dopaminergic systems involved in the nociceptive response[24]. Previous data from our group suggest the involvement of the opioid system in the hyperalgesic response induced by repeated restraint stress[12]. Conversely, it is known that the Ac and EA analgesia pathway is also related to the opioid system[25]. Therefore, we can suggest that chronic stress caused by needle insertion and electric current leads to a decrease in the analgesic effects of Ac and EA in awake rats, since theyare most likely to have reverse actions in the opioid system.

The effects of inhaled anesthetics have been previously evaluated in Ac studies with rats[20,26]. Wen et al.[20]evaluated the role of reducing the stress induced by EA and found that 0.5% halothane, at a subanesthetic minimumalveolar concentration (sub-MAC dose), reduces the influence of stress during EA, allowing strong needle stimulation and rapid recovery of the animal. Kung et al.[26] assessed the use of isoflurane and showed that 0.75% isoflurane (sub-MAC dose) is also an effective anesthetic dose for EA studies. However, an important issue regarding the use of anesthetics in animal models is motor impairment resulting from anesthetic effects. Antognini et al.[27] showed that the MAC of isoflurane (0.6-0.9%) is able to preserve the integrity of the motor system in rats receiving this anesthetic. Silva et al. 2010 [28], in a study evaluating

the effects of EA on the role of the anterior pretectal nucleus, used isoflurane at the same concentrations used in the present study for induction and maintenance of anesthesia (2% and 0.5% respectively) and, likewise, found no motor impairment in the animals.

Inhaled anesthetics hyperpolarize neuronal membranes and inhibit C-fiber latency, reducing neuronal excitability. However, isoflurane at sub-MAC doses has no action on the wind-up phenomenon, which is characterized by temporal summation. Thus, it is a good anesthetic candidate for the evaluation of the antinociceptive effects of Ac and EA[20; 29]. Moreover, isoflurane alone at sub-MAC doses does not influence motor or nociceptive responses. It is important to highlight that, in the present study, behavioral tests were performed in awake animals that had completely recovered from anesthesia.

In the open field test, all animals were able to move, indicating that they were not sedated. However, there was a significant effect of anesthesia on treatment, i.e., Ac induced an increase in locomotor activity, but anesthesia was able to reverse this effect. It is important to note that, in the present study, the anesthetic effect on locomotion was dependent on the animal state. In animals with increased locomotion, isoflurane acted to decrease it, while in sham animals, isoflurane had no significant effect on locomotion. In addition, isoflurane was used for 6 consecutive days before the test, and it can be speculated that the observed effect is the result of repeated exposure to the drug. We may suggest that isoflurane decreases locomotion and neuronal excitability by inhibiting glutamate release[30]. It is known that N-methyl-D-aspartate receptor (NMDAr), a glutamate receptor, is related to locomotoractivity[31] and nociceptionresponse[32]. We may also suggest a protective effect of isoflurane specific to animals submitted to the NP model. Another interesting aspect of this process is the increase in locomotion induced by Ac. This effect was observed in animals in the Ss and Np groups. However, after isoflurane administration, this effect was reversed, indicating an interaction of isoflurane and Ac. Manual Ac has been shown to increase dopamine release, improving

locomotor function in mice[33]. Dopamine plays an important role in peripheral and central neurons, including substantia nigra, hypothalamus, midbrain, and ventral tegmental area[34]. In the present study, rats in the NpEaAn group showed greater locomotor activity than those in the NpAn and NpAcAn groups. This improvement may be due to stimulation that reaches the motor threshold, maintaining skeletal muscle integrity in the NP model and improving locomotion. Another hypothesis is related to the increase of monoamines in the brain, which has been shown to block anesthetic action and increase locomotor activity inrabbits[35]. However, further studies are necessary to clarify this Ac effect on locomotion.

Another important finding was that the interaction of pain, treatments and anesthesia changed serum S100βlevels. It is known that chronic pain, such as fibromyalgia, correlates with higher serum S100β and BDNF levels[36], but this result was not found in our study. Some studies suggest that rats exposed to isoflurane during postnatal brain development have increased serum S100β levels[8], which could be related to brain damage. Nevertheless, according to the presentresults, isoflurane seems to prevent the increase in S100β levels in rats with NP treated with EA, at least in adult males. Although Ac and EA are known to reduce S100β levels after neural injuries[37], our results showed the opposite effect. Since the animals underwent treatment while awake, this mimicked a chronic stress condition and led to an increase in S100β levels, which is consistent with data from the literature[38]. Considering that the NpEA group had the highest serum levels of S100β, our data suggest that animals submitted to NP and EA without isoflurane show increased stress levels due to NP induction and electric stimulation while awake. The NpAc and Np groups had different results, indicating the occurrence of an electric stimulation effect. The NpEAAn group had S100β levels equal to those of the control group, indicating a protective effect of isoflurane anesthesia by maintaining serum S100β levels similar to those of controls. In the same vein, Garcia-Sanchez et al. (1993) [39] showed that isoflurane

anesthesia increases plasma beta-endorphin after surgery,supporting the present study in that it suggests a protective effect of isoflurane against stress.

Isoflurane administration, NP model and Ac and EA treatments also altered NGF levels in the left sciatic nerve. When the C and CAn groups were compared, isoflurane clearly decreased NGF levels in the left sciatic nerve. NP also decreased NGF levels, suggesting that isoflurane modifies the effect of Ac and EA in the left sciatic nerve (injured nerve). Although the groups receiving Ac or EA showed an increase in NGF levels, post-hoc tests showed that this effect was due to EA treatment, but not Ac treatment. Despite the extensive literature on the topic, there is still no consensus on the role of NGF in NP. It has been suggested that NGF, a neurotrophin involved in the growth, maintenance and apoptosis of neurons[40], is decreased in sensory neurons of the dorsal root ganglion (DRG) and of the spinal dorsal horn in diabetic NP models[41]. In contrast, Watson et al. (2008) [43] found increased NGF expression in an NP model[42]. This finding suggests that, after nerve injury, many substances, including NGF, are released mainly by the astrocytes of the central nervous system (CNS). In the dorsal horn of the spinal cord, increased NGF levels are associated with central sensitization[44], leading to NP. Similarly, increased NGF levels in the CNS[45] have been associated with an increase in pain. However, some studies suggest that the increase in NGF levelsin the peripheral nervous systemis associated with NP[46]. In contrast, another study suggests that neural mobilization increases the level of NGF in injured nerves, promoting nerve regeneration and reducing painful symptoms[47].

In the present study, treatment with EA induced an increase in the level of NGF, although a previous study showed that Ac and EA in NP decreased the level of NGF, which was associated with decreased hyperalgesia[48]. Nevertheless, studies have shown a 52% increase in NGF levels after CCI and neural mobilization in rats, indicating that NGF contributes to the regeneration of the sciatic nerve after CCI[47]. Considering this later study, the data from the present study suggest that the decrease in NGF levels in the injured nerve after NP was due to

reduced nerve regeneration involving pain. Although isoflurane also reduces pain, EA promoted an increase in the levels of this neurotrophic factor.

In conclusion, our data suggest that isoflurane administered during Ac or EA treatment in an animal model of NP decreases allodynia as determined by the von Frey test. Similarly, isoflurane prevents the increase in serum S100β levels in rats with NP treated with EA while awake and, most likely, reduces the harmful effects of chronic stress exposure. Conversely, inhaled anesthesia decreased NGF levels in the left sciatic nerve, while EA increased the levels of this neurotrophic factor. These results suggest that these effects are related to nerve regeneration rather than to increased analgesia.

Acknowledgements

This research was supported by the following Brazilian funding agencies: National Council for Scientific and Technological Development – CNPq (Dr. ILS Torres; Dr. W Caumo; Dr. VLScarabelot) and Edital Universal 475422/2013-9 (Dr. Rafael Vercelino); Brazilian Federal Agency for Support and Evaluation of Graduate Education – CAPES (LNSAdachi; Dr. LF Medeiros); CAPES/PNPD Edital PPGCR 03/2016 (Dr. R Vercelino); CAPES/PNPD Edital PPGCM 07/2016 (Dr. C de Oliveira); Graduate Research Group of Hospital de Clínicas de Porto Alegre – GPPG (Dr ILS Torres – Grant 130298).

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Figure legends

Figure 1. Acupoint

Description of the acupoint BL-24 in the rat.

Figure 2. Groups

Description of the procedures performed in the animals in each group.

Figure 3.von Frey test

Data are expressed as the mean±standard error of the mean (SEM) ofpain threshold in grams

GEE statistics showing atime×treatments×interaction (χ^2=1419.33; 52; N = 8-10 animals per group).

*Significantlydifferent from Control and Sham groups. # Significantly different from Np and NpAn groups.

Figure 4. Open field test

Data are expressed as the mean±standard error of the mean (SEM) of total number of crossings. There wasan anesthesia×treatments interaction (three-way ANOVA/SNK, $F_{(2.165)}$=3.03; P=0.05), with a significant effect of anesthesia on treatments(ANOVA/SNK, $F_{(1.125)}$=13.92; P<0.01) (N = 8-10 animals per group).

Figure 5. S100β levels in bloodserum

Data are expressed as the mean±standard error of the mean (SEM) of serum S100β levels in pg/mL. There were interactions of pain×treatments×anesthesia (three-way ANOVA/SNK, $F_{(2.68)}$=4.21; P<0.05), pain×treatments (three-way ANOVA/SNK, $F_{(2.68)}$=6.57; P<0.05), and pain×anesthesia (three-way ANOVA/SNK, $F_{(2.68)}$=8.05; P<0.05) (N= 5-7 animals per group).

Figure 6. NGF levels in the left sciatic nerve

Data areexpressed as the mean±standard error of the mean (SEM) ofleft sciatic nerve NGF levels in pg/mL. There were interactions between the independent variables: pain×treatments×anesthesia (three-way ANOVA/LSD, $F_{(2.116)}$=9.47; P<0.05). There were significant independent effects of anesthesia (three-way ANOVA/LSD, $F_{(1.116)}$=30.33; P<0.05), pain (three-way ANOVA/LSD, $F_{(1.126)}$=101.15; P<0.05), and treatments (three-way ANOVA/LSD, $F_{(2.116)}$=6.54; P<0.05) (N= 5-7 animals per group).

Figures
Figure 1

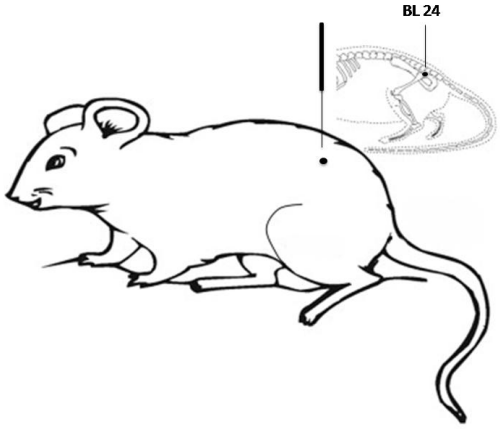
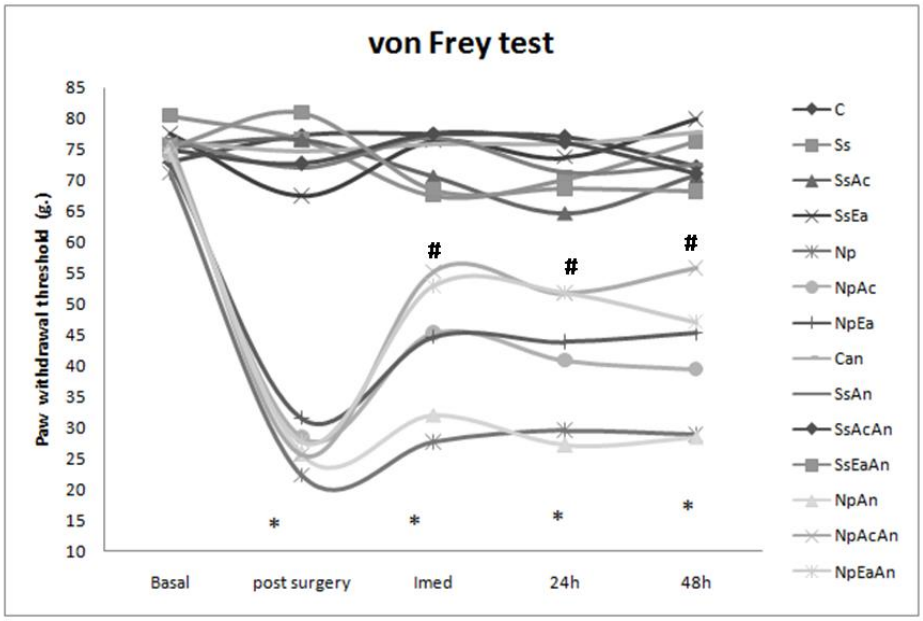


Figure 2**.**

Groups	
Control (C)	No manipulation
Sham Surgery (Ss)	surgery without nerve constriction
Sham Surgery + AC (SsAC)	surgery without nerve constriction+ 8 sessions of acupuncture
Sham Surgery + EA (SsEA)	surgery without nerve constriction+ 8 sessions of eletroacupuncture
Neuropathic Pain (Np)	surgery with nerve constriction
NeuropathicPain + AC (NpAC)	surgery with nerve constriction+ 8 sessions of acupuncture freely movements
NeuropathicPain + EA (NpEA)	surgery with nerve constriction+ 8 sessions of eletroacupuncture freely movements
Control+Anesthesia (CAn)	No manipulation + 8 days of isoflurane
ShamSurgery+ Anesthesia (SsAn)	surgery without nerve constriction+ 8 days of isoflurane anesthesia
Sham Surgery + AC+ Anesthesia (SsACAn),	surgery without nerve constriction+8 sessions of acupuncture with isoflurane anesthesia
Sham Surgery + EA+ Anesthesia (SsEAAn)	surgery without nerve constriction+8 sessions of eletroacupuncture with isoflurane anesthesia
Neuropathic Pain+ Anesthesia (NpAn)	surgery with nerve constriction+ 8 days of isoflurane anesthesia
Neuropathic Pain + AC+ Anesthesia (NpACAn)	surgery with nerve constriction+8 sessions of acupuncture with isoflurane anesthesia
Neuropathic Pain + EA+ Anesthesia (NpEAAn)	surgery with nerve constriction+8 sessions of eletroacupuncture with isoflurane anesthesia

Figure 3.



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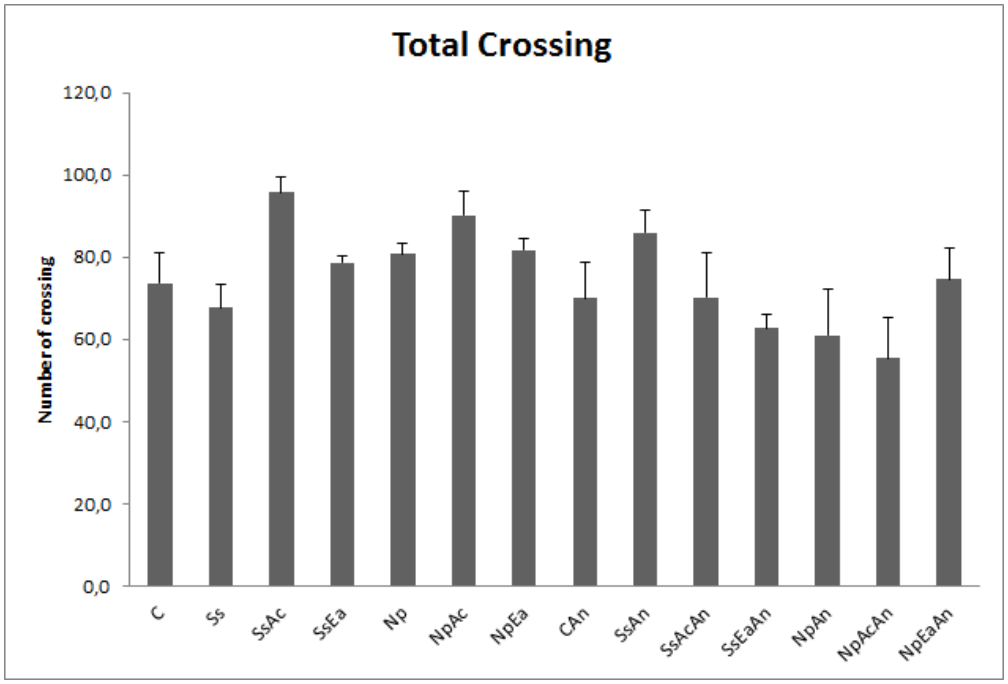

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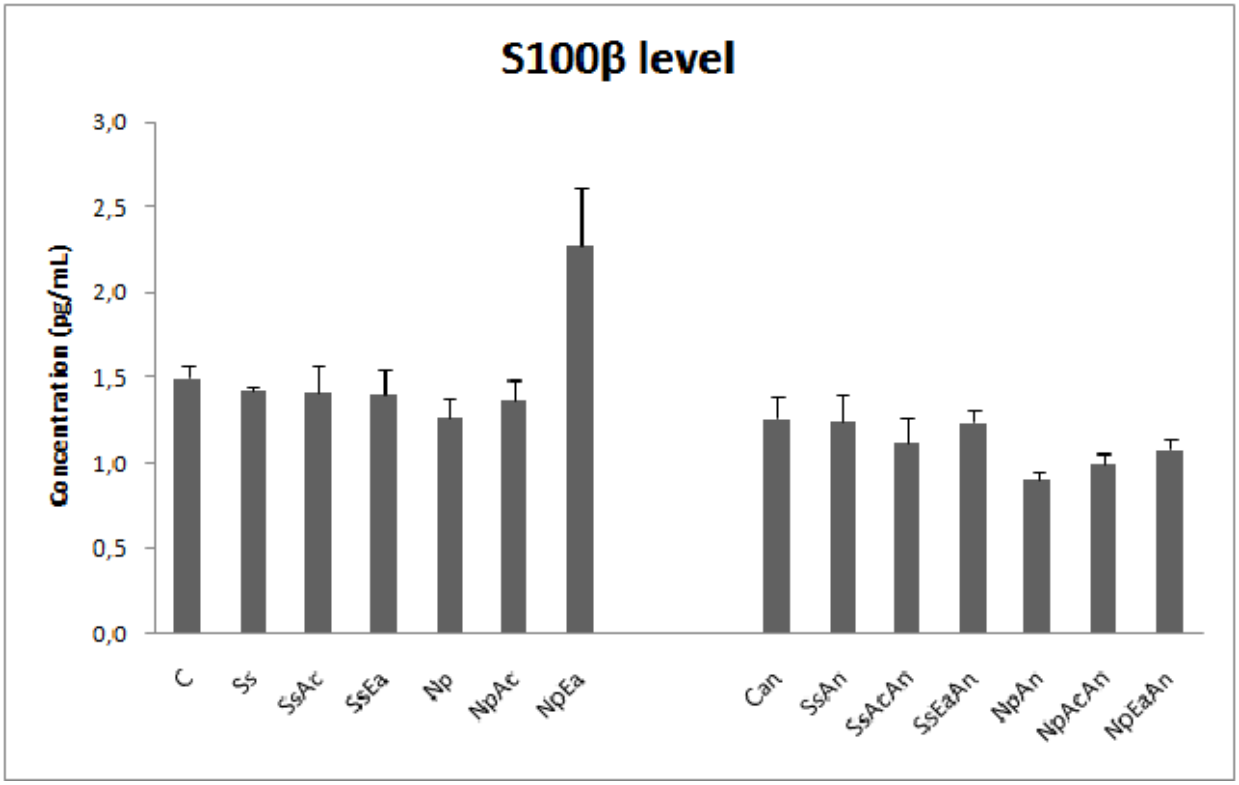
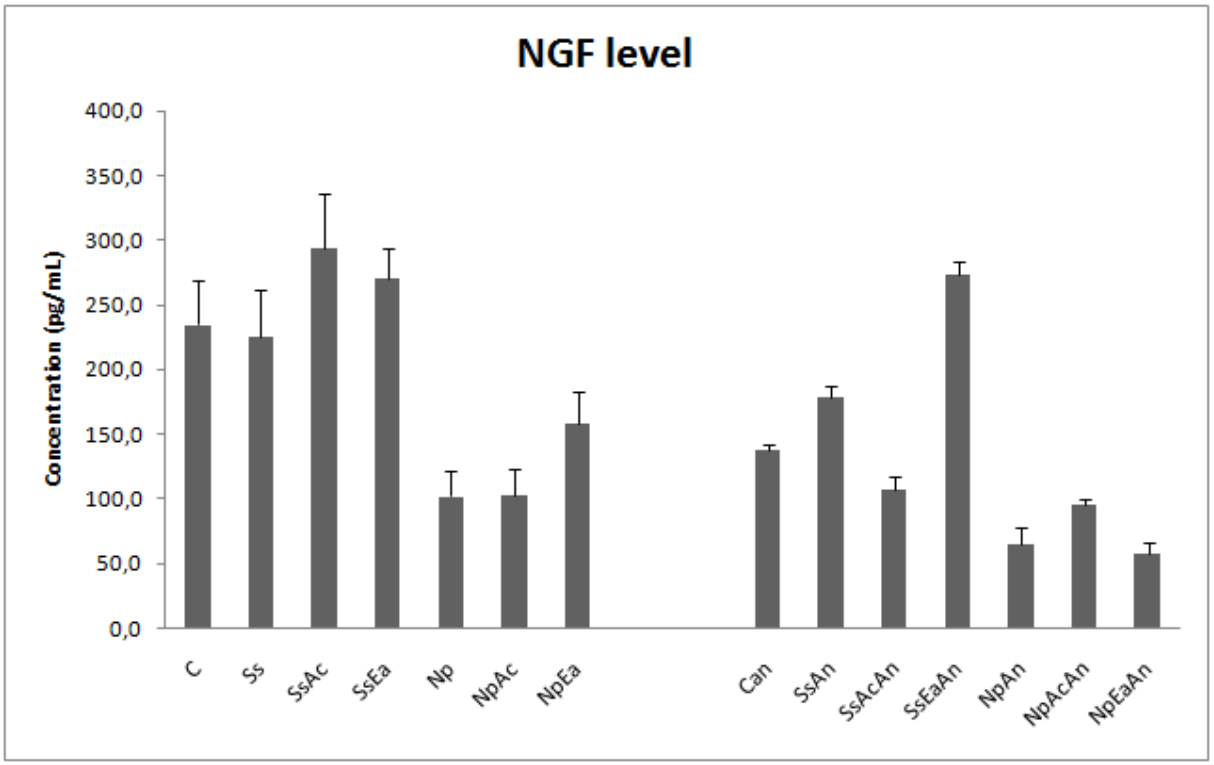


Figure 6.



6.2 Artigo 2

Isoflurane alters effects of Acupuncture and Eletroacupuncture upon locomotor activity and NMDA central levels in neuropathic pain rat model

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Abstract

Background: Acupuncture(AC) and eletroacupuncture(EA) are techniques widely used on neuropathic pain(NP) treatments. Pre-clinical studies are necessary to know the mechanisms involved, however most use restraint or isoflurane anesthesia. We hypothesize that isoflurane can alter the effect of AC and EA treatments in the locomotor activity and NMDAr concentration in central nervous system (CNS) in rats submitted to a NP model. Methods: 140 male Wistar rats, we evaluated locomotor activity using the Open Field test (OFT) and actigraphy in homecages; NMDAr level in brainstem and spinal cord. NP was induced by constricting the sciatic nerve. The treatments, with or without anesthesia using isoflurane, started 14days after surgery (20min/day/8days). The OFT was conducted 24h after the 6^{th} session, to avoid the acute effect of isoflurane; the actigraphy was measured since the beginig of the treatment until the end. Animals were put down by decapitation. Structures of CNS were collected and frozen at -80^{o}C. For statistical analysis, three-way ANOVA/SNK were conducted on the results of OFT and on biochemical analysis. Kruskal-Wallis or Mann-Whitney were performed to analyze actigraphy data. The results were considered significant when $P \leq 0.05$.Results: On OFT, AC and EA increase exploratory behaviors ($P<0.05$), however isoflurane and NP decreases it. On actigraphy, locomotion was decreased by NP and increased by isoflurane on light and dark cycles ($P<0.05$). Isoflurane also decreases NMDAr level on brainstem and on spinal cord there was an interaction between anesthesia×treatment ($P<0.05$). Conclusion: Isoflurane altered the effect of AC and EA on locomotion and NMDAr level on rats submitted to NP.

Keywords: Neuropathic pain, Acupuncture, Electroacupuncture, Isoflurane, NMDA

1. Introduction

Acupuncture (AC) and eletroacupuncture (EA) are techniques from Traditional Chinese Medicine and have been applied in Asian countries for thousands of years. Both treatments demonstrated good results in many diseases, including acute and chronic pain, as neuropathic pain (Wang *et al.*, 2016). In the last years, AC and EA analgesia have been gradually accepted due to its advantages; however its mechanism has not yet been totally described. To clarify the mechanisms underlie the treatments pre-clinical researches have great importance. Moreover, the application of these treatments in animals is known to cause some bias. The application of AC or EA in animals that are awake can serve as a stressor, because the simple insertion of the needle and the manual or electric stimulation can be harmful. Other options on literature are utilize restraint (Tu *et al.*, 2012) or anesthesia (Cha *et al.*, 2012). It is known that restraint can trigger stress-induced analgesia when applied acutely (De Medeiros *et al.*, 2003), and stress-induced hyperalgesia when applied chronically (Spezia Adachi *et al.*, 2012). However, the administration of anesthetics during Ac or EA application can be a source of bias by physiological changes inducing detrimental effects towards the efficacy of these techniques. Generally, the isoflurane is the anesthetic used in AC or EA animal studies (Cha *et al.*, 2012). Isoflurane is a inhalator anesthetic with easy route of administration, which maintains, alone, behavioral and physiological characteristics of general anesthesia (Purdon *et al.*, 2015). However, However, it is not know whether isoflurane can alter the treatment response of AC or EA.

Neuropathic pain (NP) is a chronic pain condition that causes suffering and decrease quality of life. Its treatment remains slightly effective, related to adverse effects, which increases the importance of research new therapies. The NP is characterized by central sensitization, and dysfunction of glutamatergic and opioid system (Rigo *et al.*, 2017). Peripheral nerve injury increases spinal N-methyl D-aspartate receptor (NMDAR) activity, and this increase is directly

related to the development of NP (Chen *et al.*, 2014); (Li *et al.*, 2017). The analgesic effect of acupuncture also is associated to changes in expression of many ionotropic or voltage gated receptor channels including NMDARs (Nagy *et al.*, 2004). It is known that volatile anesthetics, as isoflurane, augment the activity of inhibitory glycine receptors, and they inhibit postsynaptic AMPA and NMDA receptors on spinal motor neurons (Campagna *et al.*, 2003). NMDAR is an ionotropic receptor activated by glutamate, an excitatory neurotransmitter. Different heteromeric proteins called subunits compose it, and different combinations of subunits determine specificities. NMDA receptors control the conductance from Na^+, K^+ and especially Ca^{2+} through the neuronal membrane (Bressan *et al.*, 2003). This receptor is linked to peripheral pain signaling and central sensitization through long-term potentiation (LTP) and long-term depression (LTD) (Lu *et al.*, 2017). Concomitant with that, it is known that NMDAr is also related to locomotor activity; in neonatal mice, the receptor blocked induces reduced frequency and amplitude in pharmacologically induced locomotor activity (Acton e Miles, 2017b).

Taking this into account, the activity/rest rhythm can also be disrupting by NP, AC and EA. Circadian rhythm can be modified by chronic and prolonged stress, as well as hypothalamic-pituitary-adrenal (HPA) axis dysfunction (Dickmeis, 2009). This condition is commonly seen in chronic pain, as NP, and it can be revert by AC or EA treatments.

Based on these findings, we hypothesize that isoflurane can alter the effect of AC and EA treatments in the locomotor activity and NMDAr concentration in central nervous system (CNS) in rats submitted to a neuropathic pain model. To test this hypothesis, we evaluated the locomotor and exploratory activities using both, open field test and actigraphic monitoring (actigraphy) of circadian locomotor activity of rats submitted to a neuropathic pain model and treated with AC or EA. Additionally, we evaluated the NMDA level in brainstem and spinal cord of these animals.

2. Methods

2.1 Animals

We used Male Wistar rats (weight ≥250 g) aged between 55 and 65 days at the beginning of the experiment. The animals (N=140) were housed individually in polypropylene cages (49 × 34 × 16 cm). Based on previous studies from our research group, the number of animals needed to produce reliable scientific data was 140 (Medeiros *et al.*, 2011; Spezia Adachi *et al.*, 2012; Scarabelot *et al.*, 2016). All animals were maintained in a controlled environment (22 ± 2 °C) under a standard light–dark cycle (lights-on at 0700 h and lights-off at 1900 h), with water and chow (Nuvital, Porto Alegre, Brazil) provided *ad libitum*. All experiments and procedures were approved by the Institutional Committee for Animal Care and Use (GPPG-HCPA protocol no. 13-0298) and conformed to the Guide for the Care and Use of Laboratory Animals (8th ed., 2011). The maintenance of the animals followed the Brazilian law 11794, which establishes procedures for the scientific use of animals. The experimental protocol complied with the ethical and methodological standards of the ARRIVE guidelines (Kilkenny *et al.*, 2010).

2.2 Neuropathic pain model: chronic constriction injury (CCI) of the sciatic nerve

The chronic constriction (CCI) of the sciatic nerve was performed, as described by Bennett and Xie (Bennett e Xie, 1988), to induce neuropathic pain. The animals were anesthetized with isoflurane (5% for induction, 2.5% for maintenance), and the surgical region was trichotomized. The skin was cleaned with 2% alcoholic iodine (Bennett e Xie, 1988). The sciatic nerve was accessed at the mid-thigh level by removing part of the biceps femoris muscle. Three ligatures (4-0 Vicryl) were tied at 1-mm intervals close to the sciatic trifurcation. Ligation was tightened until a muscular contraction of the leg could be seen, ensuring epineural blood flow. The same investigator performed the ligatures in all animals. The sham surgery groups

were anesthetized and the left sciatic nerve was exposed, but not constricted. The control group did not undergo any surgical procedures.

2.3 Acupuncture (AC)

We used two stainless steel acupuncture needles with guide tubes (Suzhou Huanqiu Acupuncture Medical Appliance Co. Ltd., 218, China) that were 0.18mm in length and 0.8 mm in diameter. The needles were inserted approximately 2–3 mm to the BL24 point, which is located on the side depression of the lower edge of the L3 spinous process of the third lumbar vertebra (Fig.1).

----------*Insert Figure 1*----------

2.4 Electroacupuncture (EA)

The acupuncture needles were connected to an electro stimulator device (Model EL 608 NKL, Brusque, SC, Brazil) with an alternating frequency of 2 Hz and 100 Hz (2/10 Hz, 0.3 ms width) for 20 minutes. Procedures were conducted in rats that were freely moving or anesthetized with isoflurane (in oxygen flow; 2% for induction, and 0.5% for maintenance).

2.5 Open Field test

The behavioral test was performed 24h after the sixth session and before the seventh session. It was performed in a 60 × 40 × 50 cm cage with the floor divided into twelve 13 × 13 cm squares by lines. Each test started immediately after the animals had been placed in the back left corner and allowed to explore the surroundings for 5 min (Medeiros *et al.*, 2012); (Marques *et al.*, 2014). The measures evaluated were: number of external and internal line crossings (all paws crossing the boundary into an adjacent marked-out area), number of rearing behavior, time in

seconds of grooming behavior and number of fecal bolus. Open field test was performed 24h after the sixth session of AC or EA, before the seventh session.

2.6 Activity-Rest Rhythm Assessment

Actigraphic monitoring (actigraphy) of circadian locomotor activity is a quantitative method, non-invasive, for measurement of motor activity. In the present study we used actigraphy to examine diurnal and nocturnal variations in locomotor activity of rats submitted to a neuropathic pain model and treated with AC or EA, with and without isoflurane anesthesia. The animals were housed individually in polypropylene cages (49 × 34 × 16 cm). The rest-activity circadian rhythm was determined by the number of touch-detections per hour, during water intake (NTD) using an automatic activity data recording system (Cecon *et al.*, 2010); (Moraes *et al.*, 1997). The animals were analyzed since the first day of AC or EA treatment until the last day of experiment.

2.7 Experimental Design

The animals were acclimated to the study environment for 2 weeks before the beginning of the experiment, after which they were randomly allocated into the 14 following groups: Control (C), Sham Surgery (Ss), Sham Surgery+AC (SsAC), Sham surgery+EA (SsEA), Neuropathic Pain (Np), Neuropathic Pain+AC (NpAC), Neuropathic pain +EA (NpEA), Control+Anesthesia (CAn), Sham Surgery+Anesthesia (SsAn), Sham Surgery+AC+ Anesthesia (SsAcAn), Sham surgery+EA+Anesthesia (SsEaAn), Neuropathic Pain+Anesthesia (NpAn), Neuropathic Pain+AC+Anesthesia (NpAcAn), and Neuropathic Pain+EA+Anesthesia (NpEaAn). Subsequently, the NP and Ss received their respective interventions (CCI or sham surgery). 14 days later, von Frey test was conducted to evaluate hyperalgesia in order to confirm that

neuropathic pain was established. The animals were then treated for 8 days according to the specific protocol for each group (AC, EA or no treatment).

---------- Insert Figure 2 ----------

2.8 NMDA levels on brainstem and spinal cord

Brainstem and spinal cord were collected and frozen at -80°C until the time of testing. NMDA levels were measured by an enzyme-linked immune sorbent assay (Competitive ELISA) kit (My Biosource, California, USA), according to manufacturer's instructions.

2.9 Statistical analysis

Data were expressed as the mean ± standard error of the mean (SEM). Data of open field test and NMDA level were compared using a three-way analysis of variance (ANOVA) followed by Student-Newman-Keuls (SNK) to compare all groups, considering as independent variables: pain, treatment, and anesthesia. The results were reported as the mean ± standard error and considered significant if $P \leq 0.05$. Data from activity/rest were compared using non-parametric test of independent samples Kruskall-Wallis or Mann-Witney U test. The results were reported as median=P50 (P25 – P75) and considered significant if $P \leq 0.05$. SPSS 20.0 for Windows was used for the statistical analyses.

3. Results

3.1Effects of isoflurane, NP, AC and EA in the locomotor activity in the open field test

3.1.1 External Crossings

The statistical analyses did not demonstrated interactions among the independent variables: pain×treatment×anesthesia (three-way ANOVA/SNK, $P>0.05$); however there was interaction between anesthesia×treatment (three-way ANOVA/SNK, $F_{(2,125)}=3,03$; $P=0.052$). This result

showed that AC and EA lead to an increase in the locomotor activity, and anesthesia, to a decrease. Significant effects of anesthesia (ANOVA/SNK, $F_{(1,125)}$=14,63; P<0.01) as an independent variable was observed, showing that animals exposed to anesthesia had a decrease in locomotor activity, compared to groups without anesthesia. There was no difference between AC and EA treatments in this parameter (Fig. 3).

---------- *Insert Figure 3* ----------

3.1.2 Internal Crossings

As well as external crossings, the statistical analyses did not demonstrated interactions among the independent variables: pain×treatments×anesthesia (three-way ANOVA/SNK, P>0.05); however there was interaction between anesthesia×treatment (three-way ANOVA/SNK, $F_{(2,125)}$=3,21; P=0.04). A significant effect of anesthesia (ANOVA/SNK, $F_{(1,125)}$=5,34; P<0.01) as an independent variable was observed, showing that animals exposed to anesthesia showed a decrease in locomotor activity, compared to that of the groups without anesthesia. There were no difference between A and EA treatments (three-way ANOVA/SNK, P>0.05, Fig. 4).

---------- *Insert Figure 4* ----------

3.1.3 Rearings

There was interaction between anesthesia×treatments (three-way ANOVA/SNK, $F_{(2,125)}$=3,35; P≤0.05). While the treatments lead to an increase in exploratory activity, the anesthesia showed a decrease in this parameter. There were significant effects of anesthesia (ANOVA/SNK, $F_{(1,125)}$=57,14; P<0.01) and of pain (ANOVA/SNK, $F_{(1,125)}$=6,13; P<0.01)

were observed, showing that animals exposed to anesthesia and pain presented a decrease of rearings, compared to groups without anesthesia and pain(Fig. 5).

---------- *Insert Figure 5*----------

3.1.4 Fecal Bolus

There was no interaction among pain×anesthesia×treatments (three-way ANOVA/SNK, $P \geq 0.05$). Significant effects of anesthesia as an independent variable were observed, showing that animals exposed to anesthesia had a increase in number of fecal bolus, compared to groups without anesthesia (ANOVA/SNK, $F_{(1,125)}=8,05$; $P<0.05$, Fig. 6).

---------- *Insert Figure 6* ----------

3.2 Effects of isoflurane, NP, AC and EA in the Activity-Rest Circadian Rhythm

3.2.1 Light cycle

To demonstrate the pain effect in the locomotor activity, non-parametric tests was used to compare groups with (yes) or without (no) pain. A significant difference between groups was observed; the groups submitted to NP model decrease significantly NTDs during light cycle (Mann-Whitney U test, Yes = 16,63 (10,17-27,83); No= 21,05 (16,33-31,63) $P<0.006$). To observe the anesthesia effect we compare the groups with (yes) or without (no) anesthesia. A significant effect of anesthesia increasing NTDs was observed (Mann-Witney U test, Yes = 21,32 (15,91-36,42); No= 18,25 (11,01-24,60) $P<0.006$). When we analyzed treatments, we compare all groups that receive AC, EA, or no treatment. Significant difference between AC and EA was observed (Kruskall-Wallis, AC= 16,93 (11,03-29,40); EA= 26,00 (18,57-34,65) No= 18,21 (14,33-26,78) $P<0.02$). EA increase significantly the locomotor activity in comparison to AC, although groups that do not receive treatments (fig.7)

3.2.1 Dark cycle

To demonstrate the pain effect in the locomotor activity, non-parametric tests was used to compare groups with (yes) or without (no) pain; significant difference between groups was observed (Mann-Whitney U test, Yes = 72,48 (50,16-105,07); No= 106,11 (83,28-146,25) P<0.000). Groups submitted to NP model decrease significantly NTDs during dark cycle. To observe the anesthesia effect we compare groups with (yes) or without (no) anesthesia. A significant effect of anesthesia increasing NTDs was observed (Mann-Whitney U test, Yes = 99,43 (74,96-145,82); No= 83,78 (61,51-117,75) P<0.05). When we analyzed treatments, it was compared all groups that receive AC, EA, or no treatment. Significant difference between EA and no treatment was observed (Kruskall-Wallis, AC= 88,17 (64,75-117,22); EA= 79,61 (55,59-104,35) No= 109,68 (76,77-146,45) P<0.02). EA decrease significantly the locomotor activity in relation to groups that not receive treatments; although groups that receive AC were not different from EA or no treatment (Fig. 7).

---------- *Insert Figure 7* ----------

3.3 NMDA levels in the Brainstem

There was no interaction among pain×anesthesia×treatments (three-way ANOVA/SNK, P≥0.05). There was significant effect just anesthesia (ANOVA/SNK, $F_{(1,73)}$=5,91; P<0.05) showing that animals exposed to anesthesia present decrease in pg NMDA/mg protein (Fig. 8).

---------- *Insert Figure 8* ----------

3.4 NMDA levelsin the Spinal cord

There was no interaction among pain×anesthesia×treatments (three-way ANOVA, P>0.05). However, an interaction between anesthesia×treatment was observed (three-way ANOVA/SNK,

$F_{(2,73)}$=3,94; P≤0.05). There was significant effect of independent variables anesthesia (ANOVA/SNK, $F_{(1,73)}$=42,68; P<0.01),showing that animals exposed to anesthesia present decrease in pg NMDA/mg protein; and of treatments (ANOVA/SNK, $F_{(2,73)}$=3,41; P<0.01), showing that EA increase level of NMDA(Fig. 9).

---------- *Insert Figure 9*----------

4 Discussion

In this study, we demonstrated that repeated exposure to isoflurane during AC and EA treatments altered the locomotor and exploratory behavior effect of those treatments. While AC and EA induce to an increase on external and internal crossings and rearings in open field test, the anesthesia decreases these measures, and altered the effect of AC and EA.NP also decreases rearing behavioral without interaction with anesthesia or treatment. The NP model used can explain this result, since that the CCI model causes muscle atrophy impairing the support on hind legs (Daemen *et al.*, 1998). It is important to note that the behavior of rearing is considered an exploratory activity, since that the animal supports its weight on the hind legs and lifts the front of the body. CCI model did not alter crossing number, probably because rats use all four legs to support body weight, reducing the discomfort caused by pain, at least when they are exploring a new place. Previous study also did not show alteration on locomotor activity by CCI model on open field test, 21 days after the surgery (Filho *et al.*, 2016). AC and EA increase locomotor and exploratory activities, also in animals that underwent CCI model as in sham groups. Interestingly, a study demonstrated the effect of AC reverts morphine withdrawal-induced behavior, increasing locomotors activity in the open field test. This study suggests a possible activation of noradrenergic system during protracted abstinence following chronic morphine exposure (Lee *et al.*, 2014). Another important find was that the number of fecal bolus in open

field test was increased by repeated anesthesia. This measure is considered anxiety-like behavior, since the higher defecation in the OF test is contrary to the decreased motility in rats (Crumeyrolle-Arias *et al.*, 2014).

On activity-rest circadian rhythm, animals that underwent CCI model decrease thenumber of touch-detections per hour during water intake (NTD), both in light (7am - 7pm) and dark (7pm - 7am) cycles. Our study is the first one to demonstrate that neuropathic pain model decreases locomotion during day and night in homecages. Is important to note that this measure starts fourteen days after NP induction, highlighting the effect of NP condition and not from surgery effect. Our results are ambiguous taking into account that on open field test (OFT), NP did not show effect. We can suggest that OFT is performed just once, in one new cage, stimulating animals to explore the environment and producing stress-induced analgesia, while the actigraphy was measure on their homecages. On the other hand, isoflurane anesthesia increases NTDs also on light and dark cycle, also contradicting OFT results. The OFT was performed 24h after the sixth session of AC or EA, to avoid the acute effect of exposure to the treatments and the anesthesia, when applied. It is important to note that OFT and actigraphy evaluate different systems, since OFT evaluates locomotor and exploratory activities and actigraphy evaluate basal locomotor activity. It is know that isoflurane decreases glutamate release, decreasing the locomotion on OFT and neuronal excitability (Sandstrom, 2004). For the other hand, demonstrates isoflurane-increasing NTDs during day and night period. Kikushi et al. demonstrated that isoflurane increase locomotion in rest phase (light cycle) and suppress on acetylcholine (ACH) release in the rat hippocampus, after 24h after isoflurane exposure (Kikuchi *et al.*, 2013). The ACH release on hippocampus has been related to increase on locomotion (Mitsushima *et al.*, 2009) suggesting that isoflurane disrupts the correlation between ACH release and locomotor activity, and it probably is regulated by other mechanisms (Kikuchi *et al.*, 2013).

On light cycle EA increase NTDs/h, and this result was significantly different from AC, but not different from No treatment groups. On the other hand, on dark cycle, EAdecreases NTDs/h and was significantly different from groups without treatments, but not different from groups that receive AC.Is important to note that light cycle is considered the rest phase, and dark cycle is considered the activity phase. The OFT, performed on light cycle, and actigraphy in the same phase, presented the same result, showing EA increasing locomotor activity. EA is related to decrease nociceptive behavior and increase locomotion (Pei *et al.*, 2016). It is known that EA produce analgesia by stimulating opioid system (Zhao, 2008); in the same way, rats submitted to morphine during 5 days consecutively increase locomotors activity (Lee *et al.*, 2014). However, on night cycle, EA decreases locomotion. We can suggest that EA presented contrary effects based on the different hormone releases according to circadian rhythm. More studies are necessary to clarify these effects.

Another important find of present study was the alteration on NMDA levels in brainstem and spinal cord induced by anesthesia. Both structures presented decreased NMDAr level caused by isoflurane anesthesia; this result corroborates the literature data that suggests that isoflurane decreases glutamate release in the neuromuscular junction model (Sandstrom, 2004). However, our results, in spinal cord, showed an interaction of anesthesia and treatments, suggesting that isoflurane reverts the NMDA level increase induced by EA. In other hand, it was expected that NP increases NMDA level, as described in previous studies (Qiu *et al.*, 2013; Xie *et al.*, 2016); but this result was not seen in the present study. When EA was applied without anesthesia, it was able to increase significantly the concentration of NMDA in pg/mg protein. Many studies using pain animal models show a decrease in NMDAr expression induced by EA (Zhang *et al.*, 2014; Liu *et al.*, 2017; Tu *et al.*, 2017). Although studies demonstrate that EA inhibits transmission of noxious messages at the spinal level by dampening glutamate receptor activities (Wang *et al.*, 2006; Tao, 2010); however, it necessarily does not happen due a decrease in NMDA level. This

result corroborates the increase in locomotor activity on OFT induced by EA, since glutamate and its NMDA receptor, are related to locomotor activity. Previous study provide evidence that NMDARs within spinal locomotor networks determine the frequency and amplitude of locomotor activity, demonstrating how excitatory transmission can alter the spinal locomotor networks in mammals (Acton e Miles, 2017a).

In conclusion, isoflurane anesthesia alters directly the effect of EA and Ac treatment on locomotor activity and NMDA level in spinal cord of animals submitted to NP model. This information is very important, taking into account that many studies use isoflurane during Ac and EA apply in animals. Besides that, while isoflurane and NP alter basal locomotion in both cycles; EA also alter this measure; however it presents opposite behavior according to diary cycle. More studies are necessary do elucidated the mechanism involved in these factors, and they interactions.

Acknowledgements

This research was supported by the following Brazilian funding agencies: National Council for Scientific and Technological Development – CNPq (Dr. ILS Torres; Dr. W Caumo; Dr. VLScarabelot); Brazilian Federal Agency for Support and Evaluation of Graduate Education – CAPES (LNSAdachi; Dr. LF Medeiros); CAPES/PNPDEdital PPGCR 03/2016 (Dr. R Vercelino); CAPES/PNPD EditalPPGCM 07/2016 (Dr. C de Oliveira); Graduate Research Group of Hospital de Clínicas de Porto Alegre – GPPG (Dr ILS Torres – Grant 130298).

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Legends

Figure1. Acupuncture in animal sedated

Animals receiving Ac or EA with isoflurane.

Figure2. Table groups

Description of the procedures performed on the animals in each group.

Figure 3. External crossings

Data are expressed as the mean±standard error of the mean (SEM) of total number of crossings and considered significant if $P \leq 0.05$.There interaction between anesthesia×treatments(three-way ANOVA/SNK, $F_{(2,125)}=3,03$; $P=0.052$) and effect of independent variable anesthesia (ANOVA/SNK, $F_{(1,125)}=14,63$; $P<0.01$; N= 8-10/ group).

Figure 4. Internal crossings

Data are expressed as the mean±standard error of the mean (SEM) of total number of crossings and considered significant if $P \leq 0.05$.There was interaction between anesthesia×treatments (three-way ANOVA/SNK, $F_{(2,125)}=3,21$; $P=0.04$) and effect of independent variable anesthesia (ANOVA/SNK, $F_{(1,125)}=5,34$; $P<0.01$; N= 8-10/ group)

Figure 5. Rearings

Data are expressed as the mean±standard error of the mean (SEM) of total number of rearings and considered significant if $P \leq 0.05$.There was interaction between anesthesia×treatments(three-way ANOVA/SNK, $F_{(2,125)}=3,35$; $P \leq 0.05$) and effect of independent variable anesthesia (ANOVA/SNK, $F_{(1,125)}=57,14$; $P<0.01$) and NP (ANOVA/SNK, $F_{(1,125)}=6,13$; $P<0.01$; N= 8-10/ group).

Figure 6. Fecal Bolus

Data are expressed as the mean±standard error of the mean (SEM) of total number of fecal bolus and considered significant if $P \leq 0.05$. There was no interaction between

pain×anesthesia×treatments (three-way ANOVA/SNK, P≥0.05). Significant effects of anesthesia (ANOVA/SNK, $F_{(1,125)} = 8,05$; P<0.05; N= 8-10/ group).

Figure 7. Activity-Rest Rhythm

Data are expressed as the median followed by interquartile range [P50 (P25-P75)] of the number of touch-detections per hour during water intake (NTD) if P≤0.05. There was difference between yes and no groups on pain and anesthesia factors (Mann-Witney U test, P<0.05) in light and dark cycle. Significant difference between Ac and EA was observed in light cycle (Kruskall-Wallis, P<0.05) and significant difference between EA and No treatment was observed (Kruskall-Wallis, P<0.02) in dark cycle (N= 8-10/per group).

*Significant different from "No pain" groups; # Significant different from "No anesthesia" groups; $ Significant different from Ac groups; $$ Significant different from "No treatment" groups.

Figure 8. NMDA concentration in the Brainstem

Data are expressed as the mean±standard error of the mean (SEM) of concentration of NMDA in pg/mg protein and considered significant if P≤0.05. Significant effect of anesthesia (ANOVA/SNK, $F_{(1,73)}=5,91$; P<0.05(N= 5-7/goup).

Figure 9. NMDA concentration in the Spinal Cord

Data are expressed as the mean±standard error of the mean (SEM) ofconcentration of NMDA in pg/mg protein and considered significant if P≤0.05. There was interaction between anesthesia×treatments (three-way ANOVA/SNK, $F_{(2,73)}=3,94$; P≤0.05). There was significant effect of independent variables anesthesia (ANOVA/SNK, $F_{(1,73)} = 42,68$; P<0.01), and treatments (ANOVA/SNK, $F_{(2,73)}=3,41$; P<0.01) (N= 5-7/goup).

Figures

Figure 1.

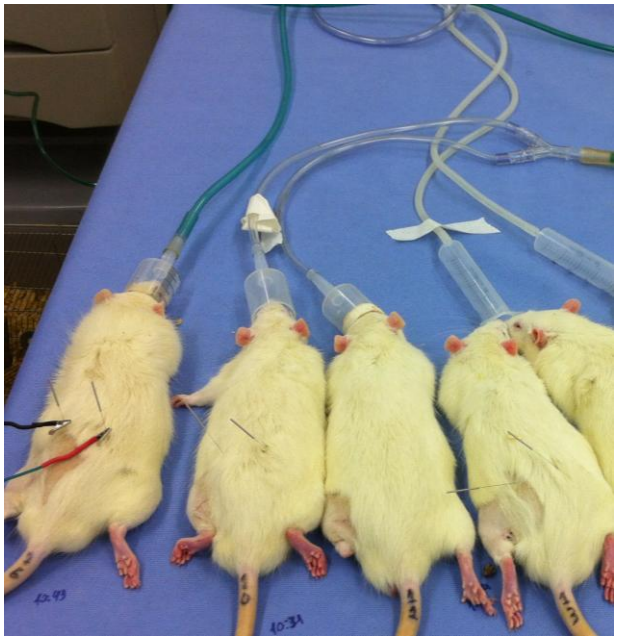


Figure 2.

Groups	
Control (C)	No manipulation
Sham Surgery (Ss)	surgery without nerve constriction
Sham Surgery + AC (SsAC)	surgery without nerve constriction+ 8 sessions of acupuncture
Sham Surgery + EA (SsEA)	surgery without nerve constriction+ 8 sessions of eletroacupuncture
Neuropathic Pain (Np)	surgery with nerve constriction
NeuropathicPain + AC (NpAC)	surgery with nerve constriction+ 8 sessions of acupuncture freely movements
NeuropathicPain + EA (NpEA)	surgery with nerve constriction+ 8 sessions of eletroacupuncture freely movements
Control+Anesthesia (CAn)	No manipulation + 8 days of isoflurane
ShamSurgery+ Anesthesia (SsAn)	surgery without nerve constriction+ 8 days of isoflurane anesthesia
Sham Surgery + AC+ Anesthesia (SsACAn),	surgery without nerve constriction+8 sessions of acupuncture with isoflurane anesthesia
Sham Surgery + EA+ Anesthesia (SsEAAn)	surgery without nerve constriction+8 sessions of eletroacupuncture with isoflurane anesthesia
Neuropathic Pain+ Anesthesia (NpAn)	surgery with nerve constriction+ 8 days of isoflurane anesthesia
Neuropathic Pain + AC+ Anesthesia (NpACAn)	surgery with nerve constriction+8 sessions of acupuncture with isoflurane anesthesia
Neuropathic Pain + EA+ Anesthesia (NpEAAn)	surgery with nerve constriction+8 sessions of eletroacupuncture with isoflurane anesthesia

Figure 3.

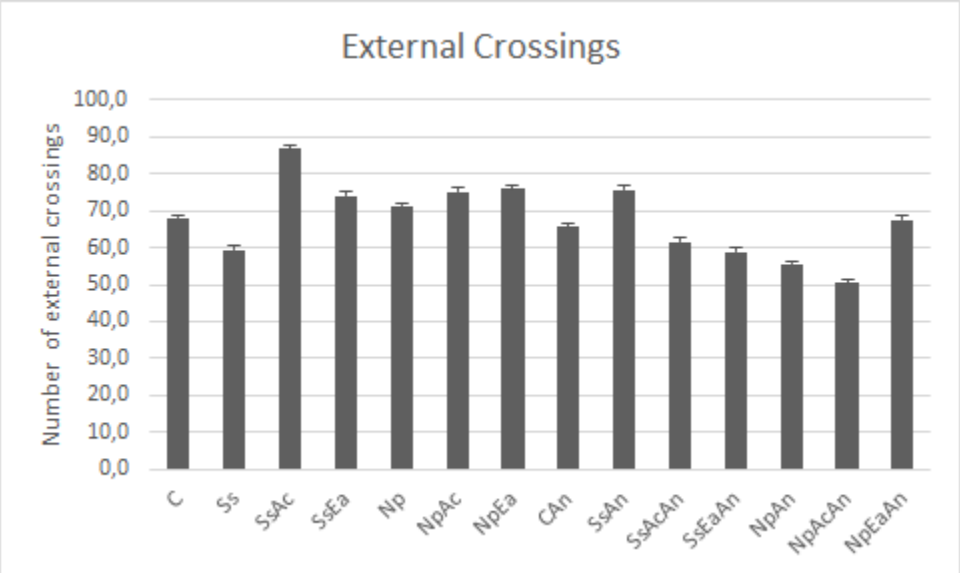
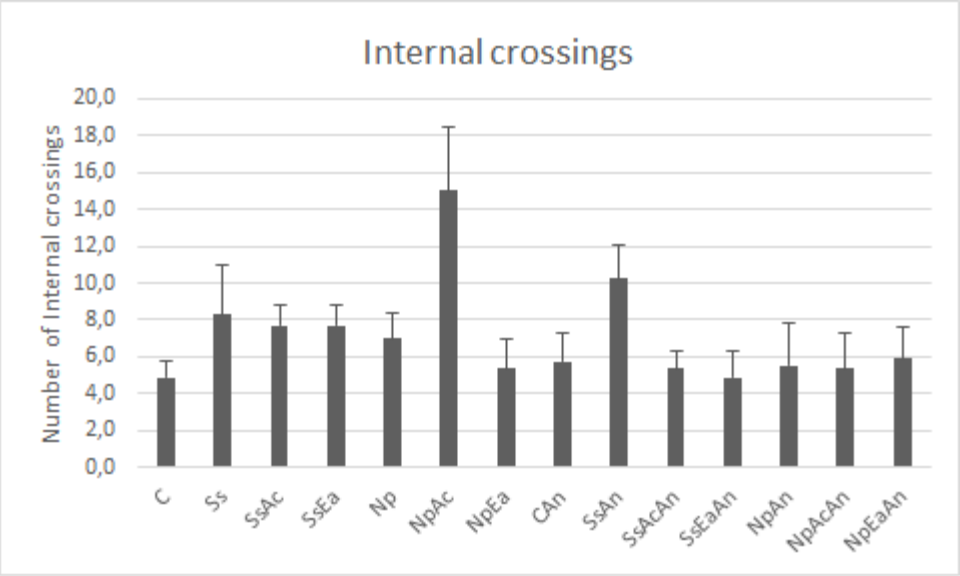


Figure 4.



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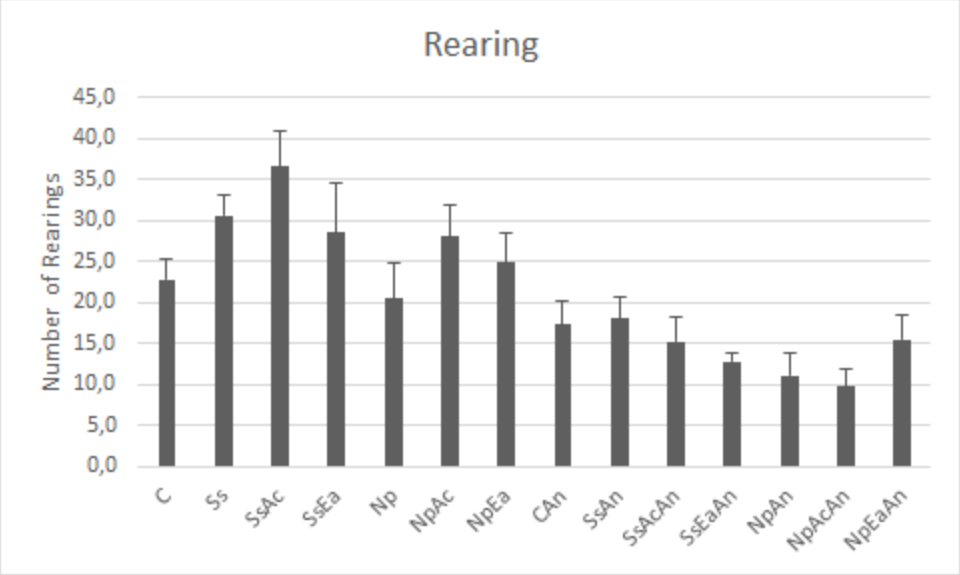


Figure 6.

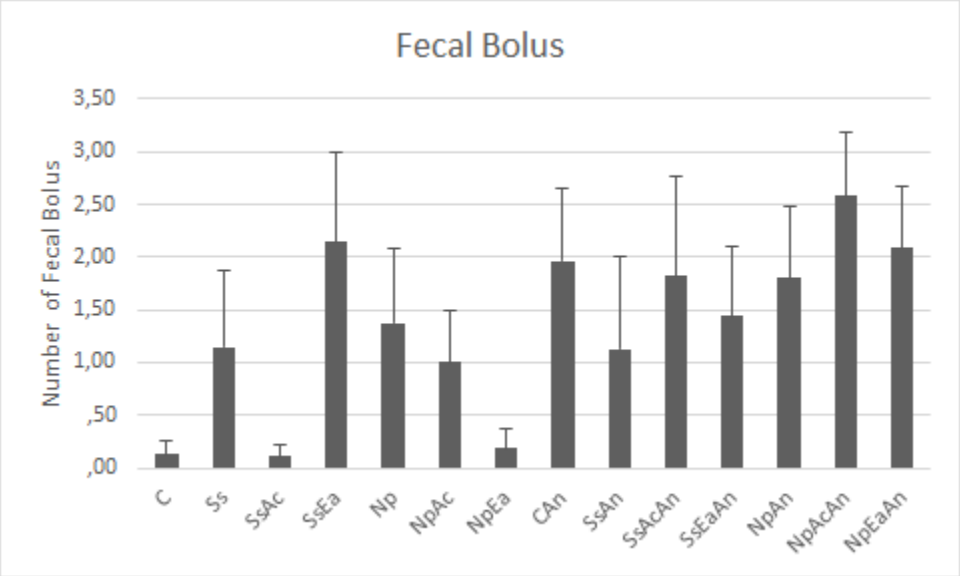
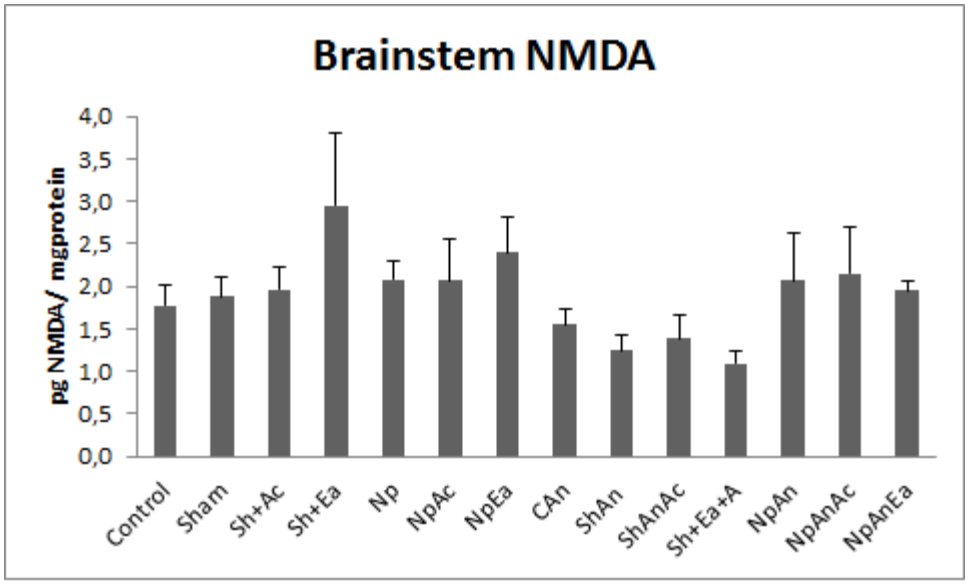


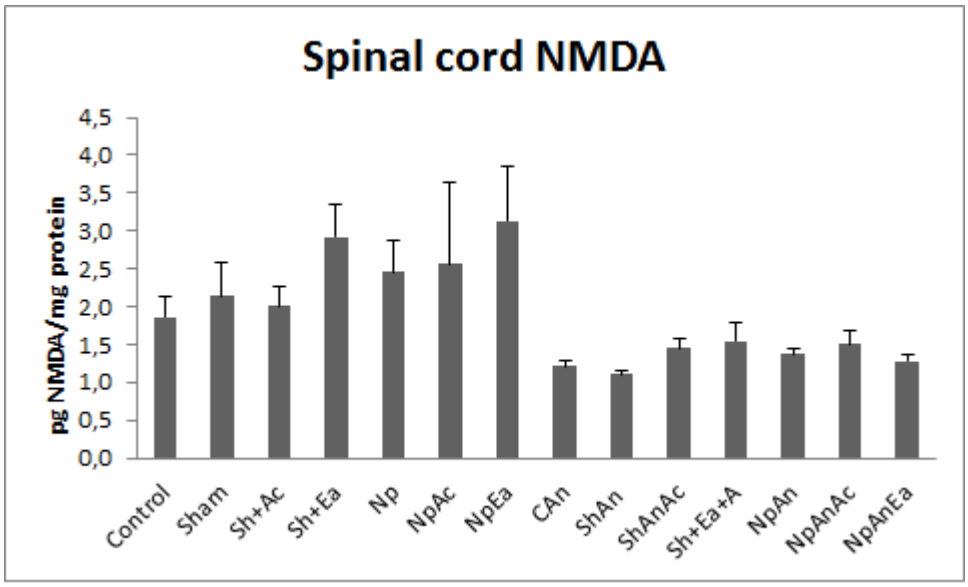
Figure 7

	Light Cycle NTDs = P50(P25 to P75)	DarkCycle NTDs = P50(P25 to P75)
Pain		
Yes	16.63 (10.17 to 27.83)*	72.48 (50.18 to 105.07)*
No	21.05 (16.33 to 31.63)	106.11 (83.28 to 146.25)
Anesthesia		
Yes	21.32 (15.91 to 36.42)#	99.43 (74.96 to 145.82)#
No	18.25 (11.01 to 24.60)	83.78 (61.51 to 117.75)
Treatments		
Ac	16.93 (11.03 to 29.40)	88.17 (64.75 to 117.22)
Ea	26.00 (18.57 to 34.65)$	79.61 (55.59 to 104.35)$$
No	18.21 (14.33 to 26.78)	109.68 (76.77 to 146.45)

Figure 8.



Fihgure 9.



6.3 Artigo 3

Acupuncture induces greater analgesia than Eletroacupuncture in rats submitted to neuropathic pain model.

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Conflict of Interest: All authors declared no financial or commercial interests in the outcome of the study.

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Abstract

Introduction: Acupuncture (AC) and electroacupuncture (EA) are recognized techniques for neuropathic pain (NP) treatment. Although, there are few studies comparing which of those are the best treatment for this pain condition and their possible effects on gastrocnemius muscle. The purpose of this study was to investigate the comparison of repeated Ac or EAc on nociceptive behavior, TNF–α imunostaining, cross-sectional area measure (CSA) and oxidative stress parameters in the gastrocnemius muscle on chronic constriction injury (CCI) model. **Methods**: seventy male Wistar rats with 60 days-year-old were used. The nociceptive response was evaluated in the Hot plate and Randall Selitto test, at baseline; 14 days post-surgery, immediately and 24h after the last session of treatment. NP was induced by sciatic nerve constriction, and the AC or EA treatments started 14days after-surgery (20min/day/8days). Animals were killed by decapitation, and gastrocnemius muscle was collected for histological analyses or DCFH measure. The statistical analysis GEE/Bonferroni were conducted to nociceptive tests, and three-way ANOVA/SNK, for histological and biochemical analysis. The results were considered significant with $P \leq 0.05$. **Results**: At baseline test, there was no difference among groups in the nociceptive threshold. Fourteen days after-surgery, the groups submitted to NP presented a decrease in nociceptive threshold compared to other groups, confirming the establishment of NP ($P<0.05$). Both treatments enhanced nociceptive threshold immediately after the last session, however AC induces greater analgesia than EA immediately in the hot plate ($P<0.05$) and Randall Selitto tests ($P<0.05$). In the CSA of gastrocnemius muscle, the frequency histogram showed a significant increase in fibers 1000 μm^2 of Pain groups ($P\leq 0.000$) and a significant decrease in fibers 4000 um^2 and 6000 μm^2 of same groups ($P\leq 0.000$). The DCFH levels were increased on gastrocnemius muscle of rats submitted to CCI model ($P\leq 0.000$), and Ac and EA were not able to revert this effect. **Conclusion:** AC and EA revert mechanical and thermal hyperalgesia, although AC induces greater analgesia than EA. CCI model changes CSA and DCFH measures on gastrocnemius muscle; however AC or EA were not able to revert these effects.

Keywords: Neuropathic pain, Acupuncture, Eletroacupuncture, gastrocnemius, CSA, DCFH.

1. Introduction

Acupuncture (Ac) is a procedure in which needles are inserted into distinguished points, described in China around 2,000 B.C. (Goldman *et al.*, 2010). Among various acupuncture indications, pain relief of different etiologies is the most known. The acupuncture can be applied with different techniques. The insertion of needles can be superficial or deep, and it can have manual manipulation or not, or electric current stimulation. The use of needles in Ac with electric current devices is known as eletroacupuncture (EA), which already demonstrated good results on pain treatment in human or animal study (Tu *et al.*, 2012). Although, the analgesic effects of Ac and EA are well known, the mechanisms by which these techniques relieve pain conditions are not been clarified yet. There are few pre clinical studies comparing manual AC and EA, and in these the methodology is not consistent to compare both techniques directly (Langevin *et al.*, 2015). The choice for the best treatment depends on the disease, clinical parameters and practitioner experience. In the neuropathic pain, for example, either AC or EA have good results, but is still necessary to know about possible differences between its effects on painful condition.

Studies have shown that AC and EA act activating peripheral and central nervous system (PNS and CNS, respectively), mainly activating opioid pathway in the treatments of all types of pain (Li et al., 2013). The opioid system activation promotes desensitization of peripheral nociceptors, combined to decrease of pro-inflammatory cytokines in PNS and CNS; and also activates the descending inhibitory system, releasing norepinephrine and serotonin (Zhang et al., 2014).

Neuropathic pain (NP) is a type of pain caused by peripheral and/or central nerve injury, leading to symptoms like spontaneous pain, allodynia and hyperalgesia (Florio *et al.*, 2009). Besides, this condition is responsible to cause persistent suffering and contributes to decrease quality of life, thus, being a public health problem. Considering that the neuropathic pain

treatment remains limited and often frustrating, it is very important to search new alternative therapies.

NP models have been widely used to study pain mechanisms and analgesic effects from several approaches, like acupuncture. Central sensitization is considered the main pathophysiological mechanism under NP conditions. First, the neuronal sensitization caused by a sequence of nociceptive peripheral stimuli repeated by a single nociceptive stimulation, increases the A, C and Aβ fiber responses. This phenomenon occurs as a consequence of the release of excitatory amino acids and neurotrophins in the dorsal horn of the spinal cord (Woolf and Salter, 2000). After, the activation of the second messenger cascade, it promotes the opening of calcium channels and, consequently, the production of prostaglandins and nitric oxide, which induces the release of glutamate, aspartate, SP and CGRP, contributing to the amplification of the algic process (Ji et al., 2002). Besides this, the induction and maintenance of chronic pain are also related to non neuronal glial cells, mainly microglia and astrocytes, increasing the release of neurotrophic and inflammatory factors, including tumor necrosis factor alpha (TNF-α), interleukin-1 beta (IL-1β) and interleukin IL- 6 (Guo *et al.*, 2007), essentials to induce pain process in the spinal cord. So, it is well described that these cytokines, mainly TNF-α, are increased in both CNS and PNS (Wang *et al.*, 2016). Other mechanism involved in the induction and maintenance of NP condition are reactive oxygen species (ROS) (Geis *et al.*, 2017), which are described as involved in the pathophysiology of neuropathic pain (Kim *et al.*, 2004; Yowtak *et al.*, 2011). The spinal nerve ligation (SNL), a sort of nerve injury, causes significant oxidative stress, indexed by an increase of the lipid peroxidation and nitrite concentration, GSH, SOD and catalase depletions in sciatic nerve (Pottabathini *et al.*, 2015). In the same way, it is known that Ac and EA treatment also alter TNF-α and oxidative stress in CNS and PNS (Wang *et al.*, 2016; Geis *et al.*, 2017).

Although many studies describe the effect of NP, AC or EA on the CNS and PNS, few

studies describe what happens on peripheral muscle gastrocnemius, directly affected by nerve constriction. Thus, we hypothesize that EA treatment promotes a powerful analgesia compared to Ac treatment, and both treatments can revert TNF-α and the oxidative stress increase on gastrocnemius muscle induced by CCI model. Therefore, the purpose of this study was to investigate the effect of repeated AC or EA on painful behavior, TNF-α and intracellular ROS production in the gastrocnemius muscle on CCI animal model.

2. Methods

2.1 Animals

Male Wistar rats (weight, ≥250 g) aged between 55 and 65 days old at the beginning of the experiment were used. The animals (n=70) were housed individually at a polypropylene cage (49 × 34 × 16 cm). All animals were maintained in a controlled environment (22 ± 2 °C) under a standard light–dark cycle (lights-on at 0700 h and lights-off at 1900 h), with water and chow (Nuvital, Porto Alegre, Brazil) *ad libitum*. All experiments and procedures were approved by the Institutional Committee for Animal Care and Use (GPPG-HCPA protocol no. 13-0298) and conformed to the Guide for the Care and Use of Laboratory Animals (8th ed., 2011). The maintenance of the animals followed the Brazilian law 11794, which establishes procedures for the scientific use of animals. The experimental protocol complied with the ethical and methodological standards of the ARRIVE guidelines (Kilkenny *et al.*, 2010). The experiment used the number of animals necessary to produce reliable scientific data.

2.2 Neuropathic pain model: chronic constriction injury (CCI) of the sciatic nerve

The sciatic nerve chronic constriction (CCI) was made as described by Bennett and Xie (Bennett e Xie, 1988), inducing the neuropathic pain condition. The animals were anesthetized

with isoflurane (5% for induction, 2.5% for maintenance) and the surgery region was trichotomized, then the skin antisepsis was made with 2% alcoholic iodine (Bennett e Xie, 1988). The sciatic nerve was accessed at the mid-thigh level, by removing part of the biceps femoris muscle. Three ligatures (4-0 Vicryl) were tied at 1 mm intervals close to the sciatic trifurcation. Ligation was tight until a muscular contraction of the leg could be seen, ensuring epineural blood flow. The same investigator performed the ligatures in all animals. The sham surgery groups underwent the anesthesia and the left sciatic nerve was exposed, but not constricted. The control group did not undergo any surgical procedure, but was submitted to anesthesia in 8 sessions of 20 minutes.

2.3 Acupuncture (AC)

Two stainless steel acupuncture needles with guide tubes (Suzhou Huanqiu Acupuncture Medical Appliance Co. Ltd., 218, China) with 0,18mm in length and 0.8 mm in diameter were used. The needles were inserted to a depth of approximately 2–3 mm at the BL24 point, which is located on the side depression the lower edge of the spinous process L3 of the third lumbar vertebra. The needles were stimulated every 5 minute with rotation during the 20 minutes session. Procedures were conducted in rats anesthetized with isoflurane (in oxygen flow; 2% for induction, and 0.5% for maintenance).

2.4 Eletroacupuncture (EA)

The acupuncture needles were connected to an electro-stimulator device (Model EL 608 NKL, Brusque, SC, Brazil) at an alternating frequency of 2 Hz and 100 Hz (2/10 Hz, 0.3 ms width) for 20 minutes. Procedures were conducted in rats anesthetized with isoflurane (in oxygen flow; 2% for induction, and 0.5% for maintenance).

2.5 Hot plate test

The hot plate test was carried out to assess the effects of Ac and EAc on the thermal nociceptive threshold adapted from Woolfe and Macdonald, 1944 (Woolfe e Macdonald, 1944). We used similar methodology as described before by (Cioato et al., 2016). The hot plate was pre-heated and kept at a temperature of 55±0.5°C. All rats were acclimated to the hot plate for five minutes, 24 hours prior to testing, as the novelty of the apparatus itself could induce antinociception (Netto *et al.*, 1987). Rats were placed in glass funnels on the heated surface, and the nociceptive threshold was assessed by recording the time taken to first response (foot licking, jumping, or rapidly removing paws), as described by Minami et al. 1994 (Minami *et al.*, 1994). We used the hot plate test to determine changes in latency as an indicator of modifications of the supraspinal pain process (Ossipov *et al.*, 1995), as licking or jumping responses while this test are considered to be the result of supraspinal sensory integration (Caggiula *et al.*, 1995; Rubinstein *et al.*, 1996). The response was recorded in seconds(s) and a cut off time of 20 s was used. The baseline measure was performed before starting the groups' protocols. This test was applied before and 14 days post-surgery, immediately and 24h after the first and the last session of AC or EA.

2.6 Randall Selitto test

The rats were subjected to mechanical stimuli in paw withdrawal test described by Randall and Selitto (Randall e Selitto, 1957). We used similar methodology as described before by Nunes et al., 2017. Analgesymeter, progressively increasing pressure stimulus (type 7200, apparatus Ugo-Basile Biological Research, Comerio-Varese, Italy) was used. For mechanical stimulation, which gradually increases the pressure was applied to the dorsal surface of the rat paw. The nociceptive threshold was defined as the force in grams at which the rat tried to withdraw its hind paw or vocalization, and the pressure values were recorded at this time. A cut-off value was used for 100g. This was recorded as the nociceptive threshold value. For each

animal, two recordings were made for each hind paw, and the data were reported as the mean of both hind paw values. The baseline measure was performed before starting the groups' protocols. This test was applied before and 14 days post-surgery, immediately and 24h after the first and the last session of Ac or EA.

2.7 Experimental design

The animals were acclimated to the study environment for 2 weeks before the beginning of the experiment, after this time they were randomly allocated into 7 groups: Control (C), Sham Surgery (Sham), Sham Surgery + Ac (Sham+Ac), Sham surgery + EAc (Sham +EA), Neuropathic Pain (Pain), Neuropathic Pain + Ac (Pain+ Ac), and Neuropathic Pain + EA (Pain+EA). After, the Np and Ss was submitted to respective interventions (CCI or sham surgery). 14 days later, von Frey test was conducted to evaluate mechanic hyperalgesia to confirm the establishment of neuropathic pain condition. The animals were then treated for 8 days according to the specific protocol for each group (AC, EA or no treatment). Then, the Hot Plate and the Randall Selitto test were conducted in baseline, 14 days post-surgery, immediately and 24h after the last session of treatments.

---------- Insert Figure 1 ----------

2.8 Histological, morphometrical and immunohistochemical analysis

Animals were decapitated 48h after the end of treatments and the left gastrocnemius muscle was dissected, fixed in 10% formalin, embedded in paraffin and transverse sections (4 μm) were obtained using a microtome. Sections were stained for routine hematoxylin and eosin (H&E) or were used for immunohistological detection of TNF-α.

Images of the H&E muscles were captured and digitalized (initially 20× and further amplified 50% for analysis) using a Nikon Eclipse E-600 microscope (Japan) coupled to a digital camera. For morphometric measurement, a set of six images was chosen using random sampling

of one slice and the mean fiber cross-sectional area (CSA) was estimated with the software Image Pro Plus 6.0 (Media Cybernetics, Rockville, MD, USA) using a point-counting technique as described by Marcuzzo et al. 2008 (Marcuzzo *et al.,* 2008). It was used an area of interest (AOI) with 78,682.40 μm^2, a grid with a density of one point per 63.3 μm^2 (Marques *et al.*, 2014).

For immunostaining, antigen retrieval was performed by heating sections in 0.01M sodium citrate buffer (pH 6.0) in a thermostatic bath at 92 °C for 20min. Sections were washed in PBS and the endogen peroxidase was inactivated with 3 % hydrogen peroxide dissolved in PBS for 30 min. Then, were washed in PBS and pre-incubated with 1 % bovine serum albumin (BSA; Sigma Aldrich, USA) in PBS with 0.1 % Triton X-100 (PBS-Tx), for 30 min and incubated with primary polyclonal rabbit anti-TNF-α antibody (1:200;Catalog#: AMC3012, Invitrogen, USA) at 4°C overnight. After washing in PBSTx, sections were incubated with the REVEAL Biotin-Free Detection System (Polyvalent HRP Conjugate; Spring Bioscience, Catalog#: SPB-999) secondary antibody at room temperature for 10 min. Following application of streptavidin-biotinylated horseradish peroxidase conjugate for 15 min, the reaction was revealed using a solution of 0.06 % 3,3-diaminobenzidine (DAB) and 10 % hydrogen peroxide for 5 min. Finally, the sections were rinsed in PBS, dehydrated in ethanol, cleared with xylene and covered with synthetic Canada balsam and coverslips.

Images of the TNF-αimmunostaining were captured, digitalized (20×) and the number of TNF-α-immunoreactive macrophages per mm^2 in the perimysium and endomysium of gastrocnemius muscle was also estimated, using the same software employed to morphometric measurement. An AOI square of 128,571.9 μm^2 was overlaid on the image, and TNF-α-immunoreactive macrophages located inside this square or intersected by the upper and/or right edges of the square were counted. TNF-α-immunoreactive macrophages intersected by the lower and/or left edges of the square were not counted. Ten images were analyzed for each animal

(Viola e et al. 2009).

2.9 DCFH oxidation

DCFH oxidation was used to measure intracellular ROS production. DCFH-DA (29-79-dichlorofluoresceindiacetate) is hydrolyzed by intracellular esterases to dichlorofluorescin (DCFH), which is trapped within the cell. This non-fluorescent molecule is then oxidized to fluorescent dichlorofluorescin (DCF) by the action of cellular oxidants. Gastrocnemius tissues were treated with DCFH-DA (10 mM) for 30 min at 37 ºC. Following DCFH-DA exposure, the homogenates were placed into PBS with 0.2% Triton X-100. Fluorescence was measured in a plate reader (Spectra Max M5, Molecular Devices, USA) with excitation at 485 nm and emission at 520 nm (Bellaver *et al.*, 2016).

2.10 Statistical analysis

Data were expressed as the mean ± standard error of the mean (S.E.M). A generalized estimating equation (GEE) followed by Bonferroni was performed to analyze the results of nociception. For the histology, three-way analysis of variance ANOVA followed by Tukey test was used to compare all groups. For the oxidative stress, a three-way analysis of variance (ANOVA) followed by Bonferroni was performed to compare data of all of the groups. DCFH data were expressed as percentage of control± standard error of the mean (S.E.M). The results are considered significant at $P \leq 0.05$. SPSS 20.0 for Windows was used for the statistical analysis.

3.0 Results

3.1. Effects of AC or EA upon thermal hyperalgesia evaluated in the Hot Plate test

The generalized estimation equation on Hot Plate test presented interaction time x treatment (χ^2=505,984; 20) P<0.001(Fig. 1). On Basal measure, all groups presented pain

threshold without statistical difference with control group (P>0.001). Fourteen days post-surgery, the control and sham groups were different from the pain groups, showing that the neuropathic pain was established (P<0.001). Immediately after the last session of treatment (21 days after surgery), AC and EA treatment enhanced the thermal pain threshold of groups submitted to neuropathic pain model. However, Ac induced better analgesia than EA. Pain+EA presented no difference from Control and Pain groups immediately, and 24h after end of treatment (P≥0.05), showing some analgesia, but not enough to revert this condition. The increase on pain threshold was significant higher in the Pain+AC than Pain group immediately after the last session of treatment (P<0.001), however this result did not remain until 24h after the last session of treatment (22 days after surgery).

3.2. Effects of AC or EA upon mechanical hyperalgesia evaluated in the Randall Selitto test

On Randall Selitto test, the generalized estimation equation (GEE) presented interaction time x treatment (χ^2= 138,609; 21) P≤0.000 (Fig. 2). On basal, all groups presented mean statistically equal (P≥0.001). Fourteen days after surgery, the control and sham groups were different from Pain groups (P<0.001). Immediately after the last session of treatment (21 days after surgery), both AC and EA treatments enhanced the mechanical pain threshold of animals submitted to neuropathic pain model with significant different from Pain group (P<0.001). The analgesic effect maintained until 24h (22 and 23 days after surgery) only in the Pain+Ac group, since the Pain+EA group presented pain threshold similar to Sham and Pain groups(P≤0.05).

3.3 Gastrocnemius muscle histology

On TNF-αimmunostaining on gastrocnemius muscle there were no interactions between the independent variables: NP, AC and EA (three way ANOVA, P≥0.05), and no significant

effect of independent variables on the number of TNF-α immunoreactive macrophages per mm^2 ($P \geq 0.05$) (Fig. 3). In the CSA measure in gastrocnemius muscle there were no interactions between the independent variables: pain, acupuncture and eletroacupuncture (three way ANOVA, $P \geq 0.05$). However, it was observed significant effect of the independent variable Pain (three way ANOVA/Bonferroni, ($F(1,30) = 41,30$; $P < 0.01$). The frequency histogram also showed a significant effect of the independent variable Pain leading to increase in fibers 1000 um^2 on Pain, Pain+Ac, Pain+EA groups (three way ANOVA/Bonferroni, $F(1,33) = 55,91$; $P<0.01$), and a significant decrease in fibers 4000um^2 and 6000um^2 on Pain, Pain+Ac, Pain+EA groups ($F(1,33)=26,87$; $P<0.01$; $F(1,33)=6,41$; $P<0.01$) (Fig. 4).

3.4 Oxidative Stress

The DCFH measure did not demonstrate interactions between the independent variables: pain, acupuncture and eletroacupuncture (three way ANOVA, $P>0.05$). However, it was observed significant effects of the independent variable Pain (three way ANOVA/Bonferroni, ($F(1,23) = 18,63$; $P<0.01$). The animals submitted to CCI model, Pain, Pain+Ac, Pain+EA presented an increase in the DCFH measures in the gastrocnemius muscle (Fig. 5).

4. Discussion

This study shows that Acupuncture induces greater analgesia than Eletroacupuncture on neuropathic pain condition. After 8 sessions by 20 minutes of stimulating bilateral BL24 both treatments were effective in revert thermal and mechanical hyperalgesia induced by CCI model. However, Ac treatment increases nociceptive threshold immediately after last session on hot plate test, and it maintains for longer time on Randall Selitto test. Generally, practitioners believe that EA promotes better analgesia than manual AC; however the literature does not assert this information. There are few basic studies comparing manual acupuncture and eletroacupuncture;

however the methodology is not consistent to compare both techniques directly (Langevin *et al.*, 2015). Some studies suggest that both treatments decrease back pain (Tao, 2000) and promotes analgesia (Guo, 2003), but EA is much more effective than manual AC. Another study suggests the same effectiveness of both techniques (Mackenzie *et al.*, 2011).

In the present study, the electroacupuncture application occurred through the association of two types of frequencies, low (2Hz) and high (100Hz) frequency. At the time of application, it was opted for the observation of the appearance of gentle muscle contraction of the affected paw, to be sure that the motor threshold was reached. After this, Aβ fibers are recruited or even motor innervation of the lombar trunks to perform the contraction. So, it was opted for this mechanism, since the application of currents with analgesic objective lie the sensory, motor and nociceptive thresholds, being that the most powerful analgesic effect occurs in the motor and nociceptive thresholds. However, it is a neuropathic pain model, the recruitment of Aβ fibers by electroacupuncture, may be, in some way impair or reduced analgesia by the behavior of the disease, which is characterized by an allodynia and the direct involvement of Aβ fibers in this pathological phenomenon. In contrast, manual acupuncture recruits only Aδ fibers, favoring the analgesic mechanism by the release of opioids and the greater reduction of the nociceptive threshold (Mackenzie *et al.*, 2011).

Another important find was in the number of TNF-α immunoreactive macrophages per mm^2 when there is not different among groups, thus neither neuropathic pain condition, nor Ac and EA treatments were able to change TNF-α on gastrocnemius muscle. This result is very significant because, it is well established that after nerve injury, microglia increase release of neurotrophic factors, while astrocytes release inflammatory factors including tumor necrosis factor alpha (TNF-α) (Guo *et al.*, 2007). The cytokines are essential to induce pain on spinal cord. So, it is well described that TNF-α is incresed both on CNS and PNS on neuropathic pain (Wang *et al.*, 2016). However, this is not seen on gastrocnemius muscle despite the lateral

gastrocnemius be innervated by sciatic nerve (Daemen *et al.*, 1998). According to literature, EA is effective in decreasing TNF-α level on dorsal root ganglion (DRG) after CCI on sciatic nerve, however no changes were observed on TNF-α immunoreactive in the gastrocnemius muscle.

On cross-sectional area measure (CSA), the frequency histogram showed that CCI model was effective in induce muscle atrophy in gastrocnemius muscle; however both Ac or EA treatment were not able to revert this condition. It is known that CCI model described by Bennet & Xie, cause atrophy on soleus, extensor digitorum longus (Moes e Holden, 2014) and gastrocnemius muscle, and also altered the fiber type in the last one (Daemen *et al.*, 1998). Nevertheless, our study is the first one to show the effect of CCI on density fiber of different diameters, using the frequency histogram. Through in this histogram we showed a significant increase of fibers 1000 um^2, and a significant decrease on fibers 4000um^2 and 6000um^2 on neuropathic pain groups. It shows that the CCI model was effective in produce atrophy, but the analgesia induced by AC or EA did not have influence on muscle condition. In this way, it is possible to conclude that CCI model induces strong muscular changes in gastrocnemius that are not reversed by Ac and EA treatments. However, it is important to note that we used the acupoints BL24, maybe if we had chosen acupoints like ST36 or GB34 could have had some change in these parameters.

Another important find was that the groups submitted to pain model presented an increase on DCFH level, a direct marker of oxidant production and oxidative stress, on gastrocnemius muscle. This result is very interesting, because is known that neuropathic pain condition has been related to increased levels of ROS on sciatic nerve, spinal cord and pre-frontal cortex (Pathak *et al.*, 2014). However, there are no studies about ROS on muscle atrophy caused by CCI model. On the other hand, the increase of ROS levels, inclusive DCFH, is present on atrophy muscles caused by inactivity (Lawler *et al.*, 2003). Considering that after CCI model, generally the animals cannot unload weight on the hind limb affected, causing inactivity. The

mechanism of action of ROS on muscle atrophy is not full elucidate, it is not known if they act as second messengers to control muscle atrophy or if they are required for oxidant regulation of muscle atrophy (Powers et al., 2006). It is important to note that AC and EA were not able to revert the increase in the DCFH measure induced by NP model.

In summary, AC and EA treatments were effective in reduce mechanical and thermal hyperalgesia, but AC demonstrates greater analgesia than EA on neuropathic pain model. In addition, both treatments did not influence on muscle condition. CCI model did not change TNF imunohistochemistry on gastrocnemius muscle, but altered the CSA fiber frequency and increase DCFH in this structure, and these measures were not affected by AC or EA treatments. It is important to note that the acupoints used were not injury local, which may have contributed for this result. In this way, we can suggest that EA in BL24 bilateral acupoints with 2 and 100 Hz, activating the motor threshold is not the best treatment for NP condition. In addition, considering the activation of Aβ fibers by EA, and that there are still committed by pain model, AC promotes better analgesia probably by activation of Aδ fibers, demonstrating be the best treatment in this case. More studies are still necessary to improve acknowledge on this field.

Acknowledgements

This research was supported by the following Brazilian funding agencies: National Council for Scientific and Technological Development – CNPq (Dr. ILS Torres; Dr. W Caumo; Dr. VL Scarabelot) and Edital Universal 475422/2013-9 (Dr. Rafael Vercelino); Brazilian Federal Agency for Support and Evaluation of Graduate Education – CAPES (LNS Adachi); CAPES/PNPD Edital PPGCR 03/2016 (Dr. R Vercelino); CAPES/PNPD Edital PPGCM 07/2016 (Dr. C de Oliveira); Graduate Research Group of Hospital de Clínicas de Porto Alegre – GPPG (Dr ILS Torres – Grant 130298).

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Figure Legends:

Figure 1: Hot Plate test

Data were expressed as the mean ± standard error of the mean (S.E.M) of nociceptive threshold in seconds. GEE statistics presented interaction time x treatment (χ^2=505,984; 20. P≤0.00; N= 10 per group).

*Significant difference from C and Sham groups.

#Significant difference from C group.

$Significant difference from other groups.

Figure 2: Randall Selitto test

Data were expressed as the mean ± standard error of the mean (S.E.M) of nociceptive threshold in grams. GEE statistics presented interaction time x treatment (χ^2= 138,609; 21. P≤0.000; N= 10-11 per group)

*Significant difference from C and Sham groups.

#Significant difference from C,Pain+Ac, Pain+EAc group.

$Significant diference from C, Pain+Ac, Sham, Sh+Ac, Sh+EAc.

Figure 3: Effects of Ac and EAc on the TNF-α immunostaining in the gastrocnemius muscle.

(A) Digitalized images of gastrocnemius muscle transverse sections showing TNF-α-immunoreactive macrophages in the perimysium and endomysium of all groups (captured at 20×). Scale bar = 50 μm. (B) Number of TNF-α-immunoreactive macrophages per mm^2 in different groups. Data are expressed as means ± SEM. There were no interactions between the independent variables: pain, acupuncture and eletroacupuncture (three way ANOVA, P≥0.05), and no significant effect of independent variables on the number of TNF-α immunoreactive macrophages per mm^2 (P≥0.05). N= 5-6 animals per group.

Figure 4: Effects of Ac and EAc on the morphometric parameters of gastrocnemius muscle.

(A) Digitalized images of H&E transverse sections of all groups (captured at 20×). Scale bar = 50 μm. (B) Fiber cross-sectional area of gastrocnemius muscle in different groups. (C) Frequency % histograms of gastrocnemius fiber cross-sectional area. Data are expressed as means ± SEM. It was observed significant effect of the independent variable Pain (three way ANOVA/Bonferroni, ($P< 0.01$). Pain increases fibers 1000 um^2 on Pain, Pain +Ac, Pain +EAc groups and significant decrease fibers 4000um^2 and 6000um^2 on Pain, Pain +Ac, Pain +EAc groups. N= 5-6 animals per group.

Figure 5: DCFH level on gastrocnemius muscle

Data were expressed as the mean ± standard error of the mean (S.E.M) of gastrocnemius muscle in percentage of control (%). There were no interactions between the independent variables: pain, acupuncture and eletroacupuncture (three-way ANOVA/Bonferroni, $P>0.05$). However, was observed significant effects of the independent variable Pain (three-way ANOVA/Bonferroni, ($F(1,23) = 18,63$; $P\leq 0.000$). N= 3-6 animals per group.

Figures

Figure 1

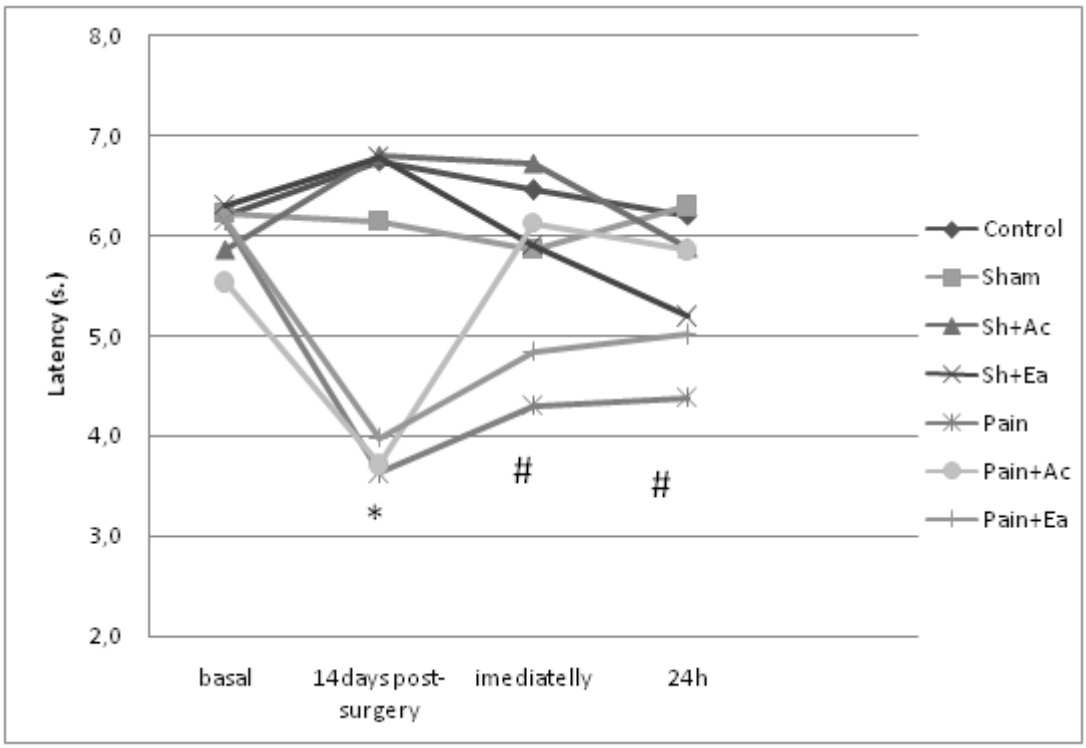


Figure 2

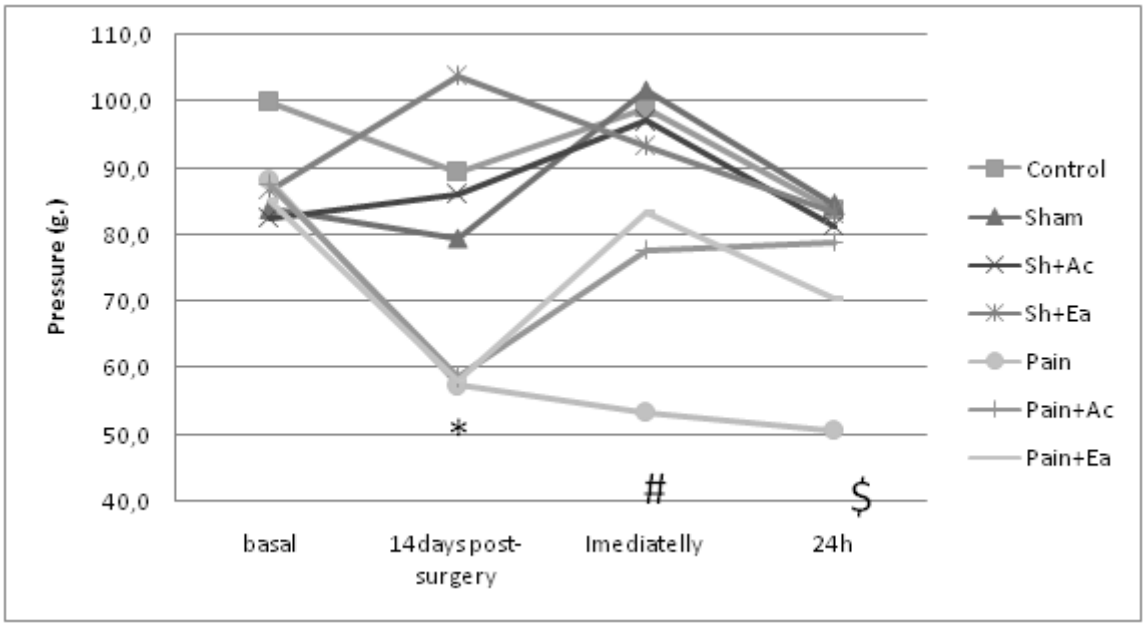


Figure 3.

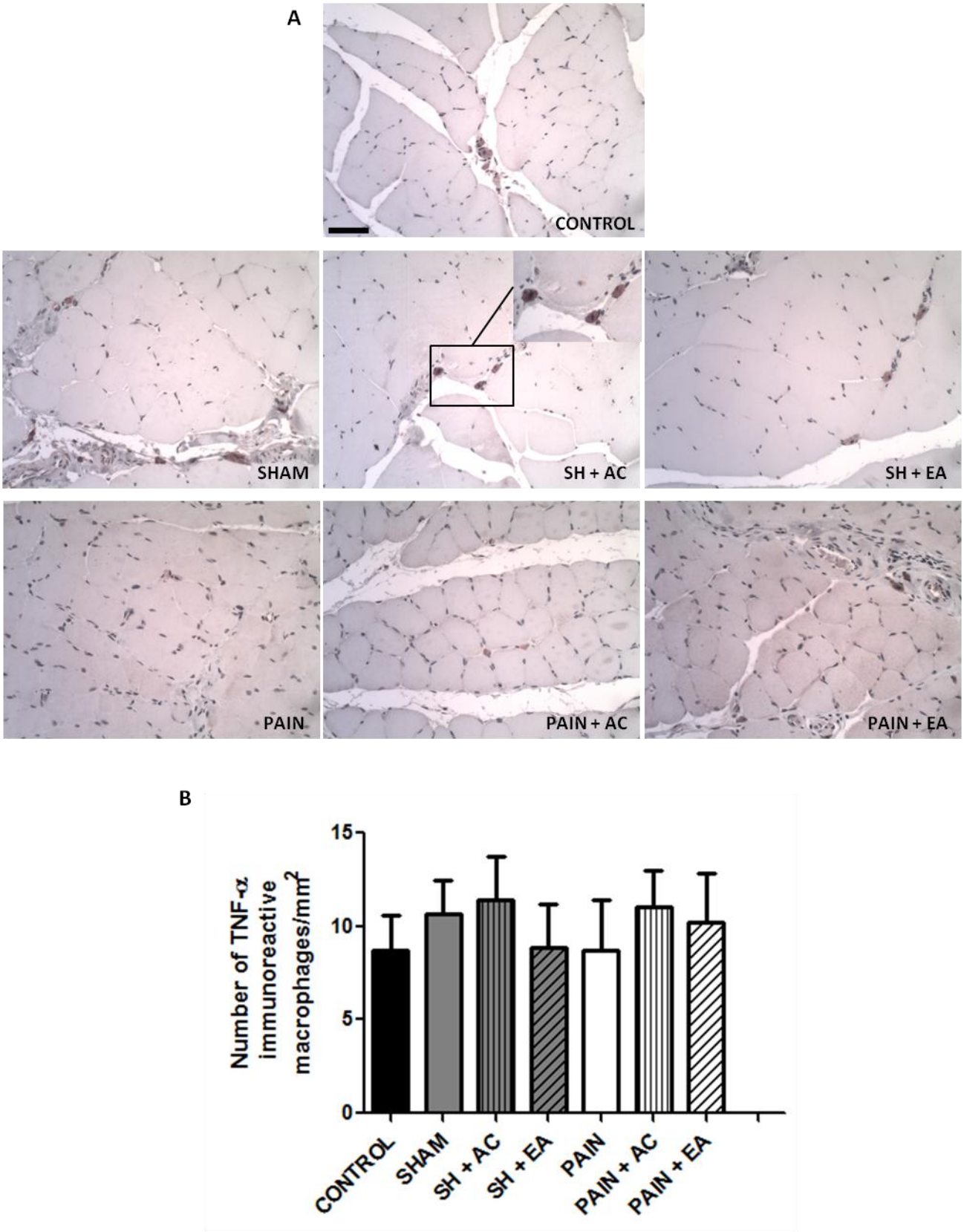

A
CONTROL
SHAM
SH + AC
SH + EA
PAIN
PAIN + AC
PAIN + EA
B
Number of TNF-α immunoreactive macrophages/mm²
15
10
5
0
CONTROL
SHAM
SH + AC
SH + EA
PAIN
PAIN + AC
PAIN + EA

Figure 4.

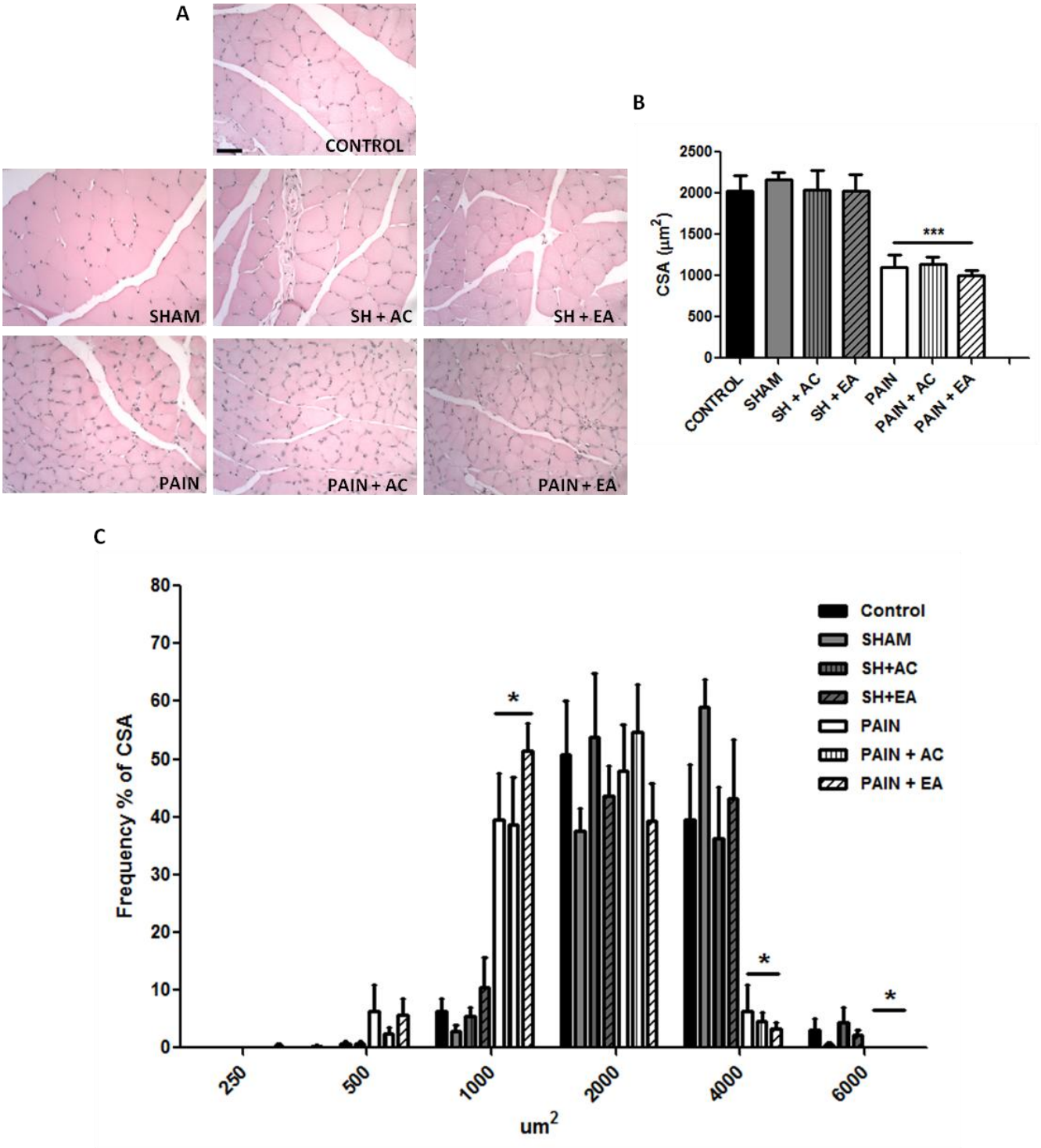
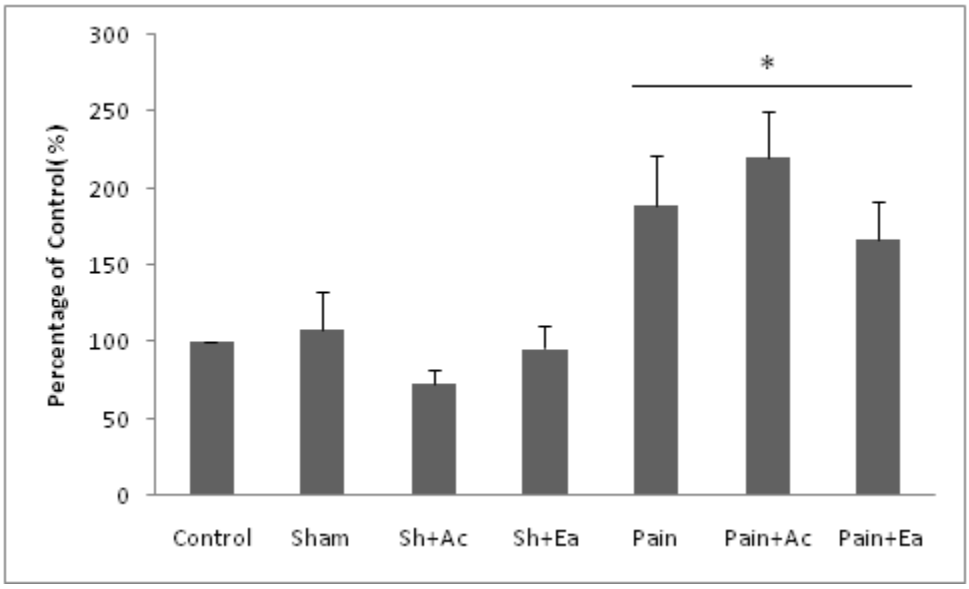

A
CONTROL
SHAM
SH + AC
SH + EA
PAIN
PAIN + AC
PAIN + EA
B
CSA (µm²)
2500
2000
1500
1000
500
0
***
CONTROL
SHAM
SH + AC
SH + EA
PAIN
PAIN + AC
PAIN + EA
C
Frequency % of CSA
80
70
60
50
40
30
20
10
0
*
*
*
250
500
1000
2000
4000
6000
um²
Control
SHAM
SH+AC
SH+EA
PAIN
PAIN + AC
PAIN + EA

Figure 5.


300
250
200
150
100
50
0
Percentage of Control(%)
*
Control
Sham
Sh+Ac
Sh+Ea
Pain
Pain+Ac
Pain+Ea

VII. CONSIDERAÇÕES GERAIS

CONSIDERAÇÕES GERAIS

Considerando a alta prevalência da dor neuropática na população mundial e o difícil tratamento desta condição, este estudo buscou abordar mecanismos neurobiológicos envolvidos nesta patologia e em possíveis tratamentos não famacológicos, como acupuntura e eletroacupuntura. Concomitante a isto, buscamos revelar o efeito do isoflurano sobre os tratamentos em questão, devido ao frequente uso deste fármaco em pesquisas pré-clínicas utilizando as técnicas de AC e EA. Abaixo listamos os resultados e conclusões obtidas nesta tese:

- Artigo 1

- Isoflurano aumentou a analgesia promovida por AC e EA no teste de von Frey;
- AC e EA aumentaram a locomoção no teste de campo aberto, porém a anestesia diminuiu este comportamento.
- Isoflurano diminuiu os níveis de S100β em soro, enquanto a aplicação de EA sem anestesia em animais com DN aumentou estes níveis;
- Isoflurano diminuiu os níveis de NGF no nervo ciático esquerdo, enquanto a EA aumentou.
- A DN e o isoflurano diminuíram os níveis de NGF no nervo ciático esquerdo, enquanto que a EA aumentou.

- Artigo 2

- AC e EA aumentaram comportamentos exploratórios, enquanto a DN e o isoflurano diminuíram.
- Isoflurano aumentou o número de bolos fecais.

- Na actigrafia, a anestesia e a EA aumentaram o número de NTDs no ciclo claro, enquanto a DN diminuiu. No ciclo escuro, o isoflurano e a DN mantiveram o mesmo efeito, porém a EA diminuiu as NTDs.
- O Isoflurano diminuiu os níveis de NMDA em tronco encefálico e medula espinhal, porém a EA aumentou estes níveis em medula espinhal.

- Artigo 3

- A AC apresentou maior analgesia em animais submetidos ao modelo de DN em comparação à EA nos testes de hiperalgesia térmica e mecânica.
- O modelo de DN aumentou a densidade de fibras musculares de 1000 μm2 e diminuiu as de 4000μm2 e 6000μm2, promovendo atrofia do músculo gastrocnemio esquerdo. Ambos os tratamentos não alteraram esta medida.
- A DN também aumentou o nível de DCFH, marcador de estresse oxidativo, no gastrocnemio esquerdo, e esta medida não foi alterada pela AC ou EA.

Considerando o exposto acima, concluímos que o Isoflurano aumenta a analgesia gerada pela AC e EA, protegendo os animais do efeito estressor dos tratamentos quando aplicado em animais acordados. Este resultado é concomitante com a diminuição do nível periférico de S100β, marcador de morte neuronal relaciona ao estresse. Por outro lado, o isoflurano diminuiu os níveis de NGF em nervo periférico, provavelmente diminuindo a regeneração neural; enquanto isso a EA aumentou esta medida. A anestesia diminuiu o comportamento exploratório, o que corrobora a diminuição nos níveis de NMDA em tronco encefálico e medula espinhal também promovido pelo isoflurano, considerando a relação entre as vias glutamatérgicas e o comportamento exploratório. Por fim, a AC mostrou-se como melhor tratamento em comparação à EA para promoção de analgesia

em animais submetidos à DN, provavelmente pela ativação de fibras Aδ. Por outro lado, demonstramos que o modelo de DN utilizado promove atrofia muscular e aumento de marcador de ROS em gastrocnemio, e ambos os tratamentos não alteram estas medidas (Fig.4).

Estes resultados demonstram a necessidade de novas pesquisas pré-clínicas demonstrando os efeitos e mecanismos de ação envolvidos em alternativas terapêuticas que possam reverter os efeitos deletérios da dor neuropática.

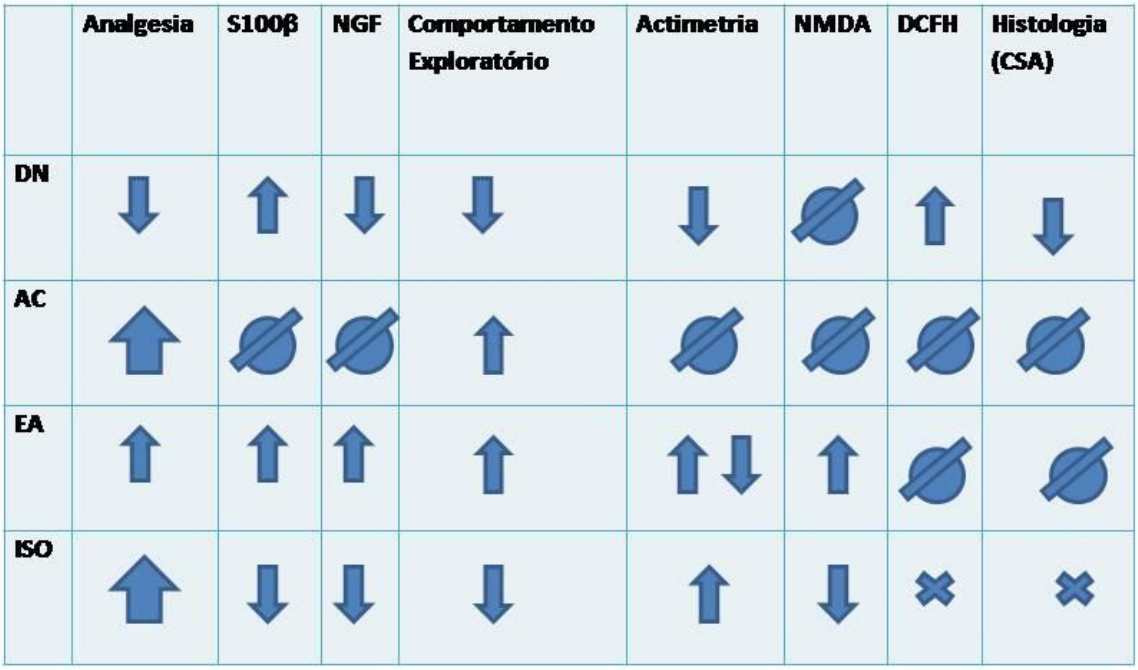


Figure 4. Resumo dos resultados apresentados nesta tese.

VIII. PERSPECTIVAS

PERSPECTIVAS

Sabe-se que AC e EA alteram vias opioide e serotoninérgica, porém a influência do isoflurano sobre estas vias não está completamente elucidada. Sendo assim, pretendemos dosar a expressão de receptores opioides em medula espinhal por meio da técnica de *Western Blotting*, bem como dosagem de serotonina e β-endorfinas por meio de ensaio imunoenzimático ELISA. Desta forma, esperamos melhor compreender a interação entre os sistemas opioidérgicos e serotoninérgicos no modelo experimental proposto, esclarecendo possíveis alterações que podem ser causadas pela sobreposição dos tratamentos e dos fármacos em questão.

Outra perspectiva é associar à AC e/ou EA a estimulação transcraniana por corrente contínua (ETCC) no tratamento da DN. Conforme estudo publicado previamente pelo nosso grupo por Cioato e colaboradores (2016), a ETCC apresentou bom efeito analgésico no tratamento da DN assim como as técnicas de neuromodulação periféricas apresentadas nesta tese. A associação entre a neuromodulação central e periférica pode aumentar os efeitos analgésicos e alterar diferentes vias envolvidas no processo nociceptivo.

IX. ANEXOS

A) APROVAÇÃO DO COMITÊ DE ÉTICA

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

COMISSÃO DE ÉTICA NO USO DE ANIMAIS

A Comissão de Ética no Uso de Animais (CEUA/HCPA) analisou o projeto:

Projeto: 130298
Data da Versão do Projeto: 30/06/2013

Pesquisadores:
IRACI LUCENA DA SILVA TORRES

Título: AVALIAÇÃO DE EFEITOS DA ACUPUNTURA E DA ELETROACUPUNTURA EM MODELO ANIMAL DE DOR NEUROPÁTICA: PARÂMETROS COMPORTAMENTAIS E BIOQUÍMICOS

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, 07 de novembro de 2013.

Dr. Alessandro Osvaldt
Coordenador CEUA/HCPA

B) DIVULGAÇÕES

2014:

ADACHI, L. N. S.; VERCELINO, R.; SCARABELOT, V. L.; MARQUES, P. R.; OLIVEIRA, C.; MEDEIROS, L.; CIOATO, S.; QUEVEDO, A. S.; CAUMO, W.; TORRES, I. L. S. AVALIAÇÃO DO EFEITO DA ACUPUNTURA E ELETROACUPUNTURA EM MODELO ANIMAL DE DOR NEUROPÁTICA - ESTUDO PILOTO. In: 34º Semana Científica do Hospital de Clínicas de Porto Alegre, 2014, Porto Alegre. Anais da 34º Semana Científica do Hospital de Clínicas de Porto Alegre, 2014. v. 34. p. 95-96

2015:

SPEZIA ADACHI, L.N.; VERCELINO, R.; OLIVEIRA, C.; SCARABELOT, V. L.; RIZZO, T. L.; MEDEIROS, L.F.; MARQUES FILHO, P.R.; CIOATO, S. G.; CAUMO, W.; TORRES, I. L. S. Efeito do uso da anestesia na aplicação de Acupuntura (AC) em ratos submetidos ao modelo de dor neuropática (DN). In: 35º Semana Científica do Hospital de Clínicas de Porto Alegre, 2015, Porto Alegre. Anais da 35º Semana Científica do Hospital de Clínicas de Porto Alegre, 2015.

VERCELINO, R.; SPEZIA ADACHI, L. N.; OLIVEIRA, C.; SCARABELOT, V. L.; RIZZO, T. L.; CIOATO, S.G.; CAUMO, W. ; TORRES, I. L. S. Acupuntura reduz a hiperalgesia mecânica e térmica induzida por compressão de nervo isquiático. In: VII SIMPÓSIO INTERNACIONAL EM NEUROMODULAÇÃO, 2015, São Paulo. Anais do VII SIMPÓSIO INTERNACIONAL EM NEUROMODULAÇÃO, 2015.

SPEZIA ADACHI, L.N.; VERCELINO, R.; OLIVEIRA, C.; SCARABELOT, V.L.; RIZZO, T. L.; MEDEIROS, L. F.; MARQUES FILHO, P.R.; CIOATO, S.G.; CAUMO, W.; TORRES, I. L. S. Comparação entre a aplicação de Acupuntura (AC) com e sem anestesia em ratos submetidos ao modelo de dor neuropática (DN). In: VII SIMPÓSIO INTERNACIONAL EM NEUROMODULAÇÃO, 2015, São Paulo. Anais do VII SIMPÓSIO INTERNACIONAL EM NEUROMODULAÇÃO, 2015

2016:

MUNERETTO, C. S.; SPEZIA ADACHI, L.N. ; VERCELINO, R. ; OLIVEIRA, C. ; SCARABELOT, V.L.; MEDEIROS, L.F. ; SOUZA, A. ; CIOATO, S. G.; CAUMO, W. ; TORRES, I. L. S. Isoflurano altera parâmetros em teste de Campo Aberto de animais submetidos a um modelo de dor neuropática tratados com Acupuntura ou Eletroacupuntura.. In: 36º Semana Científica do HCPA, 2016, Porto Alegre. Anais da 36º Semana Científica do HCPA, 2016

SPEZIA ADACHI, L.N.; VERCELINO, R.; OLIVEIRA, C. ; MUNERETTO, C. S. ; SCARABELOT, V. L. ; MEDEIROS, L. ; SOUZA, A. ; CAUMO, W. ; TORRES, I. L. S. Anestesia altera níveis séricos de S100β em modelo animal de dor neuropática (DN) tratado com Acupuntura (A) ou Eletroacupuntura (EA). In: 36ª Semana Científica do HCPA, 2016, Porto Alegre. Anais da 36ª Semana Científica do HCPA, 2016.

SPEZIA ADACHI, L.N.; VERCELINO, R.; OLIVEIRA, C.; SCARABELOT, V. L.; MEDEIROS, L. F.; SOUZA, A.; CIOATO, S.G.; CALLAI, E.; CAUMO, W.; TORRES, I. L. S. ISOFLURANE ALTERS OPEN FIELD TEST AND S100B SERUM LEVEL ON ANIMAL MODEL OF NEUROPATHIC PAIN SUBMITTED TO ACUPUNCTURE AND ELETROACUPUNCTURE TREATMENT. In: 16th World Congress on Pain, 2016, Yokohama. 16th World Congress on Pain, 2016.

VERCELINO, R.; SPEZIA ADACHI, L.N.; OLIVEIRA, C.; SCARABELOT, V.L.; CIOATO, S.G.; TORRES, IRACI L. S. ACUPUNCTURE CHANGES HYPERALGESIA INDUCED BY SCIATIC NERVE COMPRESSION. In: 16th World Congress on Pain, 2016, Yokohama. 16th World Congress on Pain, 2016.

OLIVEIRA, C.; SPEZIA ADACHI, L.N.; VERCELINO, R.; SCARABELOT, V.L.; CIOATO, S.G. ; TORRES, I. L. S. . APPLICATION EFFECT OF ACUPUNCTURE (AC) AND ELETROACUPUNCTURE (EA) IN RATS WITH AND WITHOUT ANESTHESIA IN A MODEL OF NEUROPATHIC PAIN (NP). In: 16th World Congress on Pain, 2016, Yokohama, Japan. 16th World Congress on Pain, 2016.

C) ARTIGOS PUBLICADOS NO PERÍODO DO DOUTORADO

a) Autoria

Adachi, L. N. S. ; Caumo, Wolnei ; Laste, G. ; Medeiros, L. F. ; Rozisky, Jr ; Souza, A. ; Fregni, F. ; Torres, I. L. S. Reversal of Chronic Stress induced Pain by Transcranial Direct Current Stimulation (tDCS) in an Animal Model. Brain Research, v. 1489, p. 17-26, 2012

Adachi, L. N. S. ; Quevedo, A. ; Souza, A. ; Scarabelot, Vl ; Rozisky, J. R. ; Oliveira, C. ; Marques, P.; Medeiros, L.F. ; Fregni, F. ; Caumo, W. ; Torres, I.L.S. . Exogenously induced brain activation regulates neuronal activity by top-down modulation: conceptualized model for electrical brain stimulation. Experimental Brain Research, v. 233, p. 1377-1389, 2015

b) Artigo aceito para publicação

Adachi, L. N. S.; Oliveira, C. **;** Vercelino R; Macedo, I.C.M.; Laste, G.; Quevedo, A.S.; Scarabelot, V.L.; Caumo, W.; Torres, I.L.S. Evaluation of different procedure involved in the Transcranial Direct Current Stimulation (tDCS) technique experimental application. Clinical & Biomedical Research.

c) Coautoria

Rozisky, J. R. ; Laste, G. ; Medeiros, Liciane F. ; Souza, Vs ; Adachi, L ; Macedo, I. C. ; Caumo, W. ; Torres, Ils . Morphine Treatment in Neonate Rats Increases Exploratory Activities: Reversal by Antagonist D2 Receptor. British Journal of Medicine and Medical Research, v. 4, p. 351-367, 2014.

Souza, Andressa ; Dussan-Sarria, Jairo Alberto ; Medeiros, Liciane Fernandes ; Souza, Ana Cláudia ; Oliveira, Carla ; Scarabelot, Vanessa Leal ; Adachi, Lauren Naomi ; Winkelmann-Duarte, Elisa Cristiana; Philippi-Martins, Bárbara Beatriz ; Netto, Carlos Alexandre ; Caumo, Wolnei ; Torres, Iraci L.S**.** Neonatal hypoxic-ischemic encephalopathy reduces c-Fos activation in the rat hippocampus: evidence of a long-

lasting effect. International Journal of Developmental Neuroscience , v. 38, p. 213-222, 2014

Marques Filho, P. R. ; Vercelino, R. ; Cioato, S. G. ; Medeiros, L.F. ; Oliveira, C. ; Scarabelot Vl ; Souza, A. De ; Rozisky, Jr ; Quevedo, A. ; Adachi, L. N. S. ; Sanches, P. R. ; Fregni, F. ; Caumo, Wolnei ; Torres, I.L.S. . Transcranial direct current stimulation (tDCS) reverts behavioral alterations and brainstem BDNF level increase induced by neuropathic pain model: Long-lasting effect. Progress in Neuro-Psychopharmacology & Biological Psychiatry, v. 64, p. 44-51, 2015.

Cioato, Stefania Giotti ; Medeiros, Liciane Fernandes ; Marques Filho, Paulo Ricardo ; Vercelino, R ; De Souza, Andressa ; Scarabelot, Vanessa Leal ; De Oliveira, Carla ; Adachi, Lauren Naomi Spezia ; Fregni, Felipe ; Caumo, Wolnei ; Torres I Da S. Long-Lasting Effect of Transcranial Direct Current Stimulation in the Reversal of Hyperalgesia and Cytokine Alterations Induced by the Neuropathic Pain Model. Brain Stimulation: basic, translational and clinical research in neuromodulation, v. 9, p. 209-217, 2016

Scarabelot, Vl ; Oliveira, C. ; Souza, A. ; De Freitas Js ; Macedo, I. C. ; Quevedo, A. ; Caumo, Wolnei ; Cioato, S. G. ; Adachi, L. N. S. ; Torres, I. L. S**.** Melatonin Alters the Mechanical and Thermal Hyperalgesia Induced by Orofacial Pain Model in Rats. Inflammation, v. n/a, p. n/a-n/a, 2016.

D) ARTIGO 1 SUBMETIDO AO PERIÓDICO PHARMACOLOGICAL RESEARCH

Manuscript Details

Manuscript number	YPHRS_2017_512
Title	Isoflurane enhances the analgesic effect of acupuncture and electroacupuncture on neuropathic pain in rats
Article type	Research Paper

Abstract

Introduction: Due to the difficulty of applying acupuncture (Ac) and electroacupuncture (EA) to the treatment of neuropathic pain (NP) in awake and freely moving rats, most studies use restraint or anesthesia. However, both conditions could be a potential source of bias. The purpose of the present study was to determine whether isoflurane interferes with the analgesic effects of Ac and EA using an NP rat model. We also investigated the effect of isoflurane on the levels of S100β protein in serum and nerve growth factor (NGF) in the left sciatic nerve of the animals. Methods: Using 140 male Wistar rats, we evaluated the nociceptive response induced by isoflurane using the von Frey test, serum levels of S100β, and NGFlevelsinthe left sciatic nerve. NP was induced by constriction of the left sciatic nerve. The treatments, with or without isoflurane anesthesia, started 14days after surgery (20min/day/8days). The von Frey test was performed at baseline, 14 days postoperatively, and immediately, 24h and 48h after the last treatment session. Animals were killed by decapitation. Serum and constricted nerves were collected and frozen at -80oC. Generalized estimating equations/Bonferroni were used to analyze the results of the nociceptive test and three-way analysis of variance/SNK or Fisher's LSD test was used to analyze the results of the biochemical analysis. Results were considered significant if P≤0.05. Results: At baseline, there were no differences in the nociceptive response threshold among all groups. Fourteen days after surgery, the groups with NP had a decreased pain threshold compared to the other groups, showing that NP was established (P<0.05). Ac and EA enhanced the mechanical pain threshold immediately after the last session in the groups with NP and without anesthesia. In addition, when all groups received isoflurane, the nociceptive threshold significantly increased (P<0.001). These results were maintained for 48h after the last treatment session. There was an interaction between the independent variables: pain x treatments x isoflurane in serum S100β levels (P<0.001) and NGF levels in the left sciatic nerve (P<0.001). Conclusion: Isoflurane enhanced the analgesic effects of Ac and EA and altered serum S100β levels and NGF levels in the left sciatic nerve in rats with NP.

Keywords	Isoflurane; Neuropathic pain; Acupuncture; Electroacupuncture
Corresponding Author	Iraci Lucena da Silva Torres
Corresponding Author's Institution	Universidade Federal do Rio Grande do Sul
Order of Authors	Lauren Naomi Adachi, Rafael Vercelino, Carla de Oliveira, Vanessa Scarabelot, Andressa Souza, Tizye Rizzo, Liciane Medeiros, Stefania Cioatoi, Wolnei Caumo, Iraci Lucena da Silva Torres

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Dear Ms. Adachi,

You have been listed as a Co-Author of the following submission:

Journal: Pharmacological Research

Title: Isoflurane enhances the analgesic effect of acupuncture and electroacupuncture on neuropathic pain in rats

Corresponding Author: Iraci Lucena da Silva Torres

Co-Authors: Lauren Naomi Adachi, Rafael Vercelino, Carla de Oliveira, Vanessa Scarabelot, Andressa Souza, Tizye Rizzo, Liciane Medeiros, Stefania Cioatoi, Wolnei Caumo

Iraci Lucena da Silva Torres submitted this manuscript via Elsevier's online submission system, EVISE®. If you are not already registered in EVISE®, please take a moment to set up an author account by navigating to http://www.evise.com/evise/faces/pages/navigation/NavController.jspx?JRNL_ACR=YPHRS

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If you did not co-author this submission, please contact the Corresponding Author directly at 87605@ufrgs.br.

Thank you,

Pharmacological Research

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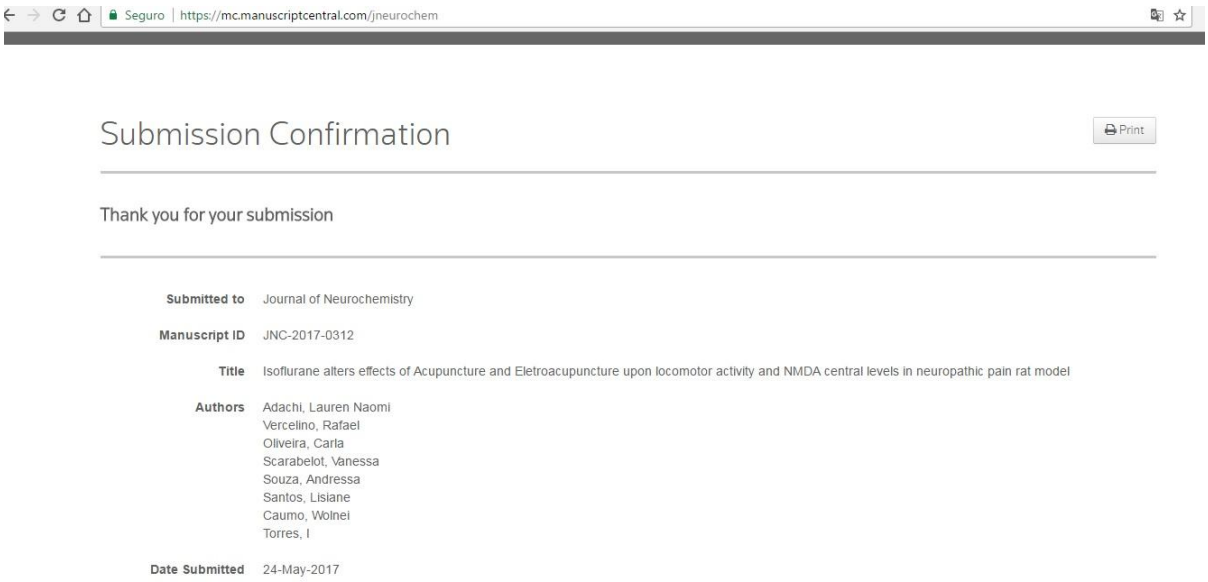
E) ARTIGO 2 SUBMETIDO AO PERIÓDICO JOURNAL OF NEUROCHEMISTRY



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Submission Confirmation

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Thank you for your submission

Submitted to	Journal of Neurochemistry
Manuscript ID	JNC-2017-0312
Title	Isoflurane alters effects of Acupuncture and Eletroacupuncture upon locomotor activity and NMDA central levels in neuropathic pain rat model
Authors	Adachi, Lauren Naomi Vercelino, Rafael Oliveira, Carla Scarabelot, Vanessa Souza, Andressa Santos, Lisiane Caumo, Wolnei Torres, I
Date Submitted	24-May-2017

Journal of Neurochemistry

Isoflurane alters effects of Acupuncture and Eletroacupuncture upon locomotor activity and NMDA central levels in neuropathic pain rat model

Journal:	*Journal of Neurochemistry*
Manuscript ID	Draft
Manuscript Type:	Original Article
Date Submitted by the Author:	n/a
Complete List of Authors:	Adachi, Lauren Naomi; Universidade Federal do Rio Grande do Sul Vercelino, Rafael; Universidade Federal de Ciencias da Saude de Porto Alegre Oliveira, Carla; Universidade Federal do Rio Grande do Sul Scarabelot, Vanessa; Universidade Federal do Rio Grande do Sul Souza, Andressa; Universidade Federal do Rio Grande do Sul Santos, Lisiane; Universidade Federal do Rio Grande do Sul Caumo, Wolnei; Universidade Federal do Rio Grande do Sul Torres, I ; Universidade Federal do Rio Grande do Sul, Pharmacology
Area/Section:	Neuronal Plasticity & Behavior
Keywords:	Acupuncture, Neuropathic pain, Eletroacupuncture, Isoflurane, NMDA

F) ARTIGO 3 SUBMETIDO AO PERIÓDICO MOLECULAR NEUROBIOLOGY

Molecular Neurobiology

Acupuncture induces greater analgesia than Eletroacupuncture in rats submitted to neuropathic pain model.

--Manuscript Draft--

Manuscript Number:	
Article Type:	Original Article
Keywords:	Neuropathic pain, Acupuncture, Eletroacupuncture, gastrocnemius, CSA, DCFH.
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Abstract:	Introduction: The purpose of this study was to investigate the comparison of repeated Acupuncture or Eletroacupuncture on nociceptive behavior, TNF-α imunostaining, cross-sectional area measure (CSA) and oxidative stress parameters in the gastrocnemius muscle on chronic constriction injury (CCI) model. Methods: seventy male Wistar rats with 60 days-year-old were used. The nociceptive response was evaluated by Hot plate and Randall Selitto test, at baseline; 14 days post-surgery, immediately and 24h after the last session of treatment. Neuropathic pain (NP) was induced by sciatic nerve constriction, and the Ac or EA treatments started 14days after-surgery (20min/day/8days). The statistical analysis GEE/Bonferroni were conducted to nociceptive tests, and three-way ANOVA/SNK, for histological and biochemical analysis. The results were considered significant with P ≤ 0.05. Results: Fourteen days after-surgery, groups submitted to NP presented decrease in nociceptive threshold compared to other groups, confirming the establishment of NP (P<0.05). Both treatments enhanced nociceptive threshold immediately after the last session, however Ac induces greater analgesia than EA immediately in the hot plate (P<0.05) and Randall Selitto tests (P<0.05). In CSA of gastrocnemius muscle, Pain groups showed a significant increase in fibers 1000 um2 (P≤ 0.000) and a significant decrease in fibers 4000 um2 and 6000 um2 (P≤ 0.000). The DCFH levels were increased on gastrocnemius muscle of rats submitted to CCI model (P≤ 0.000), and AC and EA were not able to revert this effect. Conclusion: AC induces greater analgesia than EA, however were not able to revert changes caused by CCI model on gastrocnemius muscle.

Re: "Acupuncture induces greater analgesia than Eletroacupuncture in rats submitted to neuropathic pain model."

Full author list: Iraci Torres; Lauren Naomi S Adachi; Rafael Vercelino; Francele V Piazza; Carla de liveira; Vanessa L Scarabelot; Natalia P Silveira; Bruna Belaver; Wolnei Caumo; Andre Quincozes-Santos

Dear Ms Lauren Naomi Adachi,

We have received the submission entitled: "Acupuncture induces greater analgesia than Eletroacupuncture in rats submitted to neuropathic pain model." for possible publication in Molecular Neurobiology, and you are listed as one of the co-authors.

The manuscript has been submitted to the journal by Dr. Dra. Iraci Torres who will be able to track the status of the paper through his/her login.

If you have any objections, please contact the editorial office as soon as possible. If we do not hear back from you, we will assume you agree with your co-authorship.

Thank you very much.

With kind regards,

Springer Journals Editorial Office

Molecular Neurobiology