ANA PAULA VARELA BROWN MARTINS

"Indução do Estímulo Nociceptivo na Região da ATM: mínima concentração efetiva de Piperina em Condições de Normalidade, Inflamação Local Crônica e Estresse Crônico"

"Induction of nociceptive stimulus in TMJ region: minimum effective concentration of Piperine in Normality, Local Chronic Inflammation and Chronic Stress Conditions"

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"Induction of nociceptive stimulus in TMJ region: minimum effective concentration of Piperine in Normality, Local Chronic Inflammation and Chronic Stress Conditions"

Orientadora: Profa. Dra. Célia Marisa Rizzatti Barbosa

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"O valor das coisas não está no tempo que elas duram,
mas na intensidade com que acontecem.
Por isso existem momentos inesquecíveis,
coisas inexplicáveis e pessoas incomparáveis."
Chico Xavier

"O que vale na vida não é o ponto de partida e sim a caminhada.

Caminhando e semeando, no fim terá o que colher".

Cora Coralina.

RESUMO

A disfunção temporomandibular pode afetar músculos mastigatórios, articulação temporomandibular (ATM) ou ambos; possui elevada prevalência nas mulheres e sintoma mais comum é a dor. Foi proposto determinar mínima concentração efetiva da piperina para ativar o Potencial Receptor Transiente Vanilóide da subfamília 1 (TRPV1) na região da ATM direita de ratas *Wistar*, nas condições: normalidade, inflamação crônica na ATM, estresse crônico e associação destas. Foram desenvolvidos 2 estudos experimentais, randomizados, duplo-cegos (protocolo nº 2633-1). No estudo I, 48 animais foram distribuídos aleatoriamente em seis grupos, e cada grupo recebeu 30 µl na ATM de uma das soluções: solução padrão (10% de álcool etílico, 10% de Tween 80 e 80% de solução salina estéril) ou 1, 2, 3, 4 e 5 µg de piperina diluída em 100 ml da solução padrão. No estudo II, 144 ratas foram aleatoriamente distribuídas em grupos: A - inflamação crônica na ATM direita induzida pelo Adjuvante Completo de Freund; B - estresse crônico provocado pelo modelo crônico de estresse; C - associação dessas condições. Esses grupos foram subdivididos (n = 8), e injetados na ATM 30 µl das mesmas soluções descritas previamente. Nos estudos, as ratas, na fase diestro do ciclo hormonal, após injeção de uma das soluções, foram avaliadas quanto ao comportamento nociceptivo, que consistia em quantificar o número de vezes que as ratas levantaram a cabeça abruptamente e tempo dispendido, em segundos (s), para coçar a região orofacial. Cada levantar da cabeça seguiu padrão uniforme de 1 segundo de duração, assim, os comportamentos foram expressos em função do tempo, possibilitando somatória. Para comparação estatística, foi empregado análise da variância e teste de Tukey-Kramer ($P< 0,05$). No estudo I, existiu diferença significante para comportamento de coçar a região orofacial entre os grupos de 2 µg e 5 µg (100,37 ± 63,81 s; 100,0 ± 60,5 s, respectivamente) e o controle (33.37 ± 18,48 s), e somatória dos comportamentos entre 2µg (130,87 ± 257,88 s) e o controle (62.75 ± 14,81 s). No estudo II, no grupo A, houve diferenças estatísticas significativas para os comportamentos de levantar a cabeça no grupo de 5µg (69,5 ± 16,44 s) e o controle (41,13 ± 15,06 s); coçar a região orofacial entre 4µg (51,5 ± 28,73 s) e o controle (14,71 ± 7,54 s),

e na somatória entre 4 e 5µg (105,37 ± 22,64 s; 115,50 ± 35,14 s, respectivamente) e o controle (52,86 ± 17,46 s). No grupo B, nos comportamentos de levantar a cabeça entre 4 µg (85,87 ± 19,21 s) e o controle (49,87 ± 10,70 s); coçar a região orofacial entre 5 µg (48,25 ± 27,25 s) e o controle (35,75 ± 12,69 s). No grupo C, não houve diferença significante nos subgrupos. Assim, 0,02 µg/ml de piperina mostrou-se a concentração mínima eficiente para provocar estímulo nociceptivo na condição de normalidade; a solução de 0,04 µg/ml de piperina, para os grupos de inflamação crônica articular e de estresse crônico e para o grupo de associação das condições não foi evidenciado diferença estatística significante.

Palavras-Chaves: *Articulação temporomandibular – Dor facial – Comportamento animal – Pimenta do Reino – Estresse psicológico*

ABSTRACT

Temporomandibular Dysfunction may affect the masticatory muscles, temporomandibular joint (TMJ) or both, has high prevalence in women and most common symptoms is pain. It was proposed to determine minimal effective concentration of piperine to activate the transient receptor potential vanilloid subfamily 1 (TRPV1) in the right TMJ in Wistar female rats under the conditions: normal, chronic inflammation in the TMJ, chronic stress and inflammation and stress combination. It was developed two randomized double-blind experimental studies (n. 2633-1). In study I, 48 animals were randomly divides into six groups, each group received 30 µl into the TMJ one of this solutions: standard solution (80% sterile saline, 10% Tween 80 and 10% ethyl alcohol) or 1, 2, 3, 4 and 5 µg of piperine diluted in 100 ml of standard solution. In study II, 144 rats were randomly divided into groups: A – chronic inflammation in the right TMJ induced by Freund's Complete Adjuvant; B – chronic stress caused by chronic stress model; C – association of these conditions. These groups were divided (n = 8), and it was injected into the TMJ 30 µl of the same solutions as previously describe. In both studies, rats in diestrous phase of the hormonal cycle, after injection of the solution, were assessed for nociceptive behavior, which consisted in quantify how many times the rats flinched its head and the time spent, in seconds (s), to rub the orofacial region. Each head flinch followed uniform pattern of 1 second duration, and the behaviors were expressed as time function, allowing the sum. For statistical comparison, it was used variance analysis and Tukey-Kramer (P<0.05). In study I, there was a significant difference for rubbing orofacial region between groups of 2 µg and 5 µg (100,37 ± 63,81 s, 100 ± 60,5 s, respectively) and control (33,37 ± 18,48 s), and the sum of the behaviors between 2µg (130,87 ± 257,88 s) and control (62,75 ± 14,81 s). In study II, in group A, there were statistical differences for head flinch behavior between 5µg (69,5 ± 16,44 s) and control (41,13 ± 15,06 s) groups; for rubbing orofacial region between 4µg (51,5 ± 28,73 s) and control (14,71 ± 7,54 s), and for the sum, among 4 and 5µg (105,37 ± 22,64 s, 115,50 ± 35,14 s, respectively) and controls (52,86 ± 17,46 s). In group B, for head flinching behavior there was significant difference between 4 µg

(85,87 ± 19,21 s) and control (49,87 ± 10,7 s); rubbing orofacial region, between 5 μg (48,25 ± 27,25 s) and control (3,.75 ± 12,69 s). In group C, there was no significant difference in the subgroups. Thus, 0,02 μg/ml of piperine showed the lowest concentration effective to cause noxious stimulation in normal condition, the solution of 0,04 μg/ml of piperine, for groups of chronic joint inflammation and chronic stress and for association conditions group there was not statistical significant difference.

Key-Wods: *Temporomandibular Joint – Facial Pain – Animal Behavior – Black Pepper – Psychological Stress*

SUMÁRIO

Introdução

A disfunção temporomandibular (DTM) é uma patologia que pode acometer o sistema estomatognático e envolve músculos mastigatórios, articulações temporomandibulares (ATM), músculos cervicais e outras estruturas correlatas, como vasos sanguíneos e estruturas nervosas (LeResche & Drangsholt, 2008). Dentre os sinais e sintomas mais prevalentes desta disfunção encontram-se desvio e limitação de movimentos mandibulares e cervicais, especialmente durante mastigação e abertura bucal, ruídos condilares, desconforto nas estruturas envolvidas, e com certa frequência intensa dor crânio-cervical (LeResche & Drangsholt, 2008). Estas características da DTM aliadas a outros sinais também prevalentes, como hiperatividade muscular e o aparecimento de pontos-gatilho miofasciais nos músculos mastigatórios, desencadeiam fortes dores locais e referidas capazes de conduzir a importantes limitações físicas, psíquicas e sociais ao paciente.

Apesar da fisiopatologia da dor presente nas DTM ser pouco compreendida, é considerada uma condição clínica relevante, principalmente devido à sua elevada prevalência (Lipton *et al*, 1993; LeResche *et al*, 2005; Isong *et al*, 2008; LeResche & Drangsholt, 2008). Com relação à prevalência da DTM, estudos epidemiológicos apontam uma ampla variação entre 11 a 50%, refletindo as diferenças de metodologia entre os mesmos (Lipton *et al*, 1993; LeResche, 1997; Manfredini *et al*, 2006). No Brasil foi comprovado que 39,2% dos indivíduos adultos apresentam um dos sintomas da DTM, como sons articulares, dor articular ou dos músculos mastigatórios, e dificuldade de abertura da boca e destes, 25,5% estão relacionados à dor (Gonçalves et al, 2010).

Fatores de risco, como condições de dor pré-existente, hormônios sexuais, fatores psicológicos e depressão parecem contribuir para a progressão e exacerbação da sintomatologia dolorosa da DTM (Suvinen *et al*, 1997; Drangsholt & LeResche, 1999). Estudos clássicos investigaram a relação entre estresse psicológico e DTMs (Parker, 1990; Grzesiak, 1991; Vanderas, 1994; Wexler & Steed, 1998). Parker (1990) considerou que

influência do estresse sobre a DTM seria explicada pelo aumento da tensão muscular durante as condições emocionais estressantes. Entretanto, também se considera que a disfunção muscular induzida por trauma local (Rizzatti-Barbosa *et al,* 2010; Farias Neto *et al*, 2012) ou estresse (Uhac *et al*, 2003) secundariamente produzirá alterações nas ATM, causando modificações sensíveis na biomecânica articular, promovendo microtraumas à cápsula e disco articulares, com consequente aumento na percepção de dor. Inicialmente, acreditava-se que a influência do estresse no desenvolvimento da DTM estivesse relacionada com hiperatividade muscular crônica recorrente, que progressivamente danificava os tecidos (Laskin, 1969). Hoje se sabe que o estresse é capaz de afetar profundamente os processos biológicos de transmissão e percepção da dor (Vedolin *et al*, 2009). Assim, respostas adaptativas inadequadas a condições de estresse podem agir como auto-estressores (a dor orofacial é um forte elemento estressor), alimentando um ciclo vicioso de dor-estresse-dor (Vedolin *et al*, 2009). As condições psicológicas envolvidas no estresse ainda não são totalmente compreendidas, e como os fatores psicológicos *per si* influenciam na fisiopatologia da DTM ou como refletem no impacto da doença em um indivíduo ainda é um fato desconhecido pela ciência (Vedolin *et al*, 2009). A compreensão desta influência é dificultada pela inviabilidade do uso de modelos experimentais que evidenciem esta fisiopatologia em humanos, cujo comportamento frente ao estresse não é o mesmo daquele observado nos animais. Por esta razão, a carência de estudos humanos, por questões éticas, incita o aprimoramento dos modelos animais que mais se aproximem da realidade clínica. Assim sendo, a correlação entre estudos clínicos e experimentos em modelos animais, pesquisas translacionais, torna-se cada vez mais necessária frente às respostas exigidas para a compreensão e consequente intervenção no processo de desencadeamento e manutenção da dor, de modo particular, aquela envolvida nas DTM (Roveroni *et al*, 2001).

Pesquisas comprovaram, sob determinadas condições experimentais (estresse agudo e crônico), os efeitos hiperálgicos do estresse em animais (Vidal & Jacob, 1982; Satoh *et al*, 1992; Quintero *et al*, 2000; Imbe *et al*, 2004), contrariando outros estudos que demonstraram que o estresse promove analgesia (Wiedenmayer & Barr, 2000; Lapo *et al*., 2003). Experimentos confirmaram que exposição a um estresse emocional agudo, como a

exposição sistemática a novos ambientes, é capaz de produzir hiperalgesia imediata e transitória (Vidal & Jacob, 1982), enquanto que o estresse prolongado induz hiperalgesia que persiste por até 28 dias após a suspensão do estresse crônico (Torres *et al*, 2003).

Modelos experimentais de indução de dor e/ou inflamação em ratos utilizando óleo de mostarda ou formalina, por exemplo, já foram desenvolvidos e validados. Por meio destes modelos, foi possível estudar e compreender alguns dos mecanismos envolvidos nas condições de dor craniofacial superficial e profunda, algumas das mudanças nos sistema nervoso central e periférico, e o envolvimento dos diversos mediadores inflamatórios neste processo (Fiorentino *et al*, 1999; Roveroni *et al*, 2001; Gameiro *et al*, 2005; Bonjardim *et al*, 2009). Entretanto, esses modelos foram desenvolvidos utilizando animais do gênero masculino. O desenvolvimento de modelos específicos em fêmeas torna-se interessante, pois parece existir uma diferença na percepção à dor entre os gêneros pela influência dos hormônios sexuais, tanto em humanos quanto em animais. Está comprovado que presença, duração e severidade da dor na DTM são maiores em mulheres (Flake *et al*, 2006; LeResche & Drangsholt, 2008), o que sugere a influência dos hormônios femininos, particularmente o estrógeno, na fisiopatologia da dor na DTM (Flake *et al*., 2006), por estarem ambos, níveis maiores de estrógeno e maior prevalência da DTM, mais presentes durante a fase reprodutiva da mulher (LeResche *et al*, 1997; Carlsson, 1999). As possíveis explicações sobre a influência deste hormônio no desenvolvimento e manutenção da DTM, é que ele participe na modulação do processo inflamatório associado ao dano local da ATM, ou no aumento da excitabilidade das terminações na articulação regulando a expressão e liberação local de neuropeptídeos inflamatórios (Flake *et al*, 2006). Em trabalhos anteriores, foi evidenciado que polimorfismos relacionados aos receptores de estrógeno são mais prevalentes nas mulheres que sentem mais dor diante do quadro clínico de DTM[1] (Rizzatti-Barbosa *et al*, 2009; Meloto *et al*, 2011).

Agentes irritantes naturais, por desencadearem um poderoso estímulo nocivo em neurônios sensoriais primários, podem ser empregados em experimentos em animais no intuito de avaliar o comportamento nociceptivo (Geppetti & Trevisan, 2004; Martins *et al*,

[1] Projeto FAPESP # 2004/07258-4: "Análise do Polimorfismo em gene receptor α de estrógeno em mulheres com sinais e sintomas de desordem temporomandibular".

2011). A piperina (1-peperoilpiperidina) é um alcalóide que consiste no principal princípio ativo da pimenta do reino (Piper nigrum), e que promove uma sensação de ardência supostamente mediada pela ativação do receptor de potencial transiente vanilóide, membro da subfamília vanilóide 1 (TRPV1) ((McNamara *et al*, 2005). Este foi inicialmente descrito como um receptor para capsaicina (Fu *et al*, 2010), mas sabe-se que a piperina também compartilha sítios de ligação em comum a outros produtos naturais de plantas, como a resiniferatoxina (Liu & Simon, 1996; Szallasi & Blumberg, 1999) e da sua habilidade em ativar correntes em neurônios sensoriais de ratos, isolados do gânglio trigeminal (Liu & Simon, 1996; Szallasi & Appendino, 2004).

Além da diferença estrutural pela substituição do grupo vanilil pelo metilenodioxi (McNamara *et al*, 2005; Szallasi, 2005), a piperina é mais eficiente em induzir dessensibilização dos receptores (McNamara *et al*, 2005). Foi comprovado que as ações da piperina sobre o TRPV1 são coerentes com as ações de um agonista para este receptor, mas também que ela exibe uma clara propensão para induzir dessensibilização do receptor (McNamara *et al*, 2005). Acredita-se que o TRPV1 funcione como um integrador molecular de estímulos nocivos, incluindo calor, ácidos, poluentes com mudança elétrica negativa, e substâncias endógenas pró-inflamatórias (Szallasi & Blumberg, 1999). Expresso em tecidos neuronais, tais como medula espinhal (primariamente em fibras eferentes sensoriais) hipotálamo, hipocampo e substância negra, e não-neuronais, tais como mastócitos e glia (Cortright & Szallasi, 2004). O TRPV1 também é altamente expresso em neurônios primários sensoriais, fibras A-δ e C, nociceptores polimodais que respondem a diversos estímulos químicos, mecânicos e térmicos (Geppetti & Trevisan, 2004). Linfócitos e mastócitos também expressam o TRPV1, evidenciando uma interação entre os sistemas nervoso e imune (Szallasi, 2005).

Mecanismos ainda pouco esclarecidos, mantêm o TRPV1 no estado inativo (Szallasi, 2005). Possivelmente, o TRPV1 esteja sob o controle inibitório do fosfatidilinosito (4,5)-bisfosfato [Ptd Ins (4,5) P_2], que na presença de agonistas, fosfoflipase C (que cliva o Ptd Ins (4,5) P_2) ou proteínas cinases (especialmente a cinase C) podem ativar esse receptor e acoplá-lo a outros receptores da dor, como o receptor da bradicinina B_2 (Di Marzo, 2002). A ativação do TRPV1 resulta em rápido aumento dos

níveis de Ca^{2+} intracelular (Cortright & Szallasi, 2004) e esse evento iônico resulta em um efeito excitatório dos terminais de neurônios sensoriais primários com a subsequente despolarização da fibra nervosa e o início da propagação do potencial de ação (Geppetti & Trevisan, 2004). Influxo de Ca^{2+} em terminações nervosas, impulsionada tanto pela condução antidrômica do potencial de ação ou diretamente pela propagação do TRPV1, provoca a liberação de neuropeptídeos, incluindo peptídeo relacionado ao gene da calcitonina (PRGC) e as taquicininas, substância P (SP) e neurocinina A (NKA) (Geppetti & Trevisan, 2004). A liberação de SP e de PRGC pode contribuir para o componente neurogênico da inflamação, por terem propriedades pró-inflamatórias (Khan *et al*, 2008). Esses mediadores podem provocar vasodilatação, extravasamento plasmático, liberação de histamina, prostaglandina E2 (PGE2), citocinas (interleucina-1, interleucina-6, fator de necrose tumoral-α) contribuindo para a dor e inflamação neurogênica (Khan *et al*, 2008). Foi comprovada a participação do TRPV1 no desenvolvimento de hiperalgesia pós-inflamatória, pois a expressão desse receptor encontrar-se aumentada em várias desordens humanas como doenças inflamatórias intestinais e trato urinário, e condições de dor crônica (Szallasi & Appendino, 2004).

Deste modo, o objetivo do presente estudo foi elaborar modelos experimentais de dor na região da ATM de ratas, utilizando solução de piperina em diferentes concentrações para determinar sua mínima concentração efetiva para provocar o estímulo nociceptivo, sob as seguintes condições: normalidade, presença de inflamação crônica na ATM, condição de estresse crônico e a associação de ambas as condições, avaliando o comportamento nociceptivo do animal no curso do tempo. Este experimento evidenciou a possibilidade de estimular o TRPV1 na região da ATM através a piperina, demonstrando o envolvimento deste receptor no mecanismo da dor nesta articulação, e poderá servir de base para o desenvolvimento de fármacos específicos e de ação direta para o alívio da dor nas DTM.

Capítulo 1

Piperine solution: effective concentration to promote nociception in Temporomandibular Joint and its use in female rats

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Abstract: The objective of this study was to develop an animal model of orofacial pain in temporomandibular joint region in females Wistar rats using different concentrations of piperine in order to determinate its minimum effective concentration to induce nociception. Forty eight *Wistar* female rats were randomly divided in six groups (n=8), that received an injection in the right TMJ region of 30 µl of one of the following solutions: control group – ethyl alcohol, Tween 80, sterile saline and the other experimental groups: 1; 2; 3; 4 or 5 µg of piperine diluted in 100 ml of ethyl alcohol, Tween 80, sterile saline. The rats had their hormonal cycle analyzed and the animals in diestrous cycle were immediately evaluated for the nociceptive behavior, after the injection of one of the solutions. The nociceptive behavior consisted in counting how many times the animals flinched their head and how long they rubbed their orofacial region. As the first behavior followed a uniform pattern of 1 movement per second, both behaviors were expressed in seconds. The data were submitted to the one-way ANOVA and Tukey-Kramer´s test (*post-hoc, P<0.05)*. The higher means (±SE) for time (seconds) of orofacial rubbing were gotten in groups II (100.37 ± 30.42) and V (100 ± 27.82); for the head flinches, the highest mean was in group III (44.87 ± 4.76); finally, the highest mean for the sum of both behavior was gotten in group II (130.87 ± 27.53) with significant difference when comparing with the control group. The results suggest that induced pain in the TMJ region with 0,02 µg/ml has been proved to be the minimum effective concentration to cause pain in this region and also it can be used as a valuable model in the study of orofacial pain.

Keywords: Piperine - nociceptive behavior - Temporomandibular Joint

Introduction

The prevalence of chronic pain in the population is high (8-15%) (LeResche *et al*, 2003) and its mechanisms are not still completely understood. Experiments in animal models have been important to elucidate its pathophysiology and modulation (LeResche *et al*, 2003), and to ensure the effectiveness of treatment for Temporomandibular Disorders (TMD). Higher prevalence in women, especially during the reproductive age, suggests the influence of female sex hormones in the painful symptoms of TMD (Flake *et al*, 2005). It was proved that TMD pain in women is highest at times of lowest estrogen during the hormonal cycle (LeResche *et al*, 2003).

Studies using substances that cause noxious stimuli (glutamate, mustard oil, formalin, capsaicin) were also proposed mainly to verify the effectiveness of determined drugs. Each substance has specific mechanism on the nociceptive nerve fibers endings, either to provoke local inflammation or increase muscle activity, and also to promote the conduction of noxious stimulation and its processing in higher centers related to pain. Piperine acts on nerve endings, and it has higher efficacy than capsaicin in the activation and desensitization of nociceptors, depending on its concentration (Cairns, Sessle & Hu, 1998; Szallasi, 2005; Fiorentino, Cairns & Hu, 1999).

Piperine, the main pungent of black pepper (Piper nigrum) is employed for various purposes, since food condiments and to increase nutrient absorption, till as an metabolic accelerator (Szallasi, 2005). Piperine can be employed in the study of neuronal function by its ability to activate (Liu & Simon, 1996) or block noxious stimuli in sensory neurons of the trigeminal ganglion. The action of this agent is initiated by the activation of the transient receptor potential vanilloid 1 (TRPV1) (McNamara, Gunthorpe & Randall, 2005), which presents a common binding site for other natural activating substances of the nerve fiber, such as capsaicin and resiniferatoxin (Szolcsanyi, 1983; Patacchini, Maggi & Meli, 1990; Szallasi & Blumberg, 1991). After activation of TRPV1, there is an influx of sodium and calcium ions into the sensory neurons (Marsh *et al*, 1987), and it begins the depolarization and axonal conduction of nociceptive stimuli (Patacchini, Maggi & Meli, 1990). Due to its sensitivity to heat and protons (Caterina *et al*, 1997; Tominaga *et al*,

1998), the receptor acts as a molecular integrator of chemical, physical and thermal stimuli (Caterina *et al*, 1997). Furthermore, its expression in human sensory neurons involved in pain pathways and gastrointestinal function (Hayes *et al*, 2000; Ward *et al*, 2003) makes the TRPV1 a good target for pharmaceutical intervention. This interest is due to the possibility to develop new drugs that can directly act on this receptor for the treatment of various pathological conditions since from inflammatory and neuropathic pain till bladder dysfunction and irritable bowel syndrome (Szallasi & Appendino, 2004).

The involvement of TRPV1 in the transmission of nociceptive information from peripheral nerve endings to the central nervous system (CNS) through the primary afferent neurons is duly proven, and studies indicate a broad pharmacological action of piperine as an agonist or antagonist for this receptor, depending its concentration (Yang *et al*, 2011; Szallasi, 2005). However, there are no studies that indicate the effective concentration of piperine to investigate, in an animal model, the effectiveness of nociceptive transmission of these nerve endings located in the Temporomandibular Joint (TMJ).

Thus, the objective of this study was to verify, using an animal model, the use of piperine to induce nociception in TMJ region, and also, to determine the minimum concentration of this substance required for maximum nociceptive response in female rats after its application in TMJ.

Materials and Methods

A. Animals

For this study, 48 female Wistar rats from Multidisciplinary Center for Biological Research (CEMIB – UNICAMP) were used. These animals were housed in plastic cages (05 per box) in a temperature-controlled environment (23 °C ± 1 °C), light and dark cycle of 12 hours (06 am - 06 pm) and access to water and food *ad libitum*. The rats remained in this condition until they reach maturity and weight (250-300 g) to start the research (Roveroni *et al*, 2001). This study was approved by the Local Ethics Committee on Animal Use (# 2633-1).

B. Determination of Hormonal Cycle

The estrous cycle phases of rats were determined by the vaginal smear (Long & Evans, 1922) which consists in characterizing each phase based on the proportion between the epithelial cells, leukocytes and cornified cells (Mandl, 1951). After reaching maturity, vaginal secretion was collected between 7am and 8am, using an automatic micropipette (Micropipette automatic LabMate HT 20 µl), plastic tip (Axygen Scientific, Union City, CA, USA) and sterile saline (Braun, São Gonçalo, RJ, Brazil). This procedure was performed in a separate room. Each cage was transported at a time in order to prevent stress and increased aggressiveness of females that would be later evaluated (Marcondes *et al*, 2002).

To collect vaginal fluid, 10 µl of saline solution was aspirated and deposited the initial portion of the vaginal conduit, and the secretion obtained from each animal was placed on glass slides for examination under a light microscope (Binocular Biological Microscope N 101 B Coleman) with 40x objective lens. After this analysis, the animals that were in diestrous phase, in which the estrogen levels are low, with a predominance of leukocytes cells (Marcondes *et al*, 2002), were separated for the subsequent steps of the experiment.

C. Application of Piperine

The 48 animals used for this study were randomly divided into six groups: control and experimental groups (table 01). Each animal had the respective solution injected into the right TMJ region.

Table 01: Distribution of animals in the control and experimental groups with the respective solutions, which were injected into the right TMJ.

Groups	Injected Solution
Control (n = 8)	control solution - 10% ethyl alcohol (Chemco - Indústria e Comércio Ltda, Campinas, SP, Brazil), 10% Tween 80 (Dynamic - Chemistry Contemporary Ltda, Diadema, SP, Brazil) and 80% sterile saline (Braun, São Gonçalo, RJ, Brazil) (Bölcskei *et al*, 2010; Bellinger *et al*, 2007).
I (n = 8)	1 µg of piperine (Sigma-Aldrich, St. Louis, MO, USA) diluted in 100 ml of the control solution
II (n = 8)	2 µg of piperine (Sigma-Aldrich, St. Louis, MO, USA) diluted in 100 ml of the control solution
III (n = 8)	3 µg of piperine (Sigma-Aldrich, St. Louis, MO, USA) diluted in 100 ml of the control solution

IV (n = 8)	4 µg of piperine (Sigma-Aldrich, St. Louis, MO, USA) diluted in 100 ml of the control solution
V (n = 8)	5 µg of piperine (Sigma-Aldrich, St. Louis, MO, USA) diluted in 100 ml of the control solution

After the identification of the estrous cycle phase, animals were moved to the Laboratory of Pain of the Physiology Department of the Piracicaba Dental School, and kept into a free noise room and temperature controlled at 23 °C. Individually, the animals were placed in the mirror chamber (30x30x30 cm) for behavior analyses for a period of 10 minutes without water and food, in order to accommodate themselves and reduce the stress that could interfere in the behavioral analysis (Roveroni *et al*, 2001).

After this adaptation period, the animals were lightly anesthetized with halothane (Tanohalo - Cristália Pharmaceutical Chemicals Ltd., Itapira, SP, Brazil) in which 30 µl of the following solutions were injected: control solution (mixture of 80% of sterile saline, 10% Tween 80 and 10% ethyl alcohol) and 1, 2, 3, 4 or 5 µg of piperine diluted as previously mentioned, according to their respective group. These solutions were applied by the same examiner who performed the assessment of nociceptive behavior, although not know which group the animals belonged.

For the injection of solutions, disposable 30-gauge needle (injected Dental Products, Sao Bernardo do Campo, SP, Brazil) (a needle for each animal) was coupled to a polyethylene tube (PE-50; Intramedic, Clay Adams, Becton -Dickinson, Franklin Lakes, NJ, USA), in which was attached to a Hamilton syringe for 50 µL (Hamilton, Reno, NV, USA). The needle was inserted in the posterior-anterior direction in the pre-auricular region on the intersection of imaginary lines drawn from palpebral commissure to the lower edge of the hearing canal and the tangent perpendicular line to the anterior portion of the conduit. The needle reached the upper portion of the lateral pole of the mandibular condyle, for subsequent deposition of the solution.

D. Behavior Testing

Tests for behavior analysis were performed between 8 am and 17 am, by a blinder and calibrated examiner (*Kappa* = 0,82) relative which group each animal

belonged. After injection of the solution, the animal was placed back into the mirrored chamber and quickly his conscience was reestablished to start the assessing of the nociceptive behavior. Each animal was evaluated for a period of consecutive 45 minutes divided into 9 blocks every 5 minutes. The nociceptive behavior consisted of rubbing the orofacial region (Clavelou *et al*, 1989 and 1995) on the right side (the region of application of the substances) and flinching the head (Roveroni *et al*, 2001).

For each block of 5 minutes, it was recorded the seconds spent by the animal to rub the orofacial region and the number of times that the animal flinched the head. The head flinches followed an uniform pattern of 1 second duration and at the end, this behavior was also expressed in seconds, leaving both behaviors expressed in function of time (Roveroni *et al*, 2001). Thus, we evaluated the nociceptive behavior of the sum of behaviors performed by the rats.

E. Statistical Analysis

The data with homogeneity of variance were analyzed by one-way ANOVA and post hoc multiple comparisons were performed using Tukey-Kramer, with the significance level of 5%. The data are presented in figures and in the text as mean ± SEM.

Results

When analyzing the sum of nociceptive behaviors, head flinching and orofacial rubbing, the experimental groups II and V (130.87 ± 27.53, 125.00 ± 27.55, respectively) were significantly different when compared to the control group (61.87 ± 2.98) (Fig. 01). Comparing the experimental groups with each other, even in figure 02, groups II and V differed significantly from group I (66.250 ± 3.480). The highest means of the sum of the nociceptive behavior was at concentration of 2 x 10^{-2} µg/ml and even with the increased concentration of piperine in the solution, there was a decrease in the sum of the behaviors in groups III and IV. In group V, there was a new peak in the sum of these behaviors, although no significant difference was observed in relation to group II.

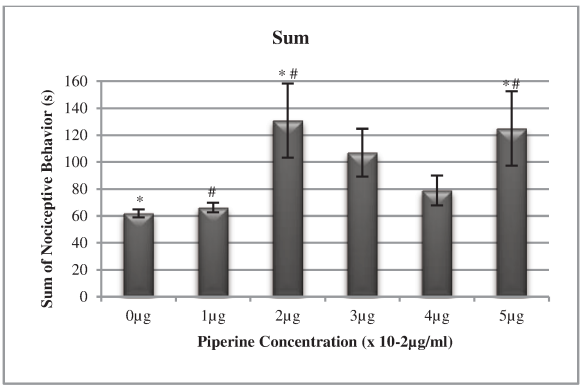

Fig. 01 – Representing the sum of nociceptive behaviors, head flinching and orofacial rubbing, after injection of different concentrations of piperine solution. The error bar indicates the SEM. The significant difference between the groups is shown (*) when compared with the control group and (#) when compared between the experimental groups (*P* <0.05 for all comparisons, Tukey-Kramer).

Different piperine concentrations had no significant effect (*P* <0.05, Tukey-Kramer) in the flinching when comparing experimental and control groups (Fig. 02).

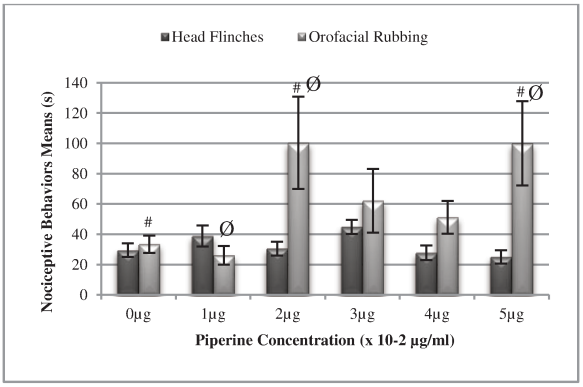

Fig. 02 – Results of flinching the head and rubbing the orofacial region behaviors after injection into the right TMJ region of increasing concentrations of piperine. The error bar indicates the standard error mean (SEM). To orofacial rubbing behavior, (#) indicates significant difference with control group and (Ø) with the 01 µg group (P <0.05 for all comparisons, Tukey-Kramer).

For the orofacial rubbing behavior, as evident in Figure 02, the groups that received 2 µg and 5 µg showed significant difference (100.37 ± 30.43, 100.00 ± 27.82, respectively) in the control group (33.37 ± 5.74). Among the experimental groups, it can be observed significant difference between those same groups and the group I which received 1 µg (26.125 ± 6.112).

The injection of piperine on the TMJ induced only one peak in the sum of nociceptive behavior, as shown in Figure 03 by the group II, in the course of 45 minutes of the analysis (Fig. 03). The behaviors sum showed increasing in the course of time to reach the maximum value between the periods of 20-25 minutes. Subsequent to this peak, there was a decline until reaching a plateau in times of 35-40 and 40-45 minutes. On the other

hand, the control group exhibited an alternation of peaks during the 45 minutes of evaluation.

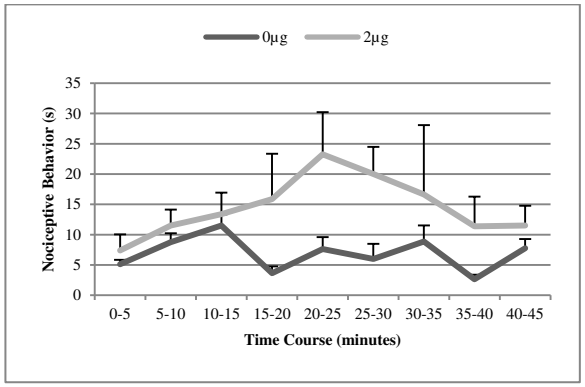

Fig. 03 – Time course of the sum of nociceptive behaviors of control and experimental II groups (2 µg). The error bars show the SEM.

Evaluating separately the nociceptive behaviors (head flinching, orofacial rubbing) and the sum of the behaviors of the group that received 2 µg, different pattern in the course of time was observed (Fig. 04). The behavior of head flinching showed almost constant levels, with values lightly greater in the first half of the total time. Thus, the differing from the orofacial rubbing and the sum of behaviors, both showed increasing profiles until they reached the peak at half the total time of the assessment and subsequent descent.

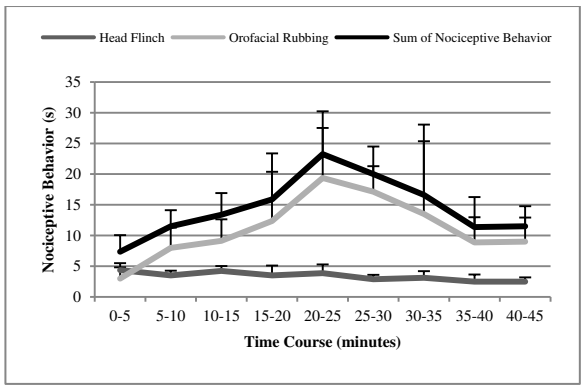

Fig. 04 – Time course of nociceptive behavior of group II (2 µg) characterized by head flinching and orofacial rubbing and the sum of the behaviors, separated in 9 blocks of 5 minutes each. The error bars indicated the SEM.

Discussion

Pain is a sensory modality response to various noxious, chemical, physical and / or thermal stimuli, in which nociceptive primary afferent neurons detect and conduct nervous impulses to higher centers responsible for processing this information (Sessle, Iwata & Dubner, 2008; Caterina & Julius, 2001; Caterina *et al*, 1997). Conditions of TMJ pain are considered important manifestations of changes in this joint, but its pathogenesis, diagnosis and treatment remain poorly understood (Roveroni *et al*, 2001). As a result, the development of experimental models becomes important to clarify the mechanisms related pain conditions.

In this study, the animals in the experimental group II that received 0,02 µg/ml of piperine solution showed the highest average of orofacial rubbing behavior, differing from the control group and the experimental group I. Groups III and IV, which received solutions with higher concentrations showed a reduction in mean values for this behavior, possibility because of the piperine action in desensitize the TRPV1. In group V, an increase of the average values was observed, while it was not enough to overcome the values reached by the group II. This behavior may have occurred as a result of some secondary mechanism of piperine as increased release of neuropeptides (Geppetti & Trevisani, 2004, Black, 2002; Caterina & Julius, 2001) that activated the receptor, but not enough to overcome the averages of the group that received 2 µg.

The higher prevalence of TMD pain in women in the reproductive phase is suggested by the influence of hormonal fluctuation in pain perception (LeResche, 1997). The average levels of pain intensity rise at the end of the cycle and reach the peak during the first three days of the menstrual cycle and during ovulation (LeResche, 1997). Because of these aspects, researches related to pain are performed in female animals and nociceptive behavior assessment is performed during the diestrous hormonal phase (Marcondes, Bianchi & Tanno, 2002).

The present study showed the nociceptive behavior of female rats characterized by head flinching and rubbing the orofacial region (Roveroni *et al*, 2001; Clavelou *et al*, 1995, 1989) after injection of different concentrations of piperine. The selection of piperine as promoting substance of the nociceptive stimulus is justified because it has more efficacy

than capsaicin for both activation and desensitization of this receptor in experimental animals and *in vitro* (Szallasi, 2005; McNamara, Gunthorpe & Randall, 2005; Liu & Simon, 1996). It is suggested that activation of TRPV1 mediated by piperine involves a larger number of binding sites than capsaicin, by recruiting more subunits in TRPV1 in the input mechanism or by having other binding sites on this receptor (McNamara, Gunthorpe & Randall, 2005). Besides this, TRPV1 has some features, such as expression in the C fiber and activation by pro-inflammatory cytokines that make this receptor suspect to be involved in TMD.

The TRPV1, a channel non-selective cation permeable to sodium and calcium (Caterina *et al*, 1997) is expressed in peptidergic and non-peptidergic neurons of the trigeminal ganglion (TG) and dorsal root ganglion (DRG), but is more associated with non-myelinated fibers than the myelinated sensory root of the brainstem (Yang *et al*, 2011; Immke & Gawa, 2006; Ward *et al*, 2003; Hou *et al*, 2002; Ichikawa & Sugimoto, 2001; Valtschanoff *et al*, 2001; Hayes *et al*, 2000; Guo *et al*, 1999). These afferent neurons, which express TRPV1 and innervate the orofacial region, are projected to the sensory trigeminal nucleus, most predominantly in laminae I and the outer portion of the laminae II of Caudal Trigeminal Nucleus (CTN) (Bae *et al*, 2004), the main local to relay of nociceptive information from the orofacial region (Sessle, Iwata & Dubner, 2008). Little is known about the organization of synaptic terminals of axons that express TRPV1 in CTN, as well as its predominance by non-myelinated fibers in the sensory root of the TG, its arrangement in the CTN and its connectivity with one or two postsynaptic dendrites (Yeo *et al*, 2010).

The polymodal nature of TRPV1 is the result of their sensitivity to surrounding pH and temperature variations by extracellular acidification (protons) as well as endogenous activators such as pro-inflammatory mediators, as well as piperine, capsaicin and resiniferatoxin (McNamara, Randall & Gunthorpe, 2005; Geppetti & Trevisani, 2004; Ward *et al*, 2003; Caterina & Julius, 2001; Jordt, Tominaga & Julius, 2000; Szallasi & Blumberg, 1991; Tominaga *et al*, 1998). Activation of TRPV1 results in a rapid increase of the levels of intracellular Ca^{+2} (Cortright & Szallasi, 2004; Geppetti & Trevisani, 2004), and causes depolarization and propagation of the action potential to the dorsal horn (Immke

& Gawa, 2006; Geppetti & Trevisani, 2004). The importance of these receptors was demonstrated by their involvement in post-inflammatory hyperalgesia, and it stimulates pharmaceutical research to identify new antagonists for TRPV1 (Bley, 2004; Szallasi & Appendino, 2004; Valenzano & Sun, 2004). Associated with this, its expression is increased in many human diseases, including inflammatory bowel disease, disorders of intestinal motility (fecal urgency), and chronic pain conditions such as chronic breast pain (Caterina & Julius, 2001). The Ca^{+2} influx into the nerve endings, after sensitization TRPV1 causes a local release of neuropeptides, such as gene-related peptide calcitonin (CGRP), and substance P (SP), which causes inflammatory responses, as neurogenic inflammation (Geppetti & Trevisani, 2004, Black, 2002; Caterina & Julius, 2001).

However, it seems that the neuronal response to the transmission of nerve impulse after stimulation of TRPV1 receptor is dose dependent of stimulatory agent. In the present study, piperine was used as a noxious agent. While in the higher concentration this substance inhibits the receptor, at lower concentrations apparently stimulates the conduction through the receptor (Szallasi, 2005). However, there are no studies that identify the optimal concentration of this agent to promote the nociceptive stimulus in animal models for studies of TMD. Therefore, in the present study it was used various concentrations of piperine in order to identify the most effective dose for this purpose. It was observed that injection of different concentrations of piperine promoted nociceptive responses in different intensities. Analyzing separately the behaviors data, the head flinching behavior was similar in all groups, with no statistical difference. Even for the experimental group III, that showed the highest mean values, although no difference was observed, when compared with the control group or the others experimental groups. This finding suggests that the nociceptive behavior of head flinching may have been influenced more by increasing the volume inside the joint (Hu *et al*, 1994) rather than to the action of piperine, since all groups received the same volume of the respective solution.

We conclude that piperine can be used as a substance in an animal model to promote nociceptive behavior in the TMJ region, and the minimum effective concentration to get the highest nociceptive response is 0,02 µg/ml.. In future studies, it will be possible

to investigate the role of TRPV1 in the underlying mechanisms of pain associated with TMD.

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Capítulo 2

Effective concentration of piperine solution in rat TMJ region in chronic joint inflammation, chronic stress and their association to induce nociceptive stimulus

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Abstract: The aim was to analyze the animal behavior of orofacial pain in temporomandibular joint region in females *Wistar* after the injection of different concentrations of piperine to determinate its minimum effective concentration in: chronic inflammation, chronic stress and the association of both conditions. The chronic inflammation was induced by Complete Freund's Adjuvant and the stress exposure followed a chronic model during 40 days. One hundred forty four female rats were randomly divided in tree groups (n=48), according to the three proposed conditions. Each group was divided in six groups, and each subgroup received an injection in the right TMJ region of 30µl of one of the following solutions: control solution – ethyl alcohol, Tween 80, sterile saline and the other experimental subgroups: 1; 2; 3; 4 or 5 µg of piperine diluted in 100 ml of ethyl alcohol of the control solution. The rats in diestrous cycle, after the injection of one of the solutions, were evaluated for the nociceptive behavior, which consisted in counting how many times the animals flinched their head and how long they rubbed their orofacial region. As the first behavior followed a uniform pattern of 1 movement per second, both behaviors were expressed in seconds. The data were submitted to the two- way ANOVA test and Tukey-Kramer test (*post-hoc, $P<0.05$)*. For the inflammation group, the control and the others experimental subgroups of piperine injected animals, there was significant difference among the groups that received 0,04 µg/ml for all the behavior and the sum of them. For the stressed group, the same significant difference was noted between the subgroup that received 0,04 µg/ml for all the behavior and the sum of them. For the association group, no difference was observed among the groups. The results suggest that, for chronic inflammation and chronic stress, the minimum effective concentration to causes TMJ pain was 0,04 µg/ml and also it can be used as a model in studying of orofacial pain.

Keywords: Chronic Inflammation - Chronic Stress - Temporomandibular Joint - Piperine – Nociceptive Behavior.

Introduction

Pain is defined as an unpleasant sensory and emotional experience associated with actual or potential damage tissue (Merskey & Bogduk, 1994). Many studies of chronic pain in humans are related to assessment, perception and response to pain, which involves the emotional-affective system, cognition, learning principles, pain behavior and social, environmental and biological factors, and vary considerably from patient to patient. (Suvinen & Reade, 1995; Melzack, 1999). All these factors explain the different pain responses that patients show for similar clinical situations. Besides ethics questions, all these factors may confound pain clinical studies, justifying the development of animals' research.

Temporomandibular Disorder (TMD) pain is the most evidenced symptom, involved in Temporomandibular Joint (TMJ), masticatory muscles or both (Cairns, 2010; Scrivani *et al*, 2008; LeResche *et al*, 2003; LeResche, 1997). Besides the muscle and/or joint pain, TMD may be associated to headache, limitation of mandibular movements and high level of pain disability (de Leeuw, 2008; Scrivani *et al*, 2008; Wadhwa & Kapila, 2008; Yap *et al*, 2002).

Orofacial pain is more prevalent in females during the reproductive age (Scrivani *et al*, 2008; LeResche, 1997; Lipton *et al*, 1993), specially during mandibular function or under palpation (LeResche, 1997). This high prevalence in adult women indicate that biological, behavioral, psychological and social problems associated with female increase the risk of pain in the TMJ region (LeResche, 1997). The TMD, as well as other diseases, has its onset resulting from the interaction between individual factors like individual social context (Schimitter *et al*, 2010).

Although the etiology of TMD has not been established, psychological aspects have been associated with predisposition, initiation and perpetuation of TMD (Suvinen *et al*, 2005; Lindroth *et al*, 2002; List *et al*, 2001), in which stress, depression and multiple somatic symptoms are risk factors (LeResche, 1997). Stress seems to exert an important influence on the development of pain (Fricton, 2007) and intensity of symptoms in TMD (Gameiro *et al*, 2006). The stress response and the mechanism of pain modulation have

some common molecules, and stress may be involved in biological process of pain (Gameiro *et al*, 2006).

Similarly to stress, inflammatory processes, as the Internal Joint Disorders, promote the release of proinflammatory cytokines in the synovial fluid, such as interleukins (IL) -1 and 6, tumor necrosis factor (TNF) -α, IL -6 and others (Takahashi *et al*, 1998; Whiteside, 1994). Those cytokines contribute to the synovitis pathogenesis, degenerative changes in TMJ cartilage and bone, and interfere in the clinical symptoms of TMJ pain (Takahashi *et al*, 1998). In the synovial fluid, cytokines stimulate production, release and activation of enzymes that degrade cartilage matrix and lead to the production of inflammatory mediators such as prostaglandins and leukotrienes (Pelletier *et al*, 1993; Arend & Dayer, 1990). The symptom becomes prolonged and resistant to treatment (Cairns, 2010). The persistence pain results a peripheral mechanisms within the joint that prolongs the sensitization of neurons in the central nervous system (CNS), resulting in local increase of calcitonin gene-related peptide (CGRP), substance P, and proinflammatory neuropeptides (Cairns, 2010). It can be induced by tissue injury caused by inflammatory processes, but also is associated to excessive loads on the joint under stress conditions (Cairns, 2010).

Through the application of different concentration of piperine, this experimental research in animals aimed to: (i) develop an experimental model of pain in the TMJ under chronic inflammation, under chronic stress condition, and under the association of both conditions; and (ii) compare the influence of these conditions in the perception of the nociceptive stimuli.

Materials and Methods

Animals

For this study, 144 female *Wistar* rats were used, from the Multidisciplinary Center for Biological Research (CEMIB - University of Campinas, Unicamp, Brazil). This study was approved by the Local Ethics Committee on Animal Use (# 2633-1). The animals were kept in a room with controlled temperature (23 ± 1 °C), and housed in plastic

cages (five animals per cage) with 12 h light/dark cycle (lights at 07:00 am) and availability of feed and water *ad libitum* for at least two months before the experiments, until they reached the ideal weight for onset of behavior assessment (200-250 g).

Experimental design

After reaching body weight of 200-250g the 144 animals were randomly divided into three groups: Group I (n = 48) that was induced by chronic inflammation in the right TMJ with Complete Freund's Adjuvant (CFA), Group II (n = 48), where the animals were subjected to chronic stress for 40 days, Group III (n = 48), the animals were submitted to both conditions, chronic TMJ inflammation and chronic stress. Each group was divided into six other subgroups (n = 8), in which one of the following solutions was injected: 80% sterile saline solution, 10% Tween 80 and 10% ethanol (control subgroup); 0; 1; 2; 3; 4 or 5 µg of piperine diluted in 100 ml of 80% sterile saline, 10% Tween 80 and 10% ethyl alcohol.

Articular Inflammation

The rats were initially anesthetized with a mixture of 55 mg/kg ketamine (Dopalen, Vetbrands Ltda Brazil, Goiania, GO) and 5.5 mg/kg xylazine (Rompum, Bayer, São Paulo, SP) (Flake *et al*, 2005). Chronic inflammation of the right TMJ was induced by intra-articular injection of 50 µl CFA (15 µg dead heat mycobacterium) (Sigma-Aldrich, St. Louis, USA) in an oil/saline suspension (1:1) (Wang *et al*, 2009). For injection of all intra-articular solutions, a cannula was used, in which one extremity was connected to a disposable needle 0.45 mm x 13 mm, and the other to 50 µl Hamilton syringe (Hamilton Company, Reno, NV, USA) (Roveroni *et al*, 2001). For evaluation of animal behavior, a period of 10 to 12 days was required for the development of chronic inflammation in the animal TMJ (Hutchins *et al*, 2000).

Stress Exposure

The animals were stressed based on a chronic model during 1 h a day, five days per week for 40 days (Ely *et al*, 1997). The procedure was performed by placing the animal

inside a small plastic perforated box to isolation (Gameiro *et al*, 2005). The process was conducted in a quiet room separate from 08:00 to 10: 00 h.

Analysis of Phase Hormonal Cycle

The diestrous phase of the estrous cycle was identified by the rat vaginal secretion, characterized by the predominance of leukocytes (irregular cells) (Mandl, 1951). Briefly, in the morning, before starting the experiment, vaginal smear was collected using an automatic micropipette and 10 ml of saline (0.9% NaCl). After arresting the rat, the pipette tip was inserted into the vagina, released saline and the vaginal secretion was aspirated. The fluid collected was placed on a glass slide and observed under a microscope 40x objective lens (Marcondes *et al*, 2002). The animals in the diestrous phase followed for the next stage of the experiment, whereas the others were evaluated on subsequent days until they were in the hormonal phase of interest. Therefore, TMJ pain in women is higher during periods of low circulating levels of ovarian hormones (LeResche *et al*, 2003).

Piperine Injection

The six solutions of piperine were prepared from commercially available stock of piperine (Sigma-Aldrich, St. Louis, USA) containing 0, 1, 2, 3, 4 or 5 µg diluted in 10% ethyl alcohol (Chemco, Ltda, Sao Paulo, Brazil), 10% Tween 80 (Dynamic, Ltda, Sao Paulo, Brazil), and sterile saline (Med Flex ®, Rio de Janeiro, Brazil), totaling a final volume of 100 ml for each solution.

Animals in the diestrous phase of the hormonal cycle were randomly distributed into subgroups, and anesthetized with halothane in order to inject into the TMJ region one of this different concentrations of piperine solution: 0 (ethyl alcohol, Tween 80 and sterile saline, control subgroup) (n = 8), $1x10^{-2}$ µg/ml (n = 8), $2\ x10^{-2}$ µg/ml (n = 8), $3\ x10^{-2}$ µg/ml (n = 8), $4\ x10^{-2}$ µg/ml (n = 8), $5\ x10^{-2}$ µg/ml (n = 8). The injections were performed by an examiner who was blinded to the experimental design, which determined the injection site by palpation of the zygomatic arch area, and the needle was inserted just below the edge of the posterior lateral condyle. The needle was introduced in the antero posterior direction until reaching the posterior lateral condyle surface, in which 30 µl of the selected solution

was injected. This procedure used a cannula prepared as previously described (Roveroni *et al*, 2001).

Assessment of Nociceptive Behavior

Rats nociceptive behavior was evaluated by a blinded calibrated examiner during the light phase (8:00 - 17:00 h),. The animals were individually placed in a mirrored chamber (30 x 30 x 30 cm) during 10 minutes for a habituation, and no feed or water was available. After this period, the animals were lightly anesthetized by inhalation of halothane (Sigma-Aldrich, St Louis, USA) for the TMJ injection. One investigator performed all the different injections in the right TMJ, using a solution of 30 µl of 0, 1, 2, 3, 4 or 5 µg of piperine diluted in 100 ml of control solution, depending on the group. After recovering consciousness, the animal was placed back into the mirrored chamber for nociceptive behavior evaluation. The nociceptive behavior characterized by rubbing the orofacial region (Clavelou *et al*, 1995) and flinching the head (Roveroni *et al*, 2001) was counted for 45 minutes divided into 9 blocks of 5 minutes. For each block, the amount of time that the animal spent rubbing the orofacial region was quantified in seconds. The head flinches were counted in number of times that happened, and it followed a uniform pattern of a second duration per movement (Gameiro *et al*, 2005; Roveroni *et al*, 2001). The behaviors were quantified in seconds (Roveroni *et al*, 2001). The nociceptive behaviors counted during the 45 minutes observation were evaluated separately, and then their sum was also used for statistical analysis (Roveroni *et al*, 2001). After the behavior test, and according to the International Association for the Study of Pain, all animals were sacrificed.

Statistical Analysis

For evaluation of nociceptive behaviors, the data with homogeneity of variance were compared by two-way ANOVA, and multiple post-hoc comparisons were performed by Tukey-Kramer, with the significance level of 5%. The data were presented as mean and standard deviation.

Results

Nociceptive behavior by Piperine under the condition of Articular Inflammation

Chronic joint inflammation induced by injection of CFA showed statistically significant difference in head flinching behavior between $5x10^{-2}$ µg/ml (69.50 ± 16.44) and control subgroups (40.13 ± 15.06). There was difference among the $1x10^{-2}$ (21.63 ± 5.15); $4x10^{-2}$ (53.87 ± 19.61) and $5x10^{-2}$ µg/ml (69.50 ± 16.44) experimental subgroups as well. The orofacial rubbing behavior had no significant difference among the subgroups. For the sum of the behaviors, there was significant difference among control (52.85 ± 17.46) and $4x10^{-2}$ (105.37 ± 22.64) and $5x10^{-2}$ µg/ml (115.50 ± 35.14) subgroups, as well as $1x10^{-2}$ (62.75 ± 21.67) and $5x10^{-2}$ µg/ml subgroups (P <0.05) (Fig. 1 and 2).

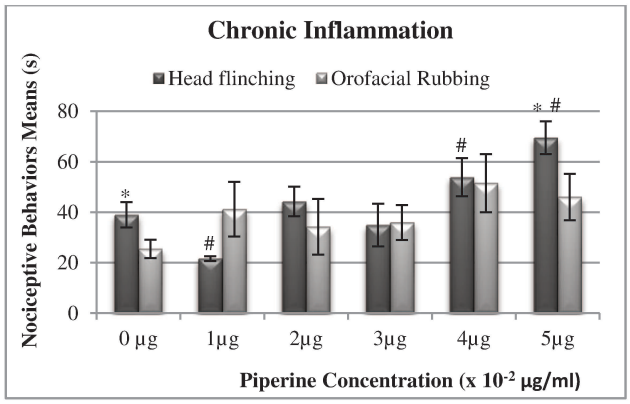

Fig 1: Mean values of behaviors of head flinching and orofacial rubbing after injection into the TMJ region of increasing concentrations of piperine in the presence of chronic inflammation. The error bar indicates the SEM. (*) indicates significant difference when compared with the control subgroup (0 µg piperine), and (#) indicates significant difference among the experimental subgroup (P <0.05 for all comparisons, test Tukey-Kramer).

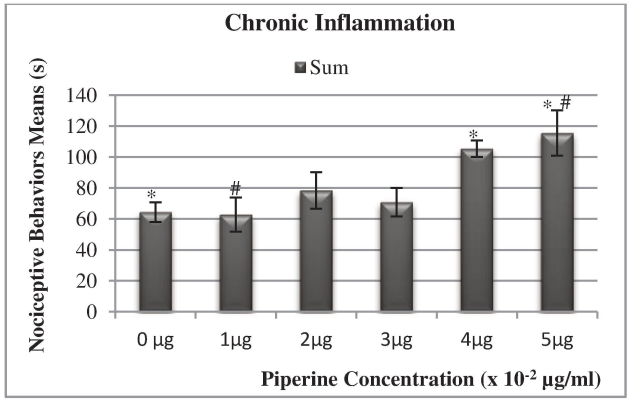

Fig 2: The mean of the sum of nociceptive behaviors, head flinches and orofacial rubbing after injection of different concentration of the solution of piperine in the right TMJ in the presence of chronic inflammation. The error bar indicates the SEM. The significant difference between the subgroups is shown (*) when compared with the control subgroup. (#) indicates significant difference between the experimental subgroups (P <0.05 for all comparisons, Tukey-Kramer).

Nociceptive behavior by Piperine under the Condition of Chronic Stress

The results presented in Figures 3 and 4 show that the head flinching behavior among control (49.87 ± 10.70) and $4x10^{-2}$ (85.87 ± 19.21) and $5x10^{-2}$ µg/ml (83.63 ± 12.69) of piperine subgroups were statistically different (P <0.05).

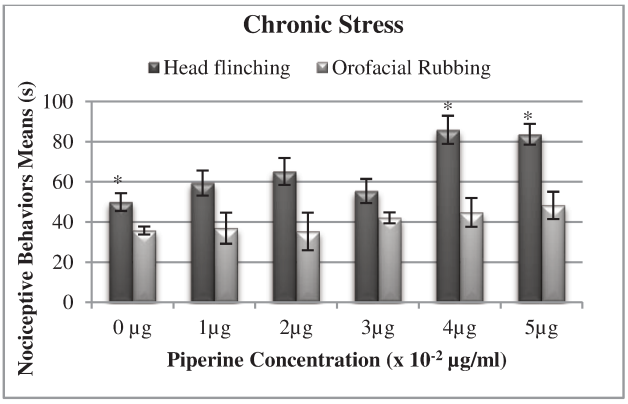

Fig 3: Results of head flinching and orofacial rubbing behaviors after injection into the TMJ region of increasing concentrations of piperine on the condition of chronic stress. The error bar indicates the SEM. For head flinching (*) indicates significant difference when compared with the control subgroup (0 µg piperine) (P <0.05 for all comparisons, Tukey-Kramer).

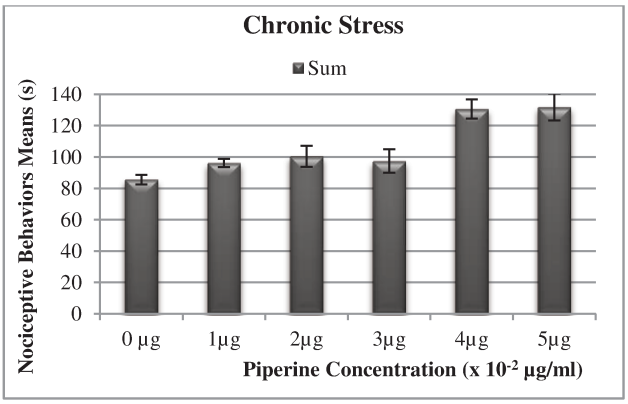

Fig 4: Representing the sum of nociceptive behaviors after injection of different concentration of the solution of piperine on the condition of chronic stress. The error bar indicates the SEM (P <0.05 for all comparisons, Tukey-Kramer).

Nociceptive behavior by Piperine under the Condition of the Association of Articular Inflammation and Chronic Stress

The association of inflammatory conditions and chronic stress had significant difference between control (60.75 ± 17.54) and $5x10^{-2}$ µg/ml (92.87 ± 18.35) subgroups for head flinching behavior. A significant statistically difference between $1x10^{-2}$ (65.13 ± 6.64) and $5x10^{-2}$ µg/ml subgroups was also observed (P <0.05).

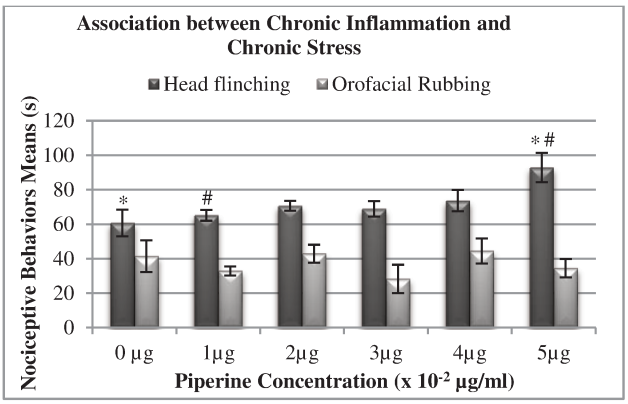

Fig 5: Results of nociceptive behaviors, head flinching and orofacial rubbing after injection into the TMJ region of increasing concentrations of piperine in the join chronic inflammation and stress. The error bar indicates the SEM. For head flinches (*) indicates significant difference when compared with the control subgroup (0 µg piperine) and (#) indicates difference with the 1x10-2 µg/ml subgroup (P <0.05 for all comparisons, Tukey-Kramer).

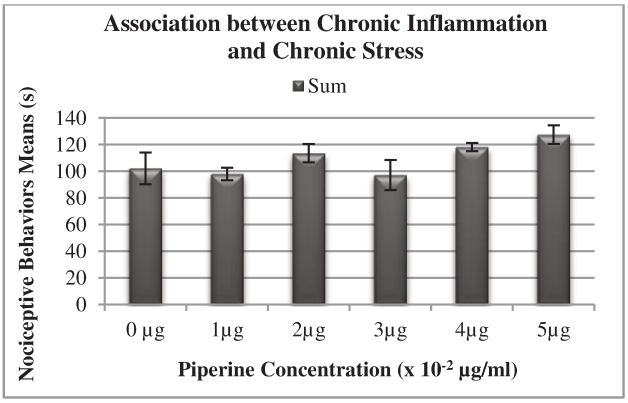

Fig 6: The mean of the sum of nociceptive behaviors, head flinching and orofacial rubbing, after injection of different concentration of the solution of piperine in the join condition of chronic inflammation and stress. The error bar indicates the SEM. (P <0.05 for all comparisons, Tukey-Kramer).

Analysis of Nociceptive Behaviors in Different Concentrations of Piperine under Chronic Inflammation, Chronic Stress and Association of both Conditions

The sub group that received $5x10^{-2}$ µg/ml showed the highest mean of head flinching behavior (P <0.05) in the combination of both treatment conditions (chronic inflammation and stress) (Table 1).

Table 1: Mean (± standard deviation) of the head flinching behavior after the injection of piperine in different concentrations and evaluated in different conditions of chronic joint inflammation, chronic stress and combination of both (P <0.05).

Concentration (x 10^{-2} µg/ml)	Condition		
	Chronic Inflammation	Chronic Stress	Association
0	40.13(±15.06) Aabc	49.87(±10.70) Ab	60.75 (± 17.54) Ab
1	21.63(±5.15) Bc	59.37(±13.66) Aab	65.13 (± 6.64) Ab
2	44.25(±23.20) Aabc	65.13(±15.60) Aab	70.63 (± 6.93) Aab
3	34.87(±19.70) Bbc	55.37(±11.71) ABb	68.87 (± 8.69) Aab
4	53.87(±19.61) Bab	85.87(±19.21) Aa	73.63 (± 19.83) ABab
5	69.50(±16.44) Aa	83.63(±12.69) Aa	92.87 (± 18.35) Aa

The average values of the orofacial rubbing behavior had no difference among treatment conditions in different piperine concentrations, as shown in Table 2.

Tabela 2: Mean (± standard deviation) of the orofacial rubbing behavior after the injection of piperine in different concentrations and evaluated in different conditions of chronic joint inflammation, chronic stress and combination of both (*P<0.05)*.

	Condition		
Concentration (x 10^{-2} µg/ml)	Chronic Inflammation	Chronic Stress	Association
0	14,71 (± 7,54) Aa	35,75 (± 12,69) Aa	41,37 (± 19,78) Aa
1	41,13 (± 20,81) Aa	36,87 (± 17,96) Aa	32,87 (± 6,75) Aa
2	34,13 (± 25,59) Aa	35,25 (± 19,58) Aa	42,87 (± 17,05) Aa
3	35,87 (± 16,14) Aa	42,00 (± 10,84) Aa	28,25 (± 18,11) Aa
4	51,50 (± 28,73) Aa	44,75 (± 20,53) Aa	44,50 (± 22,63) Aa
5	46,00 (± 22,53) Aa	48,25 (± 27,25) Aa	34,50 (± 18,45) Aa

The table 3 shows the average values of the sum of the nociceptive behaviors. The $5x10^{-2}$ µg/ml subgroup in the chronic stress exhibited higher means ($P < 0.05$).

Tabela 3: Mean (± standard deviation) of the sum of the nociceptive behaviors after the injection of piperine in different concentrations and evaluated in different conditions of chronic joint inflammation, chronic stress and combination of both conditions (*P<0.05)*.

	Condition		
Concentration (x 10^{-2} µg/ml)	Chronic Inflammation	Chronic Stress	Association
0	52,85 (± 17,46) Bc	85,63 (± 17,03) Aab	102,13 (± 22,74) Aa
1	62,75 (± 21,67) Abc	96,25 (± 15,03) Aa	98,00 (± 10,51) Aa
2	78,37 (± 32.08) Aabc	100,37 (± 15,50) Aa	113,50 (± 17,96) Aa
3	70,75 (± 24,91) Aabc	97,37 (± 17,50) Aa	97,13 (± 23,97) Aa
4	105,37 (± 22,64) Aab	130,63 (± 31,22) Aa	118,13 (± 6,20) Aa
5	115,50 (± 35,14) Aa	131,87 (± 35,05) Aa	127,37 (± 22,44) Aa

Discussion

The TMD pain is a very common symptom, involving the masticatory muscles and/or joint (Yap *et al*, 2002), with higher incidence in women, in which female hormones are one of the possible factors involved in the occurrence (Cairns, 2010). Besides the biological damage caused by the maintenance of pain sensation and imbalance in the modulation system, chronic pain often affects patients in a variety of psychosocial and behavioral comorbid conditions, such as impaired of life quality, stress, depression and

sleep disorders (Yunus, 2008). The central sensibilization, responsible for long-term pain in patients, is caused by the high magnitude and/or repetitive nociceptive impulses that can cause peripheral and central neuronal changes, leading to maintenance and exacerbation of pain sensation (Conti *et al*, 2003). This may explain the failure of treatment in some patients with TMD.

Patients with joint changes, such as disc displacement, show an increased expression of CGRP and substance P (Sato *et al*, 2007) into the TMJ, and a positive correlation with the intensity of pain (Cairns, 2010). Functional overloading on the joint can induce severe and progressive joint damage that promote increased expression of these neuropeptides (Tanaka *et al*, 2008), that contribute to the peripheral mechanisms of pain (Cairns, 2010). The TMJ inflammation results from the release of many proinflammatory cytokines, especially tumor necrosis factor-α (TNF-α) and interleukin (1, 6, 12 and 17) (Vernal *et al*, 2008), which facilitate the release of pro-nociceptive compounds which include leukotriene B4, prostaglandin (PG) E2, bradykinin, histamine (Cairns, 2010). The excitation of TMJ nociceptors by these pro-nociceptive compounds further enhances the release of CGRP and substance P (Cairns, 2010). There is also a strong relationship between joint pain and the detection of IL-1β in synovial fluid (Takahashi *et al*, 1998).

Based on data from this experimental research, the minimum concentration to provide a nociceptive behavior in the presence of chronic joint inflammation was 4×10^{-2} µg/ml of piperine. It is known that during inflammation conditions, the expression of TRPV1 is increased, as pro-inflammatory substances released during this process as well as low pH have the ability to activate TRPV1 (Di Marzo *et al*, 2002). Besides this, activation of other membrane receptors by inflammatory mediators such as bradykinin and TNF can lead to activation of TRPV1 (Prescott & Julius, 2003). It is therefore suggested that, higher amount of active receptors, the greater the amount of piperine can bind to TRPV1 and hence increases the nociceptive responses. This increased expression of TRPV1 justifies pharmacological studies in order to block the activation of this receptor, which is useful in cases of chronic pain and inflammatory hyperalgesia (Szallasi & Appendino, 2004).

Psychological factors, such as stress and depression, are related to the TMD (Sullivan *et al*, 2001) in the process of pain perception (Gameiro *et al*, 2006; Gamsa, 1994)

and implicated in the predisposition, initiation and perpetuation of TMD (Rollman & Gillespie, 2000; List & Dworkin, 1996). The stress is related to TMD pain because they can induce muscle hyperactivity and parafunctional habits, and also interfere in pain perception and response to conventional therapies in the dysfunction treatment (Dworkin & Turner, 2004; Rollman & Gillespie, 2000). In TMD, the orofacial pain is a potent stimulus for stress, as many patients have hyperactivity of the hypothalamic-pituitary-adrenal axis (Gameiro *et al*, 2006).

In this study, the group of chronic stress also exhibited the greatest results of nociceptive behavior in the minimum concentration of 4 x 10^{-2} µg/ml of piperine. We confirm that the TMD pain seems to be influenced by psychological changes (Dworkin, Huggins *et al*, 2002). The study of stress in TMD pain conditions is critical due to its strong influence on pain perception and response to treatment. Comparing the conditions of chronic inflammation and chronic stress in the same piperine concentration, 4 x 10^{-2} µg/ml, the results exhibited significant difference only for head flinches. But even though, the means values in chronic stress were higher than the chronic inflammation group, showing the stress influence over the pain perception.

The association of chronic inflammatory and stress conditions had no significant difference between the subgroups, except 5 x 10^{-2} µg/ml of piperine subgroup had only statistics difference for the head flinching behavior. This can be explained by the presence of common mediators of stress and inflammation conditions competing for the same binding site of piperine, so that the increase in the piperine concentration had no effect on nociceptive response. Another possible explanation is that many of the molecules that are involved in stress responses are the same as those associated with pain modulation, so that stress may influence the pain biological processes (Gameiro *et al*, 2006). Differing what was thought initially, group III didn't show the sum of the inflammation and stress influence over the nociceptive responses. And, as it didn't exhibited significant difference between the groups II and III, it can suggest that the stress presence, by itself, influenced the pain perception.

Pain is a complex and multidimensional experience, envolving sensory-discriminative, cognitive, emotional and motivational dimensions (Wadhwa & Kapila,

2008). Thus, pain conditions in the orofacial region can't be treated only for remission of symptoms, but also on factors that may affect the pain perception and response to subsequent therapy. According to the data obtained in this study, we can conclude that: (i) previous chronic inflammation condition, the minimum concentration to provide enhanced nociceptive behavior on orofacial region was 4 x 10^{-2} µg/ml of piperine; (ii) in chronic stress condition, the concentration of 4 x 10^{-2} µg/ml of piperine also exhibited significant differences when compared to its control subgroup; (iii) in the presence of chronic inflammation and stress, there was no significant difference among the different piperine concentrations on nociceptive response, but the concentration of 5 x 10^{-2} µg/ml showed the highest mean in this condition; (iv) comparing the conditions, the chronic inflammation group exhibited the lower values, suggesting a higher influence of the stress over the pain perception; (v) and the presence of chronic inflammation and stress in the group III didn't show the sum of the influence of these conditions on the nociceptive responses.

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Conclusão

Baseado nos dados obtidos neste estudo, para avaliar comportamento nociceptivo na região da ATM por meio da ativação do TRPV1 usando diferentes concentrações de piperina, pode-se concluir que:

- Na condição de normalidade na ATM de ratas, a concentração mínima efetiva 0,02 µg/ml de piperina pode ser utilizada para promover resposta nociceptiva;
- Na presença de inflamação crônica articular, a concentração mínima efetiva para causar resposta nociceptiva na região da ATM foi de 0,04 µg/ml de piperina;
- Em condições de estresse crônico, a solução de 0,04 µg/ml de piperina foi a concentração mínima efetiva para desencadear uma resposta nociceptiva na ATM;
- Na presença da associação das condições de inflamação e estresse crônicos não apresentou diferença estatística significante entre os grupos, mesmo a solução de 0,05 µg/ml tendo apresentado os maiores valores.

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Anexo 1

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Comissão de Ética no Uso de Animais
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C E R T I F I C A D O,

Certificamos que o projeto **"Desenvolvimento de Modelo de Comportamento de Dor na ATM em Ratos Fêmeas usando Piperina em condições de Normalidade, Inflamação Local Prévia e Estresse Animal"** (protocolo nº **2633-1**), sob a responsabilidade de **Profa. Dra. Célia Marisa Rizzatti-Barbosa / Ana Paula Varela Brown Martins**, está de acordo com os **Princípios Éticos na Experimentação Animal** adotados pela **Sociedade Brasileira de Ciência em Animais de Laboratório (SBCAL)** e com a legislação vigente, **LEI Nº 11.794, DE 8 DE OUTUBRO DE 2008**, que estabelece procedimentos para o uso científico de animais, e o **DECRETO Nº 6.899, DE 15 DE JULHO DE 2009**.

O projeto foi aprovado pela Comissão de Ética no Uso de Animais da Universidade Estadual de Campinas - CEUA/UNICAMP - em **26 de março de 2012**.

Campinas, 26 de março de 2012.

Profa. Dra. Ana Maria A. Guaraldo
Presidente

Fátima Alonso
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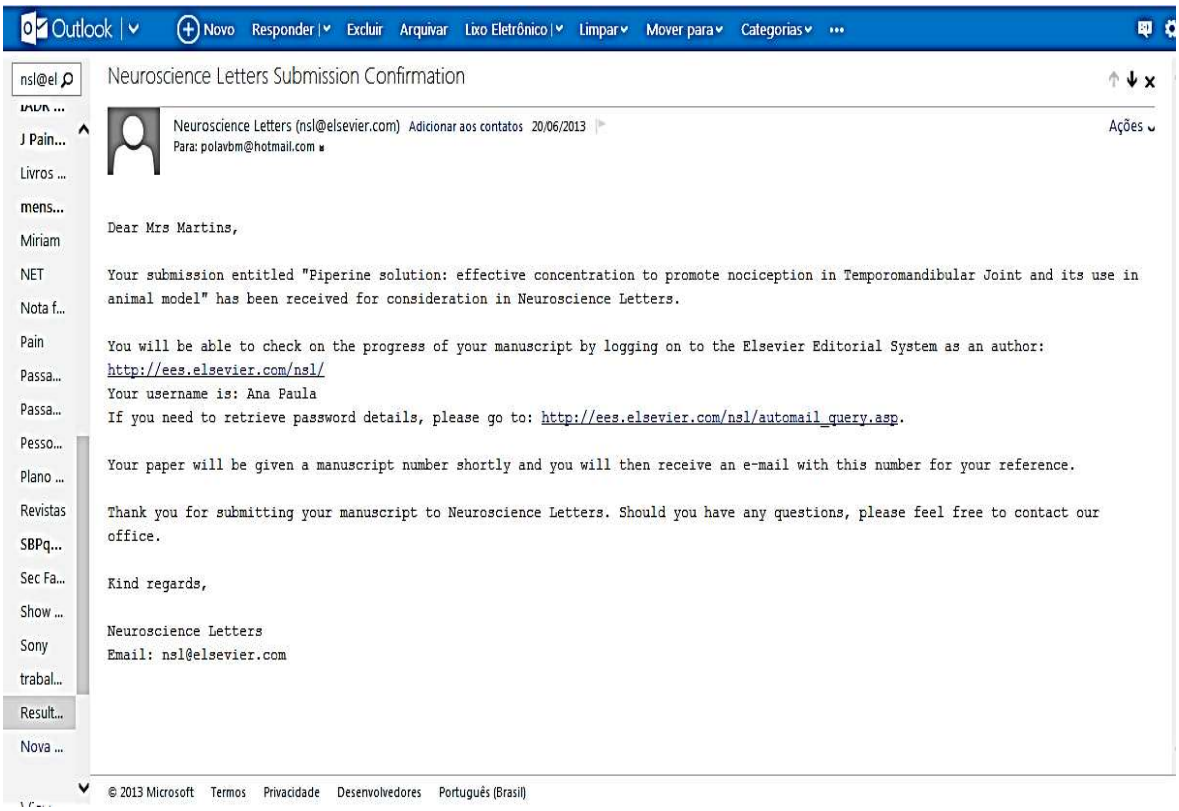

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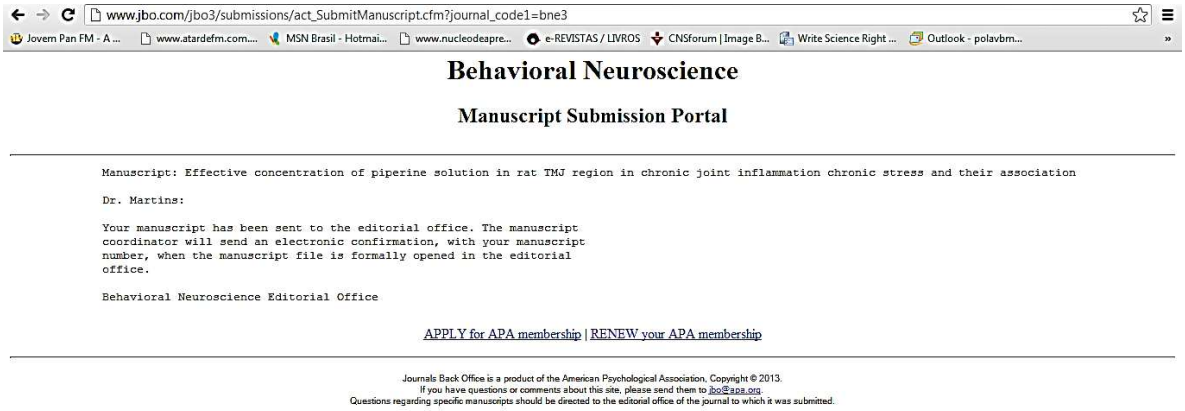

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