UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL
INSTITUTO DE CIÊNCIAS BÁSICAS DA SAÚDE
PROGRAMA DE PÓS-GRADUAÇÃO EM NEUROCIÊNCIAS

EFEITO ANTIADITIVO DE EXTRATO DE *PASSIFLORA INCARNATA* L. E TERAPIA DE ESTIMULAÇÃO TRANSCRANIANA POR CORRENTE CONTÍNUA (ETCC) EM UM MODELO DE DEPENDÊNCIA DE ÁLCOOL EM RATOS.

REBECA VARGAS ANTUNES SCHUNCK

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REBECA VARGAS ANTUNES SCHUNCK

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Tese de doutorado apresentada ao Programa de Pós-graduação em Neurociências, da Universidade Federal do Rio Grande do Sul, como requisito parcial para a obtenção do título de doutor em neurociências.

Porto Alegre

2019

A Deus, meus pais, Nei e Mara, ao Vitor e à Iasmin.

À professora Mirna.

AGRADECIMENTOS

A Deus, pela vida e pela força dada para chegar até aqui. Agradeço à minha família, meu esposo Vitor, pela compreensão e o apoio dado para que eu pudesse realizar este sonho, que, afinal, acabou tornando-se o seu sonho também. À minha pequena Iasmin, que apesar de sua pouca idade, demonstra que será esforçada e estudiosa. É uma menina preciosa e muito querida, graças a Deus por tê-la comigo. Agradeço a ela toda paciência tida nos momentos que falo a ela que tenho que estudar e trabalhar no computador (para redigir a tese). Aos meus pais, Nei e Mara, os quais sempre me incentivaram, sem medir esforços, para a realização do sonho de estudar. Tenho como umas das mais lindas lembranças de minha infância, o pai sentado ao meu lado me ajudando a fazer os "trabalhos" para a escola ou me ensinando matemática, física, etc. Essas memórias jamais se apagarão. Agradeço também aos meus sogros, Clara e Cecílio, pelo apoio, e aos meus cunhados Patrícia e Giovani, pelo incentivo e compreensão a cada vez que precisava estudar. Ao meu sobrinho Augusto, meu incentivo para que ele trilhe caminhos do aprendizado e que vá além. Quero ser inspiração para ele. Às minhas inspirações de estudo na família, Arlei (*in memoriam*, sei que se estivesses aqui vibrarias muito com este momento), à Arli (tia "Ica") por ser tão dedicada, e ao Carlos Henrique, os quais me ajudaram a descobrir como é bom estudar. Aos meus avós, Guiomar e Benjamin (*in memoriam*) pelo amor com que me cuidaram todos os anos que morei com eles para poder estudar em Porto Alegre. Quero agradecer à avó Anna e Salmo (*in memoriam*), pelo apoio e por vibrar comigo a cada etapa.

À paciência e dedicação da minha orientadora Mirna, agradeço o aprendizado e a compreensão por tudo. Agradeço por ter feito parte do seu grupo de pesquisa, e nunca vou esquecer a primeira reunião que tivemos, quando pedi para ser sua IC (iniciação científica). À professora Isabel, pela sua dedicação e destreza em artigos, sempre pronta a me ajudar. Muito querida e incentivadora. À professora Iraci, pelo seu vasto conhecimento e pela ajuda. À professora Denise, coordenadora do PPG Neurociências, pela compreensão e apoio. À Janaína, do laboratório, quem sempre teve a visão ampla de tudo o que precisávamos fazer para organizarmos os experimentos e pela sua dedicação para que os trabalhos acontecessem. Tens a minha admiração pela pessoa que és! À Marina pela ajuda e a destreza para lidar com os "ratinhos".

À Secretária de Saúde de Igrejinha, Dra. Simone Amaral, pelo apoio dado para que pudesse terminar este trabalho. Lembro da nossa primeira conversa, momento em que pedi a ela que eu queria muito continuar com o doutorado, e ela comprometeu-se a me ajudar a concluir este sonho, sempre vibrando em cada etapa que concluía. Aos chefes que passaram em minha vida, ao Alex, ao Valter e, atualmente, à Isabel, que me apoiaram, sem exceção. À minha colega de profissão, Fabiana, por gostar de realizar trabalhos e pesquisa. Aos colegas da farmácia de manipulação, por trabalharem tão bem.

Agradeço, por fim, ao Conselho de Desenvolvimento Científico e Tecnológico (CNPq) e à Coordenação e Aperfeiçoamento de Pessoal de Nível Superior (CAPES), pelo apoio financeiro.

“Se as coisas são inatingíveis... ora! Não é motivo para não querê-las...
Que tristes os caminhos, se não fora a presença distante das estrelas!”

Mário Quintana

APRESENTAÇÃO

Os resultados desta tese de doutorado estão apresentados sob a forma de dois artigos científicos, o primeiro publicado pelo periódico *Phytotherapy Research*, e o segundo a ser submetido ao periódico *Brain Research*. Os itens Introdução, Materiais e Métodos, Resultados e Discussão encontram-se nos próprios artigos.

Os itens Introdução e Discussão desta tese apresentam as bases teóricas e os comentários sobre os resultados contidos nos artigos científicos. O item Referências Bibliográficas se refere apenas às referências utilizadas nos tópicos Introdução e Discussão.

SUMÁRIO

PARTE I

LISTA DE ABREVIATURAS

Aδ – Terminações Nervosas de Fibra do Tipo A-delta

ADH – Álcool Desidrogenase

ALDH – Aldeído Desidrogenase

ATV – Área Tegmental Ventral

BDNF – Fator Neurotrófico Derivado do Encéfalo

COMT – Catecol O-Metiltransferase

CRH – Hormônio Liberador de Corticotropina

DA – Dopamina

ETCC – Estimulação Transcraniana por Corrente Contínua

GABA – Ácido Gama-aminobutírico

IASP – Associação Internacional para o Estudo da Dor

IL-6 – Interleucina-6

IL-10 – Interleucina-10

LTP – Potenciação de Longa Duração

LTD – Depressão de Longa Duração

NA – Noradrenalina

NAcc – Núcleo *accumbens*

NIDA – *National Institute on Drug Abuse*

NMDA – N-metil-D-Aspartato

NF-kB – Fator de Transcrição Nuclear Kappa B

OMS – Organização Mundial da Saúde

PeNSE – Pesquisa Nacional de Saúde do Escolar

5-HT – Serotonina

SNC – Sistema Nervoso Central

TDAH – Transtorno do Déficit de Atenção e Hiperatividade

Th1 – Linfócito T Auxiliar Tipo 1

TNF-α – Fator de Necrose Tumoral-Alfa

TrkB – Tropomiosina Cinase B

RESUMO

O transtorno relacionado ao uso de álcool é uma desordem crônica caracterizada por compulsão pela procura e ingesta de álcool, perda de controle sobre seu consumo de álcool e surgimento de estado emocional negativo como disforia, ansiedade e irritabilidade, quando o acesso ao álcool é impedido. Um dos eventos mais estressantes para pacientes dependentes de álcool é a síndrome de abstinência, cuja intensidade aumenta com repetidas tentativas de retirada e recaídas. Dor crônica e transtorno por uso de álcool são altamente prevalentes e podem ocorrer simultaneamente, com o abuso de álcool antecedendo a dor crônica ou o início de abuso de álcool após desenvolvimento de dor crônica. Diversos fatores modulam tanto a resposta nociceptiva quanto os aspectos relacionados ao alcoolismo, entre os quais podemos citar BDNF e IL-10 servindo como marcadores moleculares experimentais úteis para avaliação de ambos os aspectos. Tendo em vista as consequências adversas do uso de álcool do ponto de vista médico, social e econômico, e considerando ainda a baixa efetividade dos poucos tratamentos disponíveis para o alcoolismo, a busca por novos tratamentos é um investimento necessário. Nesse sentido, a *Passiflora incarnata* tem uma série de usos tradicionais e clínicos, entre os quais destaca-se como tratamento para transtornos de ansiedade e distúrbios do sono. Em contrapartida, o ETCC tem exibido importantes resultados no tratamento de diversos transtornos tais como depressão, ansiedade, e até adição às substâncias. Nossos resultados demonstraram que os tratamentos utilizados, *Passiflora incarnata* e ETCC, foram capazes de reverter a analgesia térmica induzida pela retirada de etanol, observados após 11 dias da finalização dos tratamentos. Os tratamentos foram capazes de restabelecer os níveis de BDNF no tronco encefálico, após 12 dias. Considerando que esta estrutura é importante na modulação da dor, nossos dados são relevantes para a procura de novos tratamentos que podem ser promissores na terapia de recaída ao uso de álcool.

ABSTRACT

Alcohol-related disorder is a chronic disease characterized by ingestion of demand and ingestion of alcohol, loss of control over the consumption of ethanol, and emergence of negative emotional state as dysphoria, anxiety, and irritability, when access to alcohol is prevented. One of the most stressful events for alcohol-dependent patients is the withdrawal syndrome, the intensity of which is increased with repeated attempts to withdraw and relapse. Chronic pain and alcohol use disorder are highly prevalent and may occur concurrently with alcohol abuse preceding chronic pain or the onset of alcohol abuse after development of chronic pain. Several factors modulate both the nociceptive response and aspects related to alcoholism, among which we can mention BDNF and IL-10 serving as useful experimental molecular markers for evaluation of both aspects. Considering the adverse effects of alcohol use from a medical, social and economic point of view, and considering the low effectiveness of the few treatments available for alcoholism, the search for new treatments is a necessary investment. In this sense, *Passiflora incarnata* has a number of traditional and clinical uses, among which it stands out as a treatment for anxiety disorders and sleep disorders. In contrast, tDCS has shown important results in the treatment of various disorders such as depression, anxiety, and even substance addiction. Our results demonstrated that the treatments used, *Passiflora incarnata* and tDCS, were able to revert the thermal analgesia induced by the ethanol withdrawal, observed after 11 days of the end of the treatments. Interestingly, the treatments were able to restore BDNF levels in the brainstem after 12 days. Considering that this structure is important in the modulation of pain, our data are relevant to the search for new treatments that may be promising in relapse therapy to alcohol use.

1. INTRODUÇÃO

1.1 Transtorno relacionado ao uso de álcool

1.1.1 Aspectos epidemiológicos

O álcool etílico é o agente psicotrópico mais antigo usado pela humanidade. O início de sua produção data de 8.000 a.C. (GATELY, 2011). Segundo a Organização Mundial da Saúde (OMS) aproximadamente dois bilhões de pessoas consomem álcool no mundo (OMS, 2018). O consumo excessivo de álcool pode resultar na dependência, que é uma doença neuropsiquiátrica muito prevalente (ROCCHITTA et al., 2012; SPANAGEL, 2009).

Dados divulgados pela OMS mostram que o nível mundial de consumo de álcool permaneceu estável entre 2010 e 2016: 6,4 litros/ano de álcool puro por pessoa com 15 anos ou mais, sendo que a Europa apresenta o consumo anual de álcool mais alto do mundo, 9,8 litros de álcool por pessoa. O consumo de álcool pela população brasileira foi o 22º maior nas Américas, com 7,8 litros de etanol por pessoa (OMS, 2018).

Segundo dados obtidos pela Pesquisa Nacional de Saúde do Escolar (PeNSE), dentre cerca de 2,6 milhões de estudantes que cursavam o 9º ano do ensino fundamental em 2015, 55,5% (1,5 milhão) já havia consumido uma dose de bebida alcoólica alguma vez. Em relação ao consumo atual de álcool, 23,8% (626,1 mil) deles já tinham usado nos últimos 30 dias antes da pesquisa (IBGE, 2015).

1.1.2 Aspectos neurobiológicos

O Manual Diagnóstico e Estatístico de Transtornos Mentais 5ª Edição (DSM-5) define o transtorno por uso de álcool como um agrupamento de sintomas comportamentais e físicos, os quais podem incluir abstinência, tolerância e fissura. Assim que um padrão de uso repetitivo e intenso se desenvolve, indivíduos com transtorno por uso de álcool podem dedicar grandes períodos de tempo para obter e consumir bebidas alcóolicas (DSM, 2014).

Fatores genéticos e ambientais interagem para o desenvolvimento do transtorno por uso de álcool (OROSZI; GOLDMAN, 2004; HIROI; AGATSUMA, 2005; LESCH, 2005; GOLDMAN et al., 2005; MATOŠIĆ et al., 2016). O alcoolismo possui componente hereditário com uma estimativa de 50% a 60% de risco para o desenvolvimento desse

transtorno (CLONINGER et al., 1981; CLONINGER, 1987; MATOŠIĆ et al., 2016). Estima-se, então, que fatores ambientais e hereditários desempenham um papel na proporção meio-a-meio no desenvolvimento da dependência ao álcool (GOLDMAN et al., 2005). Sabe-se que há uma relação entre a gravidade da dependência ao álcool e a presença do alelo A1 do receptor DRD2 de dopamina (BLUM et al., 1990). Todavia, apesar da existência de muitos estudos avaliando componentes genéticos relacionados ao alcoolismo, os resultados ainda são inconsistentes, devido à grande heterogeneidade entre pacientes, levando em consideração o grau de adição, morbidades, entre outros (MATOŠIĆ et al., 2016).

O álcool é um depressor do sistema nervoso central (SNC), induzindo sedação, decréscimo de funções cognitivas e motoras (HARPER; MATSUMOTO, 2005). Em baixas concentrações, causa euforia e redução da inibição, passando a um aumento da desorientação e perda do controle muscular voluntário assim que a concentração de álcool na circulação aumenta. Esse quadro pode levar ao coma e à morte, se a ingestão não diminuir (BURTIS et al., 2008). Quadros de intoxicação ocorrem quando a concentração de álcool no sangue alcança 50-150 mg/dL, enquanto que a concentração de 400-500 mg/dL é letal (HARPER; MATSUMOTO, 2005).

As ações do álcool se estabelecem em diversos sistemas de neurotransmissores, tais como dopamina (DA), serotonina (5-HT), noradrenalina (NA), ácido gama-aminobutírico (GABA), glutamato e opioides endógenos, entre outros (MATOŠIĆ et al., 2016; EŞEL; DİNÇ, 2017). De um modo geral, a intoxicação aguda com álcool potencializa a transmissão GABAérgica e inibe a transmissão glutamatérgica por ação direta em receptores desses neurotransmissores e via cascatas de sinalização intracelular (RON; MESSING, 2012; LOVINGER; ROBERTO, 2012). Como o álcool é um modulador alostérico positivo do receptor $GABA_A$, ocorre aumento da transmissão GABAérgica pela ligação da molécula de álcool num sítio do receptor, que resulta em complexos mecanismos de ativação de receptores $GABA_A$ pré e pós sinápticos em diversas regiões encefálicas tais como cerebelo e via mesolímbica dopaminérgica (ROBERTO et al., 2003; ENOCH, 2008). Receptores $GABA_A$ estão relacionados a muitos dos efeitos agudos do álcool e também contribuem para o desenvolvimento de tolerância, de dependência e dos sintomas da abstinência ao álcool (ENOCH, 2008). Em relação ao glutamato, a exposição aguda ao álcool causa a inibição de receptores glutamatérgicos de maneira dose-dependente. O receptor do tipo N-metil-D-aspartato (NMDA) é o receptor glutamatérgico mais suscetível aos efeitos causados pelo

álcool, e através da ação do etanol em tais receptores, ocorre a supressão da elevação intracelular de cálcio e potenciação de longa duração em neurônios hipocampais (TABAKOFF; HOFFMAN, 2013; MOST et al., 2014; EŞEL; DİNÇ, 2017). Após esses primeiros acontecimentos, uma segunda onda de efeitos indiretos do etanol em vários sistemas de neurotransmissores e neuromoduladores é iniciada, principalmente envolvendo monoaminas tais como DA, 5-HT e NA, bem como opioides e outros neuropeptídeos (VENGELIENE et al., 2008).

O transtorno relacionado ao uso de álcool é uma desordem crônica caracterizada por compulsão pela procura e ingesta de álcool, perda de controle sobre o consumo de álcool e surgimento de estado emocional negativo (por ex., disforia, ansiedade e irritabilidade) quando o acesso ao álcool é descontinuado (KOOB; VOLKOW, 2016; MASON, 2017). A compulsão por ingerir álcool, com diminuição do controle para limitar a ingesta, são resultados da associação entre propriedades reforçadoras positivas e negativas desenvolvidas com o uso crônico de álcool. As propriedades reforçadoras positivas caracterizam o efeito eufórico que levam ao aumento do consumo do álcool. Neste estágio está envolvida a via mesolímbica dopaminérgica, que é responsável pelos efeitos de reforço e recompensa associados ao consumo do álcool e consiste de projeções dopaminérgicas que se estendem da área tegmental ventral (ATV) do mesencéfalo. Várias regiões do prosencéfalo, principalmente o núcleo *accumbens* (NAcc) e o córtex pré-frontal, estão envolvidas no mecanismo de recompensa (GONZALES et al., 2004, BEAR et al., 2008; KOOB; VOLKOW, 2016; MASON, 2017). O sistema opioide também está envolvido no mecanismo de recompensa encefálico, mediado pelos peptídeos opioides no gânglio basal e a transdução da atividade via receptores mu (μ) (LE MERRER et al., 2009; MITCHELL et al., 2012; KOOB; VOLKOW, 2016; MASON, 2017). As propriedades reforçadoras negativas do abuso do álcool são responsáveis pelo desenvolvimento da ansiedade, da depressão e de outras sequelas neurológicas disfóricas que podem ser causadas pela interrupção do consumo de álcool. Neste estágio, os sistemas dopaminérgico e opioide estão prejudicados (KOOB; MASON, 2016; KOOB; VOLKOW, 2016; MASON, 2017). Estes sintomas que surgem após a retirada do álcool são responsáveis pela sua reutilização, pela manutenção do consumo e o desenvolvimento da adição ao álcool (KOOB; VOLKOW, 2016; SENAD, 2017).

Uma região encefálica muito afetada pela dependência ao álcool, entre outras, é o córtex pré-frontal, que está associado a funções executivas. Indivíduos com transtorno relacionado

ao uso de álcool apresentam déficit das funções executivas, tais como prejuízo na tomada de decisão e inibição comportamental (KOOB; MASON, 2016; MASON, 2017). Danos estruturais e funcionais ocorrem no córtex pré-frontal do dependente de álcool, culminando numa redução geral da atividade frontal com prejuízo das funções executivas (FEIN et al., 2002; FRANKLIN et al., 2002; GOLDSTEIN; VOLKOW, 2002). Outro processo importante que ocorre na presença massiva de álcool no encéfalo é o estresse oxidativo e a neuroinflamação que podem levar a apoptose e, em um estágio mais avançado, à neurodegeneração. Acredita-se que a neurodegeneração é um passo crítico para o desenvolvimento de desordens pelo uso do álcool (CREWS et al., 2011). A neuroinflamação, que está diretamente envolvida no desenvolvimento da neurodegeneração e das desordens pelo uso do álcool, está relacionada ao aumento da expressão de uma variedade de genes e citocinas pró-inflamatórias derivadas do sistema imune inato. A indução da imunidade inata em certas regiões encefálicas como o córtex pré-frontal, por exemplo, pode levar a prejuízos na tomada de decisão, que é característico da adição ao álcool (CREWS et al., 2011).

1.1.3 Síndrome de abstinência ao álcool

Um dos eventos mais estressantes para pacientes dependentes de substâncias de abuso como o álcool é a síndrome de abstinência, cuja intensidade aumenta com repetidas tentativas de retirada e recaídas (WITTE et al., 2003). A síndrome de abstinência desencadeia uma série de sintomas, em geral, opostos aos efeitos agudos e que podem ser revertidos pela administração de novas doses (SENAD, 2017). Sinais da abstinência surgem dentro de horas depois de cessar a ingestão crônica de álcool. Num primeiro momento observa-se uma hiperatividade simpatomimética, com quadros de taquicardia, sudorese intensa, tremor, hipertensão, ansiedade e agitação, efeitos que são sentidos nas primeiras 24h após a retirada do álcool. O aumento dos níveis de catecolaminas pode ser resultado do decréscimo da atividade inibitória dos receptores α2 nas células pré-sinápticas (MAYO-SMITH, 2009; CARLSON et al., 2012). A seguir (24-48h depois de cessar o consumo de álcool) podem ocorrer crises convulsivas e até delírios, sendo que este quadro pode ser observado de 3 a 7 dias depois da retirada. O terceiro conjunto de sintomas que sobrevém da interrupção do consumo de álcool caracteriza-se por alucinações visuais e auditivas, confusões, desorientações e pronunciada hiperatividade autonômica. Nesses três conjuntos de sintomas

existem vias neurais e neurotransmissores específicos que juntos são responsáveis pelos efeitos característicos da síndrome de abstinência, mas é importante ressaltar que o aumento da transmissão glutamatérgica na abstinência é responsável por muitos dos sintomas observados (WITTE et al., 2003).

A fissura que é um desejo quase incontrolável de utilizar novamente o álcool, é característica da síndrome de abstinência. Esse fenômeno causa alterações de humor e provoca sensações físicas e modificação do comportamento do usuário. É causado por desencadeadores externos (a própria droga, ambientes ou situações de uso) e internos (humor deprimido e ansiedade) (SENAD, 2017). Acredita-se que dois processos possam formar a base neurobiológica do estado emocional negativo causado pela retirada de álcool, que são: perda da função do mecanismo de recompensa e recrutamento do sistema encefálico de estresse na amígdala (KOOB; MASON, 2016; TUNSTALL et al., 2017). Sabe-se que, durante a fase de abstinência, a transmissão dopaminérgica está prejudicada pela diminuição da liberação de DA e pela diminuição de receptores de DA (VOLKOW et al., 2007; MASON, 2017), e também está comprovado que nesta fase há um recrutamento de substâncias que fazem parte do sistema de estresse encefálico, tais como: o hormônio liberador de corticotropina (CRH), norepinefrina, dinorfina, hipocretina, e substância P (KOOB; LE MOAL, 2008; MASON, 2017).

Modelos animais oferecem a oportunidade para novos achados na identificação de alvos moleculares e estratégias medicamentosas alternativas para o tratamento do alcoolismo, além de cumprirem um papel chave no entendimento dos fatores que embasem o consumo abusivo e patológico do álcool. Nesse contexto, vários modelos animais tentam mimetizar os aspectos envolvidos no processo de sensibilização que ocorre em repetidas retiradas de álcool e recaídas (KNAPP; BREESE, 2012). As formas de administração utilizadas exercem um papel muito importante no desenvolvimento dos diversos modelos de administração de álcool em animais e incluem: a dieta líquida disponível em garrafas para livre escolha, a administração por via intraperitoneal, via oral por gavagem e via inalatória por exposição a vapor de álcool (KNAPP; BREESE, 2012). Atualmente, vem sendo bastante utilizada em pesquisas a administração intermitente de álcool. Nosso grupo de pesquisa padronizou esta forma de administração por gavagem a partir do trabalho realizado por OVERESTREET e colaboradores (2002). A padronização da forma de administração do álcool consiste em três

períodos de cinco dias de administração por gavagem, com intervalos de dois dias sem administração entre eles (SCHUNCK et al., 2015).

1.1.4 Transtorno por uso de álcool e dor

Dor crônica e transtorno por uso de álcool são altamente prevalentes e podem ocorrer simultaneamente (SACKS et al., 2010; MCDERMOTT et al., 2018; POWERS et al., 2019). Indivíduos com dor crônica tendem a consumir doses excessivas de álcool e são prováveis candidatos a serem incluídos nos critérios de transtorno por uso de álcool (ZALE et al., 2015; MCDERMOTT et al., 2018; POWERS et al., 2019). O álcool produz analgesia, o que pode contribuir para o alívio da dor crônica (SHEU et al., 2008; ZALE et al., 2015). No entanto o consumo excessivo de álcool é associado com início e progressão de várias condições dolorosas (SHEU et al., 2008; ZALE et al., 2015). Indivíduos que abusam do álcool tendem a relatar maior prevalência e intensidade de dor (POWERS et al., 2019; BOISSONEAULT et al., 2019). A dor e o transtorno por uso de álcool compartilham circuitos em comum com alterações patológicas em estruturas neurais (por ex., amígdala central, córtex pré-frontal, ínsula, NAcc) que podem contribuir para o desenvolvimento e a manutenção de dor crônica e dependência de álcool (EGLI et al., 2012). O consumo excessivo de álcool pode provocar a desregulação no sistema opioide endógeno, com implicações na modulação da dor e no sistema de recompensa. A administração aguda de álcool estimula a liberação de peptídeos opioides, enquanto a exposição ao álcool em longo prazo leva a dessensibilização, resultando em deficiência central destes peptídeos (GIANOULAKIS et al., 2001; EGLI et al., 2012; ZALE et al., 2015).

A dor não é uma expressão direta de um evento sensorial, mas sim o produto elaborado de uma variedade de sinais neurais causados por estímulos nociceptivos nos receptores periféricos (nociceptores) e codificada em padrões de potenciais de ação até centros de integração. Os nociceptores são terminações nervosas livres de neurônios aferentes primários responsáveis pela transdução de estímulos térmicos, mecânicos e químicos de alta intensidade (MENESCAL-DE-OLIVEIRA; DA SILVA, 2009; KANDEL et al., 2014). O trato espinotalâmico, com suas projeções para estruturas do tronco encefálico, tálamo e córtex, pode ser considerado a via nociceptiva ascendente mais proeminente da medula espinal para condução de informações nociceptivas até as estruturas centrais envolvidas no processamento

da dor (MENESCAL-DE-OLIVEIRA; DA SILVA, 2009; KANDEL et al., 2014). A modulação descendente do processamento nociceptivo espinal tem como principais estruturas a substância cinzenta periaquedutal, o *locus ceruleus* e o bulbo rostroventromedial, que inclui o núcleo magno da rafe, as quais são responsáveis pela inibição da transmissão dos sinais de dor nos neurônios do corno posterior da medula espinal (MENESCAL-DE-OLIVEIRA; DA SILVA, 2009; KANDEL et al., 2014). Outras regiões corticais, como o córtex pré-frontal, também participam da modulação da dor, principalmente nas respostas emocionais à dor (FONOFF, 2009). Vale ressaltar que o córtex pré-frontal também desempenha um papel importante nos comportamentos comumente observados em transtorno relacionados ao uso de álcool, incluindo a propensão para sofrer recaídas (LASSETER et al., 2010). O sistema opioide também é um importante modulador da via descendente da dor, podendo estar implicado na supressão da dor (FIELDS, 2004). Peptídeos opioides endógenos e seus receptores μ, delta (δ), kappa (κ) e orfanina fazem parte do sistema opioide. Os receptores μ estão altamente concentrados na superfície do corno dorsal da medula espinal, no bulbo ventral e na substância cinzenta periaquedutal, além de outros sítios nos sistemas nervosos central e periférico (KANDEL et al., 2014).

Sabe-se que a administração crônica de álcool produz analgesia seguida pela formação de tolerância a esta analgesia. Durante abstinência ao álcool, hiperalgesia é observada em diversos estudos (GATCH, 2009), mas estes estudos avaliaram a resposta nociceptiva após poucos dias da retirada de álcool. Uma revisão de estudos de modelos animais de abstinência ao álcool, identificou como prováveis mediadores da hiperalgesia, a diminuição de receptores de adenosina e um aumento de canais de cálcio do tipo L (GATCH, 2009). Estudo recente demonstrou que a habênula lateral (LHb) está envolvida no aumento da sensibilidade hiperalgésica termal observada em ratos abstinentes, após administração crônica e intermitente de álcool, e que a inibição desta reduz a sensibilidade à dor e a recaída de consumo de álcool (KANG et al., 2019). A hiperalgesia, entre outros sintomas da abstinência, pode levar ao paciente a ter recaída novamente, para reduzir os efeitos negativos experimentados, e, portanto, pode-se afirmar que a dor pode motivar o consumo de álcool para aliviar os efeitos negativos da abstinência (DITRE et al., 2011; ZALE et al., 2015). A sensibilização que leva à hiperalgesia é uma característica de desenvolvimento de inflamação e quando há inflamação prolongada, lesão nervosa ou anormalidades teciduais, os nociceptores são sensibilizados e geram dor persistente. Sabe-se que há mediadores que levam

à instalação da inflamação e ao desenvolvimento da sensibilidade neuronal. Entre os fatores liberados no meio lesado podemos citar: a bradicinina, a histamina, a substância P, os leucotrienos, os mediadores pró-inflamatórios, as prostaglandinas, as interleucinas, as citocinas, entre outros (TEIXEIRA, 2009).

Dois métodos comumente utilizados para avaliar a nocicepção em modelos animais são os testes de *tail-flick* e placa quente (GATCH, 2009). O *tail-flick* é um teste que avalia a resposta nociceptiva a estímulo térmico nocivo medido espinhalmente e é realizado conforme técnica descrita por D'AMOUR e SMITH (1941). O segundo teste, a placa quente, é uma medida da resposta nociceptiva a estímulo térmico nocivo medido supra-espinhalmente (WOOLFE; MACDONALD, 1944).

1.1.5 Marcadores moleculares

1.1.5.1 Interleucina-10

A neuroinflamação causada pela exposição crônica ao álcool leva a neurodegeneração, que é desencadeada pelo aumento da expressão de genes pro-inflamatórios e citocinas envolvidas no sistema imune inato (CREWS et al., 2011). A exposição crônica ao álcool induz cascata de sinalização imune através da ativação do fator de transcrição nuclear kappa B (NF-kB). O aumento nos níveis de citocinas pro-inflamatórias, como interleucina-6 (IL-6) e fator de necrose tumoral-alfa (TNF-α), por exemplo, está associada com a ativação da microglia que ocorre em resposta à injúria encefálica, pois a ativação da microglia induz a secreção de citocinas e de fatores de crescimento que podem impactar o ambiente a sua volta (CREWS et al., 2011; MARSHALL et al., 2013). A família das citocinas basicamente consiste em pequenas proteínas e glicoproteínas (de peso molecular entre 8 e 30 kDa) que permitem a comunicação intercelular (FERREIRA et al., 2009). Esses mediadores, que são importantes para o recrutamento de leucócitos (neutrófilos) para o foco inflamatório podem causar danos ou proteger o ambiente dependendo do nível de ativação microglial (MARSHALL et al., 2013). Alternativamente, a microglia ativada também pode secretar interleucina-10 (IL-10), que é conhecida por suprimir a ativação da microglia e subsequente dano neuronal (SHARMA et al., 2011). Sabe-se que durante um processo inflamatório ocorre também a liberação de outras citocinas, que modulam negativamente o processo inflamatório além da IL-10, que são a IL-4, a IL-13 e também o antagonista endógeno da IL-1β, a IL-1ra,

capaz de formar um complexo estável com a citocina IL-1β, prevenindo a ativação de receptores celulares. O efeito modulador dessas citocinas sobre a inflamação parece estar associado com a inibição da liberação dos mediadores inflamatórios e também parece estar relacionado à inibição da ação das citocinas pró-nociceptivas, como é o caso da IL-1ra, que inibe a ação da IL-1β por antagonizar seu receptor (FERREIRA et al., 2009). Sabe-se que a IL-10 suprime a síntese de prostaglandinas e de algumas citocinas pró-inflamatórias (IL-1β, IL-6, IL-8, TNF-α) e limita o processo inflamatório (TEIXEIRA, 2009). É importante ressaltar que a inibição de uma (IL-1β ou TNF-α) ou de várias citocinas (pelo uso de drogas glicocorticoides) causa analgesia (FERREIRA et al., 2009).

1.1.5.2 Fator Neurotrófico Derivado do Encéfalo (BDNF)

Adaptações encefálicas após consumo excessivo de álcool são parcialmente reguladas por fatores de crescimento, em particular, as neurotrofinas. O BDNF é uma neurotrofina que tem papel importante no crescimento, na diferenciação e na sobrevivência neuronal, e é responsável, entre outras funções, pela modulação da dor (MOWLA et al., 2001). Sabe-se que também está envolvida em muitas desordens neurológicas tais como: depressão, estresse, ansiedade e a adição a drogas. O BDNF regula também da atividade de neurônios dopaminérgicos, glutamatérgicos, colinérgicos, e serotoninérgicos (DAVIS, 2008; LEE et al., 2013; LOGRIP et al., 2015). Esta neurotrofina é produzida no retículo endoplasmático de astrócitos, microglia, plaquetas, linfócitos e endotélio vascular sobre condições patológicas (DAVIS, 2008). Devido ao seu papel na plasticidade sináptica, o BDNF parece contribuir para as mudanças neuroadaptativas que ocorrem no encéfalo de quem abusa do álcool. Vários estudos tem demonstrado que esta neurotrofina é alterada pela presença e pela retirada de álcool (GHITZA et al., 2010; RAIVIO et al., 2012; ALELE; DEVAUD, 2013). O precursor, pro BDNF, é proteoliticamente clivado para ativar o BDNF que interage com o receptor tropomiosina cinase B (TrkB) (DAVIS, 2008). Estudos em roedores demonstraram que a ingestão de álcool reduz os níveis de BDNF em exposição crônica, moderada, intermitente e repetida de álcool (MACLENNAN et al., 1995; ORRU et al., 2016). Redes neuronais se adaptam à repetida presença de álcool, alterando o balaço homeostático do meio, que leva ao desenvolvimento de fissura na ausência de álcool (RON; MESSING, 2012). Estudo recente concluiu que pacientes portadores de mutações no gene do BDNF e da enzima Catecol O-Metiltransferase (COMT), consomem significativamente maior quantidade de álcool,

apresentam mais problemas de saúde e demonstram baixa motivação para a mudança dos padrões de consumo de álcool (ANNA et al., 2017). Outro estudo em ratos verificou que existem interações específicas na circuitaria encefálica de acordo com o sexo, regulando a adição ao álcool (HOGARTH et al., 2018).

1.1.6 Tratamento do transtorno por uso de álcool

O transtorno por uso de álcool é hoje visto como uma condição crônica e comparada a outras doenças crônicas, tais como asma, diabetes e hipertensão (MOAL; KOOB, 2007). Entretanto, apesar de muitos anos de pesquisas intensivas, a maioria dos tratamentos disponíveis para tratar dependência de drogas é notoriamente ineficaz (NIH/NIAAA, 2009; BLANCO-GANDÍA; RODRÍGUEZ-ARIAS, 2018).

As abordagens terapêuticas em geral estão principalmente dirigidas aos usuários compulsivos e fisicamente dependentes e o planejamento das medidas terapêuticas é orientado pela intensidade do problema. Estas abordagens terapêuticas dividem-se em medidas de desintoxicação, controle do uso compulsivo e tratamento das complicações médicas (O´BRIEN, 2005). A desintoxicação é um processo rápido e de bons resultados no alcance de um estado de afastamento das drogas; usualmente envolve a prescrição de fármacos para atenuar sintomas da síndrome de abstinência e aliviar outros problemas consequentes da ausência da droga (O'BRIEN, 2005). Porém, o tratamento da dependência exige meses ou anos para a reabilitação, pois o curso do tratamento ainda se caracteriza por uma sucessão de fases de abstinência e de recaídas. Isto indica que todos os medicamentos que visam ajudar a evitar a recaída e a diminuir ou aliviar os sinais e sintomas da síndrome de abstinência revelaram, até o momento, benefícios no máximo, modestos, devido, principalmente, ao álcool modular várias vias neurais e de neurotransmissores, tornando difícil de tratar essa desordem com um único componente. O êxito do tratamento é avaliado segundo a ocorrência de períodos de abstinência cada vez mais prolongados e recaídas menos frequentes, mais breves e menos intensas (WOLF, 1998; MOAL; KOOB, 2007; WEINSHENKER, 2008; SOYKA; MÜLLER, 2017). É evidente então que para obter resultados satisfatórios na abstinência e na prevenção de recaídas, um tratamento multidisciplinar deve ser realizado, incluindo a farmacoterapia, suporte social e intervenção psicológica (ANTONELLI et al., 2018). Também é importante levar em consideração que a dependência do álcool ocorre

muitas vezes em pacientes com outras morbidades e transtornos psiquiátricos, logo, a escolha do tratamento deve ser adequada para evitar interações e efeitos adversos (PREUSS et al., 2018; BLANCO-GANDÍA; RODRÍGUEZ-ARIAS, 2018).

Apesar das aparentes similaridades entre os sintomas psicológicos (dependência e compulsão) produzidos por várias, mas não por todas as substâncias de abuso, há tratamentos farmacológicos tradicionalmente usados para sistemas de receptores específicos, nos quais as substâncias de abuso estão provavelmente atuando (O'BRIEN, 2005; WEINSHENKER, 2008). A síndrome de abstinência de álcool é tradicionalmente tratada com benzodiazepínicos de longa ação, anticonvulsivantes e antidepressivos (JOHNSON, 2008). Porém, a fissura que leva à recaída é mais difícil de tratar. Nesse sentido, várias classes diferentes de agentes farmacológicos são usadas terapeuticamente na tentativa de prevenir a recaída. Por exemplo, o acamprosato, que tem ação antagonista glutamatérgica, foi aprovado para o tratamento do alcoolismo (MASON; HEYSER, 2010). A naltrexona, que é um antagonista não-seletivo de receptores opioides, atenua a sensação de prazer associada ao consumo de álcool, levando a diminuição do seu consumo, reduzindo a fissura e facilitando a abstinência (MONTI et al., 1999; 2001). O dissulfiram, um agente aversivo, inibe a enzima aldeído desidrogenase do fígado e do encéfalo, ocasionando aumento dos níveis de acetaldeído, e, consequentemente, causando mal-estar ao indivíduo após o consumo de álcool (HALD; JACOBSEN, 1948). As reações causadas pelo dissulfiram incluem náuseas, vômito, sudorese, hipotensão e taquicardia (SUH et al., 2006). O tratamento com dissulfiram não é de primeira escolha, pois este fármaco não age diretamente nas vias e neurotransmissores envolvidos na dependência ao álcool. Os pacientes que recebem tratamento com dissulfiram são aqueles que recebem terapia concomitante para tratar a abstinência (ANTON, 2001; BLANCO-GANDÍA; RODRÍGUEZ-ARIAS, 2018). Os inibidores seletivos da recaptação de serotonina também estão sendo utilizados no tratamento da dependência de álcool (BARTH; MALCOLM, 2010).

Tendo em vista as consequências adversas do uso de álcool do ponto de vista médico, social e econômico, não só para os indivíduos dependentes, mas também para a sociedade como um todo, e considerando ainda a baixa efetividade dos poucos tratamentos disponíveis para o alcoolismo, a busca por novos compostos antiaditivos é um investimento necessário. O desenvolvimento e teste de novos medicamentos para tratar dependência foi inclusive recomendado pelo "National Institute on Drug Abuse" (NIDA) como uma área alvo para investimentos futuros (NIDA Website, 2018).

1.2 *Passiflora incarnata* L.

Originária da América, a *Passiflora incarnata* L. pertence à família *Passifloraceae*, e é uma das mais de 500 espécies de plantas dicotiledôneas que fazem parte do gênero *Passiflora* (EMA, 2008; WOHLMUTH et al., 2010; JAWNA-ZBOIŃSKA et al., 2016). Com usos pré-históricos que datam de 8.000 a 2.000 a.C., *Passiflora* surgiu da palavra em latim "passio", porque em 1529 conquistadores espanhóis descreveram as flores desta planta como símbolo da "paixão de Cristo" (RATSCH, 1998). *Passiflora incarnata* tem uma série de usos populares, tais como: tratamento da diarreia, da hemorroida, do queimado, da síndrome pré-menstrual, sedativo (América do Norte), antiespasmódico, antiasmático, vermicida (Brasil), anticonvulsivante, tratamento da dismenorreia, da neuralgia (Turquia), tratamento da dependência de opioides (Índia), entre outros usos (MIRODDI et al., 2013). Apesar dos diversos usos desta planta, o uso mais comum e tradicional é para tratar transtornos de ansiedade e distúrbios do sono (MIRODDI et al., 2013). Em relação à fitoquímica desta planta, apesar de somente uma parte de seus constituintes serem precisamente identificados, diversos estudos, indicam que as partes aéreas de *Passiflora incarnata* são constituídas pela presença de um padrão primário de vários compostos tais como flavonoides, maltol, glicosídeos cianogênicos e alcaloides indólicos (MIRODDI et al., 2013). Os alcaloides indólicos, representados por harmano, harmol, harmina, harmalol e harmalina, são os constituintes minoritários desta planta e atuam como inibidores da monoamino-oxidase, podendo muitas vezes não ser detectáveis em certas amostras por estarem em baixa quantidade (SAMPATH et al., 2011). Os flavonoides representam 2,5% dos compostos de *Passiflora incarnata*, geralmente expressos como percentagem de vitexina. Entre outros podemos citar: isovitexina, orientina, isoorientina, canferol, apigenina, crisina, vicenina, lucenina (MIRODDI et al., 2013). Os efeitos de *Passiflora incarnata* podem ser devidos a sua atividade GABAérgica, através de ligação em receptores $GABA_A$ e opioide (ELSAS et al., 2010; APPEL et al., 2011; JAWNA-ZBOIŃSKA et al., 2016; AMAN et al., 2016). Sabe-se também que *Passiflora incarnata* apresenta certa quantidade de GABA (CARRATÙ et al., 2008). Estudos pré-clínicos e clínicos tem comprovado as ações terapêuticas desta planta, não somente nos transtornos de ansiedade e distúrbios do sono, mas também no tratamento da retirada de opioides, da menopausa, do transtorno do déficit de atenção e hiperatividade (TDAH), entre outros (MIRODDI et al., 2013). No Brasil existe uma apresentação comercial

contendo um extrato padronizado desta planta, com indicação para o tratamento de insônia e desordens de ansiedade.

1.3 Estimulação Transcraniana por Corrente Contínua (ETCC)

Na tentativa de busca de terapias antiaditivas, a Estimulação Transcraniana por Corrente Contínua (ETCC) apresenta-se como uma alternativa em potencial (SILVA, 2013). Este método não invasivo de estimulação cortical oferece pouco risco em sua aplicação e modula o potencial de repouso neuronal (FREGNI et al, 2007). O princípio da ETCC baseia-se na utilização de uma corrente elétrica fraca aplicada no couro cabeludo. A estimulação no ânodo tipicamente despolariza (aumenta excitabilidade) e a estimulação no cátodo hiperpolariza (diminui excitabilidade) dos neurônios. O efeito causado pela ETCC depende da duração da estimulação, da polaridade e da posição dos eletrodos, da intensidade da corrente aplicada e das propriedades do tecido onde ocorre a aplicação da corrente (MEDEIROS et al., 2012). Sabe-se que a ETCC tem um impacto na cognição e no comportamento (NITSCHE et al., 2011). Há estudos com resultados promissores que utilizam a ETCC para pacientes que sofrem de doenças psiquiátricas como: depressão (BIKSON et al., 2008), dor crônica (FREGNI et al., 2006), abuso de álcool (NAKAMURA-PALACIOS et al., 2012; DEN UYL et al., 2015 e 2017), abuso de tabaco (FREGNI et al., 2008) e também abuso de alimentos (GOLDMAN et al., 2011), entre outros (LEFAUCHEUR et al., 2017). Os efeitos causados pela ETCC não podem ser explicados por um único mecanismo de ação, mas o que se sabe é que altera a concentração de diversos neurotransmissores, tais como serotonina, acetilcolina e dopamina, e sabe-se também que a ETCC altera canais de sódio e cálcio (KUO et al., 2007; MONTE-SILVA et al., 2009; NITSCHE et al., 2009; MEDEIROS et al., 2012). O efeito de despolarização ou hiperpolarização causado pela ETCC pode resultar em potenciação de longa duração (LTP) ou em depressão de longa duração (LTD), respectivamente. A LTP ocorre por forte ativação do receptor NMDA (HATTORI et al., 1990; NITSCHE et al., 2003; MEDEIROS et al., 2012). Sabe-se também que a estimulação anodal está associada com a modulação de interneurônios GABAérgicos (STAGG; NITSCHE, 2011; MEDEIROS et al., 2012). Outro ponto importante é a modulação promovida pela ETCC sobre o BDNF (CHEERAN et al., 2008).

Estudos têm demonstrado que a ETCC tem efeitos positivos no tratamento do transtorno por uso de álcool, atenuando significativamente a fissura (BOGGIO et al., 2008; DEN URYL et al., 2015, SALLING; MARTINEZ, 2016; COLES et al., 2018). A estimulação anodal do córtex pré-frontal aumenta a excitabilidade e já foi demonstrado que melhora processos cognitivos, além de prover uma melhora das funções executivas, reduzindo assim a probabilidade de recaída ao uso do álcool (KUO; NITSCHE, 2012; DEN URYL et al., 2017). Sabendo da problemática associada ao transtorno por uso de álcool, e da ineficácia dos tratamentos existentes, este estudo se propôs avaliar a atividade antiaditiva de *Passiflora incarnata* e da ETCC.

2. OBJETIVOS

2.1. Geral:

Avaliar o efeito antiaditivo de extrato padronizado de *Passiflora incarnata* L. e terapia de estimulação transcraniana por corrente contínua (ETCC) na síndrome de abstinência ao álcool desencadeada por um modelo de ingestão intermitente de álcool em ratos.

2.2. Específicos:

2.2.1. Avaliar o efeito de um extrato padronizado de *Passiflora incarnata L*. na síndrome de abstinência ao álcool desencadeada por um modelo de ingestão intermitente de álcool em ratos (Artigo 1);

2.2.2. Avaliar o efeito da ETCC na síndrome de abstinência ao álcool desencadeada por um modelo de ingestão intermitente de álcool em ratos (Artigo 2);

2.2.3. Determinar os níveis dos marcadores moleculares BDNF e/ou Interleucina-10 no córtex pré-frontal, tronco encefálico e hipocampo dos ratos tratados na síndrome de abstinência ao álcool desencadeada por um modelo de ingestão intermitente de álcool em ratos (Artigos 1 e 2).

PARTE II

Capítulo I

Standardized *Passiflora incarnata* L. Extract Reverts the Analgesia Induced by Alcohol Withdrawal in Rats

Manuscrito publicado na Revista

Phytotherapy Research

PHYTOTHERAPY RESEARCH
Phytother. Res. **31**: 1199–1208 (2017)
Published online 1 June 2017 in Wiley Online Library
(wileyonlinelibrary.com) **DOI**: 10.1002/ptr.5839

Standardized *Passiflora incarnata* L. Extract Reverts the Analgesia Induced by Alcohol Withdrawal in Rats

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***Passiflora incarnata* L. (Passifloraceae) has been traditionally used for treatment of anxiety, insomnia, drug addiction, mild infections, and pain. The aim of this study was to investigate the effect of a commercial extract of *P. incarnata* in the analgesia induced by alcohol withdrawal syndrome in rats. In addition, brain-derived neurotrophic factor and interleukin-10 levels were evaluated in prefrontal cortex, brainstem, and hippocampus. Male adult rats received by oral gavage: (1: water group) water for 19 days, 1 day interval and water (8 days); (2: *P. incarnata* group) water for 19 days, 1 day interval and *P. incarnata* 200 mg/kg (8 days); (3: alcohol withdrawal group) alcohol for 19 days, 1 day interval and water (8 days); and (4: *P. incarnata* in alcohol withdrawal) alcohol for 19 days, 1 day interval and *P. incarnata* 200 mg/kg (8 days). The tail-flick and hot plate tests were used as nociceptive response measures. Confirming previous study of our group, it was showed that alcohol-treated groups presented an increase in the nociceptive thresholds after alcohol withdrawal, which was reverted by *P. incarnata*, measured by the hot plate test. Besides, alcohol treatment increased brain-derived neurotrophic factor and interleukin-10 levels in prefrontal cortex, which was not reverted by *P. incarnata*. Considering these results, the *P. incarnata* treatment might be a potential therapy in the alcohol withdrawal syndrome. Copyright © 2017 John Wiley & Sons, Ltd.**

Keywords: Passiflora incarnata extract; pain; flavonoids; BDNF; IL-10; alcohol withdrawal.

INTRODUCTION

Passiflora incarnata L. (Passifloraceae) (English common name: passionflower) is used in traditional herbal medicines, exhibiting various therapeutic properties (Dhawan et al., 2004; Miroddi et al., 2013). In European traditional medicine, *P. incarnata* has indications such as anxiety, nervousness, mild infections, and insomnia treatments. In Brazil, it is used as an analgesic, anti-spasmodic, anti-asthmatic, wormicidal, and sedative (Taylor, 1996). In North America, it is used also for the treatment of muscle cramps, hysteria, and various pain conditions (Dhawan et al., 2004). Interestingly, in India, it is used to treat morphine's dependence (Ingale and Hivrale, 2010), while in Africa, it is employed by its sedative and analgesic effects (Neuwinger, 2000). The official drugs included in the current British and European Pharmacopeias are the dried aerial parts of *P. incarnata*, and its common indication in phytotherapy is mild sedative and anxiolytic (Wohlmuth et al., 2010; Miroddi et al., 2013).

The chemistry of *P. incarnata* is complex, and the active constituents are not conclusively identified, but it is considered that the flavonoids and alkaloids play an important role in the pharmacological actions of the species (Barnes et al., 2012). Evidences of literature demonstrate a possible synergistic mechanism of action among its various constituents (Miroddi et al., 2013). The indolic beta-carboline alkaloids, which include harmane, harmol, harmine, harmalol, and harmaline, are the minority constituents of the plant, and they can act as monoamine oxidase inhibitors (Rehwald et al., 1995; Sampath et al., 2001). However, many available data suggest only flavonoids as the possible active

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Received 03 December 2016
Revised 14 April 2017
Accepted 03 May 2017

compounds in *P. incarnata* (Dhawan et al., 2001, 2002a). The flavonoids represent 2.5% of the plant compounds, and some of the *C*-glycosyl flavones are considered as marker compounds to distinguish between passionflower extracts of different origins (Raffaelli et al., 1997). Usually, they are expressed as percentage of vitexin, but they can also include isovitexin, orientin, isoorientin, kaempferol, apigenin, and chrysin (Barnes et al., 2012).

Alcohol addiction is defined as a chronic relapsing disorder characterized by compulsive alcohol seeking and drinking, loss of control over limiting alcohol intake, and the emergence of a chronic negative affective state when access to alcohol is prevented (American Psychiatric Association, 2013). The negative affective state of alcohol abuse describes the development of anxiety, depression, and other dysphoric psychiatric sequelae, which may be caused by the abrupt cessation of alcohol consumption (Pandey, 2004).

Associations between alcohol consumption and chronic pain have been reported in the episodes of alcohol abuse antedating chronic pain condition in some people, and alcohol dependence emerging after the onset of chronic pain in others (Katon et al., 1985). People experiencing chronic pain seek the alcohol presumably for its relief (Riley and King, 2009). Interestingly, oral alcohol administration increases human pain thresholds, and the withdrawal from chronic use often increases pain sensitivity as one symptom of the alcohol withdrawal syndrome (Jochum et al., 2010).

On the other hand, a previous study of our group demonstrated that alcohol withdrawal produced an analgesic effect indexed by an increase in the nociceptive threshold in rats. In addition, we suggested that these effects could be related to the increase observed in the central levels of brain-derived neurotrophic factor (BDNF) and interleukin-10 (IL-10) in these animals (Schunck et al., 2015). Noteworthy, BDNF is involved with neuronal survival, development and plasticity (Thoenen, 1995), and many psychiatric disorders such as depression, stress, anxiety, pain, and drug addiction (Horger et al., 1999; Hall et al., 2003; Murakami et al., 2005; Pandey et al., 2006; Davis, 2008; Merighi et al., 2008). The possibility of BDNF as a biomarker of alcohol abuse is corroborated by its serum levels changes after heavy alcohol intake and polymorphisms in BDNF gene in people with alcohol dependence or with alcohol-related risk phenotypes (Wojnar et al., 2009; Bus et al., 2012; Nees et al., 2015).

Altered neuro-immune signaling processes are linked to negative affects induced by alcohol, regulation of alcohol-drinking behavior, and depression-like behaviors. Immune response involves a rapid production of pro-inflammatory cytokines and chemokines, such as monocyte chemotactic protein-1, tumor necrosis factor α, and interleukin 1β (IL-1β), which serve to initiate the host's defense against pathogens and cellular damage (Crews et al., 2011, Crews, 2012). However, excessive inflammation process may give rise to disturbances, which are harmful to the organism. In this context, IL-10, a potent antiinflammatory cytokine, is essential for the regulation of immune response, because its action results in the attenuation of pro-inflammatory cytokine synthesis, such as IL-1β and tumor necrosis factor α (Clark et al., 2013). Furthermore, the neuro-immune system plays a role in the development of alcoholism, resulting in the induction of genes involved in innate immunity, which, in certain brain regions (e.g., the frontal cortex), can disrupt decision making and characterizing alcohol addiction (Qin et al., 2007; Crews et al., 2011, Crews, 2012; Blednov et al., 2012; Marshall et al., 2013).

Considering the traditional use of the aerial parts of *P. incarnata* for drug addiction and pain treatment (Dhawan et al., 2002a), the aim of this study was to evaluate the effect of a commercial extract of this species, enriched in flavonoids, in the analgesia induced by a model of alcohol withdrawal syndrome in rats. In addition, it evaluated the BDNF and IL-10 levels in the prefrontal cortex, brainstem, and hippocampus of these animals.

MATERIALS AND METHODS

Animals. Male adult Wistar rats (90 day-old) weighing 400–450 g were allocated in groups of five in 49 × 34 × 16-cm polypropylene home cages. All animals were maintained under a standard 12-h light/dark cycle (lights on at 07:00 AM and off at 07:00 PM) in a temperature-controlled environment (22 ± 2°C). Animals had free access to water and chow. All experiments and procedures were approved by the Institutional Committee for Animal Care and Use (Application No. 23651 - Graduate Research Group at Universidade Federal do Rio Grande do Sul), were compliant with Brazilian guidelines regulating the use of animals in research and adhered to the ethical and methodological standards of the Principles of Laboratory Animal Care. All possible measures were taken to minimize animal suffering and external sources of pain and discomfort. In addition, the minimum number of animals required to produce reliable scientific data was used based on previous studies of our research group that used similar methods (Spezia Adachi et al., 2012; Laste et al., 2012; Spezia Adachi et al., 2015; Schunck et al., 2015; Macedo et al., 2015; Filho et al., 2016, Cioato et al., 2016).

Drugs and extract preparation. Alcohol (ethanol, Nuclear, Diadema, SP, Brazil) was diluted daily in distilled water to prepare a 20% w/v solution, which was administrated by oral gavage in a dose of 4 g/kg of body weight. Tablets of a commercial extract of *P. incarnata* (Sintocalmy®, Aché Laboratory, Guarulhos, SP, Brazil), standardized in 21 mg (7%) of total flavonoids expressed as vitexin and exempt of beta carboline-type alkaloids, were powdered, dissolved in distilled water, filtered, and administrated by oral gavage in a dose of 200 mg/kg of body weight. The animals were treated in the same hours, all days, between 10.00 AM and 12.00 AM.

Experimental design. Rats were habituated to the maintenance room for 1 week prior to the experiment. Subsequently, animals were randomly divided into four different groups as follows: (1) water group (WAT,

$n = 9$) treated with distilled water by gavage for 19 days, 1 day without treatment, and more 8 days receiving distilled water; (2) *P. incarnata* (PAS, $n = 9$) group, treated with distilled water by gavage for 19 days, 1 day without treatment, and 200 mg/kg *P. incarnata* by oral gavage for more 8 days; (3) alcohol withdrawal (AW, $n = 9$) group, treated with 4 g/kg ethanol by oral gavage for 19 days, 1 day without treatment and distilled water for more 8 days; (4) *P. incarnata* in withdrawal of alcohol (PAW, $n = 10$) group, treated with 4 g/kg ethanol by oral gavage for 19 days, 1 day without treatment, and 200 mg/kg *P. incarnata* for more 8 days. The administrations of first 19 days of treatment were performed in three periods of 5 days with two intervals of 2 days between them (repeated alcohol withdrawals) as previously reported by our group (Schunck et al., 2015). On 20th day, there was no ethanol administration and the nociceptive tests were conducted. After, all groups were remained without treatment, and between the 21st and 28th days, the animals received *P. incarnata* commercial extract per oral. In the 29th day, the tail-flick latency test was applied. In the 30th day, the hot plate test was performed. The animals were euthanized in the 31st day (Fig. 1). During the time of treatment, all animals had free access to tap water and food.

Chemical analysis. A chemical fingerprint of the *P. incarnata* extract obtained from Sintocalmy® tablets was achieved by using a high-performance liquid chromatography coupled to a diode array detector (HPLC-DAD) (Shimadzu, Kyoto, Japan) equipment including a pump (LC-10ADVP), spectrophotometric detector module (SPD-M10AVP), and a system controller (SLA-10ADVP) with a Phenomenex Gemini RP-18 column (5 μm). An isocratic system, with a mobile phase composed by water: acetonitrile (85:15 v/v), was applied. The aqueous phase was adjusted to pH 3.0 with acetic acid. Separations were performed by using a flow of 1.0 mL/min with an injection volume of 20 μL. UV absorbance was monitored at 205, 254, 290, 330, and 350 nm. Peaks of vitexin and isovitexin were detected at 330 nm. For ultraperformance liquid chromatography (UPLC)-MS, analysis were performed on a Micromass-LCT Premier Time of Flight (TOF) mass spectrometer (Waters, MA, USA) with an electrospray interface and coupled with an Acquity UPLC system (Waters, MA, USA). The chromatography parameters were similar to those of the HPLC analysis; nevertheless, acetic acid was changed for formic acid. Total and individual flavonoids were estimated by using vitexin as a standard, peak areas were obtained at 330 nm.

Nociceptive analysis

The tail-flick latency. The tail-flick latency (TFL) apparatus was described by D'Amour and Smith (1941). Twenty-four hours before the nociceptive response evaluation, the animals were exposed to the apparatus to familiarize them with the procedure, because the novelty can itself induce anti-nociception (Netto et al., 1987). Rats were wrapped in a towel and placed on the apparatus; the light source positioned below the tail was focused on a point 2.3 cm rostral to the tip of the tail. Deflection of the tail activated a photocell and automatically terminated the trial. A cut-off time of 10 s was used to prevent tissue damage. The test was performed at 24 h and 10 days (29th day) after the last administration of the initial treatments. This test was performed by Analgesy-meter LE 71 PanLab Harvard Apparatus containing the halogen lamp for the heat stimulus.

The hot plate test. The hot plate test (HPT) was carried out to assess the effects of the study agent on the thermal nociceptive threshold (Woolfe and Macdonald, 1944). All rats were acclimated to the hot plate for 5 min, 24 h prior to the test. The temperature of the plate was kept at 50°C. The animals were placed on glass funnels over the heated surface, and the time between placement of the animals on the hot plate and onset of paw licking or jumping was recorded in seconds (s), as latency of response. The test was performed after 24 h and 11 days (30th day) after the last administration of initial treatments of water or alcohol.

Sample collection. On the 12th day after the last administration of water or alcohol, the animals were anesthetized by using ketamine/xylazine (50 and 10 mg/kg, respectively) and the animals were euthanized by decapitation. The prefrontal cortex, brainstem, and hippocampus were separated in a cold surface and

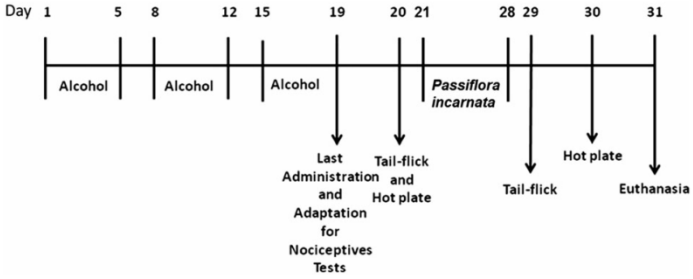

Figure 1. Experimental design. Adaptation to the test was done in the last day of the alcohol treatment; there was an interval of 5 h between the last administration of alcohol and the adaptation procedure.

immediately frozen in liquid nitrogen and kept at −80°C for subsequent analysis.

BDNF and IL-10 immunocontent. The prefrontal cortex, brainstem, and hippocampus were weighed and homogenized with a handheld homogenizer in commercial phosphate buffer (pH 7.2–7.4, Laborclin, Pinhais, PR, Brazil) containing anti-proteinases solution (1:100, Sigma-Aldrich, St. Louis, MI, USA). The resulting homogenates were centrifuged for 10 min at 4500 rpm. The BDNF and IL-10 levels were determined by using enzyme-linked immunosorbent assay kit (R&D Systems, Minneapolis, USA #DY248 and #DY522, respectively) according to the manufacturer's recommendations. Total protein was measured by Bradford's method using bovine serum albumin as standard (Bradford, 1976). The results of BDNF and IL-10 were expressed in pg/mg of protein.

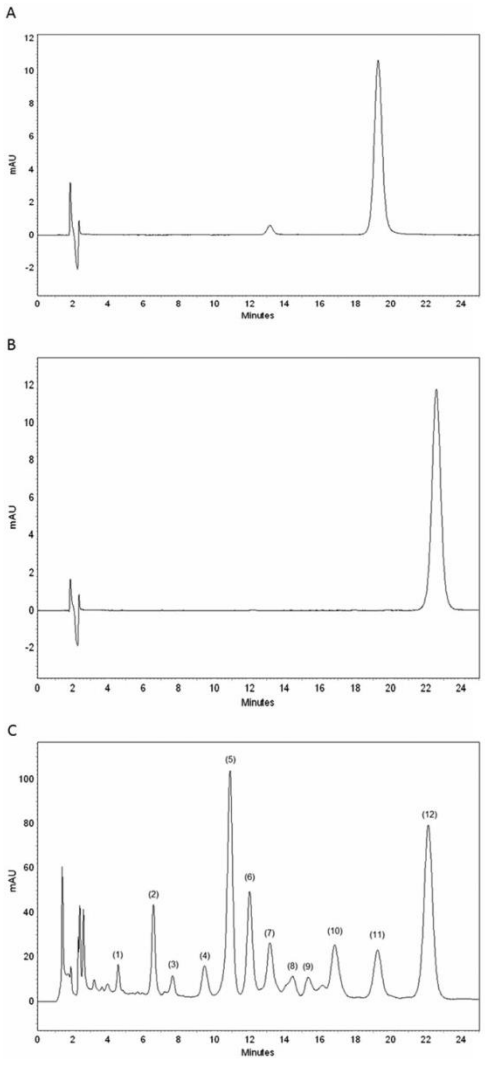

Figure 2. HPLC analysis of (A) the flavonoid standard vitexin, (B) the flavonoid standard isovitexin, and (C) *Passiflora incarnata* extract tablets.

Statistical analysis. All statistical analyses were performed by using the Statistical Package for Social Sciences software (SPSS) version 20.0 (SPSS, Chicago, IL, USA). Normality was verified for all variables by using the Kolmogorov–Smirnov test. Behavioral outcomes were analyzed with a two-way repeated measures ANOVA, while biochemical outcomes were evaluated with one-way ANOVA. Bonferroni was applied as post hoc test. All results were expressed as mean ± standard error of mean. Significance was set at $p < 0.05$.

RESULTS

Chemical analysis of commercial *P. incarnata* extract

The *P. incarnata* extract was analyzed by HPLC-DAD and UPLC-MS in order to obtain a chemical fingerprint. A typical flavonoid profile with 12 peaks was detected, as shown in Fig. 2. The identity of the major peaks (Table 1) was derived by obtaining molecular weights from UPLC-MS (data not shown), comparison of their elution order to published reports (Rehwald et al., 1994; Abourashed et al., 2002; Wohlmuth et al., 2010) and their λ of maximal absorbance (Mabry et al., 1970). In this sense, the flavonoids isoschaftoside, isoorientin, orientin, schaftoside, saponarin, vitexin, and isovitexin were identified (Elsas et al., 2010), while the other peaks remain unknown. Final amount of vitexin and isovitexin were 0.011% and 0.018%, respectively, in each tablet. As the extract used for the tablets is standardized on 7% of flavonoids and is devoid of harmane-type alkaloids, a supposition is that the other compounds may, in part, represent coumarins, whose presence is described by the supplier.

Table 1. Likely identity of flavonoid peaks in *Passiflora incarnata* extract

Peak No.	Retention time (min)	λ Max. absorvance (nm)	Likely identity of peak
1	4.56	338	Unknown
2	6.56	333	Unknown
3	7.65	333	Unknown
4	9.45	334	Isoschaftoside
5	10.90	347	Isoorientin
6	12.00	345	Orientin
7	13.18	333	Schaftoside
8	14.45	332	Unknown
9	15.35	331	Unknown
10	16.83	330	Saponarin
11	19.26	330	Vitexin
12	22.13	331	Isovitexin

Effect of *P. incarnata* in the nociceptive threshold of rats (TFL and HPT)

The results obtained in HPT are depicted in Fig. 3. At the basal measures, there was no difference between groups and the treatment with *P. incarnata* extract (PAS group) did not influence in the nociceptive threshold measured in the HPT test. Interestingly, 11 days after the end of the treatments, there was a significant increase of the nociceptive response in the HPT in the group that received alcohol [AW group, two-way repeated measures ANOVA (RMANOVA)/ Bonferroni, $F_{(3,38)} = 4.154$; $p < 0.01$] compared with control, which was reversed by the treatment with *P. incarnata* extract (PAW group) [two-way RMANOVA/Bonferroni, $F_{(3,38)} = 3.318$; $p < 0.05$].

In the TFL, there was no difference between *P. incarnata* extract (PAS group) and the control (WAT group) at the basal measures; however, the alcohol withdrawal (AW group) presented a significant influence in the nociceptive threshold [two-way RMANOVA/Bonferroni, $F_{(3,37)} = 8.930$; $p < 0.01$]. At 10 days of alcohol withdrawal, the alcohol-treated groups (AW and PAW) presented a significant increase in the nociceptive thresholds compared with other groups [two-way RMANOVA/Bonferroni, $F_{(3,37)} = 21.05$; $p < 0.001$], but, surprisingly, these effects were not reversed by *P. incarnata* extract (PAW group) (Fig. 4).

Effect of *P. incarnata* in the BDNF and IL-10 immunocontent

It was observed a significant increase in the BDNF levels in prefrontal cortex in the groups AW and PAW in relation to the other groups [one way ANOVA/Bonferroni, $F_{(3,15)} = 14.10$; $p < 0.05$] (Fig. 5A). When the BDNF content was analyzed in brainstem and hippocampus, no significant differences between groups were found [one-way ANOVA for both, $F_{(3,14)} = 2.570$; $p = 0.1029$ and $F_{(3,17)} = 0.3716$; $p = 0.7746$, respectively] (Fig. 5 BC).

The IL-10 levels in the prefrontal cortex presented a significant increase in the AW and PAW groups [one way ANOVA/Bonferroni, $F_{(3,18)} = 6.331$; $p < 0.05$] (Fig. 6A), as well as in the hippocampus of the PAS group [one way ANOVA/Bonferroni, $F_{(3,20)} = 3.610$; $p < 0.05$] (Fig. 6B). In the brainstem, a significant increase in the IL-10 levels were observed in all treated groups compared with control [one way ANOVA/Bonferroni, $F_{(3,17)} = 11.51$; $p < 0.05$] (Fig. 6C).

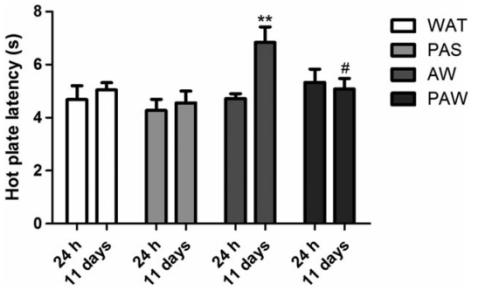

Figure 3. Effects of *Passiflora incarnata* extract in the nociceptive threshold in the hot plate test (HPT). Values represented as mean ± standard error of mean (SEM). **Significantly different from control group ($p < 0.01$, two-way repeated measure ANOVA/ Bonferroni, $n = 9–10$). #Significantly different from 11 days alcohol withdrawal (AW) group ($p < 0.05$, two-way repeated measure ANOVA/Bonferroni, $n = 9–10$). WAT = water group (control), PAS = 200 mg/kg *P. incarnata*, AW = alcohol withdrawal and PAW = 200 mg/kg *P. incarnata* in alcohol withdrawal.

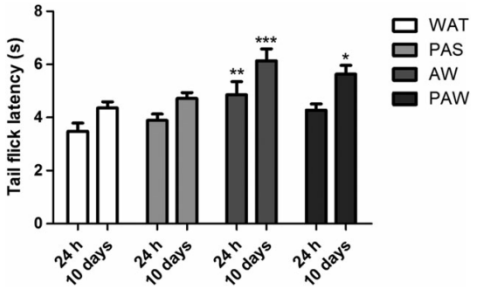

Figure 4. Effects of *Passiflora incarnata* extract in the nociceptive threshold in the tail flick test. Values represented as mean ± SEM. *Significantly different from control group (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$, two-way repeated measure ANOVA/ Bonferroni, $n = 9–10$). WAT = water group (control), PAS = 200 mg/kg *P. incarnata*, AW = alcohol withdrawal and PAW = 200 mg/kg *P. incarnata* in alcohol withdrawal.

DISCUSSION

The present study showed that the *P. incarnata* treatment reverses the analgesia induced by 10 days of alcohol withdrawal in rats (Schunck et al., 2015). In addition, it demonstrates that *P. incarnata* effect depends of the nociceptive test used because it is present in the HPT, but not in TFL test. Moreover, we observed that the group receiving alcohol presented an increase in the BDNF and IL-10 prefrontal cortex levels, corroborating previous study of our research group (Schunck et al., 2015), without *P. incarnata* effect in these parameters. Therefore, it is possible to suggest that the mechanism by which *P. incarnata* reverts the analgesia induced by alcohol withdrawal indexed by increase in the nociceptive threshold in the HPT does not have a relationship with BDNF or IL-10 central levels.

Previous studies suggested that alcohol withdrawal inducing BDNF level alterations can be sex-dependent and brain region-dependent (Alele and Devaud, 2013). Chronic alcohol abuse can lead to a reduction in BDNF levels, which returned to normal levels after prompt alcohol detoxification. These indicate that BDNF levels modifications induced by alcohol withdrawal syndrome are related to neuroadaptive processes of alcohol dependence (Huang et al., 2011; Somkuwar et al., 2016). Moreover, investigations performed by our group showed that alcohol withdrawal increased BDNF levels in prefrontal cortex (Schunck et al., 2015). Taken together, these results suggest that the BDNF levels could be used as an alcohol abuse biomarker. This is corroborated by changes in its serum levels after heavy alcohol intake and polymorphisms in BDNF gene in people with alcohol dependence or with alcohol-related risk phenotypes (Wojnar et al., 2009; Bus et al., 2012; Nees et al., 2015).

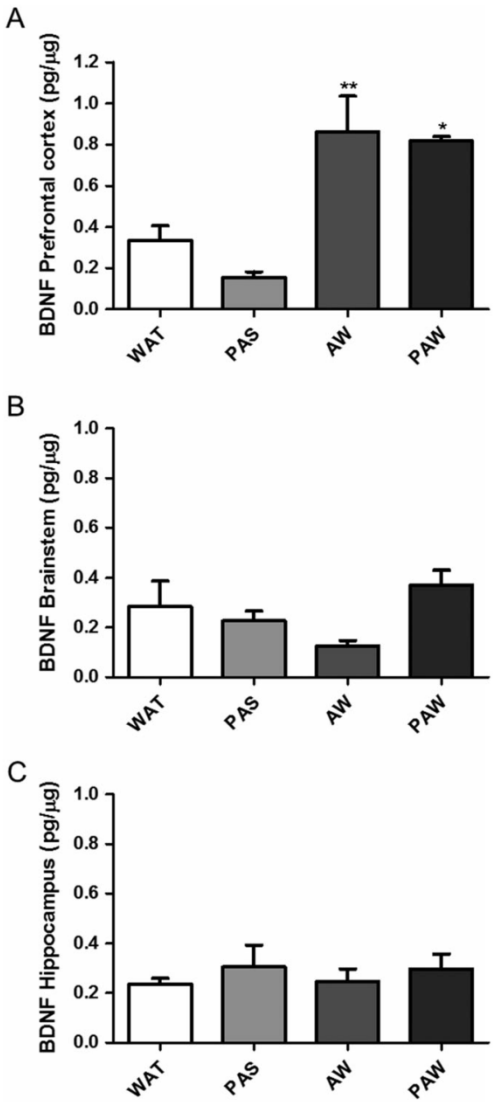

Figure 5. Effects of *Passiflora incarnata* extract in BDNF immunocontent in (A) prefrontal cortex, (B) brainstem, and (C) hippocampus. Values represented as mean ± SEM. Significant different from control group (*$p < 0.05$; **$p < 0.01$; ANOVA/Bonferroni, n = 4–5). WAT = water group (control), PAS = 200 mg/kg *P. incarnata*, AW = alcohol withdrawal and PAW = 200 mg/kg *P. incarnata* in alcohol withdrawal.

In a previous study, using the same experimental conditions, our group demonstrated that alcohol withdrawal syndrome produced an increase in the nociceptive threshold (analgesic effect) (Schunck et al., 2015). This is interesting because other studies have shown a decrease in the nociceptive threshold induced by alcohol withdrawal (Gatch and Lal, 1999; Dina et al., 2006; Gatch, 2009; Egli et al., 2012). However, it should be noted that these studies assessed nociceptive effects only by hours or few days after alcohol withdrawal, unlike our protocol, which assessed a long-term of withdrawal (10 and 11 days after alcohol exposure). This might suggest that the decrease in the nociceptive threshold observed in the PAW group is dependent of animal state, because it was not observed in control group.

Noteworthy, the differences observed in HPT and TFL measurements are somehow expected due to the different mechanisms involved in both responses. HPT involves tonic pain, a long-duration stimulus triggering a nociceptive response mainly involving C-fibers (Le Bars et al., 2001). This test produces two behavioral parameters that can be measured in terms of their reaction times, namely, paw licking and jumping. Both are considered to be supraspinally integrated responses (Le Bars et al., 2001). In contrast, the TFL evaluates phasic pain, a short-duration nociceptive stimulus related to nociceptive threshold and involving minimal surface stimulation. This thermal test involves the stimulation of Aδ fibers (Le Bars et al., 2001). The nociceptive response evaluated in the TFL test is related to the spinal cord reflex (Irwin et al., 1951; Sinclair et al., 1988), but the response remains under control of supra-spinal structures (Mitchell and Hellon, 1977). TFL test involves reflex descending supra-spinal inhibitory control that travels through the dorsolateral funiculus (Necker and Hellon, 1978). This control plays a significant modulatory role, which is accentuated by the stimuli of the heat intensity and by visual and auditory cues provided during the test (King et al., 1997). It is important to note that the light emitted by the incandescent lamp used to stimulate the tail in this experiment, might cause a learning process. The tail-flick is prone to habituation, and it is possible to occur a reduction in the response with repetitive stimulation. This behavior is generally reported for reflexes evoked by stimulation of myelinated fibers (Le Bars et al., 2001).

In addition, the nociceptive test can be altered according to the room conditions and the test temperature (King et al., 1997). As previously described, all experiments were performed in a temperature-controlled environment (22 ± 2°C), avoiding any visual or auditory cues, hence making the observations reliable. The experimental model of the alcohol consumption can induce lesion in the cell membrane (Reddy et al., 2013), more specifically neuronal lesion (Orona et al., 1988) which, after alcohol withdrawal, may be generating an adaptive response and/or the inhibitory role of the descending modulation evaluated in the nociceptive tests had been maximized. Surprisingly, the *P. incarnata* treatment only reverted the increase nociceptive threshold induced by 11 days of alcohol withdrawal in the HPT. Therefore, it might be possible that *P. incarnata* extract acts, more effectively, reversing the nociceptive threshold produced by long-duration stimulus, involving C-fibers, than the short-duration ones.

Surprisingly, *P. incarnata* extract would be restoring nociceptive threshold to the basal levels. Previous studies show that various active constituents may contribute to the reported clinical effects of *P. incarnata*, probably in a synergistic manner (Miroddi et al., 2013). The *P. incarnata* is an overall CNS depressant, long-term administration of passionflower extract affects anxiety, spatial learning, and neurotransmission in rats, with a significant dose-dependent improvement in

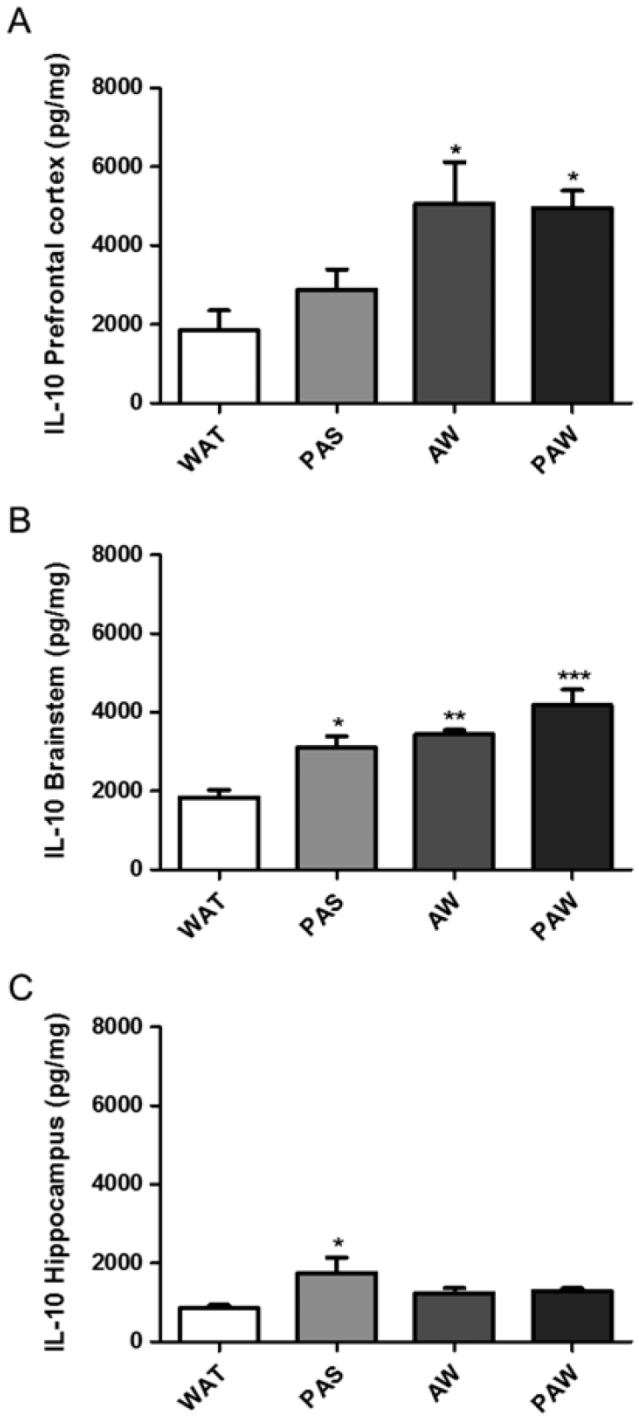

Figure 6. Effects of *Passiflora incarnata* extract in IL-10 immunocontent in (A) prefrontal cortex, (B) brainstem, and (C) hippocampus. Values represented as mean ± SEM. Significant different from control group (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ANOVA/Bonferroni, n = 4–5). WAT = water group (control), PAS = 200 mg/kg *P. incarnata*, AW = alcohol withdrawal and PAW = 200 mg/kg *P. incarnata* in alcohol withdrawal.

memory acquisition (Dhawan et al., 2004; Jawna-Zboińska et al., 2016).

Herein, several flavonoids were identified in the extract standardized in 7% total flavonoids and not presenting harmane-type alkaloids in its constituents. Indeed, the chromatographic analysis showed other peaks that remain unknown, suggesting that other compounds (e.g. coumarins) may be present in the extract. Vitexin and other flavonoids presented in the *P. incarnata* extract are c-glycosylated flavones. The presence of sugar moieties increases the molecular weight and the polarity of such compounds, which might limit the passive transport through biological barriers, such as the blood–brain barrier. Although it has been reported that vitexin has a gastric first-pass effect around 30% in rats, it is able, as well as other flavonoids, to cross the blood–brain barrier *in vivo*. The permeability of certain flavonoids is influenced by their lipophilicity; interactions with efflux transporters, such as p-glycoprotein; and active transport (Abbasi et al., 2012). In general, the bioavailability of flavonoid glycosides has been extensively studied. It has been reported that deglycosylation of such compounds by β-glucosidase in the small intestine and by the colonic microflora is often required before absorption can occur. The flavonoids aglycones, either occurring as such in plants or resulting from hydrolysis, displayed various permeability behaviors (Petit et al., 2016).

Also, extracts of *P. incarnata* were found to contain a certain amount of gamma-aminobutyric acid (GABA), suggesting that the bioactivity of this plant could result from the synergistic action of GABA with additional phytochemicals that may facilitate membrane permeation, leading to the positive modulation of $GABA_A$ receptors by flavonoids (Campbell et al., 2004; Carratu et al., 2008). Numerous pharmacological effects of *P. incarnata* are mediated by the modulation of the GABA system, including affinity to the $GABA_A$ and $GABA_B$ receptors and effects on GABA uptake. It appears to be unlikely that *P. incarnata* extract acts by binding to the benzodiazepine site. However, it is plausible that binding to the GABA-site of the $GABA_A$ receptor is one mode of action of the plant extract (Appel et al., 2011). Evaluating the attenuation effects of *P. incarnata* in neuropathic allodynia and vulvodynia, it was suggested that the methanolic extract of the plant possesses peripheral and central phasic as well as tonic anti-nociceptive activity, mediated through modulation of $GABA_A$ and opioid receptors (Aman et al., 2016).

Considering that flavonoids (vitexin, isovitexin, and orientin) present in the genus *Passiflora* showed anti-oxidant properties (Masteikova et al., 2008), the effect observed in the present study might be also related to the anti-oxidant activities. Oxidative injury increases the susceptibility of neurons to oxidative stress and hyperalgesia (Tiwari et al., 2009). Besides, some *Passiflora* species such as *P. foetida* (Sasikala et al., 2011) and *P. edulis* (Montanher et al., 2007) have antiinflammatory activity, which is also a property of *P. incarnata*, and help in the reducing pro-inflammatory cytokines and chemokines. Indeed, vitexin seems to up-regulate the levels of the antiinflammatory cytokine IL-10 (Borghi et al., 2013), which was observed in the hippocampus of PAS group. However, more studies analyzing the anti-oxidant and antiinflammatory profiles of alcohol and *P. incarnata* administration are needed to clarify a pharmacological mechanism.

A significant body of scientific literature highlighted the preclinical evidence regarding the beneficial properties of *P. incarnata* as a treatment for addictive behaviors linked to substances such as amphetamine, nicotine, cannabis and ethanol, and benzodiazepines (Dhawan et al., 2002a,b,c, Dhawan, 2003; Capasso and Sorrentino, 2005). Previous study demonstrated, in mice, that the benzoflavone moiety, a compound present in *P. incarnata* decreased the anxiety induced by chronic ethanol abuse. Additionally, mice treated with an ethanol–benzoflavone moiety combination exhibited lower dependence level and fewer withdrawal signs compared with the ethanol treated mice in a dose-dependent manner (Dhawan, 2003). In humans, *P. incarnata* had already been effective in the treatment of physical withdrawal symptoms of opioids (Akhondzadeh et al., 2001). These results corroborated our findings with *P. incarnata* extract, suggesting its efficacy in the reduction of alcohol withdrawal symptoms.

In conclusion, *P. incarnata* extract treatment seems to be effective in the management of alcohol-related withdrawal symptoms, at least in relation to reversion of the analgesia induced by our model of alcohol withdrawal syndrome, without altering BDNF or IL-10 central levels. However, complementary studies are necessary to clarify this hypothesis and find other pathways related to withdrawal conditions. Preliminarily, our results showed that the *P. incarnata* treatment could be a potential alternative therapy in the alcohol withdrawal syndrome, which justifies more studies investigating a longer treatment and also other signs and symptoms related to alcohol withdrawal.

Acknowledgements

The research was supported by the following Brazilian funding agencies: Conselho Nacional de Desenvolvimento Científico e Tecnológico - CNPq (Dr. I. L. S.Torres and J. L. O. Salomón), Coordenação de Aperfeiçoamento de Pessoal de Nível Superior - CAPES (R. V. A. Schunck, I. C. Macedo; S. Moreira, and M. D. Arbo), and Pró-Reitoria de Pesquisa/Universidade Federal do Rio Grande do Sul.

Conflict of Interest

The authors declare that they do not have any conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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Capítulo II

Transcranial Direct Current Stimulation (tDCS) or *Passiflora incarnata* Reduce Analgesia induced by Alcohol Withdrawal Model and Restore BDNF levels in Brainstem

Manuscrito a ser submetido à Revista

Brain Research

Transcranial Direct Current Stimulation (tDCS) or *P. incarnata* Reduce Analgesia induced by Alcohol Withdrawal and Restore BDNF levels in Brainstem

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Abstract

Background: Alcohol dependence is considered a disorder of difficult therapeutic management due to relapses. The Alcohol Withdrawal Syndrome (AWS) is a set of signs and symptoms that typically develop in alcohol dependent people and can be considered a key in the relapse and maintenance of the addiction The transcranial direct current stimulation (tDCS) is a non-invasive method of brain stimulation suggested as a therapeutic tool and *Passiflora incarnata L.* is used in traditional herbal medicine. This study compared the effect of tDCS and *P. incarnata* extract on nociceptive tests in rats submitted to an alcohol withdrawal model and evaluated the levels of BDNF in the brainstem. The animals were exposed to alcohol for 19 days and after this period they were treated for 8 consecutive days with active tDCS or *P. incarnata*. The nociceptive threshold was assessed by tail-flick latency test (TFL) and hot plate test (HPT) before and at 10 and 11 days after treatments, respectively. The brainstem BDNF levels was evaluated by ELISA at 12 days after the end of alcohol treatment. It was observed an increase in the nociceptive threshold in alcohol withdrawal rats in the TFT and HPT latency ($P = 0.0002$ in both tests). Also, alcohol withdrawal decreased the brainstem BDNF levels ($P<0.001$). The tDCS ($P<0.001$) and *P. incanata* ($P<0.001$) treatments decreased the threshold for HPT after 11 days of treatment and BDNF levels in brainstem ($P<0.001$). The *P. incarnata* and tDCS treatments may be promising in the therapy of relapse of alcohol abuse.

KEYWORDS: Passion flower, tail flick, hot plate, alcohol withdrawal syndrome;

1 Introduction

Alcohol dependence is characterized by reduced self-regulation, increased cravings and frequent relapses, besides sometimes the patients do not respond to the treatment (Koob and Volkow, 2010; Koob and Volkow, 2016). The Alcohol Withdrawal Syndrome (AWS) is a set of signs and symptoms that typically develop in alcohol dependent people within 8–24 h after the last drink, and can be considered a key in the relapse and maintenance of the addiction (Carlson et al., 2012).

Researches have shown an association between pain and concurrent and prospective substance abuse, such as alcohol (Egli et al., 2012). It has already been reported that, among those with alcohol dependence, the combination of pain interference and intensity was associated both with worse treatment outcomes and with relapse following treatment (Witkiewitz et al., 2015; McDermotta et al., 2018). The analgesic effect caused by alcohol with reductions in ratings of pain intensity, could explain alcohol misuse among subjects with persistent pain despite its potential consequences for longterm health (Thompson et al., 2017).

Chronic alcohol intake is known to induce selective neuronal damage (Jung et al., 2005). Adaptations to the addiction circuitry of the brain are partially regulated by neurotrophins, in particular, Brain-Derived Neurotrophic Factor (BDNF), which is produced in the endoplasmic reticulum and expressed within the central and peripheral nervous systems (Logrip et al., 2015; Hogarth et al., 2018). BDNF is also an important modulator of pain, participating in sensory neurotransmission in nociceptive pathways both at spinal and supraspinal levels (Malcangio and Lessmann, 2003; Michael et al., 1997; Pezet and McMahon, 2006; Merighi et al., 2008).

Transcranial Direct Current Stimulation (tDCS) is a method of noninvasive brain stimulation that modulates neuronal resting membrane potentials, leading to changes of cortical excitability and activity, which can outlast the stimulation for hours. These effects depend on stimulation polarity, as cathodal current decreases cortical excitability, and anodal current increases it, and persist hours after the end of stimulation (Nitsche et al., 2003; Stagg and Nitsche, 2011). Studies have found that the stimulation of the prefrontal cortex can reduce alcohol craving (Boggio et al., 2008; den Uryl et al., 2015). The prefrontal cortex is involved in executive functions (e.g. planning, flexibility and goal-directed behavior), which can be damaged in addiction disorders (Goldstein and Volkow, 2011).

Clinical investigations on *Passiflora incarnata* L. (Passifloraceae) have indicated effectiveness in the treatment of pain (Aman et al., 2016), opioid withdrawal (Akhondzadeh et al., 2001a), anxiety (Akhondzadeh et at., 2001b; Movafegh et al., 2008), and attention deficit hyperactivity disorder (Akhondzadeh et al., 2005). Phytochemical research carried out on *P. incarnata* have led to the isolation of several bioactive metabolites such as chrysin, apigenin, homoorientin, vitenxin, luteolin, quercetin, luteolin, etc. (Dhawan et al., 2004). A previous study of our group demonstrated that *P. incarnata* extract seems to be effective in the management of alcohol-related withdrawal symptoms, at least in relation to reversion of the analgesia induced by our model of alcohol withdrawal syndrome (Schunck et al., 2015; Schunck et al., 2017). In this context, the objective of this study was to compare the two treatments, tDCS and *P. incarnata*, in the treatment of AWS.

2 Materials and methods

2.1 Animals

Male adult Wistar rats (90 day-old), weighing 400–450 g, obtained from Centro de Reprodução e Experimentação de Animais de Laboratório (CREAL-UFRGS) were allocated in polypropylene cages (41 × 34 × 16 cm, 4 rats per cage) with free access to food and water on a 24-h light/dark cycle (lights on 7–19 h), in a controlled-temperature environment (22 ± 2 °C) with monitored humidity. The experiments were approved by the University Ethics Committee (Number 28118) and were carried out in accordance with current guidelines for the care of laboratory animals. All possible measures were taken to minimize animal suffering and external sources of pain and discomfort. In addition, the minimum number of animals required to produce reliable scientific data was used based on previous studies of our research group that used similar methods (Spezia Adachi et al., 2012; Laste et al., 2012; Spezia Adachi et al., 2015; Schunck et al., 2015; Macedo et al., 2015; Filho et al., 2016, Cioato et al., 2016, Macedo et al., 2016).

2.2 Extract preparation and treatments

Alcohol 99% (ethanol, Nuclear, Diadema, SP, Brazil) was diluted daily in distilled water to prepare a 20% w/v solution, which was administrated by oral gavage in a dose of 4 g/kg of body weight. Tablets of a commercial extract of *P. incarnata* (Sintocalmy®, Aché Laboratory, Guarulhos, SP, Brazil), standardized in 21 mg (7%) of total flavonoids expressed as vitexin and exempt of beta carboline-type alkaloids, were powdered, dissolved in distilled water, filtered and administrated by oral gavage in a dose of 200 mg/kg of body weight. Distilled water (1 ml/kg) was used as control. The animals were treated daily between 10:00 a.m. and 12:00 a.m. The chemical fingerprint of commercial *P. incarnata* extract was obtained by HPLC-DAD and UPLC-MS showing the presence of the flavonoids

isoschaftoside, isoorientin, orientin, schaftoside, saponarin, vitexin, and isovitexin (Schunck et al., 2017).

2.3 Transcranial direct current stimulation (tDCS) treatment

Anodal stimulation was applied using ECG electrodes (1.5 cm^2) by a battery driven constant current stimulator designed for continuous application of low currents to small mammals. The electrodes had a conductive adhesive hydrogel. Before application, the head of the animals was shaved for better adherence. The size of the electrodes was reduced to 1.5 cm^2 to fit the animals' heads. After the electrodes had been placed, they were fixed onto the head with Micropore TM adhesive tape and covered a protective mesh to prevent removal. Thus, in the present study, electrodes were placed against the skin; this placement resembled that used in human studies of tDCS for pain (Nitsche et al. 2008; Antal and Paulus 2011; Rosen et al. 2009; Fregni et al. 2006). The cathode electrode was placed at the midpoint of the lateral angle of the eyes (supraorbital area), and the anode electrode was positioned on the head using landmarks of the neck and shoulder lines as a guide (the anterior and posterior regions in the midline between the two hemispheres of the parietal cortex, as described by Takano et al. 2011). A constant current of 0.5 mA intensity was applied for 20 min (Fregni et al. 2006). According to an earlier animal safety study (Liebetanz et al. 2009), a current density higher than 142.9 A/m^2 is associated with brain lesions. Given this threshold, our stimulation parameters resulted in a current density of 33.4 A/m^2. Interestingly, we initially used a current of 66.7 A/m^2, and although it was still below the safety limits for brain injury, it did induce skin lesions. For sham stimulation, the electrodes were placed in the same positions as for real stimulation; however, the stimulator was turned off after 30 s of stimulation so as to use the same blinding methodology as used in humans (Gandiga et al. 2006). The tDCS treatment

period, consisting of daily 20-min sessions, for eight consecutive days. Immediately and 24 h after the last session, the tail-flick test and hot plate test were performed.

2.4 Experimental design

Rats were habituated to the maintenance room for 1 week prior to the experiment. Subsequently, animals were randomly divided into eight different groups as follows: (1) Water control (**WAT**, n = 9) treated with distilled water by gavage for 19 days, 1 day without treatment, and more 8 days receiving distilled water; (2) Water + *P. incarnata* (**WATP**, n = 9) group, treated with distilled water by gavage for 19 days, 1 day without treatment, and 200 mg/kg *P. incarnata* by oral gavage for more 8 days; (3) Water + Sham tDCS (**WATS** n = 10) treated with distilled water by gavage for 19 days, 1 day without treatment, and more 8 days the electrodes were placed in the same positions as for real stimulation; however, the stimulator was turned off after 30 s of stimulation; for more 8 days; (4) water group + tDCS (**WATT**, n = 9) group, treated with distilled water by gavage for 19 days, 1 day without treatment, and tDCS for more 8 days; (5) Alcohol withdrawal control (**AW**, n = 9) group, treated with 4 g/kg ethanol by oral gavage for 19 days, 1 day without treatment and distilled water for more 8 days; (6) Alcohol withdrawal + *P. incarnata* (**AWP**, n=10) group, treated with 4g/kg ethanol by oral gavage for 19 days, 1 day without treatment, and 200 mg/kg *P. incarnata* for more 8 days; (7) Alcohol withdrawal + Sham tDCS (**AWS**, n = 11) group, treated with 4 g/kg ethanol by oral gavage for 19 days, 1 day without treatment and the electrodes were placed in the same positions as for real stimulation for more 8 days; however, the stimulator was turned off after 30 s of stimulation; for more 8 days; (8) Alcohol withdrawal + tDCS (**AWT**, n = 11) group, treated with 4 g/kg ethanol by oral gavage for 19 days, 1 day without treatment, and tDCS for more 8 days. The administrations of first 19 days

of treatment were performed in three periods of 5 days with two intervals of 2 days between them (repeated alcohol withdrawals) as previously reported by our group (Schunck et al., 2015). On 20th day, there was no ethanol administration and the nociceptive tests were conducted. In the 29th day, the tail-flick latency test was applied. In the 30th day, the hot plate test was performed. The animals were euthanized in the 31st day (Figure 1). During the time of treatment, all animals had free access to tap water and food.

----------*Insert figure 1 here*----------

2.5 Nociceptive analysis

2.5.1 The tail-flick latency.

The tail-flick latency (TFL) apparatus was described by D'Amour and Smith (1941). Twenty-four hours before the nociceptive response evaluation, the animals were exposed to the apparatus to familiarize them with the procedure, because the novelty can itself induce anti-nociception (Netto et al., 1987). Rats were wrapped in a towel and placed on the apparatus; the light source positioned below the tail was focused on a point 2.3 cm rostral to the tip of the tail. Deflection of the tail activated a photocell and automatically terminated the trial. A cut-off time of 10 s was used to prevent tissue damage. The test was performed at 24 h and 10 days (29th day) after the last administration of the initial treatments. This test was performed by Analgesy-meter LE 71 PanLab Harvard Apparatus containing the halogen lamp for the heat stimulus.

2.5.2 The hot plate test.

The hot plate test (HPT) was carried out to assess the effects of the study agent on the thermal nociceptive threshold (Woolfe and Macdonald, 1944). All rats were acclimated to the hot plate for 5 min, 24 h prior to the test. The temperature of the plate was kept at 50°C. The animals were placed on glass funnels over the heated surface, and the time between placement of the animals on the hot plate and onset of paw licking or jumping was recorded in seconds (s), as latency of response. The test was performed after 24 h and 11 days (30th day) after the last administration of initial treatments of water or alcohol.

2.6 Sample collection and BDNF analysis

On the 12th day after the last administration of water or alcohol, the animals were euthanized by decapitation. The brainstem was separated in a cold surface and immediately frozen in liquid nitrogen and kept at -80°C for subsequent analysis. The BDNF levels were determined by sandwich ELISA using monoclonal antibodies specific for BDNF (R&D Systems, Minneapolis, United States). Total protein was measured by Bradford's method using bovine serum albumin as standard.

2.7 Data analysis

All statistical data analyses were performed by using the Statistical Package for Social Sciences software (SPSS) version 20.0 (SPSS, Chicago, IL, USA). Normality was verified for all variables by using the Kolmogorov–Smirnov test. Behavioral outcomes were analyzed with two-way repeated measures ANOVA, while biochemical outcomes were evaluated with one-way ANOVA. Bonferroni was applied as post hoc test. All results were expressed as mean ± standard error of mean. Significance was set at $P<0.05$.

3 Results

3.1 Effect of treatment and time on TFT latency

The tail- flick latency showed at baseline time significant effects of treatment ($F_{(7, 139)}$ = 4.33, P<0.0002) and time ($F_{(1, 139)}$ = 14.98, P<0.0002), but no interaction between factors (two way ANOVA/Bonferroni, factors: treatment × time). Alcohol withdrawal associated with Sham tDCS (P<0.05) or tDCS (P<0.01) treatment increased the TFT latency when compared to water or *P. incarnata* treatment, water + Sham tDCS (P<0.05) or water + tDCS (P<0.05). Similarly, the alcohol withdrawal associated with tDCS (P<0.05) treatment increased the TFT latency when compared with alcohol withdrawal or *P. incarnata* treatment. After 10 days the effects of the treatment and the time in this test disappeared (P>0.05) (Figure 2).

----------*Insert figure 2 here*----------

3.2 Effect of treatment and time on Hot Plate latency

The HPT latency showed after 24h and 11 days significant effects of treatment ($F_{(7, 142)}$ = 4.381, P=0.0002) and time ($F_{(1, 142)}$ = 4.102, P=0.0002) and interaction between the factors (F(7,142) = 2.124, P<0.04 (two way ANOVA/Bonferroni , factors: treatment × time). Alcohol withdrawal increase the HPT latency after 24h and 30 days. After 11 days the alcohol withdrawal remains after treatment (P>0.05), while this effect is reversed by *P. incarnata* (P<0.001), Sham tDCS (P<0.001) and tDCS (P<0.001) showing treatment x time interaction. After 24h, water + treatments showed a decrease in latency compared with alcohol withdrawal + treatments (*P. incarnata* and Sham tDCS (P<0.05); tDCS (P<0.001)) (Figure 3).

----------*Insert figure 3 here*----------

3.3 BDNF levels

Alcohol withdrawal decreased levels of BDNF in the brainstem in relation to the water group (one way ANOVA/Bonferroni, $F_{(7,30)}=16.08$; $P<0.001$). This effect was reversed in the alcohol withdrawal + Sham tDCS (one way ANOVA/Bonferroni, $F=16.08$; $P<0.05$); alcohol withdrawal + tDCS (one way ANOVA/Bonferroni, $F=16.08$; $P<0.001$); and alcohol withdrawal + *P. incarnata* (one way ANOVA/Bonferroni, $F=16.08$; $P< 0.001$).

----------*Insert figure 4 here*----------

4. Discussion

Our study showed on analgesic effects of alcohol withdrawal in the nociceptive tests reinforcing our previous studies with the same model (Schunck et al., 2015; Schunck et al., 2017). The short and long term analgesic effects induced by alcohol withdrawal in the HPT latency immediatly and lasting for 11 days may be observed by the increase in the latency of response to the animal's paw and tail heat exposure. However, tDCS (Sham tDCS and tDCS active) and *P. incarnata* treatments were able to reverse the analgesic effect induced by alcohol withdrawal after 11 days in the HPT, but not immediately after withdrawal. In other analgesic test (TFL), the baseline time corroborated the analgesic effect developed by alcohol withdrawal was prevented by *P. incarnata* treatment at short time. In a later phase of withdrawal none of the treatments reversed the alcohol withdrawal effects. The different responses to treatments observed in the tests might be related to their basic principles. HPT is used to evaluate tonic pain, whose changes in latency behaviors indicate responses at supraspinal pain process levels and supraspinal sensory integration. This involves tonic pain,

a long- duration stimulus triggering a nociceptive response mainly involving C- fibers. The observed responses include characteristic behaviors as “paw licking” and “jumping”, which are considered supraspinally integrated responses. On the other hand, the TFL is used to evaluate phasic pain involving Aδ fibers stimulation and its nociceptive responses are related to the spinal cord reflex with some degree of supraspinal control evoked by the stimulation of myelinated fibers (Le Bars et al., 2001). Therefore, we suggest that TFL might not detect the central effects produced by our treatments.

TFL test involves reflex descending supra- spinal inhibitory control that travels through the dorsolateral funiculus. This control plays a significant modulatory role, which is accentuated by the stimuli of the heat intensity and by visual and auditory cues provided during the test (Le Bars et al., 2001). It is important to note that the light emitted by the incandescent lamp used to stimulate the tail in this experiment, might cause a learning process. The tail- flick is prone to habituation, and it is possible to occur a reduction in the response with repetitive stimulation. This behavior is generally reported for reflexes evoked by the stimulation of myelinated fibers (Le Bars et al., 2001).

Other studies has demonstrated that alcohol-triggered analgesia can contribute in a relevant way to the dependence to this substance in people with chronic pain (Thompson et al., 2017), leading to increased consumption of this substance for relief of pain and contributing to addiction (Powers et al., 2019). However, these studies have shown hyperalgesia induced by alcohol withdrawal, mainly associated with chronic pain states (Egli et al., 2012; Arout et al., 2016).

Additionally, this study evaluated the BDNF levels in brainstem considering that this structure is a crucial supraspinal site for pain modulation and opioid analgesia (Fields, 2004). It should be noted that this study was pioneer in investigating the effects caused by the

withdrawal of intermittent alcohol administration in the BDNF levels in this structure. We demonstrated that alcohol withdrawal greatly decreases BDNF levels in the brainstem 12 days after the last administration of ethanol, but this effect is reverted in tDCS and *P. incarnata* treated groups. Interestingly, both treatments, sham tDCS and tDCS reversed the decrease in BDNF levels, demonstrating the sensitivity of this neurotrophin to electrostimulation and did not differ from treatment with *P. incarnata*. In humans, peripheral BDNF levels are lower in alcohol dependent patients (Joe et al., 2007), but may be elevated during alcohol withdrawal (Huang et al., 2008).

The BDNF is involved in the synaptic plasticity and survival of neurons acting as molecular mediators of the long-lasting effects of drugs of abuse, including alcohol (Siniscalco et al., 2011; Alele and Devaud, 2013). This neurotrophin is associated with the neuroadaptation in brain reward circuitry and showed a role in addiction (McGough et al., 2004; Huang et al., 2011). On other hand, BDNF levels are increased under chronic pain conditions (Pezet, 2006) demonstrating its implication in the control of nociceptive pathways (Kumamaru et al., 2008). In fact, our study showed that after 12 days both *P. incarnata* or tDCS treatments reverts the analgesia triggered by alcohol withdrawal and this result co-occurs with the return of BDNF levels in the brainstem to the levels of the total control animals (Water group). A probable mechanism of action of BDNF in the noceptive pathways includes its important role in synaptic plasticity related to nociceptive information signaling (Miletic et al., 2008) as NMDA and AMPA receptors (Caldeira et al., 2007).

P. incarnata extract restored nociceptive threshold to the basal levels corroborating our previous study using the same extract (Shunck et al., 2017). Several studies showed evidences of *P. incarnata* as a treatment for addictive behaviors linked to substances such as amphetamine, nicotine, cannabis, ethanol, and benzodiazepines (Dhawan et al., 2004).

Clinically, physical withdrawal symptoms of opioids showed satisfactory results with *P. incarnata* treatment (Akhondzadeh et al., 2001). These results corroborated our findings with *P. incarnata* extract, suggesting its efficacy in the reduction of alcohol withdrawal symptoms. These results can be associated to different compounds presented in the extract of *P. incarnata*. Among other compounds, the flavonoids (vitexin, isovitexin, and orientin) are of special interest due to their antioxidant properties (Masteikova et al., 2008). The observed effects might be related to these properties, since it is known that oxidative injury increases the susceptibility of neurons to oxidative stress and consequent hyperalgesia (Tiwari et al., 2009). Other effects can be promoted by the gamma- aminobutyric acid (GABA) found in the extract. It is know that innumerous pharmacological effects of *P. incarnata* are mediated by the modulation of the GABA system, including affinity to the $GABA_A$ and $GABA_B$ receptors and effects on GABA uptake, suggesting that *P. incarnata* extract acts by binding to the benzodiazepine site (Appel et al., 2011).

In addition, in this study tDCS, both Sham tDCS and active tDCS also reversed the analgesia and restored the BDNF levels induced by alcohol withdrawal after 12 days. Despite the tDCS action mechanism remains unclear, studies suggest that it may involve depolarization of the neural membrane, and changes in cortico-striato-thalamo-cortical connectivity (Ouyang et al. 2011; Tabas et al. 2013). The analgesic effect of tDCS treatment can be mediated by diferents systems, such as opioid, adrenergic, substance P, glutamate, and neurokinin receptors. These neurotransmitters can trigger the modulation of synaptic in the central nervous system structures as thalamic nuclei, the limbic system, brainstem nuclei, and the spinal cord (Lima e Fregni, 2008). The tDCS effect depends on the projection of fibers which down regulate processing from sensitized neurons from the motor cortex to structures related to pain processing such as brainstem or thalamus (Qin et al., 2007; Shimizu et al.,

2009; Brunoni et al., 2014). Some mechanisms appear to be elucidated, since it is known that calcium-dependent synaptic plasticity of glutamatergic neurons is thought to play a key role in the outlasting neuroplastic mechanism of action of tDCS, since blockade of N-methyl D-aspartate (NMDA) receptors diminishes tDCS effects (Liebetanz et al. 2002; Nitsche et al. 2003). The GABA neurotransmission reduction is triggered in any stimulation polarity (Stagg et al. 2009) and this effect may also impact on glutamatergic plasticity due to the close relationship between the two neurotransmitters (Lefaucheur et al., 2017).

In summary, this study corroborated our previous studies that demonstrated analgesia induced by alcohol withdrawal. Both *P. incarnata* and tDCS treatments were able to previne the thermal analgesia induced by alcohol withdrawal 11 days after treatments; and interestingly, the sham treatment did not differ from the active tDCS. Also, it was demonstrated the reestablishment of tissue levels of BDNF in the brainstem. Considering the role of this structure in the analgesia pain modulation, our results are very relevant since these treatments may be promising in the therapy of relapse of substances of abuse

Acknowledgements

The research was supported by the following Brazilian funding agencies: Conselho Nacional de Desenvolvimento Científico e Tecnológico - CNPq (Dr. I. L. S.Torres and J. L. O. Salomón), Coordenação de Aperfeiçoamento de Pessoal de Nível Superior - CAPES (R. V. A. Schunck), and Pró-Reitoria de Pós Graduação/Universidade Federal do Rio Grande do Sul.

Conflict of Interest

The authors declare that they do not have any conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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Legends

Figure 1. Experimental design and timeline showing the 31 days of the experiment: alcohol exposure (AE); alcohol exposure final (AEF); habituation to nociceptive tests (HNT); tail-flick latency (TFL); hot plate test (HPT); * treatments (ShamtDCS, tDCS active and *P. incarnata*); euthanasia (✝).

Figure 2. Effects of treatment and time in the nociceptive threshold in the TFL. Significant effect of treatment (*P<0.0002) and time (*P<0.0002) without interaction between factors (^{ns}P>0.05). Data represented as mean ± standard error of mean (SEM) (Two-way ANOVA/ two way ANOVA/Bonferroni posttests, n=9-11 group).

Figure 3. Effects of treatment and time in the nociceptive threshold in the HPT. Significant effect of treatment (*P<0.0002) and time (*P<0.0002) and interaction between factors (**P<0.04). Data represented as mean ± standard error of mean (SEM) (Two-way ANOVA/ two way ANOVA/Bonferroni posttests, n=9-11 group).

Figure 4. BDNF levels in brainstem. Significant effect of alcohol alcohol withdrawal (*P<0.0002) and treatments ($^{\#}P$<0.05, for ShamtDCS; $^{\#}$P<0.05, for tDCS active), (and $^{\#}$P<0.05, for *P.incarnata*). Data represented as mean ± standard error of mean (SEM) (One-way ANOVA/ Bonferroni posttests, n=9-11 group).

Figures

Figure 1

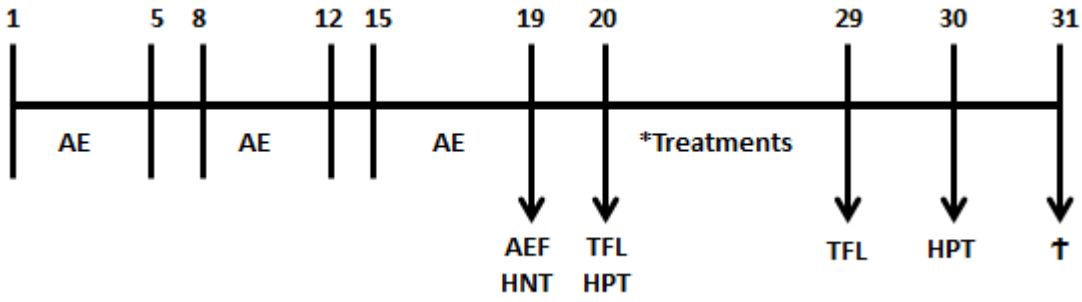


Figure 2

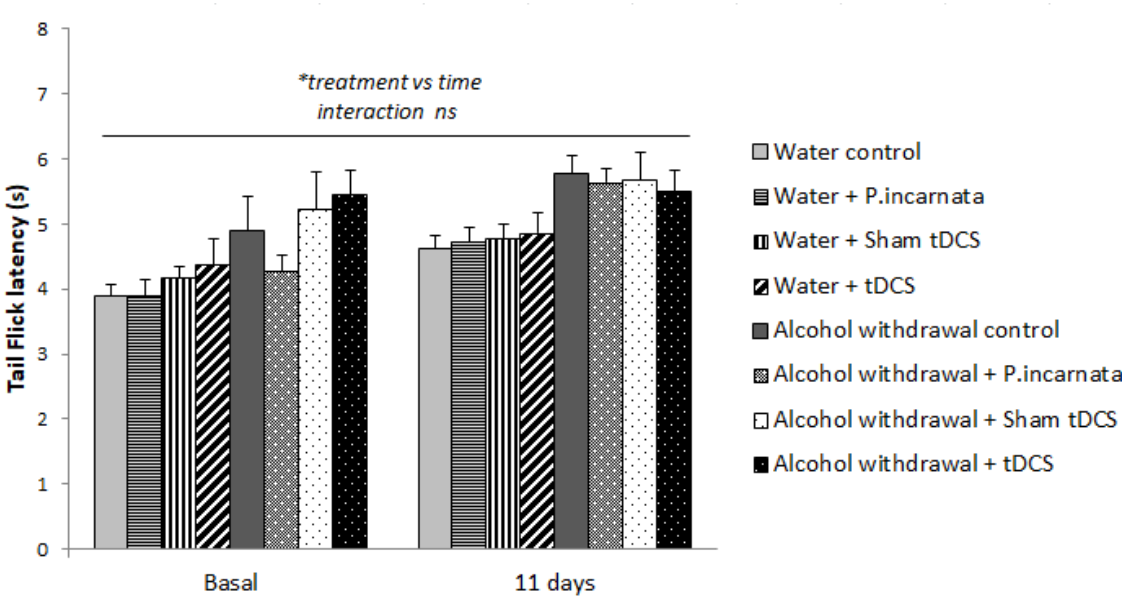


Figure 3

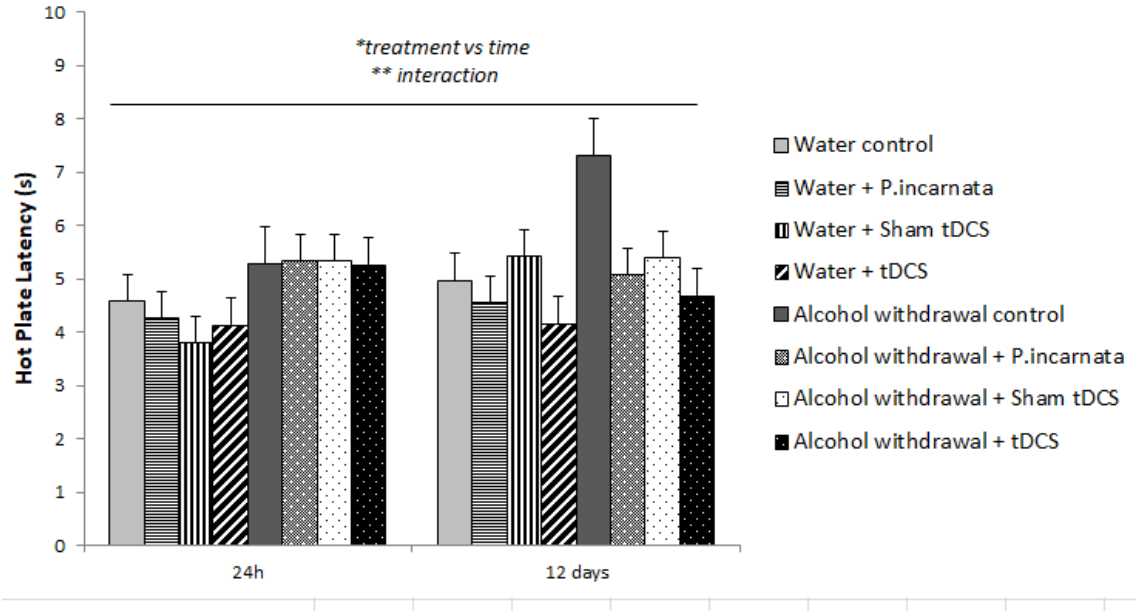
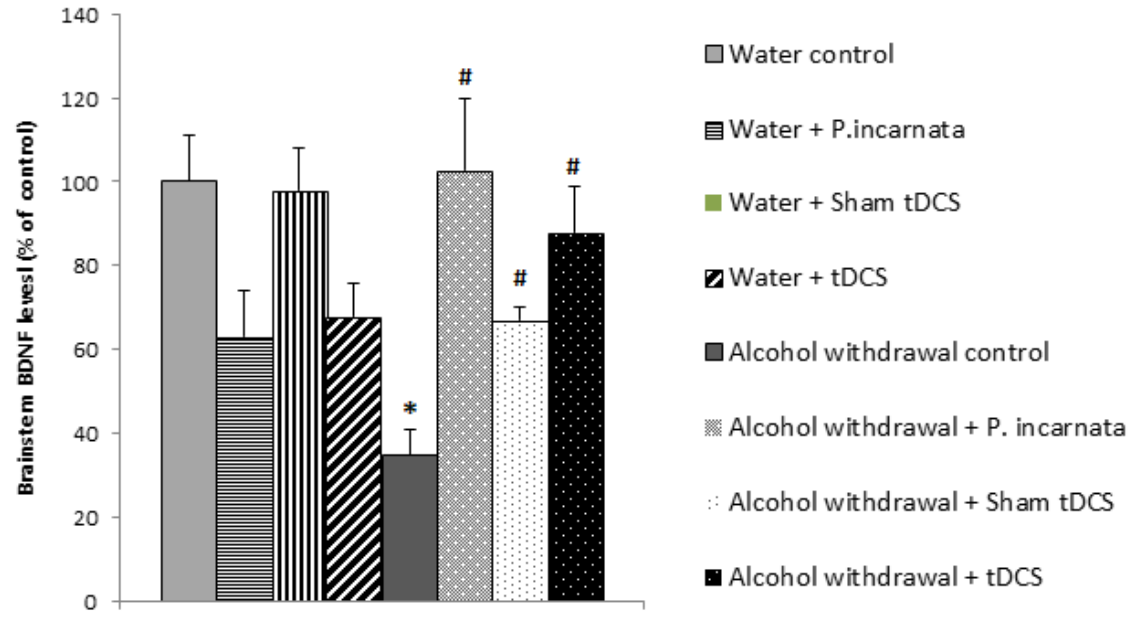


Figure 4



PARTE III

3. DISCUSSÃO GERAL

A ETCC e o extrato padronizado de *Passiflora incarnata* previnem a analgesia induzida pela abstinência ao álcool, conforme demonstrado através do teste da placa quente. Nessa avaliação verificamos que o tempo de resposta comparam-se aos níveis basais após o tratamento com a ETCC e o extrato de *Passiflora incarnata*. Vale ressaltar que essa prevenção não foi observado no teste de *tail-flick*, talvez pela via nociceptiva envolvida. Os mecanismos envolvidos nas respostas nociceptivas produzidas pelos testes da placa quente e *tail-flick* são diferentes. No teste da placa quente há a ativação de nociceptores polimodais de fibras amielinizadas do tipo C, que são caracterizadas por induzir uma dor mais difusa, por ser uma via mais lenta. Entretanto, o teste de *tail-flick* ativa nociceptores de fibras do tipo Aδ, pouco mielinizadas, caracterizadas por conduzir um estímulo nocivo mais rapidamente, provocando, assim, uma dor mais aguda (MENESCAL-DE-OLIVEIRA; DA SILVA, 2009). A placa quente produz duas respostas comportamentais que são mensuradas no tempo que promovem: o ato de lamber as patas (*paw licking*) e o ato de pular (*jumping*), ambas respostas supra espinais. A resposta nociceptiva avaliada no teste de *tail-flick* é um reflexo da medula espinal, com controle também supra espinal (IRWIN et al., 1951; SINCLAIR et al., 1988). Além disso, o teste de *tail-flick* pode gerar aprendizagem, pela luz emitida pela lâmpada incandescente usada para estimular a cauda do rato, sendo, portanto, um teste suscetível à habituação (LE BARS et al., 2001). Durante o experimento cuidou-se para que não houvesse emissão de sinais sonoros e visuais que pudessem ser percebidos como pista pelo animal. Sendo assim pode-se considerar que a ETCC e a *Passiflora incarnata* seriam efetivas em prevenir a analgesia induzida pela retirada de álcool envolvendo fibras tipo C.

A *Passiflora incarnata* tem ação sobre os receptores $GABA_A$ (CARRATÙ et al., 2008; ELSAS et al., 2010). Sabe-se que flavonoides apresentam afinidade pelo sítio benzodiazepínico do receptor $GABA_A$ (MEDINA et al., 1997), entretanto, a bioatividade da *Passiflora incarnata* pode ser devida a uma ação sinérgica entre seus vários componentes (ELSAS et al., 2010). Vale ressaltar que os componentes da *Passiflora incarnata* ligam-se ao receptor $GABA_A$ em um sítio diferente daquele ao qual o etanol ou os benzodiazepínicos ligam-se, e, através de um estudo *in vitro*, ficou demonstrado que a ação desse extrato deve-se a ligação de seus componentes fitoquímicos no mesmo sítio do GABA (APPEL et al., 2011).

A ativação do receptor $GABA_A$ pré-sináptico reduz o influxo de íon cálcio induzido pela despolarização e leva a inibição da liberação de glutamato (WALDMEIER et al., 2008). Este efeito pode explicar em parte sintomas da abstinência ao álcool, tendo em vista que ocorre uma potenciação dos efeitos glutamatérgicos na retirada do álcool. Receptores metabotrópicos $GABA_B$ pré-sinápticos, por sua vez, são responsáveis pela liberação de ácido glutâmico (neurotransmissor excitatório primário e precursor do neurotransmissor inibitório GABA) dos terminais pré-sinápticos (WALDMEIER et al., 2008). De acordo com APPEL e colaboradores (2011) a ação de *Passiflora incarnata* pode ser considerada antagonista dos receptores $GABA_B$. Sabe-se que antagonistas do receptor $GABA_B$ demonstraram ser efetivos em tratar dependência de drogas (MARTIN et al., 2009). A liberação de excessivos níveis de ácido glutâmico no encéfalo pode desencadear excitotoxicidade e pode alterar os níveis de GABA. A ação nos receptores $GABA_A$ e $GABA_B$ pode desenvolver um efeito neuroprotetor, pois o desequilíbrio entre o sistema ácido glutâmico/GABA é um importante cofator em muitas doenças do sistema nervoso central, tais como depressão, ansiedade e adição (JAWNA-ZBOIŃSKA et al., 2016). Tem-se intensificado a busca por novos tratamentos que tenham ação em receptores $GABA_A$, por sua ação neuroprotetora, e, nesse sentido, a *Passiflora incarnata* tona-se muito promissora como tratamento para a síndrome de abstinência ao álcool.

Sabe-se que a transmissão GABAérgica tem um papel muito importante na regulação inibitória do processo nociceptivo e que o teste da placa quente detecta a antinocicepção agonista dependente de GABA (BRITTO et al., 2012; SALAT et al., 2012). No nosso estudo, observamos que, 11 dias após a retirada de álcool e o início do tratamento com *Passiflora incarnata*, o grupo que não recebeu o tratamento apresentou uma diminuição da resposta nociceptiva, o que foi revertido pelo tratamento com *Passiflora incarnata*. Embora GATCH (2009) tenha demonstrado que a hiperalgesia pode ocorrer durante período agudo de abstinência ao álcool, acredita-se que a ação do extrato pode ser causada pelo retorno a níveis basais de GABA em ratos que tinham depleção deste neurotransmissor, causado pelo consumo crônico de álcool e a síndrome de abstinência, fazendo com que os níveis de GABA aumentassem logo nos primeiros dias de tratamento e retornassem aos níveis basais posteriormente.

Vale ressaltar que a antinocicepção causada por extrato metanólico de *Passiflora incarnata* foi revertida por naloxona (antagonista de receptor opioide) e também por

pentilenotetrazol (antagonista de receptor $GABA_A$), o que indica um envolvimento de mecanismos opioides e GABAérgicos na mediação da antinocicepção (AMAN et al., 2016). Agonistas opioides decrescem a transmissão de dor por ativação de fibras nervosas descendentes da substância cinzenta periaquedutal e do núcleo magno da rafe, e também pela inibição da transmissão nervosa por ligação a receptores opioides pré e pós sinápticos localizados no corno dorsal da medula espinhal (KANDEL et al., 2014). Em outro estudo foi verificado um decréscimo significativo na concentração de serotonina, noradrenalina, ácido glutâmico e uma diminuição do estresse em ratos avaliados em teste comportamental de memória, após sete semanas de tratamento com extrato de *Passiflora incarnata* (JAWNA-ZBOIŃSKA et al., 2016).

Apesar do mecanismo da ETCC ainda não ser completamente elucidado, atualmente sabe-se que a sua ação se dá em áreas corticais envolvidas tanto na circuitaria da adição, quanto no processamento da dor crônica, como o córtex pré-frontal, entre outros (APKARIAN et al., 2013; FILHO et al., 2016). Sabe-se que o sistema de recompensa encefálico está envolvido no processamento da dor crônica, principalmente o NAcc, o qual também tem participação na circuitaria de adição ao álcool. Estudo dirigido por BALIKI e colaboradores (2010) sugeriu interação entre o NAcc com o córtex pré-frontal, evidenciando interação entre as vias envolvidas na recompensa e na dor crônica, onde a circuitaria mesolímbica influenciaria e seria influenciada por condições de dor crônica. Os efeitos negativos que surgem quando a ingestão crônica de álcool é diminuída ou descontinuada, são atribuídos a mudanças encefálicas adaptativas na região da amígdala, resultado do uso crônico de álcool e a consequente abstinência a este. Sabe-se também que a ETCC influencia o sistema opioide, o qual, está desregulado na fase de abstinência ao álcool, sendo assim, o ETCC também poderia ter implicações na modulação da dor pela ação no sistema opioide (FILHO et al., 2016). A ETCC reverteu a analgesia induzida pela abstinência ao álcool após 11 dias no teste da placa quente. Este efeito pode ser devido a ação da ETCC nas vias da dor e do alcoolismo, uma vez que tem ação em ambas.

O presente estudo também demonstrou que a administração do extrato de *Passiflora incarnata* e a ETCC, restabeleceram os níveis de BDNF aos valores basais, no tronco encefálico de ratos, após 12 dias de retirada de álcool. É necessário observar que o grupo "Abstinentes ao álcool + *Sham* ETCC" também teve alteração nos níveis de BDNF. Nosso estudo foi pioneiro em investigar os efeitos causados pela retirada de álcool nos níveis de

BDNF no tronco encefálico após tratamento intermitente com álcool (SHUNCK et al, 2015). O aumento dos níveis de BDNF no tronco encefálico, após cada tratamento, encontrados neste estudo, pode ser devido a um provável mecanismo de plasticidade sináptica relacionada a sinalização do processamento nociceptivo (MILETIC; MILETIC, 2008). Também devemos ressaltar que os grupos que estavam em abstinência ao álcool, com e sem tratamento com *Passiflora incarnata*, apresentaram aumento dos níveis de BDNF no córtex pré-frontal, após 12 dias de retirada do álcool. Sabe-se que durante a fase de intoxicação aguda e crônica com álcool, os níveis de BDNF estão diminuídos no córtex pré-frontal, mas que durante a retirada, a liberação desta neurotrofina aumenta, e esta compensação foi sugerida ser uma medida neuroprotetora que regula o consumo de álcool e o dano neural. Logo, o aumento nos níveis de BDNF no córtex pré-frontal são efeitos da retirada de álcool (LOGRIP et al., 2015; FERNANDEZ et al., 2017). A retirada de álcool não produziu efeitos nos níveis de BDNF no hipocampo, e nossos dados estão de acordo com outros estudos que não verificaram diferenças nos níveis desta neurotrofina no hipocampo após retirada de exposição crônica ao álcool (MCCLAIN et al., 2014; FERNANDEZ et al., 2017). Mais estudos são necessários para avaliar e elucidar os mecanismos relacionados ao papel do BDNF na analgesia induzida pela abstinência ao álcool.

Verificamos um aumento nos níveis de IL-10 no córtex pré-frontal de ratos após 12 dias de retirada de ingestão intermitente de álcool, e, observamos também, que a *Passiflora incarnata* não reverteu este aumento. Sabe-se que, num primeiro momento, a ativação da microglia libera diversos mediadores pro-inflamatórios, e que, num segundo momento, ocorre a liberação gradual de mediadores anti-inflamatórios, como a IL-10, entre outros, que agem diminuindo a síntese de citocinas pró-inflamatórias e suprimindo a atividade das células Th1 (Linfócito T auxiliar tipo 1), produzindo, assim, uma forma de reparo do local lesionado (FERREIRA et al., 2009). Estudos demonstraram que a administração sistêmica de IL-10 diminui processos inflamatórios a nível espinhal e produz um efeito analgésico em modelos de dor inflamatória (VALE et al., 2003; ZHOU et al., 2008). Nesse contexto, a IL-10 também apresentou-se elevada no tronco encefálico de animais pertencentes aos grupos abstinentes ao álcool, com e sem tratamento de *Passiflora incarnata*, e, interessantemente, também no grupo tratado somente com *Passiflora incarnata*, sem prévio tratamento com álcool. O aumento da IL-10 também foi observado no hipocampo de ratos tratados somente com *Passiflora incarnata*, sem prévio tratamento com álcool. Estudo demonstrou a propriedade anti-

inflamatória da vitexina, um flavonoide presente na *Passiflora incarnata*, pois esta aumentou os níveis de IL-10 (BORGHI et al., 2013). O aumento nos níveis de IL-10 no tronco encefálico 12 dias após retirada de ingestão crônica de etanol pode ser responsável, entre outros mediadores, pela analgesia observada em ratos abstinentes. Este resultado aliado ao achado de que numa segunda fase do processo inflamatório, os níveis de citocinas pro-inflamatórias diminuem, e que a inibição de uma (IL-1β ou TNF-α) ou de várias citocinas causa analgesia, pode contribuir para a analgesia observada. Entretanto, mais estudos são necessários para elucidar os mecanismos da IL-10 na analgesia induzida pela abstinência ao álcool.

4. CONCLUSÕES

Nossos dados contribuem para o entendimento das alterações envolvidas na analgesia induzida pela retirada de álcool num modelo de administração intermitente. Ambos os tratamentos utilizados, extrato padronizado de *Passiflora incarnata* e ETCC, preveniram a analgesia induzida pela retirada de álcool, 11 dias após o final do tratamento com álcool. Interessantemente, os tratamentos foram capazes de restabelecer os níveis de BDNF no tronco encefálico, após 12 dias. Considerando que esta estrutura é importante na modulação da dor, nossos dados são relevantes para a procura de novos tratamentos que podem ser promissores na terapia de controle à recaída ao uso de álcool, principalmente se esta estiver associada à dor crônica.

5. PERSPECTIVAS

1) Analisar o perfil oxidativo no tronco encefálico de ratos na síndrome de abstinência ao álcool desencadeada por um modelo de ingestão intermitente de álcool e tratamento com *Passiflora incarnata* e ETCC;
2) Avaliar os níveis de IL-10 no tronco encefálico na síndrome de abstinência ao álcool desencadeada por um modelo de ingestão intermitente de álcool em ratos e tratamento com ETCC, e analisar grupo *Sham* sem aplicação de voltagem inicial (30s);
3) Avaliar o efeito da associação de extrato de *Passiflora incarnata* e ETCC na abstinência ao álcool em modelo de ingestão intermitente.

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ANEXO I

U F R G S
UNIVERSIDADE FEDERAL
DO RIO GRANDE DO SUL

PRÓ-REITORIA DE PESQUISA

Comissão De Ética No Uso De Animais

CEUA UFRGS

CARTA DE APROVAÇÃO

Comissão De Ética No Uso De Animais analisou o projeto:

Número: 28118
Título: Estudo do efeito antiaditivo de extrato padronizado de Passiflora incarnata L. e terapia de estimulação transcraniana por corrente contínua (ETCC) em um modelo de dependência de álcool em ratos.

Pesquisadores:

Equipe UFRGS:

MIRNA BAINY LEAL - coordenador desde 14/10/2014
IRACI LUCENA DA SILVA TORRES - pesquisador desde 14/10/2014
Rebeca Vargas Antunes Schunck - Aluno de Doutorado desde 14/10/2014

Equipe Externa:

Eliane Dallegrave - pesquisador desde 14/10/2014

Comissão De Ética No Uso De Animais aprovou o mesmo , em reunião realizada em 08/12/2014 - Sala I do Gabinete do Reitor - Prédio da Reitoria - Campus do Centro - Porto Alegre, em seus aspectos éticos e metolodológicos, para a utilização de 108 ratos Wistar machos adultos, de acordo com as Diretrizes e Normas Nacionais e Internacionais, especialmente a Lei 11.794 de 08 de novembro de 2008 que disciplina a criação e utilização de animais em atividades de ensino e pesquisa.

Porto Alegre, Quarta-Feira, 17 de Dezembro de 2014

STELA MARIS KUZE RATES
Coordenador da comissão de ética