UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL

FACULDADE DE MEDICINA

PROGRAMA DE PÓS-GRADUAÇÃO EM MEDICINA: CIÊNCIAS MÉDICAS

AVALIAÇÃO PRÉ-CLÍNICA DA UTILIZAÇÃO DE POTENCIAIS TERAPÊUTICOS NO TRATAMENTO DE DOR INFLAMATÓRIA CRÔNICA

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Tese apresentada ao Programa de Pós Graduação em Medicina: Ciências Médicas, UFRGS, como requisito para obtenção do título de Doutor.

Porto Alegre, dezembro de 2013.

CIP - Catalogação na Publicação

Laste, Gabriela
Avaliação pré-clínica da utilização de potenciais terapêuticos no tratamento de dor inflamatória crônica / Gabriela Laste. -- 2013.
145 f.

Orientadora: Iraci Lucena da Silva Torres.
Coorientadora: Wolnei Caumo.

Tese (Doutorado) -- Universidade Federal do Rio Grande do Sul, Faculdade de Medicina, Programa de Pós-Graduação em Medicina: Ciências Médicas, Porto Alegre, BR-RS, 2013.

1. dor. 2. inflamação. 3. melatonina. 4. estimulação transcraniana por corrente contínua (ETCC). 5. ritmo atividade-repouso. I. Lucena da Silva Torres, Iraci , orient. II. Caumo, Wolnei, coorient. III. Título.

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

Dedico a todos que me ajudaram neste trabalho, em especial minha família.

Agradecimentos

Agradeço primeiramente a minha família, em especial meu pai Romeo e irmão André. Ao meu pai pelo incentivo ao estudo e educação, apoio tanto nos momentos difíceis passados, tanto nos momentos alegres. Ao meu irmão pelo apoio.

Ao meu namorado Guilherme e família pelo apoio, companheirismo e motivação.

A minha orientadora Prof. Dra. Iraci pela oportunidade, dedicação, aprendizagem constante nesses anos de convívio, além de sugestões essenciais para a realização deste trabalho.

Ao Prof. Dr. Wolnei pela co-orientação, oportunidade, ensinamentos, e motivação constante a busca de novos conhecimentos.

Ao grupo de pesquisa Farmacologia da Dor e Neuromodulação: Modelos Animais por serem fundamentais para a realização deste trabalho. Agradeço a ajuda, amizade e apoio de todos. Em especial a Joanna, Lauren, Paulo, Isabel, Vinicius.

Ao grupo de pesquisa Dor e neuromodulação pela amizade, trocas de conhecimentos e ajuda nos experimentos. Em especial a Liliane, Aline, Alícia, Adriana, Mônica, Francislea pelos bons momentos de convívio.

A Prof. Dra. Izabel Cristina Custódio de Souza pela ajuda e pelo constante aprendizado durante esses anos de convívio.

A patologia experimental. Aos funcionários Flávia e Jorge pela confecção das lâminas para análise e por serem prestativos. A Prof. Dra. Luíse Meurer pela análise histológica e por ser prestativa em estar ensinando e analisando os resultados.

A todos os amigos fora do meio acadêmico pela amizade, incentivo, compreensão da minha ausência durante essa jornada.

A engenharia biomédica do Hospital de Clínicas pelo auxílio na monitorização da atividade dos animais e confecção dos aparelhos de estimulação utilizados.

A enfermeira Marta e veterinária Fabíola da Unidade de Experimentação Animal do Hospital de Clínicas pela ajuda e disponibilidade.

Ao funcionário Jeferson do Hospital de Clínicas – Uamp pela disponibilidade em ajudar e explicar sobre os procedimentos de análises bioquímicas.

A Vera e demais funcionários do PPGCM pelas informações e ajuda quando solicitadas.

Ao FIPE do Hospital de Clínicas pelo financiamento do projeto e ao GPPG pelas revisões dos artigos.

A CAPES pela bolsa.

A UFRGS pela oportunidade.

"É exatamente disso que a vida é feita, de momentos. Momentos que temos que passar, sendo bons ou ruins, para nosso próprio aprendizado. Nunca esquecendo do mais importante: nada nessa vida é por acaso. Absolutamente nada. Por isso, temos que nos preocupar em fazer a nossa parte da melhor forma possível. A vida nem sempre segue a nossa vontade, mas ela é perfeita naquilo que tem que ser" (Chico Xavier).

Resumo

Introdução: Os quadros de dores crônicas são prevalentes, relacionados a alterações físicas e psicológicas, que induzem prejuízos na qualidade de vida. Mais especificamente, a dor inflamatória crônica caracteriza-se por desencadear sustentada hiperexcitabilidade de neurônios no corno dorsal da medula espinhal. O processo nociceptivo da dor crônica inflamatória reduz a atividade do sistema melatonérgico que pode estar associada com dessincronização dos ritmos biológicos. **Objetivo**: Avaliar o uso pré-clínico de novas opções terapêuticas (melatonina e ETCC) para o tratamento de dor inflamatória crônica. **Métodos**: A inflamação crônica foi induzida por uma injeção de Adjuvante completo de Freund (ACF). No primeiro experimento, os ratos receberam 60 mg/kg de melatonina ou de veículo (1% de álcool em soro fisiológico), por via intraperitoneal, por três dias consecutivos. No segundo experimento, quinze dias após a injeção os animais foram tratados com injeção intraperitoneal (ip) de dexametasona (0,25 mg/kg) ou seu veículo (solução salina) por 8 dias. No terceiro experimento, os animais foram tratados com dexametasona (0,25 mg/kg) ou seu veículo, melatonina (50 mg/kg) ou seu veículo (8% de etanol em solução salina), e melatonina mais dexametasona ou seus veículos, por 8 dias. No quarto experimento, todos os ratos apresentavam inflamação crônica e foram divididos em dois grupos: estimulação transcraniana por corrente contínua (ETCC) e estimulação sham. **Resultados**: No primeiro experimento, a administração de melatonina durante 3 dias consecutivos, mostrou um efeito analgésico significativo sobre a dor inflamatória. No segundo experimento, a dexametasona produziu um aumento significativo na latência no teste da placa quente (ANOVA de uma via, $P<0,05$) e no limiar de retirada no teste de von Frey eletrônico ($P <0,005$). O grupo dexametasona apresentou aumento dos níveis de BDNF

em medula espinhal comparado aos outros grupos (ANOVA de uma via $P<0,05$). No terceiro experimento, os animais inflamados apresentaram uma desregulação do ritmo de atividade repouso que foi reestabelecido apos o tratamento farmacológico com melatonina que demonstrou ritmo atividade repouso sincronizado. Adicionalmente os animais tratados com dexametasona isolada ou associada a melatonina mostraram inibição acentuada de parâmetros inflamatórios nos achados histológicos, enquanto a melatonina mostrou uma discreta inibição nos mesmos. Ao final do tratamento foi observado um aumento significativo no limiar de retirada da pata no teste de von Frey em grupos tratados (ANOVA de uma via, $P<0,05$ para todos). No quarto experimento, após oito sessões de 20 minutos de 500 mA de ETCC anódica foi observado efeito antinociceptivo avaliado pelo teste da placa quente imediatamente ($P= 0,04$) e 24 horas após a última sessão de ETCC ($P=0,006$). Foi observado também, um aumento de latência de retirada no teste de Von Frey, 24 horas após a última sessão ($P= 0,01$). **Conclusão:** Nossos achados confirmam as propriedades antinociceptiva e anti-inflamatórias da dexametasona; e podemos sugerir uma relação entre a analgesia e o aumento nos níveis de BDNF em espinhal medula observados apos o tratamento. Por outro lado, a melatonina demonstrou fortes efeitos cronobiótico e antinociceptivo, associados a discreto efeito anti-inflamatório. A associação dexametasona+melatonina não potencializou seus efeitos. Já, a ETCC mostrou-se eficaz induzindo efeito analgésico de longa duração no modelo em estudo. Sendo assim, as propostas de novas terapêuticas abordadas nesta tese parecem ser interessantes opções como adjuvante no tratamento da dor crônica.

PALAVRAS-CHAVE: dor, inflamação, dexametasona, melatonina, ritmo atividade-repouso, estimulação transcraniana por corrente contínua (ETCC).

Abstract

Background: Chronic pain is related to physical and psychological changes that induce losses in quality of life. More specifically, chronic pain is characterized by trigger sustained hyperexcitability of neurons in the dorsal horn of the spinal cord. The nociceptive process of chronic inflammatory pain reduces the activity of melatonergic system that can be associated with desynchronization of biological rhythms. **Objective:** Evaluate the pre-clinical use of new therapeutic options (melatonin and tDCS) for the treatment of chronic inflammatory pain. **Methods:** Chronic inflammation was induced by injection of complete Freund's adjuvant (CFA). In the first experiment, rats received 60 mg/kg of melatonin or vehicle (1% ethanol in saline) intraperitoneally for three consecutive days.In the second experiment, fifteendays after the injection the animals were treated with intraperitoneal (ip) injection of dexamethasone (0.25 mg/kg) or its vehicle (saline) for 8 days. In the third experiment, animals were treated with dexamethasone (0.25 mg/kg) or its vehicle, melatonin (50 mg/kg) or its vehicle (8% ethanol in saline), and melatonin plus dexamethasone or its vehicle for 8 days. In the fourth experiment, all rats had chronic inflammation and were divided into two groups: transcranial direct current stimulation (tDCS) and sham stimulation. **Results:** In the first experiment, administration of melatonin for 3 consecutive days showed a significant analgesic effect on inflammatory pain. In the second experiment, dexamethasone produced a significant increase in latency in hot plate test (one-way ANOVA, $P<0.05$) and in withdrawal threshold in the electronic von Frey test ($P<0.005$). The dexamethasone group had increased levels of BDNF in the spinal cord when compared to the other groups (one-way ANOVA $P<0.05$). In the third experiment, the inflamed animals showed a dysregulation of the rest-activity rhythm that was restored after pharmacological treatment with melatonin. Additionally, the animals treated with

dexamethasone alone or associated with melatonin showed marked inhibition of inflammatory parameters in histological findings, while melatonin showed a slight inhibition in them. At the end of treatment there was a significant increase in paw withdrawal threshold to von Frey test in treated groups (one-way ANOVA, $P<0.05$ for all). In the fourth experiment, after eight 20-minute sessions of 500 mA of anodal tDCS, it was observed an antinociceptive effect assessed by the hot plate test immediately ($P = 0.04$) and 24 hours after the last session of tDCS ($P = 0.006$). It was also observed an increase in withdrawal latency in the von Frey test, 24 hours after the last session ($P= 0.01$). **Conclusion:** Our findings confirm the antinociceptive and anti-inflammatory properties of dexamethasone, and we can suggest a relationship between analgesia and increased levels of BDNF in spinal cord observed after treatment. Furthermore, melatonin has demonstrated strong chronobiotic and antinociceptive effects associated with mild anti- inflammatory effect. Dexametasone plus melatonin didn’t potentiate its effects. The tDCS was an effective analgesic inducing long-lasting effect. Therefore, proposals for new therapies discussed in this thesis seem to be interesting choices as an adjunct in the treatment of chronic pain.

KEYWORDS: pain, inflammation, dexamethasone, melatonin, activity-rest rhythm, transcranial direct current stimulation (tDCS).

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Lista de abreviaturas

AA-NAT ari-lalkylamine-N-acetiltransferase

AE anti-inflamatórios esteroidais

ATP adenosina-5'- trifosfato

CGRP peptídeo relacionado ao gene da calcitonina

ETCC estimulação transcraniana por corrente contínua

G6PD glicose-6-fosfato desidrogenase

GABA ácido γ-aminobutírico

GR receptores de glicocorticoides

GSHPx glutationa peroxidase

GSH-Rd glutationa redutase

HIOMT hidroxi-indol-O-metiltransferase

HPA hipófise hipotálamo adrenal

IkB proteína inibitória kapa B

IASP Associação Internacional para o Estudo da Dor

IL interleucina

MEL melatonina

MR receptores mineralocorticoide

MT	receptor melatonérgico
NAS	N-acetilserotonina
NF-kB	fator nuclear kapa B
NGF	fator de crescimento neural
NMDA	N-metil D-Aspartato
NSQ	núcleo supraquiasmático
ROS	espécies reativas de oxigênio
SNC	sistema nervoso central
SOD	superóxido dismutase
SP	substância P
tDCS	transcranial direct current stimulation
TES	estimulação elétrica transcraniana
TNF	fator de necrose tumoral

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1 Introdução

Os quadros de dor crônica são transtornos prevalentes, relacionados a alterações físicas e psicológicas, responsáveis por prejuízos na qualidade de vida (1). Consequentemente, o controle da dor é uma das mais importantes prioridades terapêuticas. No entanto, ainda há muitas limitações em relação ao seu tratamento, incluindo: dificuldades de avaliação, subestimação da dor relatada pelos pacientes, uso de analgésicos inadequados, efeitos adversos e/ou ausência de opções terapêuticas com efeito em vias neurobiológicas alternativas aos tratamentos clássicos (1,2).

A dor crônica é um processo de neuroplasticidade mal-daptativa, resultante de estímulos nociceptivos repetidos ou de superestimulação do sistema nervoso central (SNC) (3). Neurofisiologicamente, a dor crônica apresenta-se como uma hiperexcitabilidade de neurônios no corno dorsal da medula espinhal. Estes neurônios têm seu limiar de ativação diminuído, despolarizando-se espontaneamente e ampliando seus campos receptivos. Clinicamente, esta hiperexcitabilidade central se manifesta como hiperalgesias primária e secundária ao processo inflamatório (3). Vários neuromoduladores e transmissores, como substância P (SP), neurocinina A, peptídeo relacionado ao gene da calcitonina (CGRP), galanina e aminoácidos excitatórios, participam deste fenômeno de hiperexcitabilidade central (3). O processo nociceptivo da dor crônica inflamatória também envolve a atividade reduzida do sistema melatonérgico, que pode estar associada com dessincronização dos ritmos circadianos. A perturbação do ritmo se manifesta com distúrbios autonômicos, perturbações neuropsiquiátricas (depressão, ansiedade), sintomas somáticos (cansaço, fadiga, rigidez). A diminuição da secreção de melatonina (MEL) corrobora para a dessincronização e a manutenção do ciclo da dor inflamatória crônica, que é sustentado por mediadores inflamatórios que ativam o eixo hipófise-hipotálamo-adrenal (HPA).

Em relação às propostas de novas terapias para condições de dor inflamatória crônica está a melatonina como tratamento farmacológico. Entre a ampla gama de efeitos atribuídos a melatonina, os seus efeitos antinociceptivo, anti-inflamatório e cronobiotico serão estudados nesta tese. Outro tratamento proposto é a estimulação transcraniana por corrente contínua (ETCC), cujo efeito no alívio da dor depende da projeção das fibras do córtex motor para outras estruturas envolvidas no processamento da dor, como o tálamo e núcleos do tronco cerebral. Assim, este efeito pode ser consequência do estímulo propagado para vias neurais rostrocaudal inibitórias da dor, resultando em redução da percepção da dor por um efeito cortico-descendente.

Considerando a importância do problema e a escassez de opções terapêuticas no tratamento de dor inflamatória crônica, que pode estar relacionada a disfunção de sistemas regulatórios endogenos como o melatonergico, este estudo teve objetivo de avaliar o uso pré-clínico de novas opções terapêuticas (melatonina e ETCC) para o tratamento de dor inflamatória crônica

Esta tese está estruturada de acordo com s normas do Programa de Pós-Graduação em Medicina: Ciências Médicas e originou 4 artigos pulicados.

2 Revisão da literatura

2.1 Estratégias para localizar e selecionar informações

Na revisão literária, buscamos ressaltar os principais aspectos relacionados com dor, inflamação, dexametasona, melatonina, ritmo atividade-repouso, ETCC, BDNF.

A estratégia envolveu as bases de dados do MEDLINE (site PubMed), LILACS, ScIELO. Utilizaram-se artigos com datas de publicação entre 1990 e 2013. Estas referências foram revisadas para localizar outros estudos não contemplados nesta busca.

Nos sites PubMed, LILACS e ScIELO foram realizadas buscas por meio dos seguintes termos *pain, inflammation, melatonin, tDCS, dexamethasone, activity-rest rhythm, BDNF*. Em relação ao termo *pain* foram encontrados 550.591 artigos no PubMed, 8.220 artigos no ScIELO e 12.570 no LILACS. Em relação ao termo *inflammation,* foram encontrados 424.712 artigos no PubMed, 2.031 artigos no ScIELO e 5.172 no LILACS. Utilizando-se o termo *melatonin,* foram encontrados 18.614 artigos no PubMed, 97 artigos no ScIELO e 163 no LILACS. Com o descritor *tDCS,* a busca no PubMed encontrou 1.025 artigos, no ScIELO 7 artigos e no LILACS 4. Para *dexamethasone*, 56.966 artigos no PubMed, 270 artigos no ScIELO e 573 no LILACS. Para *activity-rest rhythm,* foram encontrados 117 artigos no PubMed; nenhum artigo foi encontrado nos *sites* ScIELO e LILACS. O termo *BDNF* foi encontrado no PubMed em 13.314 artigos, no ScIELO 27 artigos e no LILACS 41 artigos.

A busca de artigos com o cruzamento das palavras chaves está demonstrada no esquema abaixo (Figura 1).

Dos 1.016.678 artigos buscados pelos termos *pain, inflammation, BDNF,* 135 estão contidos nos 4 artigos e nas seções da revisão da literatura 2.2 Aspectos conceituais da dor, 2.3 Fisiopatogenia da Dor, 2.4 Fisiopatogenia da dor inflamatória, 2.5 Eixo imune-pineal desta tese.

O cruzamento dos termos *pain* e *melatonin* foram encontrados 310 artigos e 63 deram origem aos artigos 1 e 3 desta tese. E também se relacionam especificamente às seções: 2.5 Eixo imune-pineal, 2.8 Propostas de novas terapias para dor inflamatória, 2.8.1 Melatonina como agente terapêutico.

A busca pelos termos *pain* e *dexamethasone* encontrou 1.609 artigos, destes, 21 se relacionam aos artigos 2 e 3 e as seções 2.6 Tratamentos utilizados na dor crônica inflamatória, 2.7 Anti-inflamatórios esteroidais.

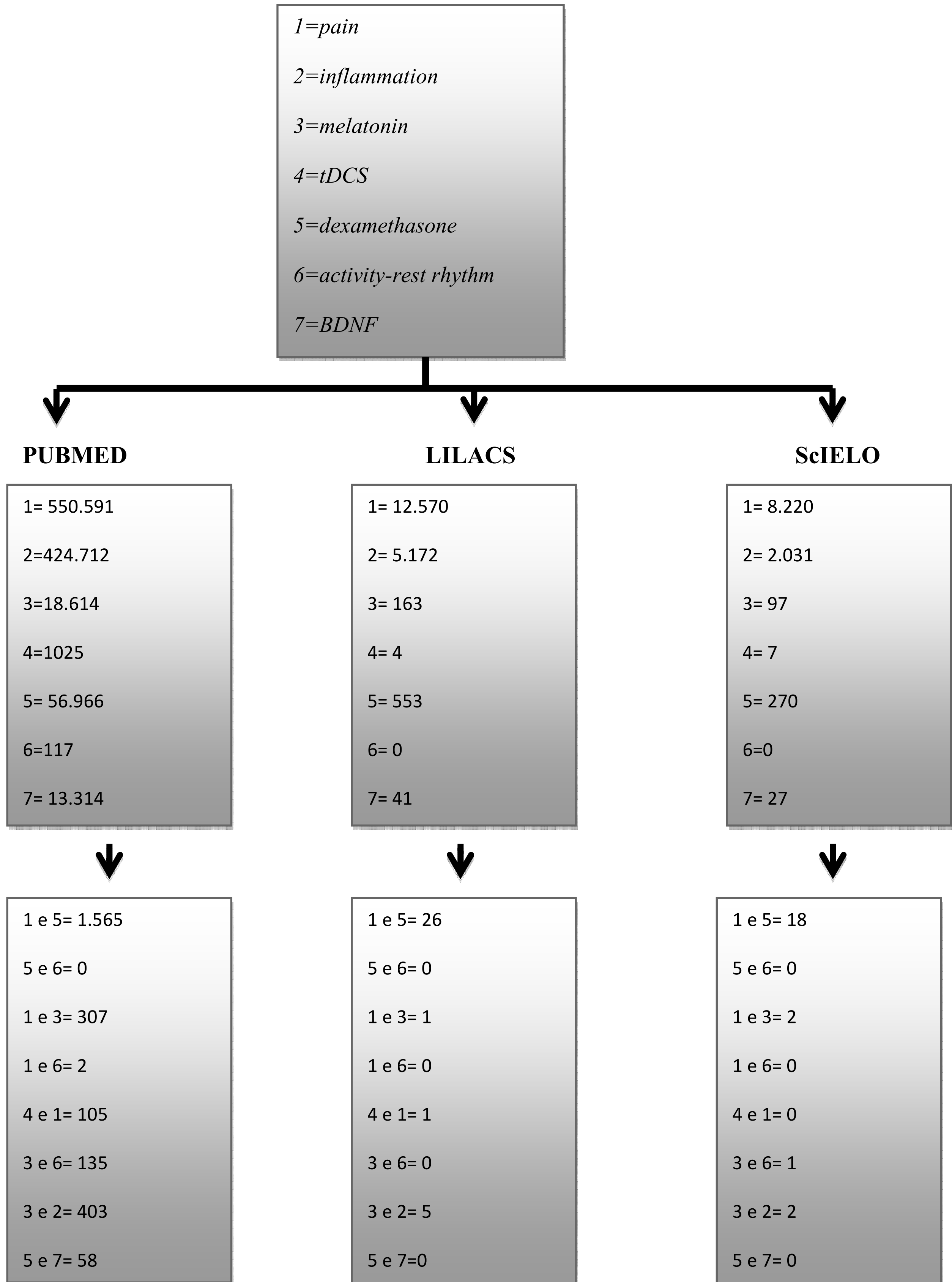
Usando-se os termos *dexamethasone* e *BDNF* foram encontrados 58 artigos. Destes, 8 artigos estavam relacionandos ao artigo 2 e também as seções 2.6 Tratamentos utilizados na dor crônica inflamatória, 2.7 Anti-inflamatórios esteroidais.

Cruzando-se *melatonin* e *inflammation, melatonin* e *activity-rest rhythm, pain* e *rest-activity-rhythm, dexamethasone* e *activity-rest rhythm* encontrou-se 1.096 artigos. Sendo que, 56 artigos foram utilizados no artigo 3 e seções 2.5 Eixo imune-pineal, 2.7 Anti-inflamatórios esteroidais, 2.8 Propostas de novas terapias para dor inflamatória, 2.8.1 Melatonina como agente terapêutico.

E, finalmente, cruzando-se *pain* e *tDCS* foram encontrados 106 artigos e 30 relacionaram-se ao artigo 4 e as seções 2.8 Propostas de novas terapias para dor inflamatória, 8.2 Eletroestimulação transcraniana por corrente contínua.

Figura 1: Fluxograma da pesquisa realizada sobre o tema nas principais bases de dados.

PALAVRAS-CHAVE



2.2 Aspectos conceituais da dor

A Associação Internacional para o Estudo da Dor (IASP) conceitua dor "como uma experiência sensorial e emocional desagradável, relacionada com lesão tecidual real ou potencial, ou descrita em termos deste tipo de dano" (4).

A dor pode ser classificada em aguda e crônica. A dor aguda desempenha papel fisiológico importante no controle da homeostasia, uma vez que ela é um dos principais sintomas clínicos de alerta para a detecção de estímulos que ameacem a integridade física do organismo (5,6). É também denominada dor fisiológica por induzir respostas protetoras, como o reflexo de retirada (ou reação de fuga), para interromper a exposição ao estímulo nocivo. Este sinal é típico da dor aguda produzida por estímulos intensos na superfície da pele (5,6). Por outro lado, a dor crônica é um processo mal adaptativo, que se manifesta como disfunção do sistema nervoso e cronologicamente persiste por pelo menos três meses além do tempo esperado para a resolução do processo ou doenca. Não tem função adaptativa às demandas do meio e causa considerável impacto negativo ao indivíduo e à sociedade (7).

Na resposta dolorosa nociceptiva, existem dois componentes a serem considerados: a reatividade à dor e a sensação dolorosa propriamente dita frente à nocicepção (8). A reatividade à dor é caracterizada por comportamentos defensivos, que compreendem desde a retirada reflexa da área afetada, para longe do fator agressor, até as respostas emocionais complexas, expressas por padrões de comportamento inatos a sensações subjetivas de desconforto e sofrimento. A reatividade emocional corresponde à interpretação afetiva da dor, de caráter individual e influenciada por estados ou traços psicológicos, experiências prévias e fatores culturais, sociais e ambientais (8). Estes fatores são capazes de filtrar, modular ou distorcer a sensação dolorosa (8). A resposta à dor pode variar marcadamente de um indivíduo para o outro, assim como em um

mesmo indivíduo, em momentos diferentes, apesar da intensidade do estimulo doloroso ser a mesma (9).

Além da experiência emocional, a dor está associada a um componente sensorial, que é a sensação dolorosa propriamente dita, denominada nocicepção. Este termo está relacionado com o reconhecimento de sinais dolorosos pelo sistema nervoso (6), constituindo uma resposta neuronal a estímulos traumáticos ou lesivos. Refere-se à atividade do sistema nervoso aferente induzida por estímulos nocivos, tanto exógenos (mecânicos, químicos, físicos e biológicos), quanto endógenos (inflamação, aumento de peristaltismo, isquemia tecidual) (9). O início do processo nociceptivo depende da ativação de estruturas periféricas específicas e vias neuroanatômicas que fazem a comunicação entre os sistemas periférico e central e integram a sensação dolorosa em níveis talâmico e cortical (9).

2.3 Fisiopatogenia da Dor

As estruturas específicas responsáveis pela detecção do estímulo nociceptivo em nível periférico são chamadas nociceptores. Estes estão localizados na porção distal dos neurônios aferentes primários, amplamente distribuídos em pele, vasos, músculos, articulações e vísceras (4,10). São receptores sensíveis a diferentes estímulos nocivos, que podem ser térmicos, mecânicos ou químicos (4,10). Os corpos celulares dos neurônios aferentes primários encontram-se nos gânglios trigeminais e nos gânglios da raiz dorsal. Após emergir de seu corpo celular, o axônio aferente primário bifurca-se, para enviar prolongamentos à medula espinhal (neurônio de segunda ordem) e outro para inervar os tecidos corporais (4,10). Além das sinapses com neurônios de segunda ordem, os neurônios aferentes primários podem fazer sinapse com interneurônios inibitórios, neurônios simpáticos e neurônios do corno ventral motor (4,10).

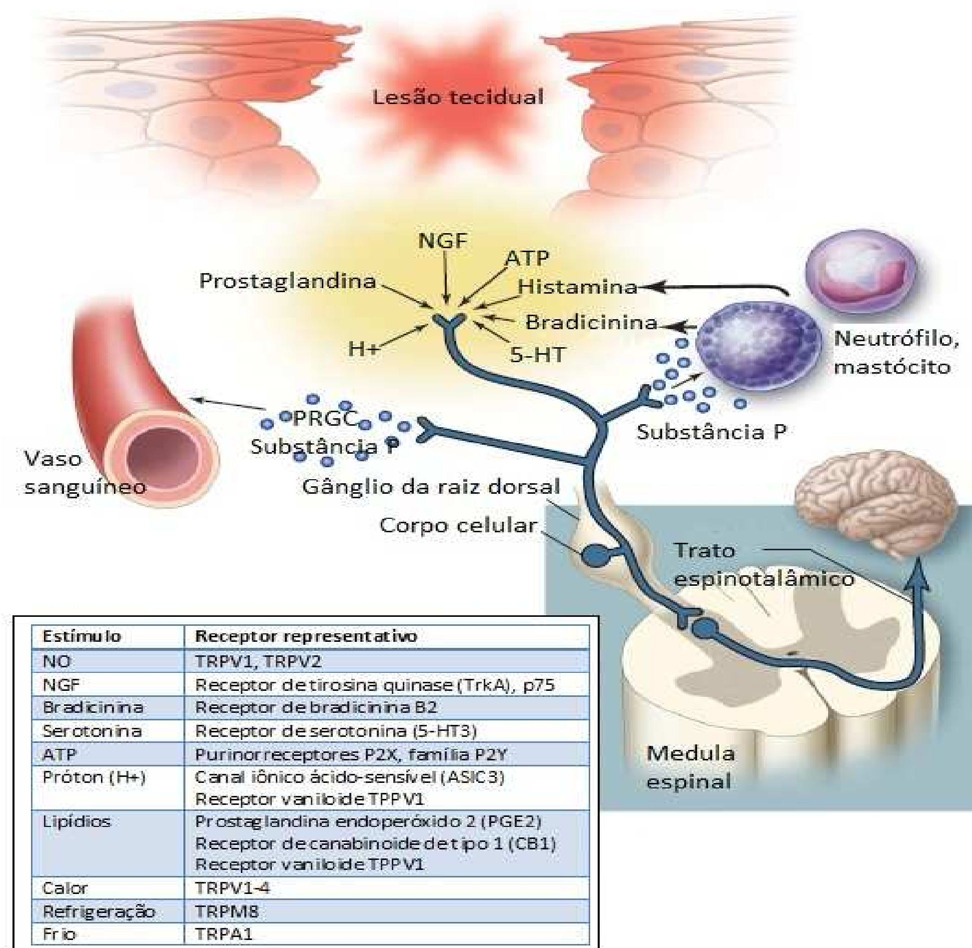
As fibras que transmitem o estímulo nocivo são de dois tipos: A e C. Fibras A delta são mielinizadas finas e de condução rápida, ativadas pelo calor, via receptores mecanotermais e mecanorreceptores de alto limiar (11). As fibras A beta são de grande diâmetro e altamente mielinizadas, e transmitem apenas propriocepção ao toque. Em contraste, fibras C são não mielinizadas e conduzem o estimulo de forma lenta. As fibras C representam a maioria dos nociceptores periféricos denominados C-polimodais (12). As fibras C e A delta no corno dorsal terminam superficialmente nas lâminas I-II, com algumas conexões para lâminas mais profundas, enquanto que as fibras A beta predominantemente terminam nas lâminas III-VI onde recebem neurônios de segunda ordem (13). Estes dão origem às vias ascendentes de condução da dor, cruzam a linha média e ascendem pelo trato espinotalâmico contralateral e podem ser de dois tipos - especificamente nociceptivos ou de faixa ampla (*wide dynamic range* ou *WDR*). Os primeiros relacionam-se apenas a estímulos nocivos, enquanto os segundos também recebem aferência não nociceptiva de fibras Aβ, Aδ e C (13).

Os neurônios especificamente nociceptivos estão arranjados somatotopicamente na lâmina I do corno dorsal, são normalmente silentes e respondem apenas à estimulação nociva de alto limiar (9,10). Os neurônios de faixa ampla constituem o tipo celular predominante no corno dorsal, sendo mais abundantes na lâmina V. Em condições de lesão grave ou persistente, as fibras C disparam repetidamente e a resposta dos neurônios do corno dorsal aumenta progressivamente, de forma gradual, em um fenômeno conhecido como *wind-up* (9,10). As fibras aferentes primárias fazem sinapse com neurônios de segunda ordem na camada superficial da medula espinhal (Figura 2).

Os neurônios de segunda ordem cruzam a medula espinhal até o lado contralateral e ascendem a múltiplas áreas supraespinhais, por meio de tratos neuronais específicos, como por exemplo, o trato espinotalâmico. Sendo assim, os neurônios de

segunda ordem ativarão, em nível talâmico, os neurônios de terceira ordem, conduzindo a informação nociceptiva até áreas sensoriais do córtex cerebral. Em nível cortical aspectos como qualidade, intensidade, localização e duração do estímulo nociceptivo serão integrados e componentes afetivos e emocionais serão interpretados e contextualizados, levando à percepção da dor (14). Salienta-se que a transmissão da informação nociceptiva na medula espinhal pode ser modulada por interneurônios inibitórios em nível de substância gelatinosa, liberando mediadores, tais como o ácido γ-aminobutírico (GABA), opióides endógenos e glicina, entre outros (3) (Figura 2).

Figura 2: Processo nociceptivo



Estímulo	Receptor representativo
NO	TRPV1, TRPV2
NGF	Receptor de tirosina quinase (TrkA), p75
Bradicinina	Receptor de bradicinina B2
Serotonina	Receptor de serotonina (5-HT3)
ATP	Purinorreceptores P2X, família P2Y
Próton (H+)	Canal iônico ácido-sensível (ASIC3) Receptor vaniloide TPPV1
Lipídios	Prostaglandina endoperóxido 2 (PGE2) Receptor de canabinoide de tipo 1 (CB1) Receptor vaniloide TPPV1
Calor	TRPV1-4
Refrigeração	TRPM8
Frio	TRPA1

Fonte:http://www.medicinanet.com.br/m/conteudos/acpmedicine/5249/dor_cronica_%E2%80%93_anne_louise_oaklander.htm

A percepção do estímulo nocivo depende de fatores periféricos e centrais. Por exemplo, o limiar de dor para estimulação mecânica diminui muito em uma área de inflamação (aferências primárias estão sensibilizadas) e em processos que envolvam hiperexcitabilidade de longa duração de neurônios nociceptivos do corno dorsal da medula espinhal (15).

Ao contrario da dor aguda que tem um caráter protetor, necessário à manutenção da integridade do organismo, a dor persistente pode gerar um quadro de dor crônica, não sendo mais considerada uma resposta adaptativa, passando a ser independente do estímulo que a gerou, caracterizando um processo de doença (6).

2.4 Fisiopatogenia da dor inflamatória

A dor nociceptiva resulta da ativação direta de nociceptores em resposta a uma lesão tecidual, acompanhada de processo inflamatório. A dor inflamatória é caracterizada por um aumento da resposta a um estímulo que normalmente seria levemente doloroso (hiperalgesia termal ou mecânica) (11). O processo inflamatório consiste em uma reação complexa do organismo a estímulos nocivos, e é acompanhado de eritema, edema, calor, dor e perda de função (16). Após injúria do tecido, uma gama de moléculas inflamatórias são liberadas por células epiteliais, mastócitos, macrófagos, etc. Entre elas, prostaglandinas E2, bradicinina, e prótons (H +), adenosina-5'- trifosfato (ATP), serotonina, fator de necrose tumoral (TNF), fator de crescimento neural (NGF). Os nociceptores são ativados por estes mediadores indutores de dor (3), amplificando o processo de inflamação local, pela indução da liberação de compostos, como a substância P e CGRP, ativação de mastócitos locais e vasodilatação (17).

Neurofisiologicamente, a dor inflamatória crônica apresenta-se como uma hiperexcitabilidade de neurônios no corno dorsal da medula espinhal. Estes neurônios têm seu limiar de ativação diminuído, despolarizando-se espontaneamente e ampliando seus campos receptivos. Clinicamente, esta hiperexcitabilidade central se manifesta como hiperalgesias primária e secundária à inflamação (3). Vários neuromoduladores e transmissores, como a substância P (SP), neurocinina A, CGRP, galanina e aminoácidos excitatórios, participam deste fenômeno de hiperexcitabilidade central (3). Glutamato e aspartato, dois aminoácidos excitatórios, têm merecido atenção especial nas alterações neurofisiológicas da dor crônica. A liberação destes aminoácidos no corno posterior da medula, ativando receptores NMDA, provoca alterações celulares e moleculares que mantém a hiperexcitabilidade celular levando a excitotoxicidade (18).

Outro importante mediador a ser considerado é o BDNF, que é uma neurotrofina abundantemente expressa no cérebro adulto e na medula espinal, que desempenha um papel importante na regulação do limiar de dor inflamatória e hiperalgesia secundária (19). Esta neurotrofina tem sido descrita como um modulador da dor, que atua sensibilizando neurônios nociceptivos do gânglio da raiz dorsal, facilitando a ativação de receptor NMDA (20). Estudo prévio demonstrou que o BDNF induz à neuroplasticidade na medula espinhal (21). O BDNF não é definido como um promotor exclusivo de inibição ou excitabilidade neuronal, uma vez que exerce um papel modulador na neurotransmissão dependente de atividade (22). Esta afirmação é corroborada por seu efeito no dimensionamento sináptico, em que desempenha um papel essencial na estabilização das sinapses excitatórias corticais decorrentes de atividade neuronal (22).

2.5 Eixo imune-pineal

A atividade reduzida do sistema melatonérgico é associada com dessincronização dos ritmos circadianos (23). Alterações do ritmo circadiano se caracterizam por transtornos autonômicos, desorientação, perda de vontade ou motivação e de sociabilidade. Perturbações neuropsiquiátricas (depressão, ansiedade) e doença inflamatória (24) são dessincronizadores clássicos.

Os ritmos circadianos são sincronizados com o ciclo claro-escuro, e a secreção periférica de melatonina fornece informações circadianas para o resto do organismo (25). Este sistema é regulado por um marcapasso circadiano central, localizado no núcleo supraquiasmático (NSQ), que recebe sinais da retina e controla os ritmos circadianos de atividade e repouso, temperatura e a secreção de melatonina (26).

A síntese de melatonina pela glândula pineal envolve a acetilação de serotonina pela enzima Ari-lalki-lamina-N-acetiltransferase (AA-NAT), seguida por metilação de N acetilserotonina (NAS) e de reação com a enzima hidroxi-indol-O-metiltransferase (HIOMT). A transcrição do gene e/ou o aumento de atividade da AA-NAT depende principalmente do controle simpático, via ativação de receptores $\beta_{1,}$ pela noradrenalina (27), e de receptores purinérgicos P2Y1 (28,29).

A melatonina (N-acetil-5-metoxitriptamina) é um hormônio sintetizado e secretado pela glândula pineal. Esta indolamina é derivada do aminoácido L-triptofano (30). Quando formada, a melatonina não é armazenada na glândula pineal ela se difunde livremente através de todas as membranas biológicas, agindo praticamente em todas as células do organismo (31). Algumas ações são mediadas por receptores melatonérgicos de membrana tipo 1 e 2 (MT1 e MT2) acoplados à proteína G (32), enquanto outras são independentes, como a ligação de receptores hormonais nucleares associados à diferenciação celular e à regulação da resposta imune (33), como, por exemplo, redução do estresse oxidativo e modulação do NF-kB (34). Inúmeros sítios extrapineais

produzem melatonina e indicam que esse hormônio pode ter ação parácrina, além de sua ação endócrina (31). Além disso, sua alta lipossolubilidade permite que ela penetre na barreira hematoencefálica (35).

A melatonina é considerada um biomarcador da ritmicidade circadiana e seus níveis estão alterados em algumas situações de desregulação circadiana, como quadros de dor crônica (36). Quadros de dor inflamatória crônica estão associados a desacoplamento (dessincronização) de ritmos biológicos, como ritmo atividade-repouso (37).

A melatonina (MEL) tem sido associada à estimulação do sistema imunitário. No entanto, alguns estudos têm mostrado que tanto o ritmo claro/escuro quanto o ritmo diário de MEL são mantidos durante a inflamação (38). Adicionalmente, em geral, o ritmo de secreção de MEL não é marcadamente diferente em pacientes com AR (39). Por outro lado, estudo prévio demonstrou que os níveis de MEL no soro atingiram seu pico 2 horas mais cedo em pacientes com AR do que em indivíduos saudáveis (39).

Estudo utilizando modelo de lesão crônica induzida por injeção de BCG (Bacilo Calmette-Guérin) em pata de camundongos demonstrou que os animais apresentaram uma redução no pico noturno de melatonina (40), sugerindo a participação da glândula pineal em respostas inflamatórias. Esta hipótese foi corroborada por outro estudo, que demonstrou que, em animais cronicamente inflamados com BCG e adrenalectomizados, a produção noturna de melatonina é suprimida, elevando a produção diurna (41). Markus et al. (2007) demonstraram que há necessidade de suprimir o pico noturno de melatonina, a fim de permitir um aumento nos mecanismos de defesa do organismo. Assim, citocinas liberadas no início do processo inflamatório suprimem a produção de melatonina pela pineal (42).

2.6 Tratamentos utilizados na dor crônica inflamatória

O uso de anti-inflamatórios não esteroides (AINES), anti-inflamatórios esteroides (corticosteroides) e fármacos antirreumáticos modificadores da doença são usualmente prescritos no tratamento de patologias caracterizadas por dor crônica inflamatória (43).

Anti-inflamatórios não esteroides (AINEs) estão entre os medicamentos mais amplamente utilizados no mundo, devido a sua comprovada eficácia na redução da dor e da inflamação (44). O principal mecanismo de ação dos AINEs ocorre por meio da inibição específica das enzimas cicloxigenases (COX) e consequente redução da conversão do ácido araquidônico em prostaglandinas e tromboxanos (45). A classe dos AINEs compreende tanto os AINEs não seletivos tradicionais, que inibem tanto a COX-1 e COX-2, quanto os inibidores seletivos da COX-2 (46). O uso prolongado de anti-inflamatórios não-esteróides está relacionado a efeitos adversos gastrintestinal, renal e risco de eventos tromboembólicos (47).

Analgésicos não-opioides e opioides também podem ser utilizados. Um exemplo de um não-opioide é a dipirona, porém tem sido relacionada a eventos de agranulocitose (potencialmente fatal) (47). Já aos analgésicos opioides não provocam estes efeitos, mas seu uso prolongado é limitado pela indução de tolerância e dependência e de outros efeitos adversos, como sedação e constipação intestinal (47).

Os fármacos modificadores de doença formam um grupo de agentes que exercem atividades anti-inflamatória e imunossupressora, sendo relacionados com elevada toxicidade, tornando essencial uma monitorização adequada do paciente durante o tratamento (48,49). O metotrexato, um antagonista do ácido fólico, é um dos fármacos deste grupo mais utilizado no tratamento de doenças reumáticas (48,49). Entre seus efeitos adversos estão o aumento das enzimas hepáticas, alopecia, fibrose hepática,

infiltrados ou fibrose pulmonar, mielossupressão e teratogenicidade (48,49). A ciclosporina, agente imunossupressor amplamente utilizado em transplantes, também é indicada para tratamento da artrite reumatoide (44,49). Este fármaco apresenta como efeitos adversos hipertensão, hipercalemia, hirsutismo, hipertrofia gengival, hiperuricemia, tremores, indução de enzimas hepáticas (48,49).

Outro fármaco utilizado no tratamento da artrite reumatoide é o antimalárico cloroquina, que atua aumentando o pH dos compartimentos intracelulares ácidos. Entretanto, seu mecanismo de ação no tratamento de doenças reumáticas não está ainda esclarecido (48-50). Como efeito adverso, destaca-se a retinopatia, o que leva à necessidade de acompanhamento por oftalmologista (48-50).

Os medicamentos imunobiológicos caracterizam-se por bloquear componentes específicos da resposta inflamatória, identificados como essenciais no desenvolvimento e na manutenção do processo patológico (48-50). Dentre estes, estão os fármacos anti-TNF, que, em longo prazo, são relacionados à indução de infecções e neoplasias (48-50).

2.7 Anti-inflamatórios esteroidais

Anti-inflamatórios esteroidais (AE) são os agentes anti-inflamatórios mais eficazes atualmente disponíveis (51), sendo uma opção para o tratamento de condições dolorosas musculoesqueléticas, inflamatórias e articulares (51). Seus eficazes efeitos anti-inflamatórios e imunossupresores são dependentes de dose e amplamente explorados em quadros clínicos refratários a outros tratamentos tradicionais (52). Entretanto, a administração destes fármacos por tempo prolongado é relacionada a efeitos adversos graves, como perda de massa muscular, osteoporose, alterações no

perfil lipídico, hiperglicemia, hipertensão, exacerbação de doenças cardiovasculares, alterações no ritmo circadiano, entre outros (53).

Os efeitos dos AE são decorrentes de ação genômica direta e indireta (transativação e transrepressão) e não genômica (53). No citoplasma, os corticoides se ligam a receptores proteicos específicos (54). Estes complexos migram até o núcleo e atuam como um fator de transcrição, alterando a expressão dos genes-alvo, em resposta a um sinal hormonal específico (55). O complexo corticoide/receptor se liga a regiões promotoras de certos genes, denominadas elementos responsivos a corticoide, induzindo a síntese de proteínas anti-inflamatórias, como a lipocortina-1 e proteína inibitória kapa B (IkB). Também é capaz de induzir a síntese de proteínas que atuam no metabolismo sistêmico (por exemplo, proteínas que promovem gliconeogênese). Este processo é chamado de transativação (56). Os AE atuam, ainda, por meio do mecanismo genômico, chamado de transrepressão, em que monômeros de moléculas de AE e receptores de corticoides interagem com fatores de transcrição, como a proteína ativadora 1 (AP-1) e o fator nuclear kapa B (NF-kB), por interação proteína-proteína e promovem efeito inibitório de suas funções. Por essa via, a síntese de mediadores pró-inflamatórios é reduzida, como a interleucina 6 (IL-6) e IL-2, fator de necrose tumoral (TNF) e prostaglandinas (56).

Os corticoides podem ter efeitos rápidos sobre a inflamação, não mediados por mudanças na expressão gênica (57), mas por meio de ligação a receptores de membrana ou por interações físico-químicas com as membranas celulares (58). Efeitos não-genômicos podem determinar a redução da ação histamínica, diminuição da síntese de prostaglandinas (diminuem a fosfolipase A2) e da ativação do plasminogênio (58).

AE também estão envolvidos na regulação de diferentes funções fisiológicas durante hiperalgesia (59). Estudos têm demonstrado que os esteróides podem controlar

o desenvolvimento, a plasticidade e atividade do sistema nervoso (60). A medula espinhal é um tecido corticoide responsivo (61), apresentando maior densidade de receptores mineralocorticoides (MR) e de receptores de glicocorticoides (GR) nas lâminas I e II (62).

Tem sido relatado também que os corticoides podem regular a expressão de fatores neurotróficos (52), como o BDNF que é um mediador de neuroplasticidade (63), e um modulador nociceptivo (64). A administração de corticoides causou atrofia dendrítica em neurônios do hipocampo e diminuição nos níveis de BDNF em estudo prévio (52). No entanto, outro estudo mostrou aumento na síntese de neutrofinas em córtex cerebral e hipocampo de ratos (65,66). Grundy et al. (2001) demonstraram que a administração de dexametasona, eleva os níveis cerebrais de BDNF em injúria cerebral (67).

2.8 Propostas de novas terapias para dor inflamatória

2.8.1 Melatonina como agente terapêutico

A melatonina desempenha um papel importante na regulação das funções fisiológicas, incluindo sono e ritmo circadiano (68). No entanto, prévios estudos demonstram que a melatonina também pode estar implicada em efeitos farmacológicos (68).

A administração de melatonina é capaz de influenciar, direta ou indiretamente, a fase e/ou o período do relógio circadiano. Estudos demonstraram que a injeção subcutânea diária de melatonina produz um arrastamento do ritmo de atividade locomotora em ratos (69). E infusão de melatonina é capaz de arrastar ritmo de hamsters, um roedor diurno, por meio da indução de avanços de fase, quando o período de locomoção foi superior a 24 horas, e atrasos de fase, quando o período foi inferior a

24 horas (70). Os receptores de alta afinidade de melatonina (MT1/MT2) localizados no SCN parecem ser condição necessária para o efeito cronobiótico da melatonina (71).

Entre a gama de feitos atribuídos à melatonina, os efeitos antinociceotivo e anti-inflamatório têm sido estudados. A associação entre dor e melatonina foi relatada primeiramente por Morris et al (72), em 1969, em um ensaio experimental, seguido por outros estudos que demonstraram que durante o escuro, quando os níveis de melatonina são elevados, os ratos estão menos sensíveis a estímulos nociceptivos e mais suscetíveis aos efeitos da morfina (73,74). Já o efeito antinociceptivo da melatonina exógena intraperitoneal em ratos foi relatado pela primeira vez em 1981, por Lakin (75), que demonstrou bloqueio de seu efeito analgésico pela naloxona, sugerindo participação de opióide endógeno.

O efeito analgésico da melatonina pode estar relacionado a pela sua ação em receptores MT1 e MT2, acoplados à proteína G, expressos na medula espinhal e em várias regiões do cérebro. Quando estes receptores são ativados, reduzem a concentração de AMPcíclico e modificam a função dos canais iônicos de cálcio e potássio, ação intracelular compartilhada pelos opióides e receptores GABA A (76). Foi demonstrado *in vitro* que a melatonina reduz a excitabilidade da transmissão da dor, ativando canais de potássio acoplados à proteína G e inibindo o potencial de ação neuronal (77), além de inibindo canais de cálcio dependentes de voltagem (78), os quais são associados à dor neuropática e à sensibilização neuronal (79).

Vários estudos em modelos de inflamação em ratos demonstraram possíveis mecanismos de ação da melatonina também em doenças inflamatórias, inibindo a produção de óxido nítrico, modulando o fator de transcrição NF-kB, reduzindo a expressão de cicloxigenase, de prostaglandinas e o recrutamento de células polimorfonucleares no sítio inflamado (80). A melatonina pode inibir as fases inicial e

crônica de respostas inflamatórias (80). Além disto, desempenha um papel importante na ativação de defesas antioxidantes, como a superóxido dismutase (SOD), a catalase (CAT), a glutationa peroxidase (GSHPx), a glutationa redutase (GSH-Rd) e a glicose-6-fosfato desidrogenase (G6PD) (81). Estes efeitos permitem diminuir a formação de espécies reativas de oxigênio (ROS) (82).

2.8.2 Eletroestimulação transcraniana por corrente contínua

Considerando possibilidades não-farmacológicas e não-invasivas para o tratamento de doenças crônicas que cursam com dor, não podemos desconsiderar a utilização de estimulação transcraniana por corrente contínua (ETCC). A ETCC induz mudanças na excitabilidade cortical, apresenta baixo risco e pouco desconforto, e com a utilização em sessões repetidas, o efeito pode ser duradouro (83). O princípio da ETCC baseia-se na utilização de uma corrente elétrica fraca aplicada no couro cabeludo. Estudos em seres humanos têm demonstrado que a estimulação do córtex motor induz mudanças na excitabilidade, de acordo com a polaridade de estimulação; estimulação anódica aumenta a excitabilidade cortical, ao passo que diminui a excitabilidade estimulação catódica (83,84).

O tratamento com ETCC para dor crônica tem como foco o córtex motor, região que representa área adjacente à área da dor (85) Em estudo com pacientes com dor crônica, condições repetidas de ETCC anodal sobre o córtex motor primário foram capazes de diminuir a dor destes pacientes (86). É plausível que esta resposta seja consequência de estímulos do córtex motor. Foi sugerido que os efeitos da estimulação transcraniana no alívio da dor depende da projeção das fibras do córtex motor para outras estruturas envolvidas no processamento da dor, como o tálamo e núcleos do tronco cerebral (85). Assim, este efeito pode ser consequência do estímulo propagado de

vias neurais rostrocaudal inibitórias da dor, resultando em redução da percepção da dor (87).

O efeito da ETCC em um curto prazo (efeito imediato) podem ser, respectivamente, devido a uma diminuição (anódica) ou aumento (catodal) do limiar de repouso neuronal (88). No entanto, os efeitos de duração a longo prazo da ETCC envolvem a participação de receptores NMDA glutamatérgicos em mecanismos de plasticidade sináptica (89), tal como demonstrado por estudo que utilizou um antagonista de NMDA (90). O papel do sistema glutamatérgico também foi demonstrado pela utilização de um agonista parcial de receptores NMDA, a D-Cicloserina, que seletivamente potencializa a excitabilidade cortical motora induzida por ETCC anódica, mas não reduz a excitabilidade induzida pela estimulação catódica (91).

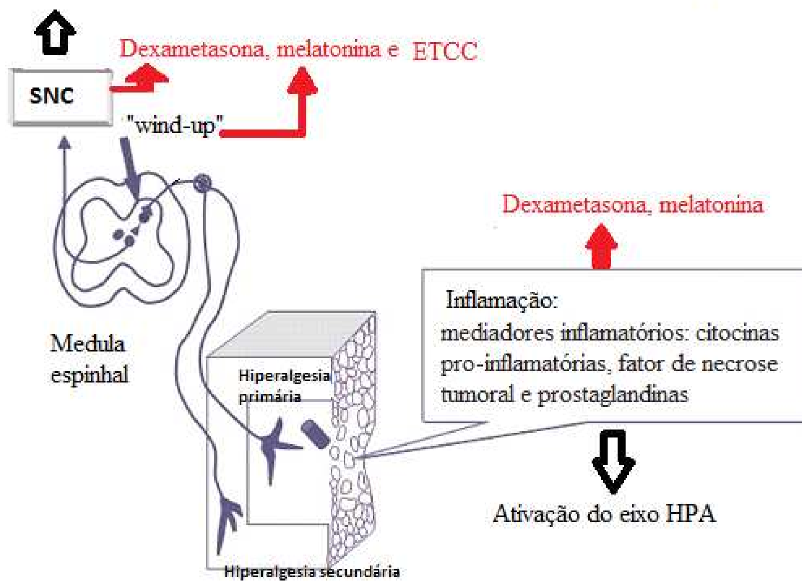
Este método simples de modulação cerebral não-invasiva, tem mostrado resultados significativos em diferentes tipos de dor crônica em humanos (86,92). Em ratos, os efeitos da ETCC foram demonstrados na memória (93), em um modelo de doença de Parkinson (94) e de epilepsia focal (95).

3 Marco teórico

A figura 3 representa um esquema do processo nociceptivo e da dor crônica inflamatória e os locais de atuação dos fármacos utilizados nesta tese. Neurofisiologicamente, a dor crônica apresenta-se como uma hiperexcitabilidade de neurônios no corno dorsal da medula espinhal. Clinicamente, esta hiperexcitabilidade central se manifesta como hiperalgesias primária e secundária ao processo inflamatório. A diminuição da secreção de melatonina corrobora para a dessincronização e a manutenção do ciclo da dor inflamatória crônica, que é sustentado por mediadores inflamatórios que ativam o eixo hipófise-hipotálamo-adrenal (HPA). Em relação às propostas de novas terapias para condições de dor inflamatória crônica, estão a melatonina e a ETCC, sendo também avaliado o efeito da dexametasona.

Figura 3: Esquema do processo nociceptivo e da dor crônica inflamatória e os locais de atuação dos fármacos utilizados nesta tese.

Processo nociceptivo e da dor crônica inflamatória e os locais de atuação da dexametasona, melatonina e ETCC.



4 Objetivos

4.1 Objetivo geral

Avaliar o uso pré-clínico de novas opções terapêuticas (melatonina e ETCC) para o tratamento de dor inflamatória crônica

4.2 Objetivos específicos

1. Avaliar o efeito agudo e repetido (3 dias consecutivos) da administração de melatonina na resposta nociceptiva em ratos submetidos a um modelo de dor inflamatória aguda.

2. Avaliar em ratos submetidos a um modelo de inflamação crônica:

- o efeito antinociceptivo e anti-inflamatório da administração de dexametasona (controle positivo) e melatonina, e da associação de ambos os fármacos;
- os níveis de melatonina e corticosterona séricos após tratamento com melatonina, dexametasona ou associação de ambos os fármacos;
- o ritmo atividade e repouso antes e durante o tratamento com melatonina, dexametasona ou associação de ambos os fármacos;
- o efeito antinociceptivo do tratamento repetido com ETCC.

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6 Artigos

6.1 Artigo publicado: Journal of Pain Research 2012 5:359-362.

Melatonin administration reduces inflammatory pain in rats

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Abstract: In view of the broad range of effects attributed to melatonin, this study evaluated its analgesic effect on inflammatory pain induced by complete Freund's adjuvant (CFA) in Wistar rats. Inflammation was induced by intradermal CFA injection in the hind paw of all animals, which were then divided into two groups that received either 60 mg/kg of melatonin or vehicle (1% alcohol in saline), intraperitoneally, for three days. The analgesic effect of melatonin was assessed by the hot-plate test, immediately and thereafter at 30, 60, 90, and 120 minutes after the first administration and 24 hours after once-daily administration for 2 more days. After CFA injection, melatonin administration increased withdrawal latency at 60 minutes after the first dose. After the end of treatment, melatonin showed a significant analgesic effect on inflam-

matory pain. This study paves the way for exploration of how brief courses of treatment could improve this analgesic effect in the late phases of inflammatory pain.

Keywords: analgesic response, complete Freund's adjuvant, hot-plate test, inflammation, melatonin, nociception.

Introduction

Melatonin (N-acetyl-5-methoxytryptamine) is a hormone synthesized and secreted by the pineal gland. This indolamine is derived from the amino acid l-tryptophan and plays an important role in the regulation of physiological functions, including sleep and circadian rhythms.[1,2] Previous studies have suggested that melatonin has a variety of pharmacological effects.[3] Among the broad range of effects attributed to melatonin, its potential antinociceptive and anti-inflammatory actions have been studied in an animal model of acute pain.[4] The analgesic properties of melatonin have been demonstrated in previous studies conducted by our group[5,6] and in other studies.[7,8] Melatonin administration has been found to raise pain thresholds.[9] In humans, melatonin helps regulate circadian rhythms and, in addition to its analgesic action, also exerts hypnotic,[10] anxiolytic,[10] anti-inflammatory,[11] and antioxidant effects.[9] To date, the precise mechanisms through which melatonin attenuates nociception have not been elucidated. Its analgesic effect has been shown to involve MT1 and MT2 receptors present in the spinal cord and various brain regions.[9,12] Another study suggested that the opioid system may be involved in this effect.[3] In inflammatory pain, the mechanism of melatonin analgesia appears to be by inhibition of nitric oxide production, reducing activation of the transcription factor NF-kB (nuclear factor kappa B), the expression of cyclooxygenase and prostaglandins, and the recruitment of polymorphonuclear cells to the site of inflammation.[4,11] However, little is known about the antinociceptive effects

of melatonin from its acute, short-term administration. In this context, the present study sought to characterize the antinociceptive effect of melatonin when given in a single acute dose and over a 3-day course of treatment in Wistar rats subjected to inflammatory pain induced by complete Freund's adjuvant (CFA).

Methods and materials

Male Wistar rats, 60 days old and weighing 250–300 g, were used. The animals were housed, four to five per cage, in 49 × 34 × 16 cm polypropylene home cages with sawdust-covered floors. Animals were kept on a standard 12-hour dark/light cycle (lights on between 0700 and 1900 hours), at room temperature (22°C ± 2°C), with access to food and water ad libitum. Animal handling and all experiments were performed in accordance with international guidelines for animal welfare. The protocol for this experimental study was approved by the Research Ethics Committee of the institution where the work was conducted (GPPG-HCPA 100013, approved March 8, 2010), and it adhered to the ethical and methodological standards of the Guide for the Care and Use of Laboratory Animals (NIH Publication No 85-23, revised 1985), the UK Animals (Scientific Procedures) Act of 1986, and the European Communities Council Directives of November 24, 1986 (86/609/EEC).

CFA and melatonin were purchased from Sigma-Aldrich (St Louis, MO). Melatonin was dissolved in 1% ethanol in saline (v/v) immediately before use and administered at a dose of 1 mL/kg.

The rats were acclimated to the maintenance room for 1 week before the start of the experiment. All animals received intradermal injections of CFA into the right hind paw to induce inflammation. Animals were randomly allocated to one of two groups (n = 11–12 per group): melatonin (treatment) or vehicle (control). Rats in the experimental

group were treated with intraperitoneal injections of melatonin (60 mg/kg; final solution 60 mg/mL),[13,14] while those in the control group received vehicle (1% ethanol in saline). Injections were administered 1 hour post-CFA and once daily for 2 more days thereafter (for a total of 3 days of melatonin administration), always at 1500 hours. Hot-plate measurements were performed at baseline (before CFA injection), 1 hour after CFA injection, and 30, 60, 90, and 120 minutes and 72 hours after the first administration of melatonin or vehicle, to assess the cumulative effects.

CFA-induced inflammation is commonly recommended as an acute and chronic animal inflammation model for the study of therapeutic approaches to pain.8 Briefly, animals were anesthetized with isoflurane, and inflammation was induced by a single intradermal injection of 100 µl CFA (heat-killed Mycobacterium tuberculosis suspended in paraffin oil, plus mannide monooleate, 1 mg/1 mL) into the right hind paw.

The hot-plate test was carried out to assess the effects of melatonin on the thermal nociceptive threshold. Twenty-four hours prior to testing, all rats were given 5 minutes to acclimate to the hot plate. The temperature of the plate was kept at 55°C. The animals were placed in glass funnels onto the heated surface. With a stopwatch, the experimenter recorded, for the latency of response in seconds, the time between placement of the animals and the onset of paw licking or jumping behavior.[15]

The results are presented in Figure 1 as mean ± standard deviation. The time course of the latency response on the hot-plate test was plotted using a repeated measures analysis of variance (ANOVA), followed by a Bonferroni post hoc test, with time as the repeated measure and the group as the independent variable. Student's t-test for independent samples was used to ascertain whether the mean hot-plate latencies at baseline and after CFA injection were similar for both groups. P-values of < 0.05 were considered significant.

There was no between-group difference in latency at baseline (V: 4.7 ± 0.6 seconds; M: 4.5 ± 0.5 seconds; Student's t-test, P = 0.10) or after CFA injection (V: 2.5 ± 0.6 seconds; M: 2.7 ± 1.3 seconds; Student's t-test, P = 0.18). The repeated measures ANOVA (Figure 1) results showed a time effect (F(1,23) = 22.8, P = 0.001), a group effect (F(1,23) = 25.0, P = 0.001), and an interaction between time and group (F(1,23) = 12.5, P = 0.001). The post hoc Bonferroni test showed an effect from melatonin both after 60 minutes and after 3 days of treatment (P<0.05 for both), but no effect at 30 minutes, 90 minutes, or 120 minutes (P>0.05 for all) (30 minutes: 2.7 ± 0.1 seconds; 60 minutes: 3.7 ± 0.3 seconds; 90 minutes: 3.1 ± 0.1 seconds; 120 minutes: 2.6 ± 0.1 seconds; 4.1 ± 0.3 seconds).

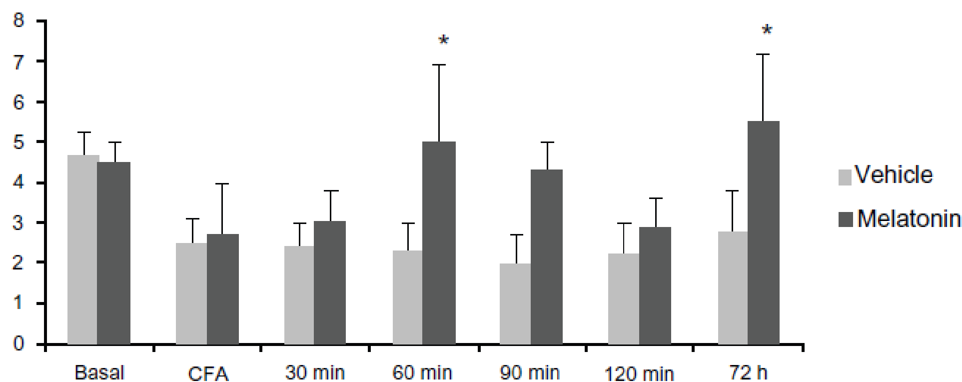

Figure 1 Analgesic effect of melatonin on inflammatory pain based on hot-plate latency.
Notes: Data presented as the mean ± standard deviation (SD) as latency of response in seconds to onset of paw licking or jumping. *Significant between-group difference (repeated measures analysis of variance [ANOVA] with post hoc Bonferroni test; $P < 0.05$, n = 11–12/group).

Discussion

The results of the present study suggest that melatonin plays a role in increasing the threshold of pain associated with CFA-induced inflammation. Our findings are consistent with those of a previous study showing that, at higher doses (60 mg/kg and

120 mg/kg), melatonin produced effective antinociception in the tail-flick test, starting 15 minutes after melatonin administration and peaking after 30 minutes, with the effect lasting over 100 minutes[16]. In addition, a previous study of intrathecal melatonin administration showed antiallodynic activity in rats with neuropathic pain, with the effect observed from 30 minutes through 240 minutes after administration.[17] In diabetic rats, the best antinociceptive effect was observed with 60 minutes' pretreatment (300 mg/kg orally) in the formalin test.[3]

The inflammatory pain model employed in this study has been widely used to test several analgesic drugs,[18] including drugs with anti-inflammatory activity.[8] Tissue damage or inflammation leads to the release of a variety of inflammatory mediators – such as histamine, serotonin, arachidonic acid metabolites (via cyclooxygenase), and kinin – from leukocytes migrating to the affected region.[19] This cascade can be induced by CFA injection. CFA-induced inflammation has been shown to increase levels of cytokines and free radicals.[20] Some of these agents activate nociceptors, while others release local algogenic agents. Algogenic substances sensitize nociceptive neurons, thereby enhancing neuronal excitability in pain transmission pathways and causing secondary hyperalgesia.[21,22] The transmission of pain by primary afferent fibers, the central processes of which are located in the dorsal horn of the spinal cord, is thought to be influenced by several neurotransmitter systems, including opioid, adrenergic, substance P, glutamate, and neurokinin receptors.[21,22]

It should be noted that we used the hot-plate test with the specific intent of evaluating centrally acting analgesics, as described by Woolfe and MacDonald.[15] This test is a neurogenic-modulated model that produces, at a constant temperature, two types of behavioral response (paw licking and jumping), both of which are considered to be supraspinally integrated.[15]

This result suggests a probable central effect of melatonin, as has been described by other authors.[2,9] The possible mechanisms of action for the antinociceptive effect of melatonin include activation of supraspinal sites[16] and inhibition of "spinal windup."[23] Thus, the reduction in secondary inflammatory hyperalgesia in our study could indicate that melatonin reduces the excitability of pain transmission in dorsal horn neurons by acting on membrane-bound MT1 and MT2 receptors.[9] There is also experimental evidence to suggest that the analgesic effect of melatonin is mediated by opioids[1] and by gamma-aminobutyric acid (GABAergic) systems.[17]

While it has been suggested that melatonin has anti-inflammatory properties, we cannot rule out that its central antinociceptive effect is mediated by these mechanisms alone. It should be noted that we did not measure paw edema or biochemical markers of inflammation, and thus cannot infer any improvement in inflammation. Furthermore, melatonin has been shown to produce a marked anti-inflammatory effect at peripheral sites by inhibiting the release of proinflammatory cytokines and thereby relieving pain.[24,25] Several studies in rat models of inflammation have demonstrated that another possible mechanism of melatonin effect in inflammatory diseases is inhibition of nitric oxide production, modulation of activation of the transcription factor NF-kB, reduced expression of cyclooxygenase and prostaglandins, and recruitment of polymorphonuclear cells at the site of inflammation.[4,18]

Conclusion

This study showed that melatonin treatment exerts an antihyperalgesic effect in an animal model of inflammatory pain. This finding paves the way for exploration of how short courses of treatment could improve analgesia in the late phases of inflammatory pain.

Acknowledgments

This study was supported by the following Brazilian funding agencies: National Council for Scientific and Technological Development, CNPq (Dr ILS Torres, Dr W Caumo); HCPA/PIBIC/CNPq (FR da Silva): Committee for the Development of Higher Education Personnel, CAPES (JR Rozisky, G Laste); Graduate Research Group of Hospital de Clínicas de Porto Alegre (Dr ILS Torres; Grant 100013).

Disclosure

The authors report no conflicts of interest in this work. The authors alone are responsible for the content and writing of the paper.

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6.2 Artigo publicado: Neuroimmunomodulation 2013 20(2):119-25.

SPINAL CORD BDNF LEVELS INCREASE AFTER DEXAMETHASONE TREATMENT IN MALE RATS WITH CHRONIC INFLAMMATION

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DECLARATION OF INTEREST: The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

ABSTRACT

Dexamethasone is widely used in the therapy of chronic inflammatory diseases for its pain-modulating effects. The objective of this study was to evaluate the effect of dexamethasone on nociception and local inflammation, the levels of brain-derived neurotrophic factor (BDNF) in the spinal cord in male rats with chronic inflammation induced by Complete Freund's Adjuvant (CFA). Rats were randomly divided in: Control group (no manipulated) and two CFA-induced chronic inflammation (in the 15th post CFA injection) groups: one injected with vehicle (saline solution) and one received dexamethasone (0.25mg/kg) for 8 days. The hot plate and electronic von Frey tests were performed 24 hours after the end of treatment. BDNF spinal cord levels were determined by enzyme-linked immunosorbent assay (ELISA). The level of inflammation in the tibiotarsal joint (the ankle region) was evaluated histologically at the end of treatment. Dexamethasone produced significantly increased latency in the hot plate test (one way ANOVA, $P< 0.05$) and withdrawal threshold in the electronic von Frey test ($P< 0.005$). The dexamethasone group showed increased spinal cord BDNF levels compared with the other groups (one way ANOVA $P<0.05$). Histological analysis showed a local inflammatory response only in animals treated with vehicle demonstrating that the dexamethasone treatment decreased the inflammatory process. Our findings corroborate the antinociceptive and anti-inflammatory properties of dexamethasone. And additionally we showed that the dexamethasone treatment increased BDNF levels in the spinal cord and this effect can be involved with its pain-modulating effects.

Keywords: Dexamethasone; BDNF; spinal cord; antinociception

1. INTRODUCTION

Synthetic glucocorticoids (GCs) are the most potent anti-inflammatory agents currently available for clinical use [1]. GCs are extensively used in a wide range of different tissues, and are often the primary nonsurgical option for treating painful and inflammatory musculoskeletal and articular conditions in humans [1]. GCs can act by inhibiting the production and release of the cytokines interleukin (IL) 1 and 6, tumour necrosis factor (TNF), and of products of arachidonic acid metabolism (prostaglandins and leukotrienes) [2]. GCs are also involved in the regulation of different physiological functions during hyperalgesia [3].

Studies have shown that steroids can control the development, activities and plasticity of the nervous system; there is particular interest in use of these compounds for the modulation of pain [4]. The spinal cord is a corticoid-responsive tissue [5] and within the spinal cord the greatest density of mineralo (MR)- and glucocorticoid receptor (GR) occurs in laminae I–II [6]. However, few studies have evaluated spinal cord brain-derived neurotrophic factor (BDNF) levels after GC treatment. The neurotrophin BDNF is abundantly expressed in the adult brain and spinal cord, and this expression plays an important role in regulating inflammatory pain thresholds and secondary hyperalgesia [7]. After binding to the TrkB (tropomyocin receptor kinase B) on the cell surface of neurons, BDNF regulates neuronal survival, promotes neurite outgrowth, and maintains synaptic connectivity in the adult nervous system [8]. This neurotrophin has been described as a pain modulator, peripherally acts sensitizing nociceptive neurons of the dorsal root ganglion by facilitating the activation of N-

methyl-D-aspartic acid (NMDA) receptors [9]. BDNF has also been shown to potentiate plasticity in the spinal cord [10]. Evidence suggests that BDNF cannot be narrowly defined as exclusively promoting excitatory or inhibitory action, but instead exerts an activity-dependent regulatory role that normalizes neurotransmission [11]. This interpretation is congruent with the role of BDNF in synaptic scaling, in which BDNF has been shown to play an essential role in the stabilization of excitatory cortical synapses following neural activity [12]. BDNF has been found to regulate activity-dependent scaling of inhibitory synapses as well [13].

Considering the wide use of GCs in inflammatory diseases and those previous studies have shown that these agents can modulate nociceptive signalling, the aim of the present study was to evaluate the effect of dexamethasone on nociceptive and inflammatory responses, as well as BDNF spinal cord levels of in male rats with chronic inflammation induced by Complete Freund's Adjuvant (CFA).

2. MATERIAL AND METHODS

Animals

Male Wistar rats weighting 250-300g were used. The animals were housed in groups of five in home cages made of Polypropylene material (49 x34x16cm) with sawdust-covered floors. Animals were kept on a standard 12-hour dark/light cycle (lights on between 0700h and 1900h), at room temperature (22 + 2°C), with free access to food and water. The experiments and procedures were approved by the Institutional Animal Care and Use Committee (GPPG-HCPA protocol No. 100013) and were compliant with Brazilian guidelines involving the use of animals in research (Law No. 11,794) and adhered to the ethical and methodological standards of the Principles of

Laboratory Animal Care (Guide Laboratory for the care and use of animals 8thed, 2011), Vigorous attempts were made to minimize suffering and external sources of pain and discomfort. In addition, the minimum number of animals required to produce reliable scientific data were used.

Experimental Design

Rats were habituated to the maintenance room for 1 week before the experiment commenced. Animals were randomly assigned either to the control group (C) or to the group receiving intradermal (i.d) injection of CFA to induce inflammation where half of the animals were randomly alocated to either the dexamethasone group (D) or the vehicle group (V). Starting on the 15th day after CFA injection, animals received intraperitoneal injection of dexamethasone (0.25mg/kg) [14], or vehicle (saline), for 8 days, at 0800h. The hot-plate and mechanic Von Frey test measures were performed 24 hours after the last administration of dexamethasone or vehicle (on the 23th day). Six to eight animals were used per group.

Drug and chemicals

Complete Freund's adjuvant was purchased from Sigma Chemical Co (F5881; St Louis, MO, USA). Dexamethasone phosphate (Nova Farma, nº1170382) was dissolved in saline (v/v) immediately before use. It was administrated at a rate of 1ml/kg.

CFA-induced chronic inflammation

The inflammatory pain model used in this study is similar to Bernardi et al. 2009 [15]. The CFA injection can elicit the release of a series of inflammatory mediators,

including cytokines and active oxygen species producing hyperalgesia and allodynia [16]. In the CFA induced inflammation there is a biphasic inflammatory response: early (acute) and late (chronic) phases, and in our study we were interested in the late phase of CFA-induced inflammation that is associated with chronic inflammatory pain [15].

Briefly, in animals anaesthetized with isoflurane, inflammation was induced via a single i.d. injection (100µl) of heat killed Mycobacterium Tuberculosis suspended in paraffin oil plus mannide monooleate (1mg\1ml) in the footpad.

Hot plate test

The hot plate test was carried out to assess the effects of the study agent on the thermal nociceptive threshold [17,18]. All rats were acclimated to the hot plate for five minutes, 24 hours prior to testing. The temperature of the plate was kept at 55°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, in seconds (s), was recorded as latency of response.

Electronic von Frey test

Mechanical nociception was assessed using an electronic von Frey (Insight, São Paulo, Brazil). This is an adaptation of the classical von Frey filaments test in which pressure intensity is recorded automatically after paw removal [19]. Rats were placed in 12 x 20 x 17 cm plexiglass cages with a wire grid floor and acclimatized for 15 minutes, 24 hours prior to the test. For testing, a polypropylene tip was placed perpendicularly underneath the mesh floor and applied to one of the five distal footpads with a gradual increase in pressure. A tilted mirror below the grid provided a clear view of the animal's hind paw. The test consisted of poking the hind paw to provoke a flexion reflex

followed by a clear flinch response after paw withdrawal. The intensity of the stimulus was automatically recorded when the paw was withdrawn. It was repeated until the animal presented three similar measurements (the difference between the highest and the lowest measurement should be less than 10 g). The paw withdrawal threshold was expressed in grams (g) [19].

Determination BDNF levels

Animals were killed on the 23th day by decapitation. The spinal cords were collected and stored at -80°C for later BDNF measure. On the date of measurement, spinal cords were weighed and homogenised with a handheld homogeniser in 1:10 Tris-buffered saline. The resulting homogenates were centrifuged for 20 min at 1000g, and a commercially available Enzyme-linked Immunosorbent Assay Kit for Brain Derived Neurotrophic Factor (BDNF) (Uscnk, Life Science Inc.) was used to detect BDNF in the supernatants.

Histology and histological scoring

On day 23, the hind paws were excised and fixed in 10% buffered formalin for 7 days. Paws were then decalcified with nitric acid 10% for 27 h. Tissues were sectioned, embedded in paraffin, and slides prepared and stained with hematoxylin and eosin. We used a described comprehensive histological scoring system.

Briefly, tibiotarsal joint (the ankle region) was histological scored evaluated for the following parameters: inflammation: were scored for the percentage of infiltrating mononuclear cells as follows: 0, absent; 1, mild (1–10%); 2, moderate (11–50%); 3, severe (51– 100%) and the number of inflammation layers was counted.

Statistical analysis

All statistical analyses were carried out using the SPSS 16.0 software (SPSS, Chicago, IL, USA). The results are presented as mean ± SEM. The ANOVA one way test followed Student-Newman-Keuls (SNK) was used to compare the dexamethasone treatment effect. P-values less than 0.05 were considered significant.

3. RESULTS

3.1 Effect of dexamethasone on hot plate test latencies and electronic Von Frey test on paw withdrawal threshold

Pain behaviour tests were performed on day 23, 24 hours after the last administration of dexamethasone or vehicle. Dexamethasone antinociceptive response was demonstrated by a significant increase in response latency in the hot plate test observed in this group when compare to CFA+vehicle group (C: 4.58±0.23s; V: 3.38±0.29s; D: 4.87±0.45s, ANOVA/1W, $F(2,22)$:5.33, $P< 0.05$) (Fig. 1a).

We also examined the effect of dexamethasone on tactile stimuli, as determined by the electronic Von Frey test (Fig. 1b). Dexamethasone administration increased the hind paw withdrawal threshold to punctuate mechanical stimuli only when compare to CFA+vehicle group (C:59.32±1.43g; V: 23.15±2.36g; D:48.31±5.86g, ANOVA/1W, $F(2,19)$:: 4.81, , $P< 0.05$).

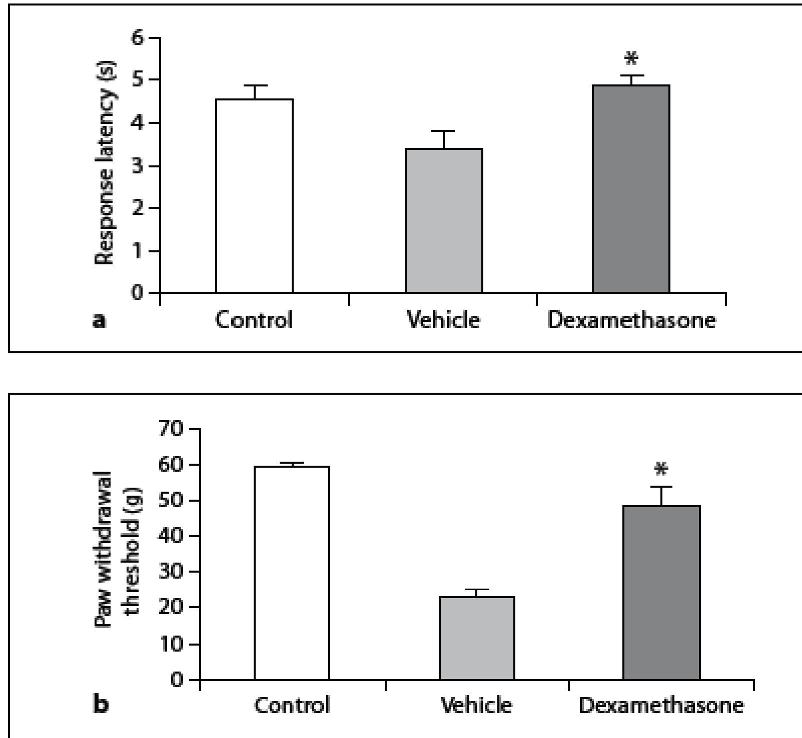

Fig. 1. Effect of dexamethasone on hot-plate test latencies (**a**) and electronic von Frey test on paw withdrawal threshold (**b**) in rats with chronic inflammation. Data expressed as mean ± standard error of mean (SEM). **a** * Significant between-group difference in response latency(s) (one-way ANOVA, $p < 0.05$, n = 6–8 per group). **b** * Significant between-group difference in withdrawal threshold (g) (one-way ANOVA, $p < 0.05$, n = 6–8 per group).

3.2 BDNF levels

BDNF levels were measured by ELISA. As shown in Fig. 2, rats in the dexamethasone group showed significantly increased spinal horn levels of BDNF as compared with other groups (C: 45.72±4.25 pg/mL; V: 51.63±1.38 pg/mL, D: 66.16±3.45 pg/mL; ($F(2,20)= 11.31$, $P< 0.05$).

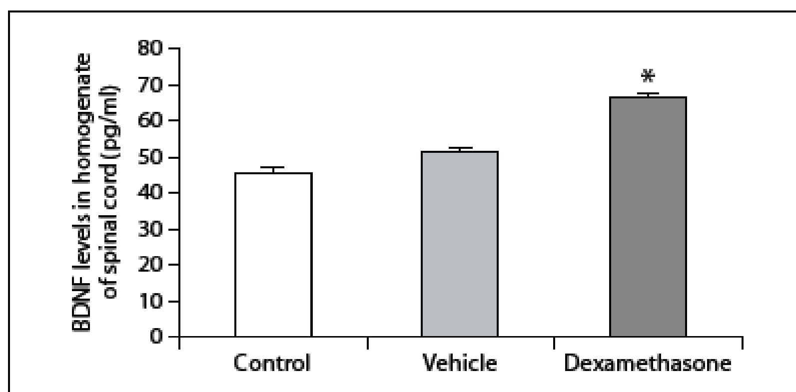

Fig. 2. BDNF levels. Data expressed as mean ± standard error of mean (SEM). BDNF levels were expressed in pg/ml. * Significant between-group difference (one-way ANOVA, $p < 0.05$, $n = 6–8$ per group).

3.3 Histological analysis

The histological findings for each group are shown in Figure 3. Additionally, two parameters of histological changes were assessed, namely the percentage of cell infiltration, and the number of inflammation layers. The CFA injection provoked an inflammation reaction with a presence of infiltrating mononuclear cells (macrophages, neutrophils, lymphocytes) in the muscle and subcutaneous tissues in the ankle joints. The control group presented normal tissue architecture of the ankle joints (Fig. 3A and B), while the vehicle group presented a highly abnormal histology of the joint, with pronounced inflammation (Fig. 3 E and F). The vehicles group presented a mean of 2.6 inflammatory layers. In contrast, the dexamethasone treated group had a remarkable inhibition of all histological findings of inflammation, presenting only mild inflammatory infiltration and nearly normal joint architecture with a mean of 1.0 inflammatory layer (Fig. 3 C and D).

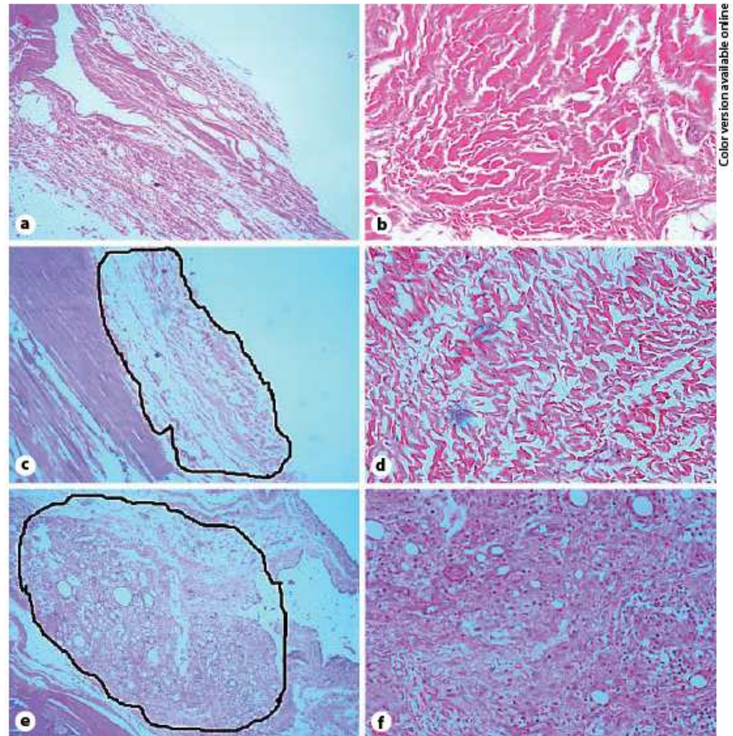

Fig. 3. Hematoxylin and eosin-stained sections from the right hind paws of rats. Circles indicate infiltrating mononuclear cells (macrophages, neutrophils and lymphocytes). **a, b** Control: normal joint architecture. **c, d** Dexamethasone: decrease of the joint inflammation with mild inflammation. **e, f** Dexamethasone's vehicle: severe joint damage with increased inflammation, cartilage infiltrating. ×100 and ×400.

4. DISCUSSION

In this study we demonstrated that dexamethasone treatment reverted thermal hyperalgesia, mechanical hyperalgesia and local inflammation. Additionally, we demonstrated for the first time an increase in spinal cord BDNF levels. In the dorsal horn of the spinal cord, it has been implicated in the control of nociceptive neurotransmission on behavioural, physiological, and pharmacological grounds [20]. Therefore, the BDNF role in pain process is still unclear. The majority of data are consistent with a pro-nociceptive role [21], whereas other research supports an antinociceptive effect [22]. Once released in the spinal dorsal horn, BDNF induces a rapid onset and relatively short lasting increase in phosphorylation of its high-affinity receptor trkB [23]. The functional consequences of BDNF induced plasticity in spinal cord will depend on the type of cells affected, their distribution, and the timing of

events. In fact, there is evidence that BDNF may serve both pro- and antinociceptive roles in different contexts [23].

Additionally, studies have shown an acute BDNF up regulation during a few days following inflammation establishmenthat BDNF is transported and released into the superficial dorsal horn in an activity-dependent manner [24,25]. These findings suggest that BDNF may act during the restricted period in the early phase of inflammation, whereas in our study, the anti-inflammatory treatment was administered during the late phase of inflammation. Thus, the BDNF increase in our study could not be related to the anti-inflammatory effect but rather as a result of spinal synaptic plasticity. Additionally, we can suggest that the increase of the spinal cord BDNF levels in induced by dexamethasone treatment can be involved with its pain- modulating effects.

GCs have been showed as regulators of the expression of neurotrophic factors in the rat brain. Webster and Pirrung (2008) [26] demonstrated that administration of dexamethasone leads to increased brain levels of NGF (nerve growth factor), BDNF, and NT-3 (neurotrophin-3) following injury, producing beneficial effects of neurotrophins that may not be limited to neuronal survival, as BDNF reduces blood–spinal cord barrier permeability after spinal cord injury and reduces leakage of serum proteins.

BDNF has been shown to potentiate plasticity in the spinal cord [10]. Evidence suggests that BDNF cannot be narrowly defined as exclusively promoting excitatory or inhibitory action, but instead exerts an activity-dependent regulatory role that normalizes neurotransmission [11]. This interpretation is congruent with the role of BDNF in synaptic scaling, in which BDNF has been shown to play an essential role in the stabilization of excitatory cortical synapses following neural activity [12]. BDNF

has been found to regulate activity-dependent scaling of inhibitory synapses as well [13].

Acting through the TrkB receptor, BDNF is known to increase the expression of the intracellular calcium-binding protein calbindin, promoting calcium homeostasis following prolonged excitation [27]. Alternatively, BDNF has been shown to act at presynaptic terminals to increase the probability of GABA release, and this effect has been suggested to indirectly suppress over excitation through GABAergic signalling [24]. By normalizing neurotransmission, and by re-establishing a basal state that opposes the excitatory effects of uncontrollable stimulation, BDNF may promote a neural environment that is permissive for adaptive spinal plasticity. More generally, BDNF's action may best be conceived as having a homeostatic function that promotes selective neural adaptations by maintaining the neural network within an optimal operational range.

The data presented above is in accordance with the dexamethasone-induced antinociceptive effects herein demonstrated in the Von Frey and hot plate tests. It is important to note that intraplantar injection of inflammatory agents such as CFA causes increased firing of peripheral afferents in the spinal cord, with consequent hyperalgesia [28]. Peripheral afferent fibres synthesize a diversity of substances that could potentially contribute to hyperalgesia, including glutamate and other excitatory amino acids, neuropeptides such as substance P, adenosine triphosphate, nitric oxide, and prostaglandins [29]. Lam and Thel (2010) [30] demonstrated that administration of dexamethasone produces analgesia by blocking substance P and glutamate receptors. Additionally, Pinto-Ribeiro et al. (2009) [31] reported that, after 3 weeks of treatment with dexamethasone or corticosterone in rats, the expression of γ-aminobutyric acid type B2 (GABAB2) in the superficial dorsal horn was significantly increased,

confirming that corticosteroid receptors play a crucial role in the mediation of pain transmission at the spinal cord level. These studies corroborate our results, demonstrating a probable central effect of dexamethasone, as shown by a significant effect in the hot plate test, which evaluates centrally acting analgesics as described by Woolfe and MacDonald (1994) [17].

Moreover, our analysis of joint histology showed dexamethasone treatment reduced local inflammation.This result corroborates previous studies that demonstrated that corticosteroids have potent immunosuppressive and anti-inflammatory effects and for this reason they are widely used in the therapy of chronic inflammatory diseases [32]. Inhibition of production of cytokines (genomic effect), prostaglandins and leukotrienes (non-genomic effect) have been suggested as their anti-inflammatory mechanisms [33]. In agreement with our study other study, using rat models of Freund adjuvant-induced arthritis, showed significantly reduction of paw oedema with corticosteroids administration [34].

5. CONCLUSION

Taken together, our findings predict a potential influence of corticosteroids in the nociceptive and local inflammatory responses and it may suggest that the BDNF levels increased can be a possible modulation pathway of spinal cord nociceptive transmission. Future studies should also consider assessing the mechanism of action in presence of selective antagonist (such as TrkB receptor antagonist and GABA antagonist) to provide a better understanding of the effects of dexamethasone administration on inflammatory pain and further elucidate and optimize this treatment effects in BDNF levels in spinal cord

ACKNOWLEDGEMENTS AND FUNDING: This study was supported by the following Brazilian funding agencies: National Council for Scientific and Technological Development - CNPq (Dr. I.L.S. Torres, Dr. W. Caumo); Committee for the Development of Higher Education Personnel - CAPES (J.R. Rozisky, G. Laste); Graduate Research Group (GPPG) of Hospital de Clínicas de Porto Alegre - HCPA (Dr I.L.S. Torres – Grant 100013).

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6.3 Artigo publicado: Chronobiology International 2013 30:1077-1088.

MELATONIN TREATMENT ENTRAINS THE REST-ACTIVITY CIRCADIAN RHYTHM IN RATS WITH CHRONIC INFLAMMATION

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CONFLICT OF INTEREST STATEMENT: The authors declare that they do not have conflicts of interest associated with the publication of this manuscript. The authors alone are responsible for the content and writing of the paper.

ABSTRACT

We assessed the therapeutic effect of exogenous melatonin (MEL), dexamethasone (DEXA), and a combination of both on nociceptive response induced by chronic inflammation and on the rest-activity circadian rhythm in rats. A total of 64 animals were randomly divided into eight groups of eight rats each: one control group and seven groups with complete Freund's adjuvant-inflamed animals (CFA; injection into the footpad). One of the CFA-inflamed groups did not receive any treatment; the other six were treated with melatonin (MEL), dexamethasone (DEXA), melatonin plus dexamethasone (MELDEXA), and their respective vehicles. Fifteen days after CFA injection, animals were treated with intraperitoneal injection of MEL (50 mg/kg) or its vehicle (8% ethanol in saline), DEXA (0.25 mg/kg) or its vehicle (saline), and MEL plus DEXA or their vehicles, for 8 days. The von Frey test was performed 24 hours after the last administration of each treatment regimen. Hind paw thickness was measured using a pachymeter during the treatment days. The degree of swelling and histological findings were analyzed. All treated groups significantly reduced the

severity of inflammation when compared with their vehicles (ANOVA repeat measures, $P<0.05$ for all analyses). Inflamed animals treated with dexamethasone alone or associated with melatonin showed marked inhibition of histological findings. On the other hand, the group treated with melatonin remained with moderate inflammation. The CFA group showed a decrease in the mean rest-activity circadian rhythm, determined by the number of touch-detections per hour during water intake in comparison with the control group; only the group treated with melatonin showed a synchronized rest-activity rhythm. At the end of treatment, a significant increase was observed in hind paw withdrawal threshold on the von Frey test in the treated groups (one-way ANOVA, $P<0.05$ for all). Our findings showed that melatonin (50 mg/kg) has strong chronobiotic and antinociceptive effects, but only mild anti-inflammatory effects. This evidence supports the hypothesis that melatonin can induce phase advance and circadian rhythm synchronization in rats with chronic inflammation.

Keywords: melatonin; rest-activity rhythm; antinociception; complete Freud's adjuvant (CFA); inflammation, dexamethasone.

INTRODUCTION

Desynchronization of circadian rhythms is presumably associated with reduced activity of the melatonergic system (Kohyama et al., 2009). Rhythm disruption manifests as autonomic disturbances, disorientation, loss of will or motivation, and loss of sociability. Neuropsychiatric disorders (depression, anxiety), somatic symptoms (tiredness, fatigue, stiffness) (Kohyama, 2011), and inflammatory disease (Swanson et al., 2011) may also be present.

Circadian rhythm disturbances concur with chronic, prolonged stress and exhaustion of the hypothalamic-pituitary-adrenal (HPA) axis (Dickmeis et al., 2009). They cause blunted cortisol reactivity and a flattened diurnal cortisol profile (Gunnarsson et al., 2004; Pruessner et al., 1999), which in turn may influence inflammatory activity (Geiss et al., 2005). This situation may give rise to a vicious cycle, in which low cortisol levels induce the secretion of pro-inflammatory cytokines (e.g., tumor necrosis factor [TNF]), which then inhibit melatonin synthesis (da Silveira Cruz-Machado et al., 2010; Carvalho-Sousa et al., 2005). If, on the one hand, a decreased melatonin secretion mantains the desynchronization, on the other hand the cycle is sustained by macrophage-derived inflammatory mediators such as TNF, interleukin-1β (IL-1β), and IL-12 (Abbas & Lichtman, 2005), which activate the HPA axis (Abbas & Lichtman, 2005). In addition to cortisol, melatonin has been associated with stimulation of the immune system, which would lead to an increase in pro-inflammatory conditions in patients with arthritis (Fildes et al., 2009).

Although, in general, the rhythm of melatonin secretion is not markedly different in patients with rheumatoid arthritis (RA) vs. healthy controls (Straub et al., 2007), one study has shown that serum melatonin levels reached its peak 2 hours earlier in RA

patients than in controls (Straub et al., 2007). This finding may have therapeutic relevance in the management of arthritis, as the nighttime peak of pro-inflammatory cytokines (TNF and IL-6) is usually observed at around 2:00 to 3:00 a.m. In fact, those results support the hypothesis that a stronger inhibition of TNF and IL-6 will lead to a significant reduction in early morning RA symptoms when corticoids are administered at 2 a.m. vs. at 7.30 a.m. (Kirwan et al., 2010). In inflammatory arthritis, corticoids have been used since the 1940s (Hench et al., 1949), and they remain a key component of RA treatment (Parker et al., 1991; Penninx et al., 2004). Although the efficacy of corticoids has been established in inflammatory chronic pain induced by arthritis, its chronic use has been shown to have several side effects, including circadian rhythm disruption (Nieman et al., 2010).

Melatonin may restore the circadian rhythm (Slotten et al., 1999; Carpentieri et al., 2006) and improve pain (Shin et al., 2011; Esposito et al., 2010; Caumo, 2009; Tanuri et al., 2009; Noseda et al., 2004). The anti-inflammatory (Ambriz-Tututi et al., 2009) and anti-hyperalgesic effects of melatonin have been demonstrated both in animals (Laste et al., 2012) and in humans (Hussain et al., 2011). Furthermore, in a recent clinical trial, we have shown that the effects of MEL on pain were independent of improved sleep quality suggesting a direct effect on pain pathways or on chemicals that regulate pain (Vidor et al., 2012). This effect might be linked to the anti-inflammatory properties of MEL (Shin et al., 2011; Esposito et al., 2010; Tanuri et al., 2009). Overall, the efficacy of MEL and corticoids alone on inflammatory pain had already been demonstrated (Shin et al., 2011; Esposito et al., 2010; Tanuri et al., 2009; Caumo et al., 2007; Caumo et al., 2009; Noseda et al., 2004). However, the combination of MEL with corticoids could result in an additional therapeutic approach to improve the

management of inflammatory pain and rhythm disruptions observed in patients with chronic arthritis.

Therefore, the objective of this study was to assess the therapeutic effect of exogenous melatonin, dexamethasone (DEXA) and their combination on nociceptive response induced by chronic inflammation and on rest-activity rhythm in rats.

METHODS

Animals

Sixty-four male, 60 days-old Wistar rats weighting 250-300 g were used. Animals were housed individually for activity recording in cages made of polypropylene (49x34x16 cm), with the floor covered with sawdust. Animals were maintained under a standard 12:12 light-dark (LD) cycle (lights turned on at 07:00 a.m., Zeitgeber time [ZT] 0, and off at 7:00 p.m., ZT 12), at room temperature (22±2°C). Animals had unlimited access to food and water.

Animal handling and experimentation were performed in accordance with international standards set forth in the Laboratory Guide for the care and use of animals (8th ed. 2011). The research protocol was approved by the Animal Care and Ethics Committee at the institution where the study was conducted (protocol no. GPPG-HCPA 10.0013) and adheres to the ethical and methodological standards for medical biological rhythm research laid down by Portaluppi et al. (2010). All possible measures were taken to minimize animal pain and discomfort. The experiment used only the number of animals necessary to produce reliable scientific data.

Experimental Design

Rats were habituated to the maintenance room for 1 week before the beginning of the experiment. Subsequently, the animals were randomly divided into eight groups, with eight rats each, as follows: one control group and seven groups with complete Freund's adjuvant-inflamed (CFA) via injection into the footpad. One of the CFA-inflamed groups did not receive any treatment; the other six were treated with melatonin (MEL), dexamethasone (DEXA), melatonin plus dexamethasone (MELDEXA), and their respective vehicles (melatonin vehicle [VMEL]; dexamethasone vehicle [VDEXA]; melatonin vehicle+ dexamethasone vehicle [VMELDEXA]). Fifteen days after CFA injections, animals were treated with a intraperitoneal injection of MEL (50 mg/kg) (Padhy & Kumar, 2005; Wilhelmsen et al., 2011) or MEL vehicle (8% ethanol in saline), DEXA (0.25 mg/kg) (Caparroz-Assef et al., 2007; Oelzner et al., 2010) or DEXA vehicle (saline), and MELDEXA or MELDEXA vehicles. Injections were administered at 08:00 a.m., for 8 days. Mechanical nociception was measured using the von Frey test 24 hours after the last administration in each group (on the 23th day).

Drugs and chemicals

CFA and MEL were purchased from Sigma Chemical Co. (St. Louis, MO, USA), and DEXA phosphate from Nova Farma. All drugs were administrated at 1 ml/kg.

CFA-induced inflammation

The protocol adopted for CFA-induced inflammation is similar to those described by other authors (Bernardi et al., 2009; Stein et al., 1988), with minor modifications, and the inflammatory pain model is similar to that described by Bernardi et al. (2009), who showed that treatment with indomethacin-loaded nanocapsules

significantly decreased serum levels of pro-inflammatory cytokines TNF-α and IL-6 in rats.

Briefly, rats were anesthetized with isoflurane and inflammation was induced via a single 100-µl intradermal injection of heat-killed Mycobacterium tuberculosis suspended in paraffin oil and mannide monooleate (1 mg/1 ml). CFA was injected into the rats' right footpad, as described by Stein et al. (1988). The contralateral left paw received 100 µl of saline.

Hind paw inflammation measurement and evaluation

Hind paw swelling was quantified by macroscopic measurement of the dorsal-plantar thickness of the hind paws using a pachymeter. Measurements were made 1 day before the beginning of treatment and after its end, and were expressed in millimeters (mm), as the difference between the right and left paws.

Electronic von Frey test

Mechanical nociception was assessed using an electronic version of the von Frey test (Insight, São Paulo, SP, Brazil). This version is an adaptation of the classical von Frey filament test, in which pressure intensity is recorded (Vivancos et al; 2004). Rats were placed in acrylic cages (12x20x17 cm) with a wire mesh floor and acclimatized for 15 minutes 24 hours before the test. For the test, a polypropylene tip was perpendicularly placed underneath the mesh floor, touching one of the footpad digits available at a gradually increasing pressure. A tilted mirror placed below the mesh provided a clear view of the animal's hind paw. The test consisted of poking the right hind paw to provoke a flexion reflex, followed by a clear flinch response after paw withdrawal. Stimulus intensity was automatically recorded. Paw stimulation was

repeated until the animal presented three similar measurements (i.e., when the difference between the highest and the lowest measurements was <10 g). Paw withdrawal threshold was expressed in grams (g) (Vivancos et al., 2004; Amaral et al., 2008).

Serum levels of melatonin and corticosterone

Animals were killed on the 23rd day following CFA injection (between 12:00 and 04:00 pm) by decapitation. Trunk blood was drawn and blood samples were centrifuged in plastic tubes for 20 minutes at 1,000 g, at 4°C. Serum samples were obtained and frozen at -80°C, until assay performance. Serum hormone levels were determined using a commercially available enzyme-linked immunosorbent assay (ELISA) kit for rat MEL or corticosterone (Uscnk, Life Science Inc., St. Petersburg, FL, USA), according to the manufacturer's instructions.

Histological scoring

After the rats were sacrificed, hind paws were excised and fixed in 10% buffered formalin for 7 days. Paws were then decalcified with 10% nitric acid for 27 hours. Tissues were sectioned and embedded in paraffin. Slides were prepared and stained with hematoxylin and eosin.

Briefly, the tibiotarsal joint (ankle region) was observed by optical microscopy and histologically graded according to the following parameters by a specialist blinded to treatment information: percentage of infiltrating mononuclear cells compared to the surrounding tissue (0, absent; 1, mild [1-10%]; 2, moderate [11-50%]; and 3, severe [51-100%], using low and medium power – 40, 50 and 100x); and number

of medium microscopic power fields (200x) occupied by the inflammatory process. Cells were identified according to their morphological characteristics.

Activity-rest rhythm assessment

The rest-activity rhythm was determined by the number of touch-detections per hour during water intake, using an automatic activity data recording system (Moraes et al., 1997; Cecon et al., 2010).

Statistical analysis

All statistic analyses were carried out using the Statistical Package for the Social Sciences (SPSS) version 16.0 (SPSS, Chicago, IL, USA). Results were expressed as means ± standard error. One-way analysis of variance (ANOVA) and Tukey test were used to analyze treatment effects. ANOVA repeat measures and Bonferroni test were used to evaluate the treatments effects in paw edema.

Non-parametric rest-activity and scores of histology measures were analyzed using independent samples Kruskal-Wallis test, followed by Dunn's test (with Bonferroni test correction) (Dunn, 1964). P values < .05 were considered significant.

The approach used to determine the rest-activity circadian rhythm was cosinor analysis (El Temps freeware, A. Díez-Noguera, University of Barcelona, 1999; available at http://www.el-temps.com).

RESULTS

Serum levels of melatonin and corticosterone

Serum levels of MEL and corticosterone were quantified using the ELISA method. As shown in Figure 1.A, both MEL and MELDEXA groups showed

significantly increased serum MEL levels as compared with the other groups (one way ANOVA/Tukey, $F[7,56]: 528.38$, $P< .05$). Figure 1.B shows a significant decrease in serum corticosterone levels in rats treated with MEL, DEXA, and MELDEXA (one way ANOVA/Tukey, $F[7,54]: 11.87$, $P< .05$). However, no differences were observed in the serum levels corticosterone and MEL between control and inflamed rats (one way ANOVA/Tukey, $P> .05$).

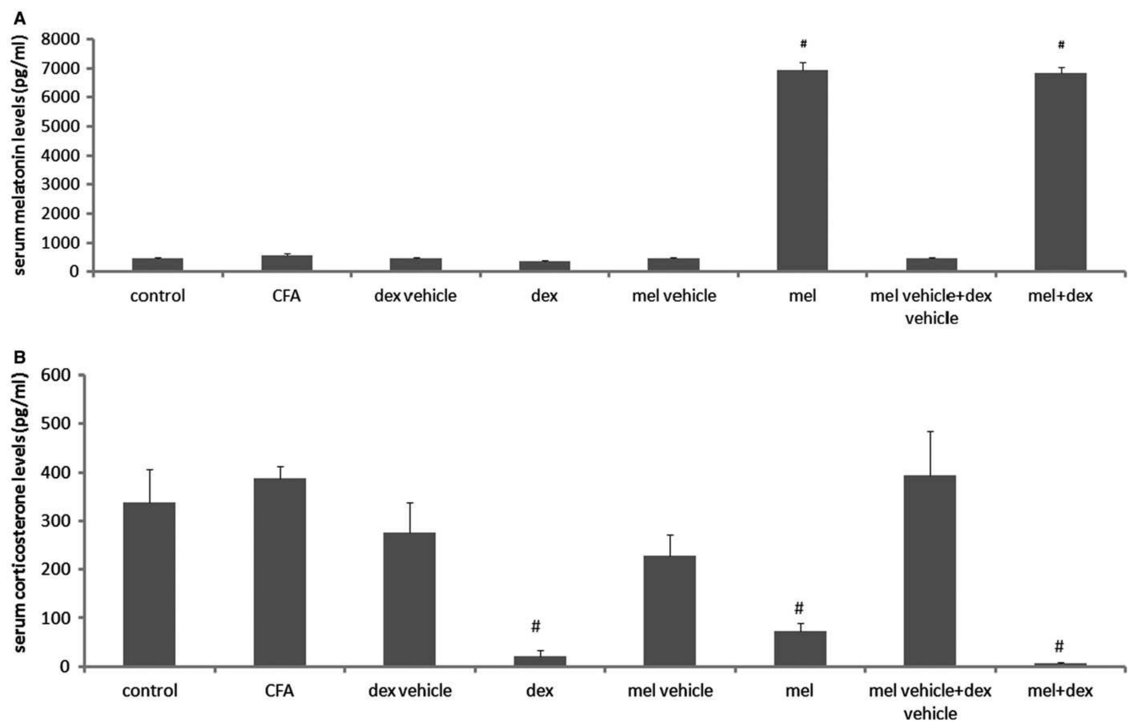

Figure 1. Serum levels of melatonin and corticosterone

Data presented as mean ± standard error. P values < .05 are considered significant. **(A) Serum melatonin levels** # Significant intergroup differences (one-way ANOVA/Tukey, $P< .05$, n=7-8/group). **(B) Serum corticosterone levels** # Significant intergroup differences (one-way ANOVA/Tukey, $P< .05$, n=6-7/group).

Treatment effects on hind paw swelling

All groups were similar at baseline (P> .05). Treatments were initiated on the 15th day and continued until the 23th day. The ANOVA repeat measures showed the effect of the time (F[6,55] = 28.41, P< .05), interaction time x group (F[6,55] = 9.81, P< .05). The treatment groups (MEL, DEXA, MELDEXA) were different to CFA and vehicle groups (P< .05). The days of treatment 4,6 and 8 were equal (Bonferroni, P> .05) and different to baseline and day 2 (Bonferroni, P< .05).

All the groups developed edema on 15th day after CFA injection (Baseline measure in Figure 2).Control rats did not show any changes in paw thickness (one way ANOVA/Tukey, P> .05).

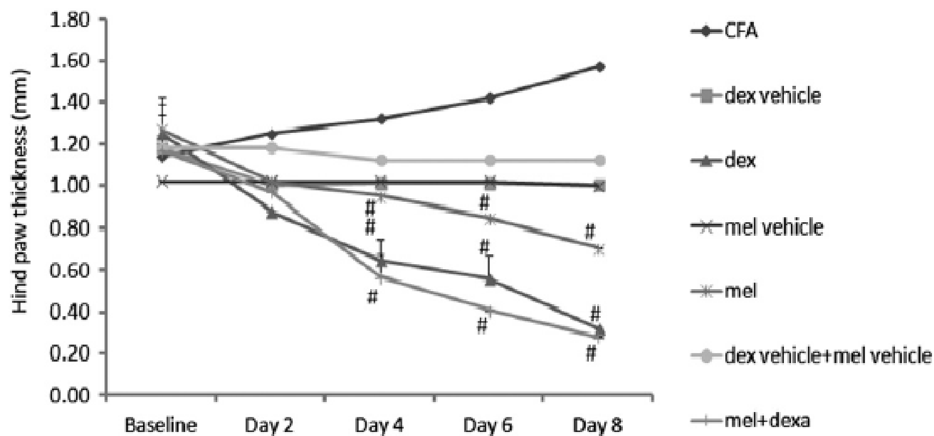

Figure 2. Treatment effects on hind paw thickness

Data presented as mean ± standard error. P values < .05 are considered significant. #Significantly different from CFA and vehicle group (ANOVA repeat measures/Bonferroni, P< .05, n=8/group).

Histological analysis

The histological findings obtained for each group are shown in Figures 3 and 4. CFA injection provoked an inflammatory reaction with lymphocytes, plasmocytes and rarely neutrophils, aggregates of macrophages, adipocytes degenerated presented in subcutaneous tissues of the ankle joints. The control group presented normal tissue architecture (Figure 3A), while the groups inflammation control and treated with vehicles presented highly abnormal histological findings, with pronounced inflammation (Figures 3B, C, D, F, I).

Groups treated with vehicles were significantly different from all other groups and presented highest numbers of macroscopic fields (one-way ANOVA/Tukey , $F[6,28]: 5.07$; $P< .05$ for all). In contrast, treatment with DEXA alone and with MELDEXA had a remarkable inhibitory effect on inflammation, showing mild inflammatory infiltration and degenerated cells permeated (Figures 3E, J). These treated groups showed reductions when compared with vehicle groups in terms of histological scoring (Kruskal-Wallis/Dunn test, $P< .05$ for all) (Figure 4B) and macroscopic fields (one-way ANOVA/Tukey ,$P< .05$ for all) (Figure 4A). The MEL group (Figure 3G) showed a moderate anti-inflammatory effect, with reductions only in the macroscopic fields (one-way ANOVA/Tukey, $P< .05$) (Figure 4A), but not in histological scoring (Kruskal-Wallis/Dunn test, $P> .05$) (Figure 4B) when compared with vehicle groups.

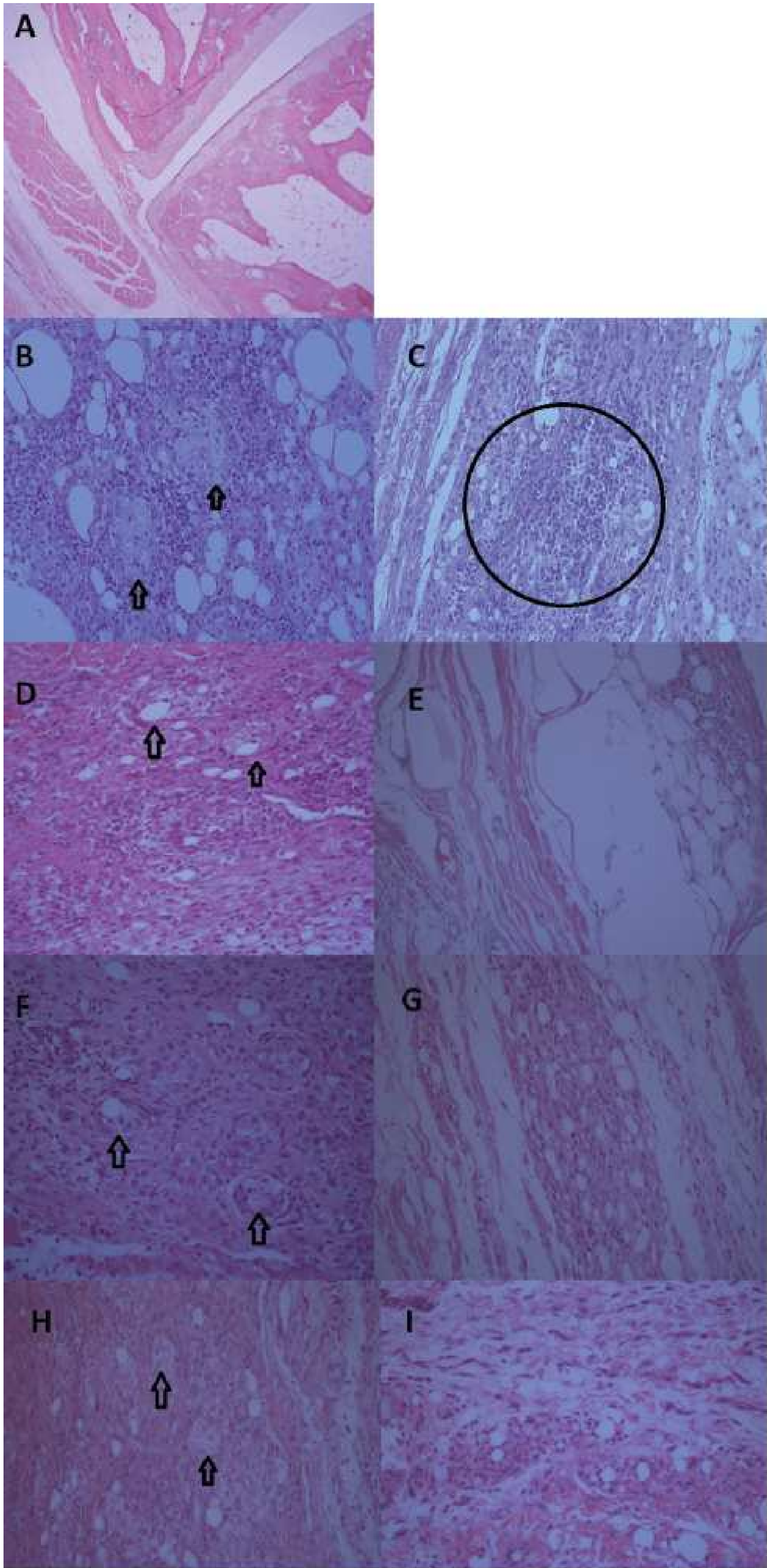
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Figure 3. Hematoxylin and eosin-stained sections of the right hind paws of rats (100x and 400x magnification)

Arrows indicate aggregates of macrophages; circle indicates lymphocytes.

A - Control: normal tissue architecture; **B, C-** CFA: severe inflammatory reaction with lymphocytes, plasmocytes and rarely neutrophils, aggregates of macrophages, adipocytes degenerated; **D-** Dexamethasone vehicle: severe inflammation reaction; **E-** Dexamethasone: mild joint inflammation with degenerated cells permeated; **F-** Melatonin vehicle: severe inflammation reaction; **G-** Melatonin: moderate joint inflammation; **H-** Melatonin plus Dexamethasone vehicles: severe inflammation reaction; **I-** Melatonin plus Dexamethasone: mild joint inflammation with degenerated cells permeated.

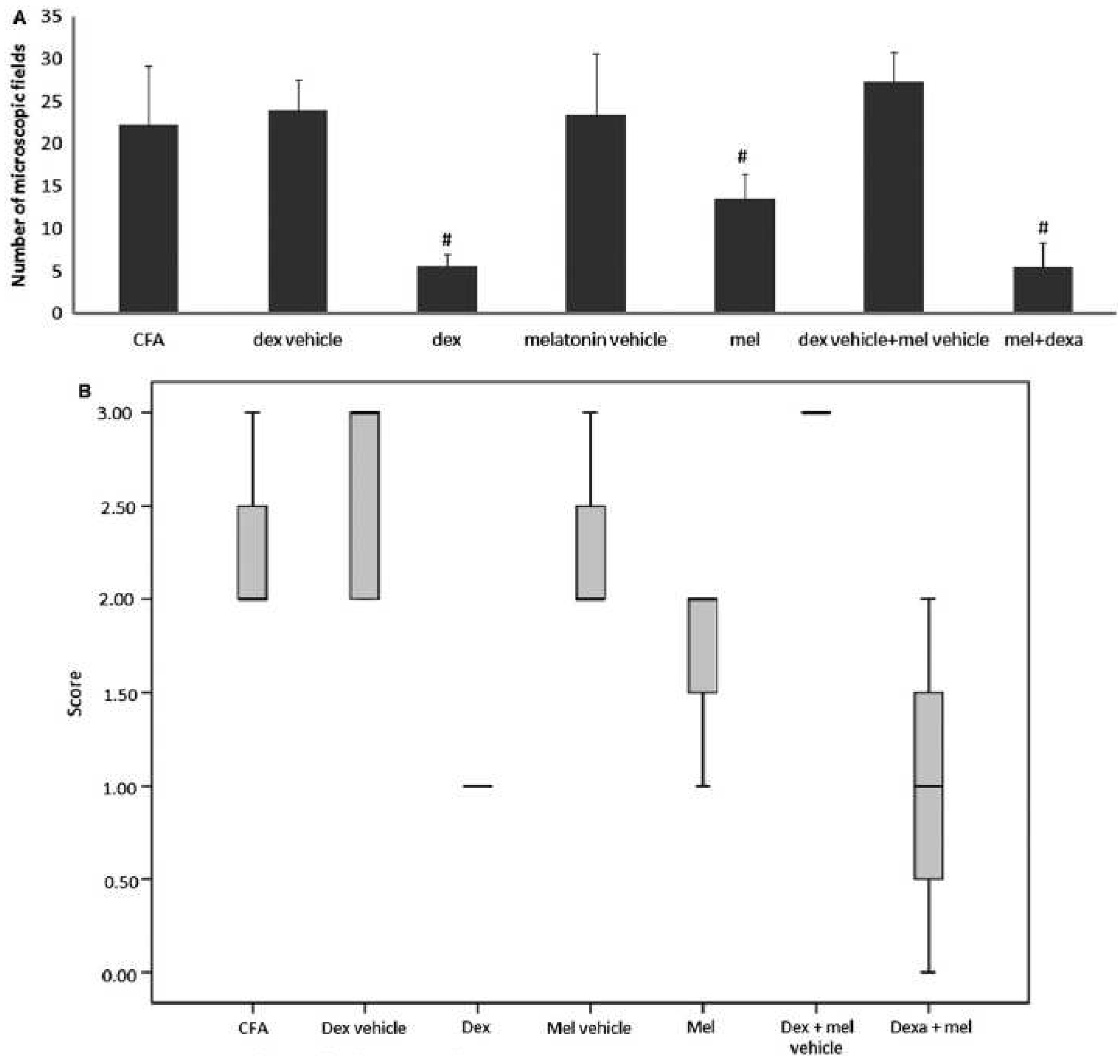

Figure 4. Histological analysis: number of microscopic fields (A) and score of infiltrating mononuclear cells (ranging from 0, absent, to 3, severe) (B).

(A)

Data expressed as mean ± standard error.

#Significantly different from vehicles and CFA groups (one-way ANOVA/Tukey, P< .05, n=3-6/group).

(B)

* Significantly different from vehicles and CFA groups (Kruskal-Wallis/Dunn test, P< .05, n=3-5)

Treatment effects on the electronic von Frey test (hind paw withdrawal threshold)

After the end of treatment (24 hours after last injection), a significant increase in hind paw withdrawal threshold was noted in groups treated with DEXA, MELDEXA, and MEL (F[7,54]: 17.79; P< .05 for all) (Figure 5). However, the MEL group was also similar to the CFA and vehicle groups (P> .05). No differences were observed among vehicle (P> .05) and among treatment groups (P> .05).

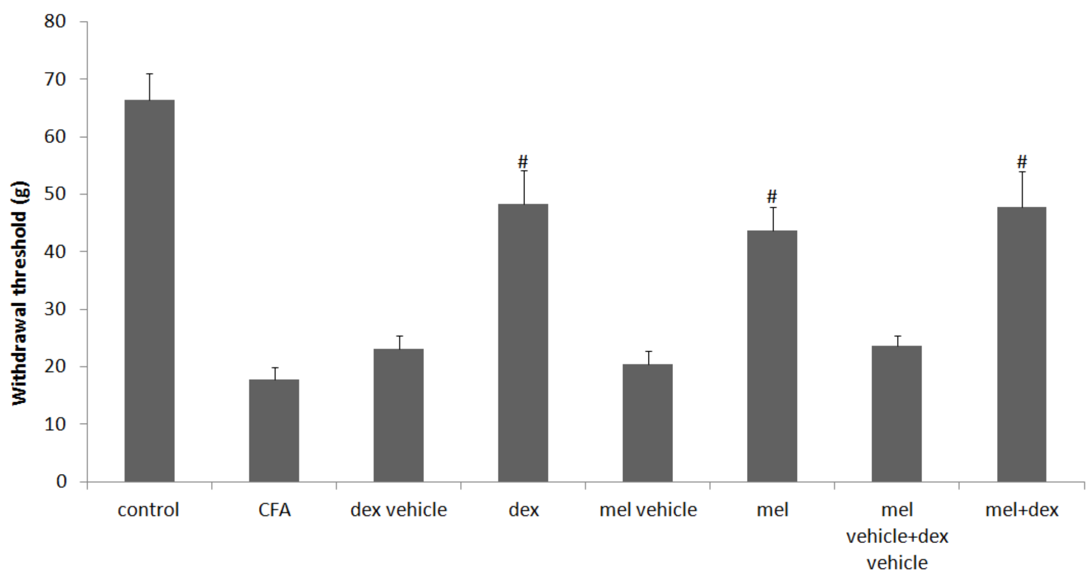

Figure 5. Treatments effects on the electronic von Frey test (hind paw withdrawal threshold) Data presented as mean ± standard error. P values < .05 are considered significant. # Significantly different from vehicle and CFA groups (one-way ANOVA/Tukey, P< .05, n=8/group).

Rest-activity rhythm

Rest-activity rhythm cosinor parameters (mesor, amplitude, and acrophase) analyzed using the El Temps freeware showed interactions between the experimental groups during the 8-day treatment period. The untreated CFA group presented a

different rhythm when compared with the control group within 24 hours, with a decrease in all cosinor parameters (Table 1: $P < .05$, Figure 6: Kruskal-Wallis/Dunn test: $P < .05$). Also, there was a phase delay in the CFA group when compared with the control group. Acrophase occurred at 6:59 p.m. in the control group, vs. 4:41 a.m. in the CFA group (Table 1: $P < .05$).

DEXA and MELDEXA vehicles disrupted the rhythm pattern shown by their respective treatment groups (Figure 6: Kruskal-Wallis/Dunn test: $P < .05$) and by the CFA group (Figure 6: Kruskal-Wallis test, $P > .05$), as no significant circadian rhythm was detected using cosinor analysis (Table 1: Kruskal-Wallis test, $P > .05$ and $P > .05$, respectively). The CFA and the MEL vehicle groups presented similar mesor and amplitude. Acrophase in the MEL vehicle group showed a phase advance in comparison with the CFA group (Table 1: Kruskal-Wallis/Dunn test: $P < .05$), but also a phase delay in comparison with the MEL group (Table 1: $P < .05$). No differences were detected between these groups in the Kruskal-Wallis test (Figure 6: $P > .05$).

The CFA and DEXA groups showed similar parameters (mesor and amplitude, see Table 1). Likewise, the DEXA and control groups showed similar mesor and amplitude parameters (Table 1), however with a phase delay in the DEXA group when compared with controls (Table 1). The MELDEXA group showed improved mesor and amplitude parameters when compared with the CFA and control groups, however also with a phase delay in the acrophase parameter when compared with the control group (Table 1). All parameters of the MEL group were improved when compared with the CFA group and the control group (Table 1: Kruskal-Wallis/Dunn test: $P < .05$). Mesor, amplitude, and acrophase between the MEL and the control groups are similar (Table 1: $P < .05$). The Kruskal-Wallis test followed by Dunn test shows significant differences between treatment groups and the CFA group (Figure 6: $P < .05$).

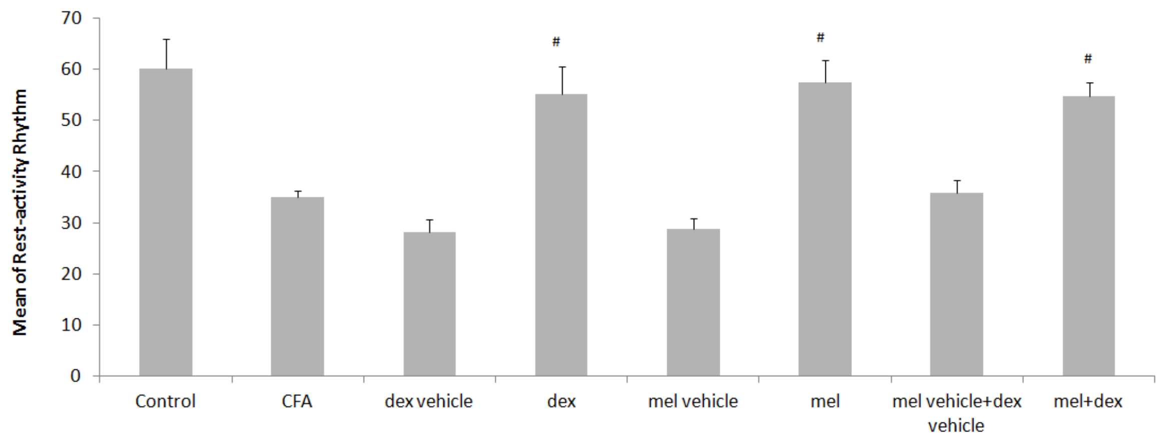

Figure 6. Treatment effects on mean of rest-activity rhythm values expressed in the number of touch-detections per hour for water ingestion. Data presented as mean ± standard error. P values < .05 are considered significant. # Significant difference in rest-activity rhythm between CFA and vehicle groups (Kruskal-Wallis/Dunn test, P< .05, n=47).

Groups	Mesor (95% CI) £	Amplitude (95%CI) £1	Acrophase (clock time)	Acrophase (radians) (95% CI) £1
Control*	52.68 (41.71 to 63.66)	21.65 (2.21 to 41.09)	18:59	4.87 (3.74 to 5.97)
CFA inflammation	28.96 (26.49 to 31.43)	6.63 (2.26to 11)	4:41	1.15 (0.43 to 1.87)
Dexamethasone vehicle	25.22 (20.63 to 29.81)	1.43	23:32	6.10 (2.97 to 9.25)
Melatonin-dexamethasone vehicle	30.10 (25.27 to 34.93)	5.07	15:09	3.95 (0.82 to 7.09)
Melatonin vehicle	24.83 (21.13 to 28.54)	9.47 (2.92 to 16.01)	0:14	0.035 (5.56 to 0.80)
Dexamethasone*	41.53 (32.53 to 50.50)	16.85 (0.92 to 32.78)	22:37	5.75 (4.52 to 6.99)
Melatonin-dexamethasone*	46.52 (41.56 to 51.48)	17.30 (8.52 to 26.08)	22:41	5.87 (5.34 to 6.4)
Melatonin*	47.59 (39.64 to 55.53)	15.09 (1.01 to 29.16)	19:53	5.11 (3.91 to 6.31)

Table 1 - Cosinor Parameters of the activity-rest rhythm during the treatment period. £ Number of touch-detections per hour for water ingestion; £1 clock

of time. * Significant different to other groups (Kruskal-Wallis/Dunn test, $P < .05$, $n=47$).

DISCUSSION

In this study, we demonstrated the antinociceptive effect of MEL, DEXA, and MELDEXA, as well as their effects on rest-activity circadian rhythm in rats with chronic inflammation. Treatment with MEL alone or combined with DEXA increased locomotor activity and induced a phase-advance entrainment of the circadian rhythm. Conversely, only MEL was able to synchronize the rest-activity rhythm (Figures 5 and 6). Also, both DEXA and MEL caused increases in pain threshold on the von Frey test (Figure 7). The DEXA suppressed peripheral inflammation, while MEL effect in this regard was mild (Figure 3 and 4). Finally, animals treated with MELDEXA showed similar improvement in pain scores and inflammation suppression as DEXA treatment group.

These findings show that the effects of MEL on paw inflammation were weaker than those of DEXA. DEXA is widely known to have an anti-inflammatory effect mediated by inhibition of the expression of multiple inflammatory genes (cytokines, enzymes, receptors, and adhesion molecules) (Zhang et al., 2004). The anti-inflammatory properties of MEL may be induced by the inhibition of pro-inflammatory cytokines (Cuzzocrea et al., 1997; Cuzzocrea et al., 1999; Pang et al., 2001), by the modification of rolling and adhesion of neutrophils to the endothelial layer (Lotufo et al., 2001), and by the inhibition of COX-2 (Deng et al., 2006) and iNOS (Alonso et al., 2006). Likewise, our findings are in agreement with previous studies that show that the anti-inflammatory effects of MEL starting at 10 mg/kg were less evident than those observed at lower doses (Mahmood et al., 2010; El-Shenawy et al., 2002) and also

show an analgesic effect at higher doses (Yu et al., 2000; Wang et al 2006). In this context, anatomical, physiological and pharmacological evidence supports the existence of bilateral interactions between the endocrine and immune systems (Blalock, 1994). Hence, higher MEL doses may suppress the pain and inflammation improving stress-induced immunosuppression and other secondary immune deficiencies.

The CFA injection used to create the RA model triggers the release of inflammatory mediators, including cytokines and active oxygen species (Rezazadeh et al., 2009). It also produces hyperalgesia and allodynia (Bolcskei et al., 2005; Helyes et al., 2004), which may persist for up to 28 days. It is important to emphasize that CFA injections have been used to test several different analgesic drugs (Lichtenberger et al., 2008), including anti-inflammatory agents (Zaringhalam et al., 2010). CFA-induced inflammatory response is biphasic, with an early (acute) and a late (chronic) phase (Philippe et al., 1997). Our study focused on the late phase of inflammation, which induces immunological events (Patil et al., 2009) and provokes pain (Lorton et al., 2000; Bernardi et al., 2009). Although the anti-inflammatory effects of MEL was weaker than those of DEXA, it can be observed a reduction in the number of inflammatory cells (macrophages, neutrophils, lymphocytes) in subcutaneous tissues of the ankle joints (see figures Figures 3 and 4). The cytokines produced by these mononuclear cells (e.g., IL-1, IL-6, and TNF) may explain the mechanical and thermal hyperalgesia phenomena (Muller-Ladner, 1996).

These anti-inflammatory effects may involve a direct interaction with specific binding sites located in lymphocytes (CD4+T, CD8 T and B cells) and macrophages (Pozo et al., 1997; Garcia-Perganeda et al., 1999). MEL modulates the proliferative response of activated lymphocytes and induces mononuclear cells to produce cytokines via nuclear factor kappa B (NFκB) (Szczepanik, 2007). In sum, the effects of MEL on

the immune system are complex and depend on several factors, including drug dose, animal's immune status (as well as its age, sex, and species), season during which the immune system was evaluated, circadian rhythm of immunity, pineal gland status, and presence of stressful conditions (Skwarlo-Sonta, 2002). In the context of the present study, a limitation was the fact that we did not measure serum biochemical markers of inflammation; however, an objective surrogate less prone to bias (i.e., histological changes) was consistent with the effects of the drug in loco. Hence, it is unlikely that the factors mentioned above would have influenced the direction of our conclusions.

Our findings showed that MEL is able to induce phase advancement and circadian rhythm synchronization in rats with chronic inflammation. This effect could be related to a direct action of MEL on the suprachiasmatic nucleus (SCN) clock (Pévet et al., 2002). Also, MEL infusion in hamsters induced phase advancement when the free-running period was longer than 24 hours and phase delays when the period was shorter than 24 hours (Slotten et al., 2002). In addition, chronic administration of MEL to rats interferes with neuroendocrine mechanisms of the negative feedback of glucocorticoids, controlled by the HPA axis. Moreover, it attenuates secretory responses to stress (e.g., pain conditions), affecting the content and release of hypothalamic adrenocorticotropic secretagogues such as corticotropin-releasing hormone (Marinova et al, 1991). Finally, daily treatment with MEL in stressed mice prevented several disturbances induced by chronic stress, including reduction in spontaneous locomotor activity (Kopp et al., 1999).

An unexpected finding of our study was that serum levels of MEL and corticosterone were statistically similar in both control and inflamed groups. We hypothesize that this similarity is related to the time at which measurements were taken, as the peaks of neither hormone coincide (Augusto et al., 2011; Barriga et al., 2001)

with the timing at which our animals were killed (early afternoon). Whereas some studies have shown that both the light/dark rhythm and the daily MEL rhythm are maintained during inflammation (Augusto et al., 2011; Cutolo et al., 2005), other authors have reported consistently and markedly increased serum corticosterone levels during chronic inflammation (Nadeau & Rivest, 2002; Padgett & Glaser, 2003). Thus, as previously shown, circulating corticosterone levels are essential for maintaining MEL rhythm especially in cases where chemical or surgical adrenalectomy has abolished the nocturnal MEL surge (Lopes et al., 1997). Similarly, previous study showed that MEL rhythm was maintained in RA (Cutolo et al., 2005), a disorder that expresses high levels of TNF and glucocorticoids.

The effects of MEL on pain corroborate a growing body of evidence suggesting an analgesic effect of this drug on pain in both animal (Laste et al., 2012) and clinical studies (Caumo et al., 2007; Caumo et al., 2009). Although the mechanisms of action underlying this effect remain unclear, it could be associated with its effects on specific receptors (MT1 and MT2) in the nervous system, including the thalamus, hypothalamus, anterior pituitary gland, and dorsal horn of the spinal cord (Park et al., 1997; Ray et al., 2004). A further explanation for the analgesic effect of MEL could lie in its modulation in nociceptive pathways, e.g., enkephalins and endorphins (Ray et al., 2004).

One finding that contradicts our hypothesis is the fact that the combination of MELDEXA potentiates anti-hiperalgesic and anti-inflammatory responses. We hypothesize that it is the result of the high doses of MEL and DEXA used. According to previous studies, no anti-inflammatory effects were observed in animals treated with MEL (10 mg/kg) or DEXA (0.025 mg/kg) alone (Gonovese et al., 2007). These findings may be explained as the MEL reduces the affinity of glucocorticoid receptors and

increases sensitivity to the negative feedback of glucocorticoid (Presman et al., 2006; Quiros et al. 2008). Accordingly, it also has been demonstrated that MEL antagonizes several effects of exogenous corticosteroids, e.g., immune depression (Maestroni et al., 1986) and catabolism, thymic involution, and adrenal suppression (Mori et al., 1984). These findings suggest that MEL may work as an anti-adrenocortical or anti-stress factor (Maestroni et al., 1986); conversely, it is also important to emphasize that high levels of glucocorticoids resulting from a disrupted rhythm of glucocorticoid release can be pathogenic. Therefore, properly timed exogenous MEL administration may entrain or reorganize this critical endocrine rhythm, providing long-term systemic benefits. In fact, it may represent an integral immune-recovery mechanism by which MEL acts as a kind of buffer against the harmful effects of stress on immune homeostasis (Malhotra et al., 2004).

In summary, our results indicated the effects of MEL in an animal model of chronic inflammatory pain in terms of activity-rest rhythm synchronization and pain improvement. We showed that MEL acts suppressing the HPA axis, suggesting that it could relieve the stress effect provoked by pain conditions. In comparison with DEXA or DEXAMEL, MEL showed a less inhibitory effect on inflammation and pain, suggesting a more central action of this agent. Our findings showed that MEL has strong chronobiotic and antinociceptive effects, but only mild anti-inflammatory effects. This evidence supports the hypothesis that MEL can induce phase advance and circadian rhythm synchronization in rats with chronic inflammation. Further studies analyzing animals with deficiencies in clockwork genes may help elucidate the effects of MEL on inflammation.

Acknowledgements:

This research project was supported by the following Brazilian funding agencies: National Council for Scientific and Technological Development - CNPq (Dr. I.L.S. Torres and Dr. W. Caumo); Coordination for the Improvement of Higher Education Personnel - CAPES (Dr. I.C. Custodio de Souza, G. Laste, L.F. Medeiros, J.R. Rozisky, and A. Souza); Graduate Research Group – Hospital de Clínicas de Porto Alegre - GPPG-HCPA (Dr. I.L.S. Torres, grant no. 100013); FAPERGS/PRONEM (Dr. I.LS. Torres, grant no. 11/2050).

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6.4 Artigo publicado: Experimental Brain Research 2012 221:75-83.

AFTER-EFFECTS OF CONSECUTIVE SESSIONS OF TRANSCRANIAL DIRECT CURRENT STIMULATION (tDCS) IN A RAT MODEL OF CHRONIC INFLAMMATION

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DECLARATION OF INTEREST: The authors report no conflicts of interest. The authors alone are responsible for the contents and writing of this paper.

ABSTRACT

Transcranial direct current stimulation (tDCS) induces cortical excitability changes in animals and humans that can last beyond the duration of stimulation. Preliminary evidence suggests that tDCS may have an analgesic effect; however, the timing of these effects, especially when associated with consecutive sessions of stimulation in a controlled animal experiment setting, has yet to be fully explored. Objective: To evaluate the effects of tDCS in inflammatory chronic pain origin immediately and 24 hours after the last treatment session. Methods: Complete Freund´s Adjuvant (CFA) was injected (100µl) in the right footpad to induce inflammation. On the 15th day after CFA injection, rats were divided into two groups: tDCS (n=9) and sham (n=9). tDCS was applied for eight days. The hot plate and Von Frey tests were applied immediately and 24 hours after the last tDCS session. Results: Eight 20-minute sessions of 500 µA anodal tDCS resulted in antinociceptive effects as assessed by the hot plate test immediately (P=0.04) and 24 hours after the last tDCS session (P=0.006), for the active tDCS group only. There was increased withdrawal latency in the Von Frey test at 24 hours after the last session (P=0.01). Conclusion: Our findings confirm the hypothesis that tDCS induces significant, long-lasting; neuroplastic effects and

expands these findings to a chronic pain model of peripheral inflammation, thus supporting the exploration of this technique in conditions associated with chronic pain and peripheral inflammation, such as osteoarthritis.

Key words: Transcranial direct current stimulation (tDCS); motor cortex; neuromodulation; inflammatory pain

INTRODUCTION

Chronic pain is characterized by plasticity within the nervous system, leading to sensitization in the response of the pain system to noxious or innocuous stimuli (Ji et al. 2003). Sensitization can occur at several levels of the nervous system. At the peripheral level, local hormones or inflammatory mediators can heighten the response of nociceptors to lower levels of sensory stimulation (Leigh et al. 2000). In addition, neurons within the spinal cord can also become sensitized (Nayef et al. 2008). Remarkably, these spinal cord neurons show a particular type of plasticity, known as long-term potentiation, which is molecularly similar to processes that are thought to be involved in the formation of memories within the brain.

Sensitization of the pain system is not limited to peripheral nociceptors or spinal cord neurons (Ji et al. 2003). Neurons in the nociceptive amygdala and in the anterior cingulate cortex (ACC) are also sensitized in chronic pain (Ji et al. 2003). In some conditions, enhanced responses to painful stimuli acquire new low threshold inputs (a neural correlate of allodynia). Thus far, the pain amplification of chronic pain has been limited to evoked stimuli; however, it is also clear that stimulation of peripheral structures (e.g. the skin) is not a necessary step for the perception of pain (Ruscheweyh et al. 2011). In fact, spontaneous pain, especially the dull ache that characterizes so many chronic pain conditions, is often the primary complaint of chronic pain patients and is the most difficult feature to treat. These central plastic changes in nociceptive pathways support the need for the assessment of central-based targeted therapeutic options for pain (Jensen et al. 2007; Bruguerolle et al. 2007). In fact, treatments that can reverse pain-related maladaptive plasticity are important to avoid pain chronification (Linton 1987).

In this context, a technique that can modulate focally induced plastic changes in pain-related neural networks, such as transcranial direct current stimulation (tDCS), may have significant therapeutic effects. This simple but powerful method of non-invasive brain modulation has shown significant results in different types of chronic pain in humans (Fregni et al. 2007; Fregni et al. 2006a; Antal and Paulus, 2006). tDCS has other potential advantages, such as a reliable placebo condition (Gandiga et al. 2006). In rats, the effects of tDCS have been demonstrated on memory (Dockery et al. 2011), rat models of Parkinson's disease (Li et al. 2011) and focal epilepsy (Liebetanz et al. 2006), and modulation of cerebral blood flow in a polarity specific manner in rats constituting a promising therapeutic option in acute stroke patients (Watcher et al. 2011). In a recent systematic review focused specifically on pain, Volz et al. (2012) found 10 studies investigating the effects of non-invasive brain stimulation on rat models of pain, but most of these (nine articles) used TES (transcranial electrical stimulation) as the main stimulation method, and only one explored the combination of TES and tDCS on pain (Nekhendzy et al. 2004).

Studies in humans have demonstrated that stimulation of the motor cortex changes motor cortex excitability according to stimulation polarity; anodal stimulation increases cortical excitability, whereas cathodal stimulation decreases excitability (Nitsche and Paulus 2000; Nitsche et al. 2003). Extensive studies show that motor cortex stimulation, whether invasive or noninvasive, is associated with improvement of pain (Carroll et al. 2000; Tsubokawa et al. 1993) The motor cortex is thought to inhibit pain perception through indirect effects via neuronal networks synapsing on pain-modulating areas (Brown and Barbaro 2003). In this study, we chose to use a montage in which the motor cortex would be located between the two electrodes so as to maximize effects over this area (see Datta et al. 2009).

Taking into account the possible benefits of tDCS in several mechanisms involved in the pain sensitization process, we assessed its effect in a model of hyperalgesia induced by chronic inflammation to understand what functional and phenotypic changes are observed immediately and 24 hours after the last tDCS treatment session. tDCS has been shown to induce potent neuroplastic changes in animal (Brunoni et al. 2011) and human models (Fritsch et al. 2010) alike, and this study will thus address an important knowledge gap by investigating the immediate and delayed effects of consecutive sessions of tDCS in a model of chronic pain.

METHODS

Animals

Male Wistar rats weighing 250-300g were used. The animals were housed in home cages made of Plexiglas (65 cm x 25 cm x 15 cm) with sawdust-covered floors and kept on a standard 12-h dark/light cycle (lights on between 0700h and 1900h) at room temperature (22 ± 2°C). The animals had free access to food and water. Animal handling and all experiments were performed in accordance with international guidelines for animal welfare. The protocol for this experimental study was approved by the Ethics Committee of the institution where the work was conducted (GPPG-HCPA: 100013), and adheres to the ethical and methodological standards of the NIH Guide for the Care and Use of Laboratory Animals (Publication No. 85-23, revised 1985), the UK Animals Scientific Procedures Act 1986 and the European Communities Council Directives of 24 November 1986 (86/609/EEC).

Experimental Design

Rats were habituated to the maintenance room for 1 week prior to the experiment. All animals received intraplantar injections of CFA to induce inflammation. After the habituation period, the animals were randomly divided into two groups (n=9/group): sham stimulation (S) and tDCS (T). tDCS sessions (20 minutes per day over 8 days) were started on the 15th day post CFA injection.

CFA-induced inflammation

Complete Freund's Adjuvant was purchased from Sigma Chemical Co. (St Louis, MO, USA) and used in a protocol similar to those described elsewhere, with minor modifications (Bernardi et al. 2009; Stein et al. 1988). The inflammatory pain model used in this study is similar to that described by Bernardi et al. 2009, which demonstrated that treatment with indomethacin-loaded nanocapsules was able to produce a significant decrease in serum levels of the proinflammatory cytokines TNF and IL-6 in rats. CFA injection can trigger release of a series of inflammatory mediators, including cytokines and reactive oxygen species (Rezazadeh et al. 2009). It also produces hyperalgesia and allodynia (Bolcskei et al. 2005; Helyes et al. 2004), which persist for up to 28 days. It is important to emphasize that the CFA model has been used to test the adverse effects and anti-inflammatory activity of analgesic drugs (Lichtenberger et al. 2008) and medicinal plant species (Zaringhalam et al., 2010). CFA-induced inflammation consists of a biphasic inflammatory response, with early (acute) and late (chronic) phases (Philippe et al. 1997). The present study focused on the late phase of CFA-induced inflammation, which induces immunological events (Patil et al. 2009) and, on day 15, is associated with chronic inflammatory pain (Lorton et al. 2000; Bernardi et al. 2009).

Briefly, animals were anaesthetized with isoflurane and inflammation was induced via a single intradermal injection (100 μl) of heat-killed Mycobacterium tuberculosis suspended in paraffin oil plus mannide monooleate (1 mg/mL). CFA was injected in the footpad as described by Stein et al. (1988).

Transcranial direct current stimulation (tDCS)

After 14 days of CFA administration, anodal stimulation was applied using ECG electrodes (1.5 cm2) 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, we made a trichotomy of the head was shaved for better adherence. The size of the electrodes was reduced to 1.5cm2 to fit the animals' heads. After the electrodes had been placed, they were fixed onto the head with Micropore™ 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. 2006c).

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 2011, Fig. 1).

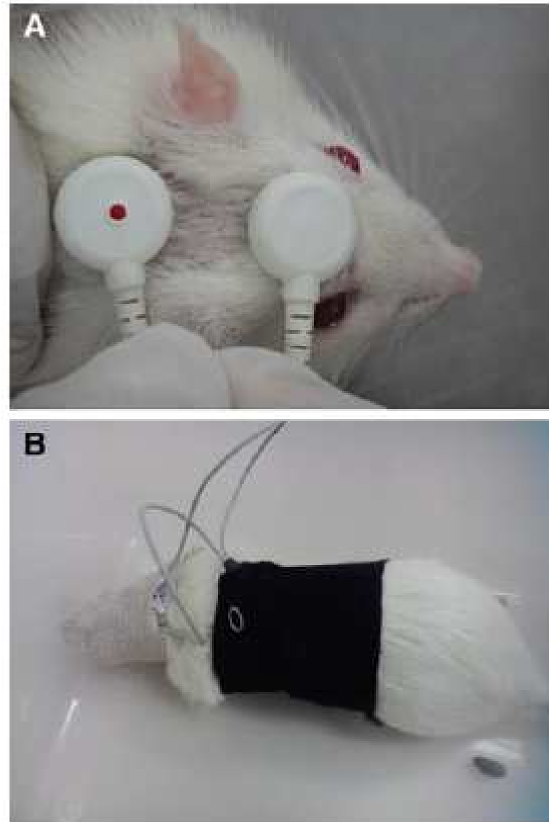

Fig. 1 The cathodal stimulus electrode was placed at the midpoint of the lateral angle of the eyes, and the anodal electrode, from the neck to shoulder areas. The stimulator was placed onto the thorax with a corset, and the electrodes were positioned. After the electrodes were in place, they were fixed onto the head with adhesive tape (Micropore^{TM}) and with a protective mesh to prevent removal. **a** Placement of tDCS electrodes. **b** tDCS stimulation

A constant current of 0.5mA intensity was applied for 20 min (Fregni et al. 2006b). According to an earlier elegant animal safety study (Liebetanz et al. 2009), a current density higher than 142.9 A/m2 is associated with brain lesions. Given this threshold, our stimulation parameters resulted in a current density of 33.4 A/m2. Interestingly, we initially used a current of 66.7 A/m2, 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 seconds 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-minute sessions, was eight consecutive days. Immediately and 24 hours after the last session, the Von Frey test and hot plate test were administered.

Hot plate test

All rats were given 5 minutes to habituate to the hot plate 24 hours prior to testing. The temperature of the plate was kept at 55°C. Animals were placed in glass funnels on the heated surface and the time between placement and onset of paw licking or jumping, in seconds (s), was recorded as the latency of response (Woolfe and Macdonald 1944).

Von Frey filament test

The Von Frey filament test is used to assess mechanical allodynia (Wegert et al. 1997). It has been proposed that tactile hypersensitivity is likely to be the consequence of a change in function and a phenotypic switch in primary afferent neurons innervating the inflamed tissue and the pattern of excitation they produce in spinal neurons, and this presumption was partially confirmed by the finding that a subpopulation of A beta primary afferent neurons began to express substance P following conditioning inflammation, thereby enhancing synaptic transmission in the spinal cord and exaggerating the central response to innocuous stimuli (Ma and Woolf 1996; Neumann et al. 1996).

Paw withdrawal threshold in response to a mechanical stimulus was determined using a series of von Frey filaments. Animals were placed in a cage with a metal mesh floor, allowing them to move freely. They were acclimatized to this environment for 15 minutes, approximately 24 hours prior to testing, to allow for behavioral

accommodation. Von Frey filaments were applied to the mid-plantar surface of the inflamed hind paw through the mesh floor. Application was only performed when the animal's paw was in contact with the floor. Each filament was applied to the paw until it was slightly bent. The interval between three consecutive filament measures was at least 5 seconds. Filaments were applied in ascending order and the smallest filament that elicited a foot withdrawal response was considered the threshold stimulus (Tal and Bennett 1994). The withdrawal threshold of the right hind paw was expressed in force: grams (g).

Statistical analysis

Considering that the mean baseline of the latency on hot plate and Von Frey were similar in both groups (Student's t-test for independent samples, $P > 0.05$), to compare the effect of tDCS for both outcomes (hot plate and Von Frey), we used Student's t-test for independent samples. Furthermore, we conducted within-group comparisons (immediately and 24 hours after the last session) using the paired t-test. The results are presented as mean ± standard error of the mean (SEM).

RESULTS

Effects of tDCS immediately and 24 hours after end of treatment on the hot plate test (fig. 2)

Between-group analysis showed a significant difference in hot plate test results immediately (S:3± 0.27 s; T:3.66±0.17 s; $F(1,17)= 0.088$; Student's t test, $P = 0.047$) and 24 hours after the last tDCS session (S:2.12± 0.12 s; T:3.33±0.33 s; $F(1,17)= 8.96$; Student's t test, $P = 0.006$). The tDCS group had a significant increase in latency time in

both time points (immediately and at 24 hours) as compared to the sham group, thus suggesting an antihyperalgesic effect.

In order to understand whether effects seen immediately after stimulation were similar to those observed 24 hours after stimulation, we performed a within-group comparison between these two time points. We did not find any significant differences between these time points in the active tDCS group (immediately vs. 24 h – paired t-test, p = 0.34 for both groups). Therefore, the antihyperalgesic effect was sustained for at least 24 hours in the tDCS group, suggesting a long-lasting anti-nociceptive effect.

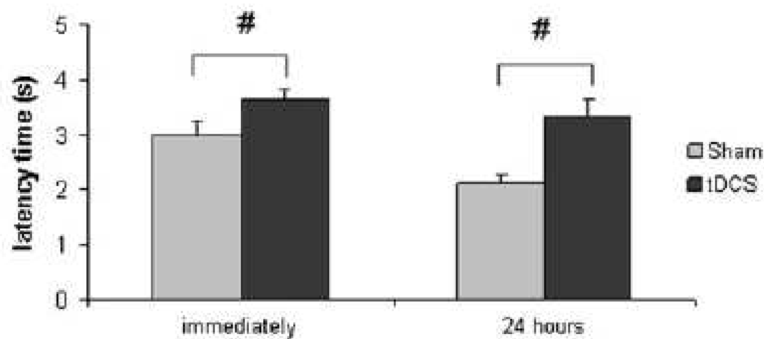

Fig. 2 Effects of tDCS immediately and 24 h after the end of treatment on inflammatory pain as evaluated with the hot plate test. Data are presented as mean ± standard error of the mean (SEM) of licking paws or jumping as latency of response in seconds (s). *Number sign* (#) denotes significant between-group differences (Student's *t* test, $P < 0.05$; Sham group, $n = 8$; tDCS group, $n = 9$)

Effects of tDCS immediately and 24 hours after end of treatment on the Von Frey test (fig. 3)

Between-group analysis did not reveal any significant differences in the Von Frey test at either endpoint, i.e. immediately (P = 0.47) and 24 hours after the last tDCS session (P = 0.19). However, in exploratory within-group comparisons, the tDCS group showed a significant increase in withdrawal threshold of the paw 24 hours after the last session vs. immediately after (paired t-test, P = 0.01).

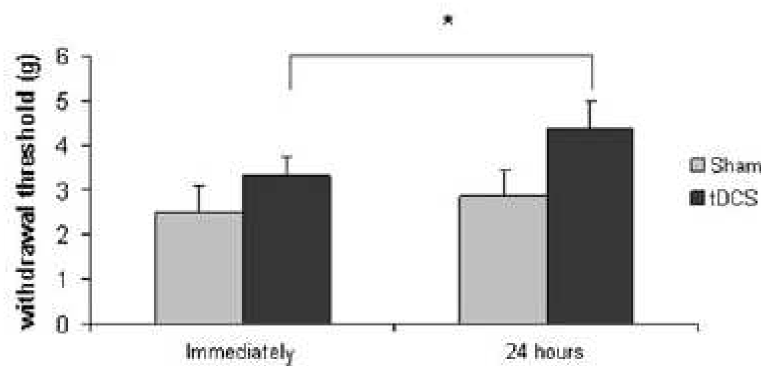

Fig. 3 Effects of tDCS immediately and 24 h after the end of treatment on inflammatory pain as evaluated with the Von Frey test. Data are presented as mean ± standard error of the mean (SEM) of withdrawal response in grams (g). *Asterisk* (*) denotes significant difference between 24-h and immediate response (paired t test, $P < 0.05$; sham group, $n = 8$; tDCS group, $n = 9$)

DISCUSSION

This study supports the notion that tDCS induces a significant, long-lasting behavioral effect in a controlled model of chronic inflammation. In tDCS-treated animals, as compared to the sham tDCS group, the hot plate test showed an antinociceptive effect immediately after stimulation that lasted 24 hours post-application. For the Von-Frey test, a similar effect was observed, but only 24 hours after the end of stimulation sessions in the tDCS treated group.

It is important to note that the hot plate test is a specific test designed to evaluate two behavioral components—namely, paw licking and jumping—that are considered supraspinally integrated responses. Because tDCS is not a focal technique, it is not possible to determine the specific neural networks involved in tDCS-induced antinociception. According to extensive evidence and also on the basis of the electrode placement used in this study, it is plausible that these effects may be due to motor cortex stimulation. It has been suggested that the effects of transcranial stimulation on pain relief depend on the projection of fibers from the motor cortex to other structures involved in pain processing, such as the thalamus and brainstem nuclei that

downregulate processing from sensitized neurons (Drouot et al. 2002; Lefaucheur et al. 2006).

A previous study showed that the short-term (immediate) effects of tDCS on motor cortex excitability are observed with anodal and cathodal tDCS, and are associated with an increase and decrease of the resting neuronal threshold respectively (Nitsche and Paulus 2000). However, the long-lasting effects of tDCS involve the participation of glutamatergic N-methyl-D-aspartic acid (NMDA) receptors (Fertonani et al. 2010), as shown by pharmacological studies using an NMDA antagonist (Liebetanz et al. 2002). This role of the glutamatergic system was also demonstrated using D-cycloserine, a partial NMDA agonist, which selectively potentiated the duration of motor cortical excitability induced by anodal tDCS, but excitability was not decreased by cathodal stimulation (Nitsche et al. 2004). It is therefore conceivable that tDCS induced long-lasting effects by synaptic modification of pain circuits.

One potential area for further exploration, given our findings, is the relationship between neural modulation with tDCS and modulation of the immune system. A recent rat study found that, following 5 days of rTMS treatment, changes occurred in BDNF–tyrosine receptor kinase B (TrkB) signaling and TrkB–NMDA receptor interaction increased in the lymphocytes (Wang et al. 2011). Also, it has been well documented that TrkB receptors are present in the lymphocytes (Schuhmann et al. 2005; De Santi et al. 2009); thus, through TrkB activation, BDNF promotes immune system maturation as well as lymphocyte development, maintenance, and survival (Schuhmann et al. 2005). The link between the immune system and the brain is no surprise, as numerous studies have shown that both systems can interact bidirectionally through common receptors and ligands, such as interleukin-1 and other proinflammatory cytokines (Derecki et al. 2010; Yirmiya and Goshen 2011). Overall, these results suggest that the induction of

plasticity-related phenomena in the brain by transcranial neuromodulation might also have an effect on inflammatory pain as mediated by the regulatory effect of BDNF on the immune system. In a previous study, we showed that dexamethasone (Laste et al. submitted manuscript) and melatonin (Laste et al. personal communication) produced analgesia in the Von Frey test. In fact, in our dexamethasone study, the same model also induced a similar increase in pain threshold levels that was reversed after a course of dexamethasone, 0.25 mg/kg daily for 8 days. In this context, 8 days of tDCS had a similar effect to an 8-day course of dexamethasone at the aforementioned dose.

Thus, one important finding in our study is that, on the basis of the cortical effects of tDCS, this technique induces a likely top-down effect on pain circuits, either by modulation of the threshold for activation of central structures associated with pain processing or by activation of descending inhibitory pathways that can induce an antinociceptive effect even in the presence of active inflammation (Lima and Fregni 2008). This finding gives additional support to the use of tDCS in acute pain syndromes, such as during the postoperative period, as recently demonstrated in another study (Borckardt et al. 2011a).

Given the effects of tDCS in pain induced by active inflammation; it is possible that its modulatory effects on pain sensation involve several neurotransmitter systems, including opioid, adrenergic, substance P, glutamate and neurokinin receptors (Morgan et al. 1994; Laurido et al. 2002; Wu et al. 2000). tDCS may lead to a cascade of events resulting in the modulation of synaptic neural chains that include several thalamic nuclei, the limbic system, brainstem nuclei and the spinal cord (Lima and Fregni 2008). In addition, evidence using magnetic stimulation has shown that an increase in noradrenergic activity can lead to improvement in pain (Maarawi et al. 2007).

Additionally, we performed the Von Frey test to evaluate peripheral nociception. A tactile stimulus was directly applied to the area of local inflammation (right hind paw). The effect observed with this test was smaller, as no significant between-group difference was shown in pain behavior; however, withdrawal latency was increased 24 hours after stimulation in the tDCS group only. Interestingly, this finding is consistent with a recent study, which showed that high-definition tDCS induces significant decreases in heat and cold sensory thresholds, but has no significant effects on mechanical pain thresholds (Borckardt et al. 2011b).

Overall, our findings support the notion that tDCS has significant effects on chronic inflammatory pain. One limitation of this study would be the fact that the experimental protocol of inflammatory chronic pain may have been relatively short and, thus, underpowered to compare with chronic inflammatory pain in humans, which sometimes lasts years (Manchikanti et al. 2009). Therefore, we used references such as Manchikanti et al. 2009 and Apkarian et al. 2009, which defined chronic pain as persistent pain. The underlying mechanisms that explain this effect remain unclear, although the current evidence suggests these effects comprise non-synaptic and synaptic mechanisms. Ardolino et al. (2005) discussed that prolonged exposure to a constant electric field, apart from locally changing ionic concentrations, could induce migration of transmembrane proteins and locally alter the tissue acid–base balance (Ardolino et al. 2005). They also discussed that changes in intracellular pH and [Ca2+] are closely correlated and that anodal polarization has been shown to increase Ca2+ and cathodal polarization might also influence [Ca2+], thus shifting the pH (Ardolino et al. 2005). The synaptic mechanisms, on the other hand, may involve neuroplastic changes such as the strength of connections, representational patterns, or neuronal properties, either

morphological or functional (Antal et al. 2006). The long-lasting after-effects of tDCS may be due to a change in NMDA receptor efficacy (Nitsche et al. 2003).

Our stimulation montage was designed to be similar to models used in humans. For this reason, we chose a cephalic montage only, as the extra-cephalic montage may be less effective (Mendonca 2011). Thus, our results could link animal model study results with human tDCS study findings. Another point to consider is that neither anesthesia nor surgical interventions were necessary, according to the traditional model used in previous tDCS studies in rats (Dockery et al. 2011; Wachter et al. 2011; Liebetanz et al. 2006). Volatile anesthesia (such as isoflurane) has been shown to decrease excitatory and increase inhibitory transmission (Gomez and Guatimosim 2003; Ouyang and Hemmings 2005), affect neuroplasticity, and possibly alter BDNF expression (Lu et al. 2006; Head et al. 2009). Taken as a whole, our animal tDCS model (adapting ECG electrodes and placing electrodes on the skin) is a close approximation of tDCS methods used in humans (Fregni et al. 2006c). Considering that inflammatory pain involves an activity-dependent facilitation in excitability of both peripheral neurons (peripheral sensitization) and spinal and supraspinal neurons (central sensitization), including the thalamus and cortex (Kubo et al. 2009), the hot plate test was carried out to assess the effects of tDCS on the thermal nociceptive threshold (Woolfe and Macdonald 1944). We used the hot plate test to determine changes in latency as an indicator of modifications of the supraspinal pain process (Ossipov et al. 1995); licking or jumping responses were considered to be the result of supraspinal sensory integration (Caggiula et al. 1995; Rubinstein et al. 1996). Finally, this model produces consistent findings that suggest an effect of tDCS on chronic inflammatory pain.

CONCLUSIONS

In summary, we confirmed the significant immediate and long-lasting effects of tDCS on a model of subacute pain due to chronic inflammation. This result supports further investigation of tDCS in the management of pain syndromes associated with inflammatory conditions, such as osteoarthritis, in humans. However, additional studies are needed to elucidate the mechanisms involved in this antinociceptive effect and whether this method of neuromodulation can have a significant clinical impact on pain associated with peripheral inflammation.

ACKNOWLEDGEMENTS AND FUNDING: This study was supported by the following Brazilian funding agencies: National Council for Scientific and Technological Development - CNPq (Dr. I.L.S. Torres, Grant no. 307772/2008-0/2008, Dr. W. Caumo); PROPESQ/UFRGS/PIBIC/CNPq (Y. Nonose): Committee for the Development of Higher Education Personnel - CAPES (J.R. Rozisky, G. Laste, L.S.N. Adachi); Graduate Research Group (GPPG) of Hospital de Clínicas de Porto Alegre - HCPA (Dr I.L.S. Torres – Grant 100013). We would like to thank the engineering staff of HCPA for developing the tDCS stimulator.

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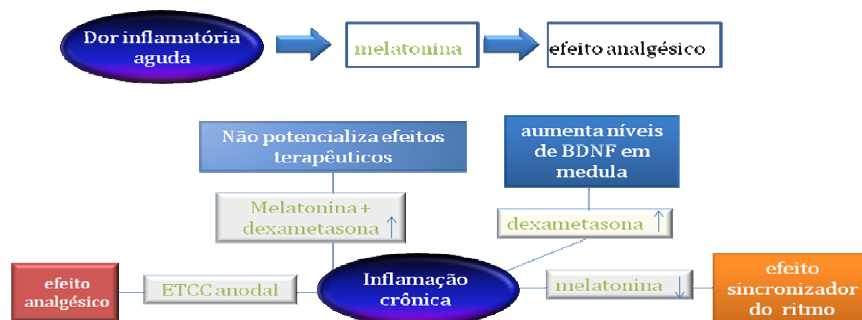
7 Considerações finais

7.1 Conclusões

Os resultados desta tese obtidos em um modelo de dor inflamatória crônica demonstram que as novas propostas terapêuticas avaliadas parecem ser interessantes opções como adjuvante no tratamento da dor crônica, uma vez que (Figura 4):

- a administração de melatonina durante 3 dias consecutivos, mostrou um efeito analgésico significativo sobre a dor inflamatória aguda;
- o tratamento por 8 dias com dexametasona produziu analgesia relacionada ao aumento nos níveis de BDNF em medula espinhal de ratos inflamados;
- o modelo de inflamação crônica induzida na pata dos animais provocou uma desregulação do ritmo de atividade-repouso e dor;
- o tratamento com melatonina reestabeleceu o ritmo circadiano e produziu efeito analgésico neste modelo, com uma discreta melhora no processo inflamatório avaliado histologicamente;
- os animais tratados com dexametasona isolada ou associada à melatonina demonstraram inibição acentuada de parâmetros inflamatórios nos achados histológicos;
- o tratamento com ETCC anódica produziu efeito analgésico nos animais submetidos ao modelo de dor crônica inflamatória.

Figura 4: Esquema relacionado às conclusões da tese



7.2 Perspectivas

- Modelo de artrite: aplicação da ETCC- níveis de BDNF e TNF, histologia, ritmo atividade-repouso.
- Efeitos na artrite: ETCC + dexametasona.
- Efeitos na inflamação crônica: ETCC+melatonina.
- Expressão de receptores melatonérgicos no SNC.
- Expressão de receptores TrkB no SNC.