Universidade Estadual de Campinas

Faculdade de Odontologia de Piracicaba

Luiz Marques da Rocha Neto

A FASE INICIAL DO DIABETES TIPO 1 INDUZ HIPONOCICEPÇÃO NA ARTICULAÇÃO TEMPOROMANDIBULAR DE RATOS.

EARLY PHASE OF TYPE 1 DIABETES INDUCES HIPONOCICEPTION IN THE TEMPOROMANDIBULAR JOINT OF RATS.

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Tese apresentada à Faculdade de Odontologia de Piracicaba da Universidade Estadual de Campinas como parte dos requisitos exigidos para a obtenção do título de Doutor em Odontologia, na Área de Fisiologia Oral.

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Orientadora: Profa. Dra. Juliana Trindade Clemente Napimoga

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RESUMO

As condições dolorosas induzidas pelo diabetes são uma consequência do dano nos neurônios nociceptivos aferentes periféricos. Este estudo estabelece modelo experimental em animais que avalia o efeito do diabetes tipo 1 em diferentes tipos de modelos nociceptivos na articulação temporomandibular de ratos (ATM). Ratos Wistar (± 150 g, n = 4 - 6 / grupo) foram tratados com injeção intraperitoneal de veículo (grupo normoglicêmico - NG) ou estreptozotocina 75 mg / kg (grupo diabético - DB). O efeito do diabetes na ativação de neurônios nociceptivos aferentes primários foi avaliado pelo comportamento nociceptivo dos animais induzido por uma injeção intra-articular de 1,5% de formalina ou 1,5% de capsaicina aos 7, 14, 21 e 28 dias após a indução diabética. Após os ensaios comportamentais nociceptivos, os animais foram eutanasiados e os seus subnúcleos caudal trigeminal, gânglio trigeminal e tecido periarticular foram removidos para análises posteriores. O diabetes induziu redução da ativação de fibras C primárias, caracterizadas pela redução do nível proteico de neuropeptídeos substância P (SP) e o peptídeo relacionado ao gene da calcitonona (CGRP) nos tecidos periarticulares. Animais diabéticos pré-tratados com inibidores de PKC-α e -β, GO6976 e LY333531 aumentaram significativamente a nocicepção induzida por capsaicina na ATM e aumento nos níveis de proteína da ATPase trocadora de sódio-potássio (Na^+ / K^+-ATPase) no gânglio trigeminal. Foi observado no subnúcleo caudal do trigeminal dos animais diabéticos um aumento do nível protéico de CX3CR1 (marcador de células microgliais) e proteína quinase ativada por mitógeno p38 (p38MAPK). Além disso, o diabetes reduziu significativamente a nocicepção induzida pela formalina na ATM. Por outro lado, animais diabéticos demonstraram um aumento nos nível proteico de citocinas pró-inflamatórias interleucina 1-β (IL1-β) e da quimiocina derivada de queratinócios (KC) no tecido peri-articular. No geral, os resultados do presente trabalho sugerem que a fase inicial do diabetes reduz a ativação primária das fibras C associada ao aumento do nível protéico das isoformas PKC α e β e à redução da Na^+ / K^+ ATPase. Por outro lado, a fase inicial do diabetes aumentou os níveis proteicos das citocinas pró-inflamatórias IL1-β e KC.

Palavras-chave: diabetes mellitus tipo 1, nociceptividade, articulação temporomandibular, ATPase trocadora de sódio-potássio.

ABSTRACT

Diabetes-induced painful conditions are a consequence of damage in the peripheral nociceptive afferents. This study stablishes animal experimental model that evaluates the effect of diabetes type 1 in different types of nociceptive models in the temporomandibular joint of rats (TMJ). Wistar rats (± 150 g, n = 4 - 6/group) were treated with an intraperitoneal injection of vehicle (normoglycemic – NG group) or Streptozotocin 75 mg/kg (diabetic – DB group). The effect of diabetes in the activation of primary afferent nociceptive neurons was assessed by the animals' nociceptive behavior induced by an intra-articular injection of 1.5% capsaicin or 1.5% formalin at 7, 14, 21, and 28 days after the diabetic induction. After nociceptive behavioral assays the animals were euthanized and their trigeminal subnucleus caudalis, trigeminal ganglion and/or periarticular tissue were removed for further analyses. Diabetes induced a reduction of activation of primary C-fibers characterized by the reduction of protein level of neuropeptides substance Pain (SP) and calcitonin gene-related peptide (CGRP) in the periarticular tissues. Diabetic animals pre-treated with PKC-α and -β inhibitors, GO6976 and LY333531 significantly increased capsaicin-induced nociception in the TMJ and elicited protein levels of sodium-potassium-exchange ATPase (Na^+/K^+ATPase) in the trigeminal ganglia. In the trigeminal subnucleus caudalis of diabetic animals it was observed an increase of protein level of CX3CR1 (microglial cell marker) and p38MAPK. In addition, diabetes significantly reduced the formalin-induced nociception in the TMJ. Otherwise, diabetic animals was demonstrated an elicited protein level of pro-inflammatory cytokines interleukin 1-β (IL1-β) and Keratinocyte chemoattractant (KC) in the periarticular tissue. Overall, the results of the present work suggest that early phase of diabetes reduce the primary C-fibers activation associated with the increased protein level of PKC α and β isoforms and reduction of Na^+/K^+ATPase. On the other hand, early phase of diabetes increased protein level of pro-inflammatory cytokines IL1-β and KC.

Keywords: diabetes mellitus type 1, nociception, temporomandibular joint, sodium-potassium-exchange ATPase

SUMÁRIO

1 INTRODUÇÃO

O diabetes mellitus (DM) consiste em um distúrbio metabólico caracterizado por hiperglicemia persistente, decorrente de deficiência na produção de insulina ou na sua ação, ou em ambos os mecanismos, ocasionando complicações em longo prazo (SBD, 2017). Em 2017, a Federação Internacional do Diabetes declarou que aproximadamente, 425 milhões de pessoas no mundo têm a doença e estima-se que, para o ano de 2045, esse número chegue a 628 milhões, com custos da ordem de 727 bilhões de dólares, somente neste ano.

Atualmente, o Brasil ocupa a quarta colocação entre os dez países com maior número de pessoas com diabetes, com cerca de 14,3 milhões de pessoas diagnosticadas com idade entre 20 – 79 anos (SBD, 2017). Trata-se, assim, de um crescente problema de saúde pública, cujos dados alarmantes preocupam, tendo em vista que as complicações da doença causam um alto índice de morbidade e mortalidade e diminuição da qualidade de vida (Lin et al., 2014; Ogurtsova et al., 2017).

A classificação do DM é baseada na sua etiologia e não mais no tipo de tratamento, como era feito no passado. A classificação proposta pela OMS e pela Associação Americana de Diabetes inclui quatro classes clínicas: DM tipo 1A e B, DM tipo 2, outros tipos específicos de DM e DM gestacional. A maioria dos casos de diabetes divide-se entre as duas principais categorias etiopatogênicas – o DM tipo 1 e tipo 2 (ADA - American Diabetes Association, 2018).

O DM tipo 1 é uma doença autoimune, poligênica, decorrente de destruição das células β pancreáticas, ocasionando deficiência completa na produção de insulina. Na maioria das vezes, essa destruição de células beta é mediada por autoimunidade, confirmada pela positividade de um ou mais autoanticorpos (tipo 1A), porém há casos em que não há evidências de processo autoimune, sendo, portanto, referidos como forma idiopática (tipo 1 B). O DM tipo 1 corresponde a 5% a 10% dos casos, e é mais frequentemente diagnosticado em crianças, adolescentes e, em alguns casos, em adultos jovens, afetando igualmente homens e mulheres. O DM tipo 2 possui etiologia complexa e multifatorial, envolvendo componentes genético e ambiental, corresponde a 90% a 95% dos casos, e caracteriza-se por defeitos na ação e secreção da insulina, sendo vinculado a sobrepeso ou obesidade (SBD, 2017).

Independente da etiologia, o DM apresenta como característica comum um quadro de hiperglicemia. A hiperglicemia crônica está associada a lesões de longa duração, a disfunções e a falha de vários órgãos, incluindo retinopatias, com potencial perda de visão; nefropatias, que possibilitam a falha renal; neuropatia periférica, com risco de pé diabético, amputação e articulação de Charcot; e neuropatias autonômicas, causadoras de sintomas gastrointestinais, uroginecológicos, cardiovasculares, além de disfunção sexual (ADA - American Diabetes Association, 2005).

As neuropatias diabéticas (ND) envolvem a perda progressiva das fibras nervosas do sistema nervoso periférico somático e autonômico que acarretam sequelas importantes entre pacientes com DM (Boulton et al., 2004). Dentre as complicações do diabetes, as neuropatias periféricas sensoriais são as mais comuns, afetando mais de 50% dos pacientes diabéticos (Boulton et al., 2004; Schreiber, 2015). Contudo, se pacientes com níveis subclínicos de distúrbios neuropáticos forem incluídos, a prevalência pode exceder os 90% (Yagihashi et al., 2011). Essa não é uma condição unitária, mas é o resultado de distúrbios no sistema nervoso periférico, como uma consequência da hiperglicemia (Boulton et al., 2004; Malik et al., 2005).

As neuropatias diabéticas induzem a uma variedade de alterações na condução nervosa, incluindo sintomas positivos e negativos (Calcutt, 2004). Os sintomas positivos incluem dor espontânea, parestesia, hiperalgesia (aumento da sensibilidade a um estímulos nocivo) e alodínia (dor causada por um estímulo inócuo). O sintomas negativos consistem na perda da percepção sensorial e motora, associadas com diminuição da velocidade de condução nervosa e amplitude de potenciais de ação em nervos sensitivos e motores, que levam a quadros de hipoalgesia (diminuição da resposta dolorosa a um estímulos nocivo) e analgesia (ausência de sensibilidade a um estímulo nocivo) (Calcutt, 2004; Dobretsov et al., 2007).

Os quadros hipoalgésicos provocados pelo diabetes estão vinculados, à alterações estruturais nas fibras neuronais, como rompimento das células de Schwann (desmielinização), degeneração, perdas axonais, lesões microvasculares, e alterações nas sinalizações bioquímicas intracelulares (Arezzo and Zotova, 2002). As complicações envolvidas com a neuropatia periférica induzida pelo diabetes, como também os mecanismos celulares e moleculares envolvidos com o início e manutenção dessas neuropatias ainda são pouco compreendidos (Daulhac et al.,

2006). A patogênese da neuropatia periférica diabética (NPD) continua sendo um assunto controverso entre os pesquisadores. Muitos trabalhos têm sido publicados nos últimos anos, porém é bastante improvável que haja apenas uma causa, mas sim uma associação de diferentes anormalidades, culminando em um quadro clínico comum de neuropatia (Tesfaye et al., 2010; Singh et al., 2014).

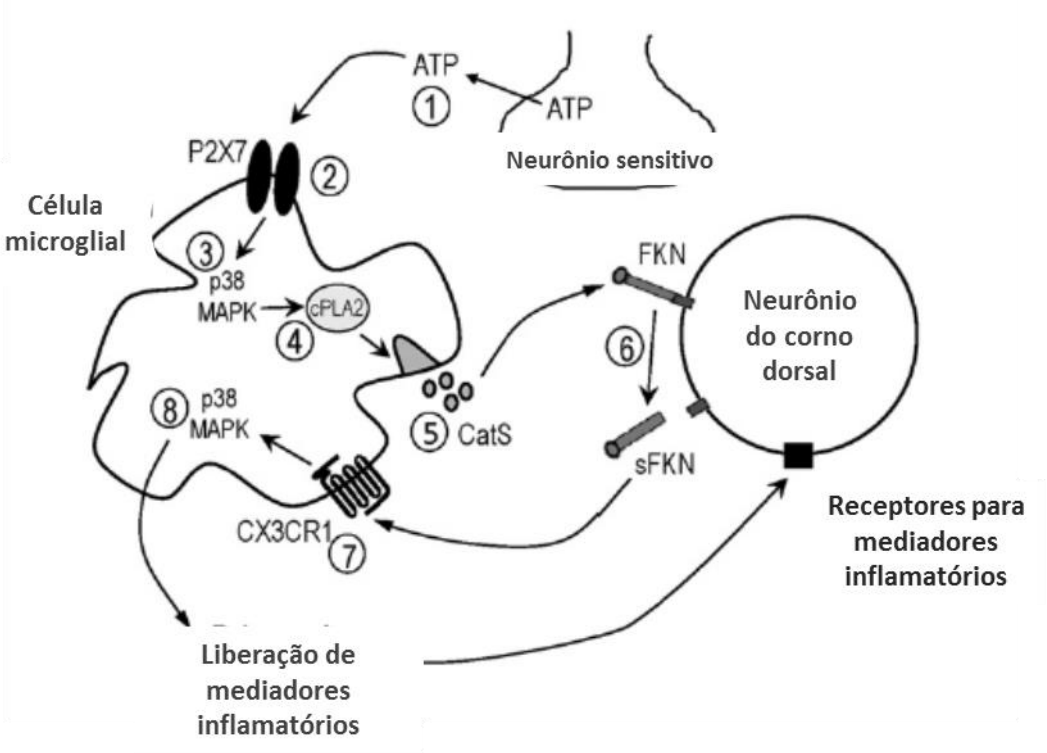
Sabe-se que mecanismos periféricos e centrais estão envolvidos na fisiopatogênese da neuropatia diabética dolorosa. As alterações periféricas estão relacionadas com a distribuição e expressão de canais de sódio e de cálcio; expressão alterada de neuropeptídios; perda do controle inibitório muscular; alteração no fluxo sanguíneo periférico; atrofia e degeneração axonal; dano em fibras finas e aumento do fluxo glicêmico. Em relação às alterações centrais, considera-se: a sensibilização central, alteração da facilitação e inibição das vias descendentes, o aumento da vascularização talâmica, entre outros (Tesfaye et al., 2010, 2013).

A sensibilização central refere-se ao processo através do qual um estado de hiperexcitabilidade está estabelecido no sistema nervoso central, levando a um aumento da transmissão das mensagens nociceptivas (Woolf, 1983). Inúmeros mecanismos têm sido implicados na sensibilização central, entre ele as interações neuro-gliais. As células gliais, principalmente micróglias e astrócitos, também contribuem para o processo de sensibilização central decorrente de uma lesão periférica ao promoverem a liberação de moléculas que vão se ligar a receptores em terminais pré e pós-sinápticos no corno dorsal da medula para modular a transmissão sináptica excitatória e inibitória (Basbaum et al., 2009; Grace et al., 2014), como no caso da neuropatia diabética periférica.

As micróglias são células fagocitárias, originadas da migração de monócitos da medula óssea, responsáveis pela imunidade inata do sistema nervoso central (SNC) (Ji et al., 2009). As células microgliais têm propriedades morfológicas e funcionais distintas que são desenvolvidas sob a influência de neurônios e outras células da glia (McMahon and Malcangio, 2009). Em muitos estudos, as células microgliais vêm sendo relacionadas como um fator importante tanto na indução quanto na manutenção de transtornos sensoriais seguidos de injúrias neurais e/ou teciduais periféricos (Daulhac et al., 2006; Xie et al., 2007; McMahon and Malcangio, 2009; Chiang et al., 2011).

Nesse sentido, tem sido demonstrado na medula espinhal que frente a uma injúria neural e/ou tecidual periférica, o aumento da concentração extracelular de ATP

ativa os receptores purinérgicos P2X7 localizados na micróglia que, por sua vez, ativa a via p38 proteína-quinase-ativada-por-mitógeno (p38 MAPK) e libera a protease Catepsina S (CatS). A CatS cliva a Fractalcina (FKN) (uma proteína transmembrana presente no neurônio) que libera sua porção solúvel, ativando o receptor CX3CR1 na micróglia. Uma vez ativado, o receptor CX3CR1 promove a fosforilação da p38 MAPK, o que leva à liberação de mediadores inflamatórios pela micróglia, resultando em uma sensibilização dos neurônios e consequente manutenção da dor (Clark and Malcangio, 2012).



Fonte: Adaptado de Clark e Malcangio, 2012*

Atualmente, muitos estudos têm ressaltado a importância da ativação da p38 MAPK no desenvolvimento e manutenção da dor (Ji and Suter, 2007; Xie et al., 2007). As proteínas quinases ativadas por mitogénio (MAPKs) constituem uma família de moléculas que desempenham um papel crucial na sinalização celular e expressão gênica (Daulhac et al., 2006). Embora sejam caracteristicamente envolvidas com regulação da proliferação, diferenciação e sobrevivência celular, atualmente, elas têm sido reconhecidas pelo seu grande papel na geração da hipersensibilidade dolorosa

* Clark, A.K., Malcangio, M., Microglial signalling mechanisms: Cathepsin S and Fractalkine, Exp. Neurol. (2012),

(Daulhac et al., 2006). A p38 MAPK é considerada como uma quinase induzida por estresse e desempenha um papel crucial nas respostas inflamatórias (Ji and Suter, 2007). Embora haja duas principais isoformas da p38 - a p38α e p38β - somente a última é expressa nas micróglias (Svensson et al., 2005).

A interação de fatores vasculares e metabólicos (hiperglicemia crônica, estresse oxidativo e nitrosativo, ativação da polimerase poli (ADPribose - PARP), hiperatividade da via dos polióis, hiperatividade proteína quinase C, glicação não-enzimática, via das hexosaminas, elevação da sinalização do fator nuclear κB e da p38-MAPK e anormalidades do crescimento neuronal) estão envolvidos em todos os estágios da neuropatia periférica diabética (Cameron, 2013; Lupachyk et al., 2013; Tesfaye et al., 2016).

A via dos polióis está presente em vários tecidos, incluindo, vasos sanguíneos e nervos periféricos. Nessa via, a glicose é convertida em sorbitol, pela aldose redutase, e adicionalmente, metabolizado em frutose, catalizada pela sorbitol desidrogenase (Ramasamy and Goldberg, 2010). A hiperatividade da via dos poliós relacionada ao esgotamento do mio-inositol, é uma das hipóteses metabólicas para as complicações do diabetes (Singh et al., 2014). O acúmulo excessivo de sorbitol, resulta em diminuição do mio-inositol em nervos periféricos, levando à redução da atividade da Na^+/K^+-ATPase e alteração da homeostase iônica, via diminuição da atividade da PKC (Greene et al., 1992).

Em destaque, tem sido demonstrado que a hiperglicemia induz as complicações neurovasculares através da via diacilglicerol/proteína quinase C (DAG/PKC) (Das Evcimen and King, 2007). O DAG é o principal ativador fisiológico da PKC. O aumento dos níveis de DAG no diabetes pode ocorrer através de múltiplas vias (Das Evcimen and King, 2007). A via DAG/PKC pode ser ativada pela hiperglicemia como resultado do aumento do estresse oxidativo, como por exemplo, pelo aumento do oxidante peróxido de hidrogênio, o qual é um conhecido ativador da PKC, seja de forma direta ou indireta pelo aumento da produção do DAG. (Konishi *et al*., 1997; Nishikawa *et al*., 2000).

A Na^+/K^+-adenosina trifosfatase (Na^+/K^+-ATPase), é um componente da bomba de sódio/potássio envolvido na contratilidade, crescimento e diferenciação celular, tem se mostrado diminuído em tecidos vascular e neuronal de pacientes diabéticos e modelos animais (Meier and King, 2000). A Na^+/K^+-ATPase é crucial para

a manutenção da homeostase iônica e potencial de repouso da membrana de e para tanto, sua atividade requer o uso de ATP (Persson et al., 2013).

Várias vias, aparentemente, influenciam a atividade da Na^+/K^+-ATPase na neuropatia diabética, como: aumento da via dos polióis, via do DAG/PKC, estresse oxidativo, entre outras. O estresse oxidativo pode promover alterações mitocondriais que favorecem a depleção de ATP em animais diabéticos e levar à ativação de vias de morte celular programada, como a via da caspase. A hiperglicemia medeia a produção de espécies reativas de oxigênio (ROS) e estresse oxidativo no gânglio da raiz dorsal (DRG) de ratos diabéticos que, paralelamente, induz a modificações no tamanho e função mitocondrial (Russell et al., 1999). Juntos, o aumento nas ROS diminuição na regulação de estressores oxidativos resulta em morte celular programada no DRG e fornece um mecanismo para explicar como a regulação prejudicada dos níveis máximos de glicose leva à lesão induzida por ROS na neuropatia diabética (Russell et al., 2002).

Nos neurônios, a maior porção de ATP disponível é utilizada pela Na^+/K^+ - ATPase no transporte dos íons de Na^+ e K^+ contra seus gradientes de concentração para manutenção do gradiente iônico através da membrana celular. Em axônios destituídos de energia, como em condições de respiração celular prejudicada e produção reduzida de ATP mitocondrial, há um aumento do influxo de Na + como resultado da capacidade reduzida de exportação de Na^+ pela Na^+/K^+ - ATPase e a consequente despolarização da membrana seria previsível. Os axônios não mielinizados seriam particularmente vulneráveis a flutuações nos níveis intra-axonais de ATP devido a sua alta razão superfície-volume, alta resistência à entrada e constante de comprimento curto difusional e eletrotônico, exigindo atividade significativa de Na^+/K^+ ATPase para manter os gradientes iônicos (Waxman et al., 1989; Donnelly, 2008).

Alguns estudos tem demonstrado que a lesão de axônios está associada a uma atividade reduzida de Na^+/K^+ ATPase, com o influxo de Na^+ excedendo a capacidade de exportação de Na^+. Aumentos subsequentes no Na^+ intra-axonal podem causar reversão do trocador de Na^+/Ca^{++}, de tal forma que este, em vez de retirar o Ca^{++} intracelular, passa a importá-lo, resultando em sobrecarga de Ca^{++} intra-axonal e no início de cascatas prejudiciais causadas por Ca^{++} (Stys et al., 1992; Lehning et al., 1996; Li et al., 2000).

Segundo Persson e colaboradores (2013), a atividade dos canais de sódio e do trocador de sódio/cálcio podem contribuir para injuria axonal de axônios periféricos, resultado de disfunção mitocondrial e prejuízo da atividade da Na^+/K^+ ATPase.

Estudos em humanos têm sugerido que os transtornos sensoriais induzidos pelo diabetes também acometem estruturas inervadas pelo sistema trigeminal (Rahim-Williams et al., 2009; Arap et al., 2010; Ogawa et al., 2017), resultando em transtornos sensoriais. Em particular, tem sido sugerido que quadros de hipoalgesia dos tecidos constituintes da articulação temporomandibular (ATM) podem provocar desordens articulares e deformidades como as articulações de Charcot (Collin et al., 2000). A articulação de Charcot é uma neuroartrite, consequência da lesão dos nervos, que inclui sintomas como fraqueza, atrofia muscular e perda de sensibilidade, especialmente nos segmentos distais dos membros superiores e inferiores (Rezende et al., 2013), como no caso da neuropatia induzida pelo diabetes, a qual impede a percepção da dor articular, acarretando lesões e fraturas insignificantes e repetidas (de forma despercebida) até a deterioração permanente da articulação (Rogers et al., 2011; Larson and Burns, 2012).

A ATM é inervada principalmente pelo nervo trigêmeo, cujos corpos celulares dos aferentes primários estão localizados no gânglio trigeminal, que se projetam para o complexo nuclear sensitivo do trigêmeo no tronco encefálico: núcleo sensorial principal e núcleo do trato espinhal do trigêmeo (subnúcleos oral, interpolar e caudal) (Sessle, 2011). A maioria das evidências indica que o subnúcleo caudal é o primeiro e principal sítio de retransmissão da informação nociceptiva trigeminal (Sessle, 2011; Chichorro et al., 2017).

Um estudo piloto demonstrou a fase inicial do diabetes induz hiponocicepção na ATM de ratos (Muzilli, 2014). Neste estudo, ratos Wistar tiveram diabetes induzida por uma injeção intraperitoneal de estreptozotocina (75 mg/Kg) e nos dias 7, 14, 21, 28, 35 e 42, após a indução do diabetes receberam uma injeção do agente nociceptivo formalina (1,5 %, 30 µl/ATM). Os resultados demonstraram que os animais diabéticos apresentaram uma resposta comportamental nociceptiva significativamente menor em relação aos animais normoglicêmicos.

Processos inflamatórios estão envolvidos no início do diabetes e na progressão de suas complicações (King, 2008). Tanto no DM tipo 1, como no DM tipo 2 são caracterizados por um aumento crônico das citocinas pró-inflamatórias (Pradhan

et al., 2009). Todas as vias clássicas do diabetes como a vias dos polióis, PKC, MAPK e o aumento da produção de produtos finais da glicação avançada podem direta ou indiretamente iniciar e manter a produção de mediadores inflamatórios. Especialmente o acúmulo dos produtos finais da glicação avançada que estimulam a ativação do fator de transcrição NF-kB, um potente indutor de processos inflamatórios (Sandireddy et al., 2014).

É bem estabelecido na literatura que a dor é um dos sinais clássicos do processo inflamatório instalado, apresentando como denominador comum, a sensibilização dos neurônios nociceptivos aferentes primários. Decorrente de estímulos inflamatórios ou lesões teciduais, a liberação de citocinas e quimiocinas pró e anti-inflamatórias desencadeia a liberação de prostanóides e aminas simpatomiméticas que, por sua vez, atuam diretamente em nociceptores causando hipernocicepção, resultado da redução do limiar de excitabilidade devido à modulação de canais de sódio voltagem-dependentes (Verri et al., 2006; Gold and Gebhart, 2010).

Sendo assim, é possível sugerir que a hipoalgesia induzida pela fase inicial do diabetes na ATM de ratos seria resultado de uma alterações do sistema nervoso periférico e/ou central, relacionadas com a diminuição do reflexo antidrômico no tecido periarticular, assim como redução na atividade do DAG/PKC e Na^+/K^+/ATPase no tecido gânglio trigeminal, e também redução da participação das micróglias do subnúcleo caudal trigeminal, independente do aumento da resposta inflamatória local.

Consideramos que o trabalho proposto é de relevância clínica, uma vez que auxilia no melhor entendimento dos mecanismos envolvidos no desenvolvimento dos transtornos sensoriais induzidos pelo diabetes, uma vez que, pode auxiliar no diagnóstico precoce e escolha de condutas terapêuticas mais efetivas, prevenindo lesões e deterioração da articulação temporomandibular.

2 ARTIGO

Early phase of type 1 diabetes induces hypoalgesia in the temporomandibular joint of rats

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Running title: Diabetes-induced hypoalgesia in temporomandibular joint

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Abstract

Background: Diabetes in early phase is known to result a variety of painful conditions induced by a modification in the neuronal activation and transmission. This study evaluates the effect of diabetes type 1 in different types of nociceptive models in the temporomandibular joint of rats (TMJ). Methods: Male Wistar rats (± 150 g, n = 5/group) were treated with an intraperitoneal injection of vehicle (normoglycemic) or Streptozotocin (STZ) 75 mg/kg (diabetic). The nociceptive behavior induced by an intra-articular injection of 1.5% capsaicin or 1.5% formalin at 7, 14, 21, and 28 days after STZ-induced diabetes. After nociceptive behavioral assays, the animals were euthanized and their trigeminal ganglion, trigeminal subnucleus caudalis and periarticular tissue were removed for further analyses. Results: Diabetic animals showed a reduction of primary C-fibers activation characterized by the reduction of protein level of Substance P and CGRP in the periarticular tissues. Diabetic animals pre-treated with PKC-α and -β inhibitors, significantly increased capsaicin-induced nociception in the TMJ and elicited protein levels of Na^+/K^+-ATPase in the trigeminal ganglia. In the trigeminal subnucleus caudalis of diabetic animals it was observed an increase of protein level of CX3CR1 and p38MAPK. In addition, diabetes significantly reduced the formalin-induced nociception in the TMJ, but elicited protein level of IL1-β and KC. Conclusion: Overall, the results of the present work suggest that early phase of diabetes reduce the primary C-fibers activation associated with the increased protein level of PKC α and β isoforms and reduction of Na^+/K^+-ATPase.

1 Introduction

Diabetic peripheral neuropathy (DPN) is one of the most frequent complications of diabetes mellitus (Tesfaye et al., 2010), and it is associated with considerable morbidity, mortality, and diminished quality of life (Tesfaye et al., 2016). DPN affects between 28% and 49% of patients (Abbottet al., 2011)**,** and between 25% and 50% of patients with DPN develop neuropathic pain (Abbott et al., 2011; Bouhassira, Letanoux, & Hartemann, 2013; Van Acker et al., 2009).

There is increasing pre-clinical and clinical evidence that diabetes may be related to alterations in the transmission of orofacial sensory information in the trigeminal system (Arap et al., 2010; Kazamel & Dyck, 2015; Nones et al., 2013; Rahim-Williams et al., 2009; Rodella et al., 2000; Xie et al., 2015). The presence of orofacial pain was reported by 55.2% of patients with type 2 diabetes (Arap et al., 2010). In addition, Collin et al. (2000) have observed that patients with diabetic neuropathy present hypoalgesia, suggesting that loss of sensitivity in TMJ may induce joint disorders and deformities, such as Charcot's joints.

Diabetic peripheral neuropathy induces a variety of sensations including spontaneous pain, hiperalgesia and allodynia, as well as hypoalgesia and analgesia (Calcutt, 2004; Yamamoto et al., 2009). The hypoalgesic conditions caused by diabetes are linked to structural changes in the neuronal fibers, such as Schwann cell disruption, degeneration, axonal losses, microvascular lesions, and alterations in intracellular biochemical signals (Arezzo & Zotova, 2002).

Several theories have been proposed to explain the sensory alterations related to the diabetic neuropathy. Such as changes in the blood vessels that supply the peripheral nerves; metabolic and autoimmune disorders accompanied by glial cell activation; altered expression of neuropeptides; changes in Na^+/K^+- ATPase activity, sodium and calcium channels expression; axonal atrophy and degeneration, damage to fine fibers and increased glycemic flow. More recently, central pain mechanisms, such as increased thalamic vascularity and imbalance of the facilitatory/inhibitory descending pathways, among others (Schreiber, 2015; Tesfaye, Boulton & Dickenson, 2013).

Hyperglycemia is considered to be a major pathophysiological factor in the development of diabetic neuropathy. Several biochemical mechanisms are proposed for the development of structural and functional abnormalities related to the prolonged exposure of neuronal tissues to hyperglycemia. These include elevated polyol pathway

activity, oxidative stress, the formation of advanced glycation end products, activation of protein kinase C isoforms, increased hexosamine pathway flux and various pro inflammatory changes such as elevated nuclear factor κB and p38 mitogen activated protein kinase signaling (Brownlee, 2005; Cashman & Höke, 2014; Tesfaye et al., 2016; Tomlinson & Gardiner, 2008). Other mechanisms include increased poly (ADP-ribose) polymerase (PARP) activity. These mechanisms do not work alone but strongly interact in a mutually facilitatory fashion (Cameron, 2013; Lupachyk, et al., 2013; Sytze Van Dam et al., 2013).

The purpose of the present study was to evaluate the effect of streptozotocin-induced diabetes on capsaicin and formalin-induced nociception in rat TMJ, as well as the peripheral and central mechanisms involved in this process.

2 Materials and methods

2.1 Animals:

Male Wistar rats (150-250 g) were kept in plastic cages (3 per cage) containing wood shavings, in a controlled light environment (light/darkness cycles of 12h) and food and water *ad libitum*. All animal procedures were approved by the University of Campinas' Animal Research Committee (CEUA #3384-1) and are in accordance with the guidelines of the National Council for Control of Animals Experimentation (CONCEA), ARRIVE guidelines (Kilkenny et al., 2010) and the International Association for the Study of Pain (IASP) for the study of pain in conscious animals (Zimmermann, 1983). The number of animals used was 5 per group and each animal was used once.

2.2 Drugs:

Streptozotocin, capsaicin, carrageenan and an aqueous solution of 37% formaldehyde were obtained from Sigma-Aldrich (Sigma-Aldrich, USA), PKC-α e PKC-β inhibitor (GO6976) were obtained from Bio-Techne (Bio-Techne, USA) and PKC-β inhibitor Ruboxistaurin (LY333531) were obtained from Cayman Chemical (Cayman Chemical, USA) Streptozotocin was dissolved in 0.1M of sodium citrate buffer (pH 4.5) (Courteix et al., 2007). Formalin solution was prepared from commercially available formalin further diluted in 0.9% NaCl to a final concentration of 1.5%. Carrageenan was dissolved in 0.9% NaCl solution. Capsaicin solution was prepared from 10%

capsaicin in ethanol, tween 80 and sterile saline in a 1:1:8 ratio by volume (Lam, Sessle, Cairns, & Hu, 2005) PKC-α e PKC-β (GO6976) and PKC-β inhibitor Ruboxistaurin (LY333531) were dissolved in vehicles consisting of 1% dimethyl sulfoxide (DMSO) in phosphate-buffered saline (PBS).

2.3 Type 1 diabetes induction:

The animals were randomly divided into two groups. The first group (Normoglycemic group – NG) was treated with intraperitoneal injection of 0.1M of sodium citrate buffer (pH 4.5), and the second group was treated with an intraperitoneal injection of 75 mg/kg of Streptozotocin (STZ®; Sigma-Aldrich, St Louis, MO, USA) freshly dissolved in 0.1M of sodium citrate buffer (pH 4.5) (Courteix et al., 2007). Streptozotocin, an antibiotic extracted from *Streptomyces achromogenes*, is one of the most commonly used chemical agents to induce experimental diabetes in rodents (Cheng et al., 2014; Courteix et al., 2007; Daulhac et al., 2006; Szkudelski, 2001). The rats were fasted for 8 h prior to STZ injection. Three days after induction, plasma glucose in the blood samples collected from the tail vein was measured using a glucose-oxidase enzymatic method (*Optium Xceed*; Abbott; Alameda, CA, USA). STZ-induced diabetes was confirmed by a blood glucose concentration of > 300 mg/dl after 8 h of fasting (Braga et al., 2011). Six animals in each group were used for testing procedure for TMJ pain 7, 14, 21 and 28 days after STZ-induced diabetes. At the moment of the experiments, body weight and blood glucose level were measured.

2.4. Experimental design

2.4.1. Effect of diabetes on capsaicin-induced TMJ nociception: To evaluate the effect of diabetes on primary C-fibers activation, groups of diabetic or normoglycemic rats (n=6/group) were treated with an intra-TMJ injection of the agonist of primary C-fibers capsaicin (1.5%, 30 µl/TMJ) 7, 14, 21 and 28 days after diabetes induction. All animals received a total volume of 30 µl the solution into their TMJ, after which, the nociceptive behavior was evaluated over a period of 30 min (Lamana et al., 2017). At the end of this test, the animals were euthanized and their periarticular tissues and trigeminal subnucleus caudalis were removed to evaluate the release of neuropeptides and activation of microglial cells.

2.4.2. Role of PKC isorforms αand βon diabetes-induced hyponociception in the TMJ: Another group of diabetic or normoglycemic rats (n=6/group) of all periods analyzed (7, 14, 21 and 28 days after diabetes induction) were pre-treated (15 min) with an intra-TMJ injection of the PKC-α e PKC-β inhibitor (GO6976) (50.5 µg/TMJ) or PKC-β inhibitor Ruboxistaurin (LY333531) (50.5 µg/TMJ) and then capsaicin (1.5%, 30 µl/ TMJ) was injected. All animals received a total volume of 45 µl into their TMJ, after which the nociceptive behavior was evaluated over a period of 30 min (Lamana et al., 2017). At the end of this test, the animals were euthanized and their trigeminal ganglia were removed to evaluate the protein level of Diacylglycerol (DAG) and Na^+/K^+ ATPase.

2.4.3. Effects of diabetes on formalin-induced nociception and on the inflammatory response in TMJ: To evaluate the effect of diabetes on formalin-induced TMJ hyponociception, groups of diabetic or normoglycemic rats (n=6/group) were treated with an intra-TMJ injection of the 1.5% formalin (30 µl/ TMJ) 7, 14, 21 and 28 days after diabetes induction. All animals received a total volume of 30 µl the solution into their TMJ, after which, the nociceptive behavior was evaluated over a period of 45 min (Clemente, Parada, Veiga, Gear, & Tambeli, 2004). To evaluate the effect of diabetes on inflammatory response in TMJ, groups of diabetic or normoglycemic rats (n=6/group) were treated with an intra-TMJ injection of the carrageenan (100 µg/ TMJ) 7, 14, 21 and 28 days after diabetes induction. Carrageenan is a polysaccharide widely used for the induction of experimental inflammation on animal model (de Castro et al., 2015). Previous studies have demonstrated that inflammatory hypernociception induced by carrageenan results in release of a cascade of mediators initiated by the production of the hypernociceptive cytokines tumoral necrosis factor-α, interleukin-1β (IL-1β), and chemokines, such as keratinocyte chemoattractant (KC) (Napimoga et al., 2008). After 60 minutes, the animals were euthanized and their periarticular tissues were removed to evaluate the release of pro-inflammatory cytokines (IL-1β and KC).

2.5. Testing procedure for capsaicin- or formalin-induced hypernociception in the temporomandibular joint:

The nociceptive assay was done during the light cycle (between 9:00 AM and 5:00 PM) in a quiet room at 23°C (Rosland, 1991). Each animal was then individually accommodated in a test chamber (30 x 30 x 30 cm wooden chamber internally lined with mirrors on all sides and a clear glass on the front side) for 15-min to reduce stress. After that rats were anesthetized by isoflurane inhalation (1.5%, 30-s period) and a 30-gauge needle connected by a polyethylene tube (P20) to a Hamilton syringe (50 µl) was penetrated into the lower portion of posterior-edge of the zygomatic arch, and advanced into an anterior direction until contacting the posterior lateral condyle (Clemente et al., 2004). After temporomandibular joint (TMJ) injection, the animals immediately returned to the test chamber to measure the nociceptive response over 30 min or 45 min. The nociceptive response score was defined as the cumulative total number of seconds that the animal spent rubbing the orofacial region asymmetrically with the ipsilateral fore or hind paw plus the number of head flinches counted during the observation period as previously described (Clemente et al., 2004; Roveroni, Parada, Cecília, Veiga, & Tambeli, 2001). The occurrence of a given behavior is proportional to the length of time in which that behavior is observed to occur. Since head flinches followed a uniform pattern of 1-s duration, each flinch was expressed as 1s (Roveroni et al., 2001). At the conclusion of the nociceptive behavior testing, animals were euthanized by isoflurane inhalation (5%, 30 s) followed by cervical dislocation and their TMJ periarticular tissues, trigeminal ganglia and trigeminal subnucleus caudalis were removed for further analysis.

2.6. Protein extraction:

Periarticular tissues were removed by dissection of the temporalis and posterior deep masseter muscles, with careful attention to anatomical landmarks (zygomatic arch and tympanic bulla) until exposure of the condylar process. The samples contained all the condylar process surrounding tissues, including the masticatory muscles (temporalis, posterior deep masseter and pterygoideus externus), articular cartilage, fibrocartilage of the disc and lateral ligaments. The standard sample size was 1 x 1 x 0.5 cm (Lamana et al., 2017).

For trigeminal ganglia extraction, the neurocranium was opened and brain removed. The trigeminal ganglion was localized in the Meckel's cavel in the dura mater near the apex of the petrous part of the temporal bone.

Trigeminal subnucleus caudalis was removed by the dissection of the caudal part of the medulla oblongata guided by the Swanson's Atlas (Swanson, 2004). The standard sample size was 0.3 cm3.

The samples from TMJ periarticular tissues, trigeminal ganglia or trigeminal subnucleus caudalis were homogenized individually in 500 µl of Ripa Lysis Buffer containing protease inhibitors (Santa Cruz, Biotechnology, Dallas, Texas, USA) followed by centrifugation at 10,000 g for 10 min at 4°C. The total protein amount was measured by using the BCA protein assay kit (Thermo Scientific, Rockford, IL, USA). The supernatants were stored at -20°C until further analysis.

2.7. Western Blot analysis:

Aliquots containing 80 µg of total proteins were separated by polyacrylamide gel electrophoresis SDS-10% PAGE and transferred to nitrocellulose membranes (Bio-Rad, Hercules, CA, USA). The membranes were incubated "overnight" at 4°C with blocking buffer [PBS 5% (w/v) skim milk and 0.1% Tween 20], being washed three times with PBS 0.1% Tween 20. They were then incubated in a PBS solution containing 5% skim milk and 0.1% Tween 20 containing primary antibody for the detection of microglia expression (CX3CR1; GeneTex, USA; 1:3000), p38 MAPK (GeneTex, USA; 1:1000) in the trigeminal subnucleus caudalis. After washing, membranes were incubated with conjugated secondary antibody specific HRP and washed again. The membranes were then developed with chemiluminescence kit (Amersham ECL Western Blotting Detection Reagent, Amersham Pharmacia Biotech, Little Chalfont, UK) as described in the instruction manual. To measure the bands by optical density the software ImageJ 1.49v (National Institute of Health, USA) was used. To confirm uniform protein loading, membranes were stripped and blocked overnight at 4 °C, and then incubated for 2 h with and alpha-tubulin (Santa Cruz, USA: 1:1000) followed by secondary antibody conjugated with peroxidase (1:10,000; Sigma- Aldrich, St. Louis, MO, USA). Banding specificity was determined by omission of the primary antibody from the western-blotting protocol.

2.8. Enzyme-linked immunosorbent assay (ELISA):

The levels of DAG, Na^+/ K^+ ATPase (Blue Gene Biotech, Shanghai, China) and FKN (RD Systems®, Minneapolis, MN, USA) in trigeminal ganglion; substance P (Phoenix Pharmaceuticals®, Inc., Burlingame, California, EUA), calcitonin gene-related peptide (CGRP) (Phoenix Pharmaceuticals®, Inc., Burlingame, California, EUA), KC and IL-1β (RD Systems®, Minneapolis, MN, USA) in periarticular tissue were determined by capture enzyme-linked immunosorbent assays (ELISA) using protocols supplied by the manufacturer.

2.9. Statistical analysis:

After checking if the data fitted the normal distribution, the two independent variables Protocol (at 2 levels – normoglycemic and diabetics; or at 4 levels – normoglycemic, diabetics, diabetics treated with GO6976 and diabetics treated with LY333531) and Time (at four levels – 7, 14, 21 and 28 days after treatments) were tested using a two-way ANOVA. Multiple comparisons were performed by Tukey's test (GraphPad Prism 6.0). The significance level was set at 0.05.

3. Results

3.1. Induction of type 1 diabetes in rats

The animals treated with STZ (Diabetic) significantly lost weight after 14 days ($P<0.05$: Two-way ANOVA, Tukey's test) (Fig. 1A). The animals treated with citrate buffer (Normoglycemic) significantly increased the body weight in a time-dependent manner ($P<0.05$). Animals treated with STZ (Diabetic) showed blood glucose level up to 300 mg/dl that allowed us classify these animals with type 1 diabetes (Fig. 1B). The two-way ANOVA showed a significant effect of the interaction of the independent variables protocol and time. After 7 days, the blood glucose level scores found for diabetic animals are significantly higher than normoglycemic animals (Fig. 1B).

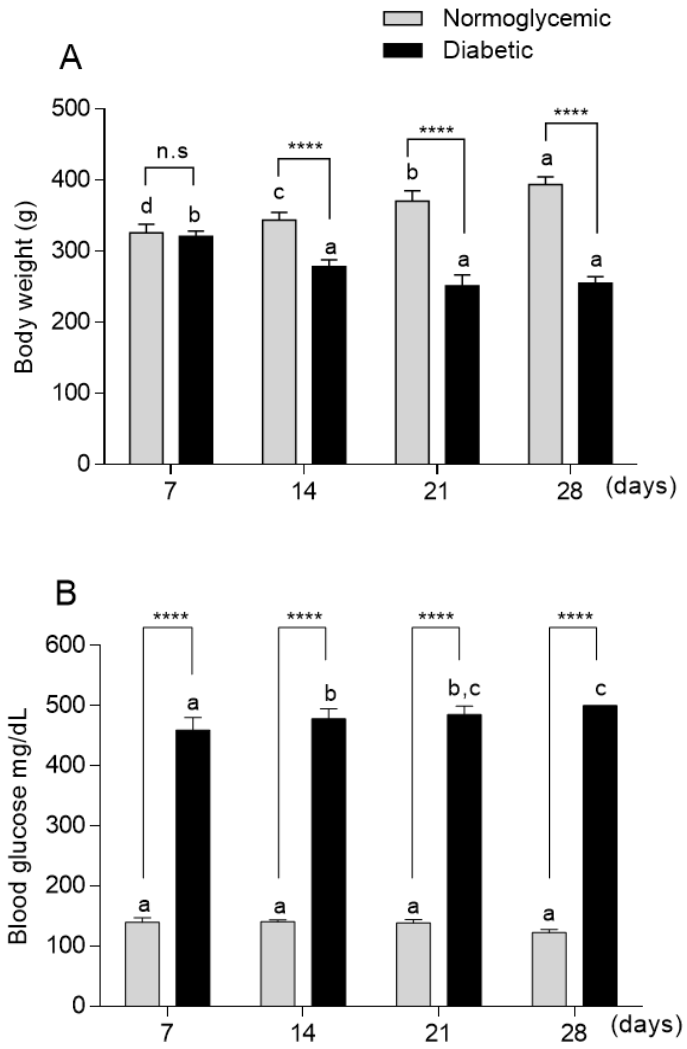


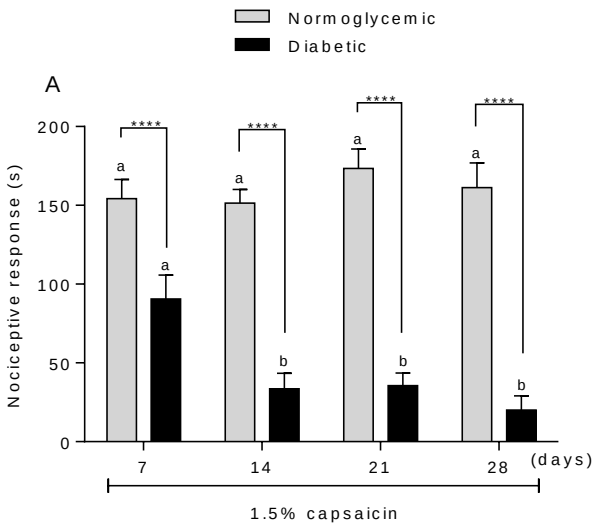
Figure 1 - Body weight and blood glucose levels of normoglycemic and diabetic rats. **(A)** Body Weight (g) **(B)** Blood glucose mg/ml. Values are expressed as mean ± SD. Bars followed by different lowercases letter differ from each other within the same protocol (P <0.05: Two-way ANOVA, Tukey's test). The symbol (****) indicates P<0.0001, and (n.s) means not significant between protocols in the same time point.

3.2. Diabetes reduced capsaicin-induced nociception in the TMJ.

The two-way ANOVA showed a significant effect of the interaction of the independent variables Protocol and Time (p<0.0001). After 7 days, significantly lower nociceptive scores were observed in the diabetic animals (p<0.0001) (Fig. 2A). For diabetic animals it was observed that the nociceptive scores after 14, 21 and 28 days were significantly lower than that observed at 7 days. On another hand, normoglycemic animals showed not difference among different time points (Fig. 2A).

To confirm these results, it was evaluated the effect of diabetes on the release of neuropeptides, substance P (SP) and calcitonin gene-related peptide (CGRP), of primary C-fibers stimulated by capsaicin in the periarticular tissues. The two-way ANOVA showed a significant effect of the interaction of the independent variables Protocol and Times ($p<0.0001$) for SP protein level in the periarticular tissues (Fig. 2B). After 7 and 14 days, the protein level of SP found for diabetic and normoglycemic animals did not significantly differ from one another, while at 21 and 28 days, significantly lower protein level of SP were observed in diabetic animals (Fig. 2B). For diabetic animals, it was observe that the protein level of SP after 21 and 28 days were significantly lower than that observed at 7 and 14 days, which did not differ between one another. Normoglycemic animals showed not difference among different time points (Fig. 2B).

The two-way ANOVA showed a significant effect of the interaction of the independent variables Protocol and Times ($p<0.0001$) for CGRP protein level in the periarticular tissues (Fig. 2C). After 7 days, the protein level of CGRP found for diabetic and normoglycemic animals did not significantly differ from one another, while at 14, 21 and 28 days, significantly lower protein level of CGRP were observed in diabetic animals (Fig. 2C). For diabetic animals, it was observed that the protein level of CGRP after 14, 21 and 28 days were significantly lower than that observed at 7 days, which did not differ among one another. Normoglycemic animals showed not difference among different time points (Fig. 2B).



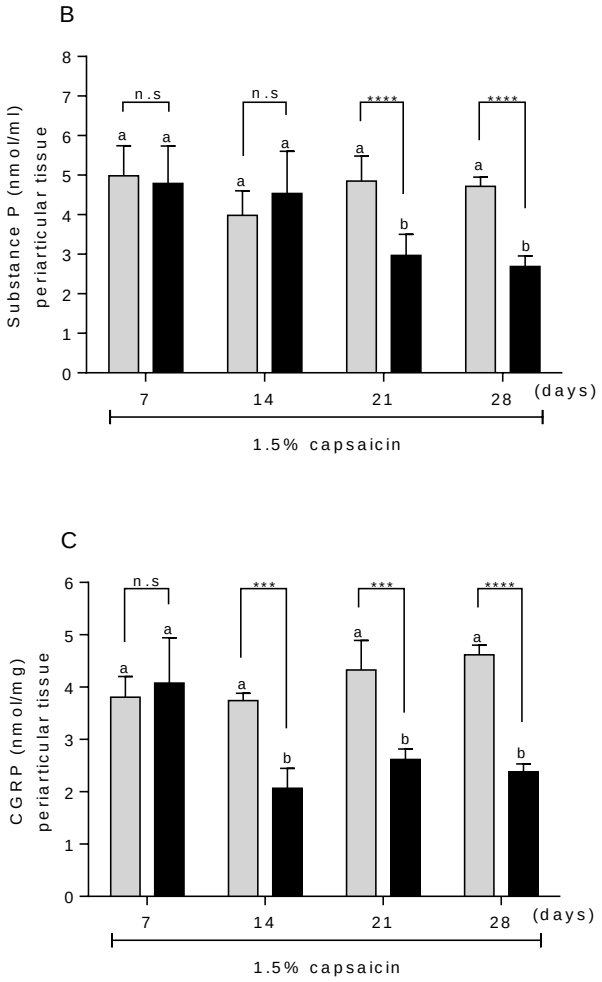


Figure 2 – Diabetes reduced capsaicin-induced nociception in the TMJ. (A) Nociceptive score induced by 1.5% capsaicin; **(B)** Protein level of substance P (SP) in the periarticular tissues; **(C)** Protein level of calcitonin gene-related peptide (CGRP) in the periarticular tissues. Values are expressed as mean ± SD. Bars followed by different lowercases letter differ from each other within the same protocol (P <0.05: Two-way ANOVA, Tukey's test). The symbol (****) indicates P<0.0001, (***) indicates P<0.001 and (n.s) means not significant between protocols in the same time point.

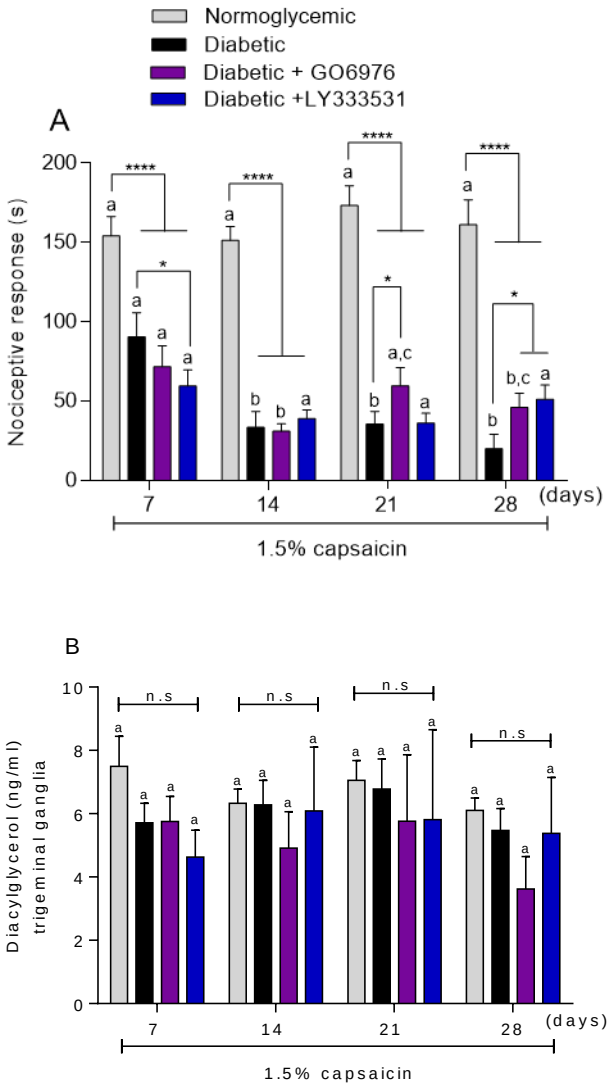
3.3. The hyponociception induced by diabetes is associated with PKCα, PKCβ and Na^+/K^+ ATPase.

It was observed a significant effect of the interaction of the independent variables Protocol and Time (p<0.0001). After 7 days, significantly higher nociceptive scores were observed in the normoglycemic animals (p<0.0001) (Fig. 3A).For normoglycemic animals it was observed not difference among different time points (Fig. 3A). For diabetic animals treated with GO6976 (inhibitor of PKC-α and –β) it was

observed a significantly higher nociceptive score after 21 and 28 days ($p<0.05$) than that observed at diabetic animals, which did not differ between one another (Fig. 3A). On the other hand, diabetic animals treated with LY333531 (inhibitor of PKC–β) it was observed a significantly higher nociceptive score after 28 days ($p<0.05$) than that observed at diabetic animals (Fig. 3A).

It was not observed a significant effect of the interaction of the independent variables Protocol and Time for substance diacylclycerol in the trigeminal ganglia (Fig. 3B).

In addition, it was observed that diabetic animals treated with GO6976 or LY333531 was showed protein level of Na^+/K^+ ATPase significantly higher in the trigeminal ganglia after 7, 14, 21 and 28 days ($p<0.05$) than that observed at diabetic and normoglycemic animals (Fig. 3C).



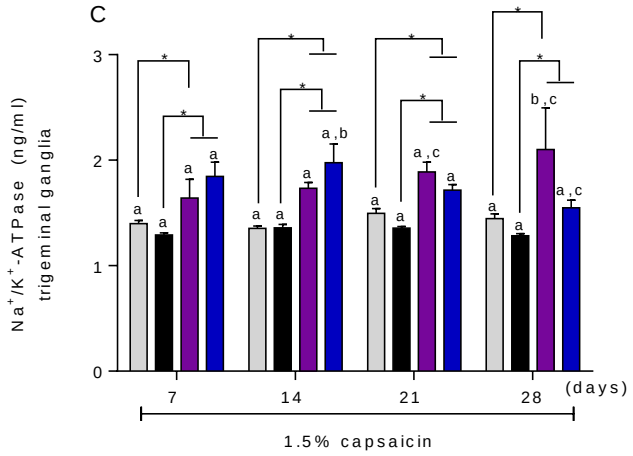


Figure 3 – The hyponociception induced by diabetes is associated with PKCα, PKCβ and Na+/K+ ATPase. (A) Nociceptive score induced by 1.5% capsaicin; **(B)** Protein level of substance diacylclycerol in the trigeminal ganglia; **(C)** Protein level of Na^+/K^+/ATPase in the trigeminal ganglia. Values are expressed as mean ± SD. Bars followed by different lowercases letter differ from each other within the same protocol (P<0.05: Two-way ANOVA, Tukey's test). The symbol (****) indicates P<0.0001, the sympol (*) indicates P<0.05, (n.s) means not significant between protocols in the same time point.

3.4. The increase of CX3CR1, p38 MAPK and fractalkine in the trigeminal subnucleus caudalis signal diabetes-induced neural damage

Trigeminal nerve injury amplify the excitability of neuron in the trigeminal subnucleus caudalis and upper cervical spinal cord mediated by neuro-immune interaction (Iwata et al., 2017). At 7 and 28 days of protocol, significantly higher protein level of CX3CR1 (microglial cells marker) (Fig. 4A) and p38MAPK (Fig. 4B) in the trigeminal subnucleus caudalis were observed in the diabetic animals (p<0.05).For normoglycemic animals it was observed not difference among different time points (Fig. 4A). In addition, diabetic animals was demonstrated significantly protein level of fracktalkine in the trigeminal subnucleus caudalis after 14 and 28 days, than that observed at normoglycemic animals (Fig. 4C).

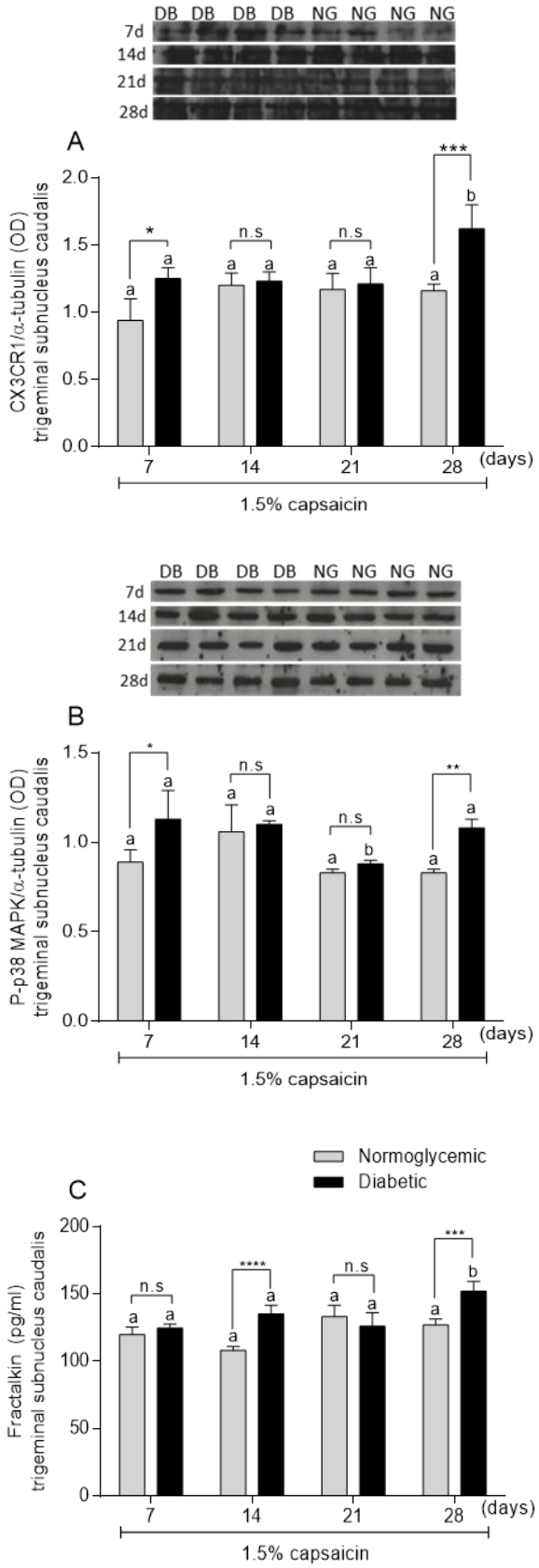
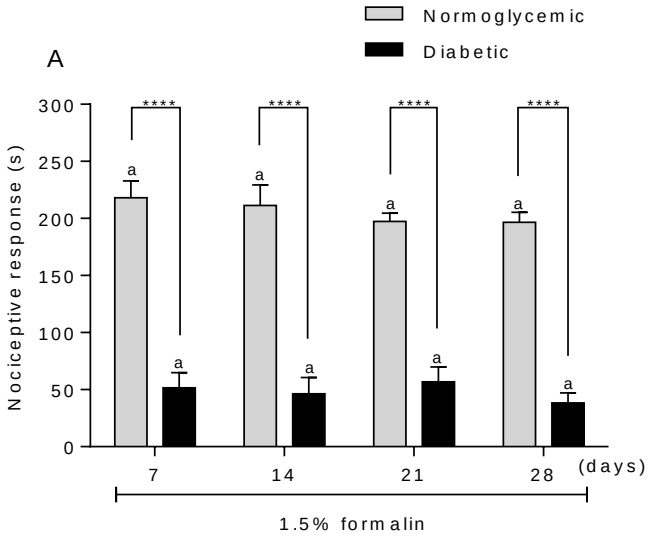


Figure 4 - The increase of CX3CR1, p38 MAPK and fractalkin in the trigeminal subnucleus caudalis signal diabetes-induced neural damage. (A) Protein level of

CX3CR1 (microglial marker) in the trigeminal subnucleus caudalis; **(B)** Protein level of p38MAPK in the trigeminal subnucleus caudalis **(C)** Protein level of fractalkin in the trigeminal subnucleus caudalis. Values are expressed as mean ± SD. Bars followed by different lowercases letter differ from each other within the same protocol (P<0.05: Two-way ANOVA, Tukey's test). The symbol (***) indicates P<0.001, (**) indicates P<0.01, the sympol (*) indicates P<0.05, (n.s) means not significant between protocols in the same time point.

3.5. The effect of diabetes on formalin-induced nociception and on the inflammatory response in the TMJ.

The two-way ANOVA showed a significant effect of the interaction of the independent variables Protocol and Time (p<0.0001). After 7 days, significantly lower nociceptive scores were observed in the diabetic animals (p<0.0001) (Fig. 5A).For normoglycemic animals it was observed not difference among different time points. In addition, diabetic animals showed not difference among different time points (Fig. 5A). Despite of these data, it was observed that in the diabetic animals treated with an intra-TMJ injection of carrageenan, protein level of pro-inflammatory cytokine KC was significantly higher in diabetic animals than that observed at normoglycemic animals (Fig. 5C). The protein level of the pro-inflammatory cytokine IL1-β demonstrated a tendency to increase in periartiuclar tissues of diabetic animals (Figure. 5B).



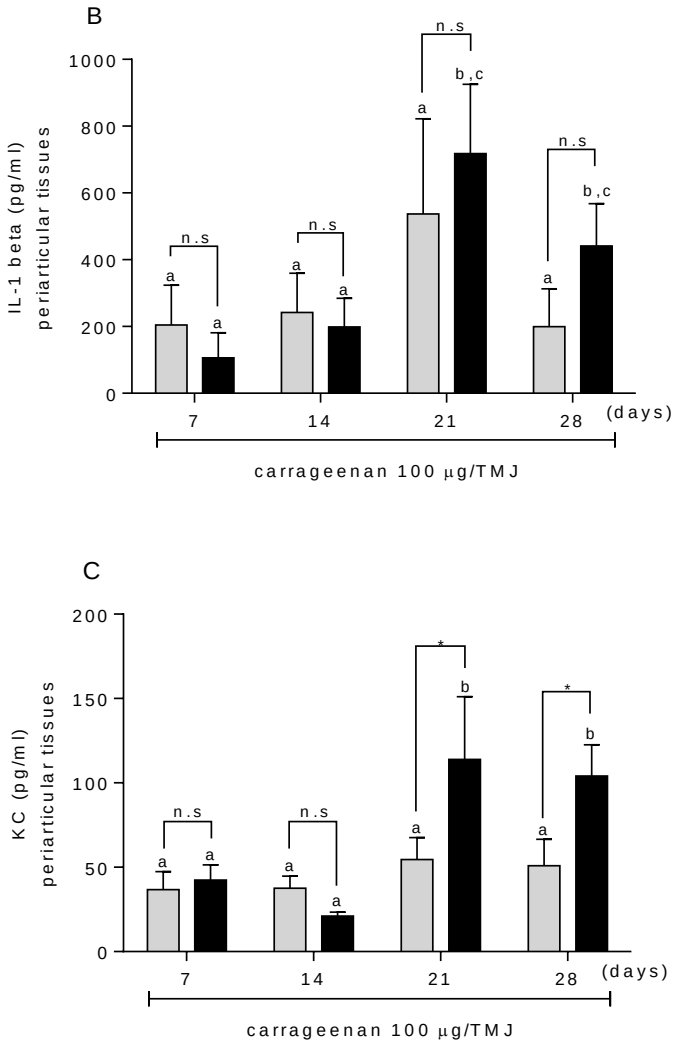


Figure 5 - The effect of diabetes on inflammatory response in the TMJ. (A) Nociceptive score induced by 1.5% formalin; **(B)** Protein level of IL-1β in the periarticular tissues; **(C)** Protein level of KC in the periarticular tissues. Values are expressed as mean ± SD. Bars followed by different lowercases letter differ from each other within the same protocol (P <0.05: Two-way ANOVA, Tukey's test). The symbol (****) indicates P<0.0001, the symbol (*) indicates P<0.05, and (n.s) means not significant between protocols in the same time point.

4. Discussion

This study demonstrated that STZ—induced diabetes in rats results in developing of hyponociception in temporomandibular joint of rats. STZ-induced diabetes is considered an experimental model commonly used for the evaluation of nociceptive alterations diabetes—induced, both in the paw and in the orofacial region (Araya et al., 2017; Courteix et al., 2007; Daulhac et al., 2006; Nones et al., 2013;

Pabreja, Dua, Sharma, Padi, & Kulkarni, 2011; Patel & Udayabanu, 2013; H. Y. Xie et al., 2015). Although, in the orofacial region, the number of pre-clinical studies is still scarce and the results contradictory.

Most pre-clinical studies with rodents show thermal hyperalgesia in the orofacial region (Araya et al., 2017; Nones et al., 2013; Rodella et al., 2000; H. Y. Xie et al., 2015). However, our study demonstrated that STZ-induced diabetes resulted in hyponociception in rat TMJ 7 days and persisted up to 28 days after disease induction. The difference between the results of these studies can be explained by the variability of techniques and tissues used for the evaluation. These studies evaluated distinct regions: superficial and subcutaneous tissue (face and lip) and deep tissue (TMJ), predominantly innervated by different subtypes of primary afferent neurons. Deep inputs may be more effective in inducing central neuronal excitation than cutaneous inputs, greater sensory disturbances may occur in pain conditions involving deep tissues than in those involving cutaneous tissues (Imbe et al., 2001).

In addition, different tests were used to evaluate the nociceptive response in the orofacial region. The thermal test detects, principally, activation of superficial nociceptive fibers (Vivancos, Parada, & Ferreira, 2003), while the present study used the chemical test with intra-articular application of capsaicin. This substance is known to activate TRPV1-type vanyloid receptors in C-nociceptive peripheral fibers, which increase Na^{+} and Ca 2^{+} chain conductance, and release neuropeptides from primary afferent neurons that induce hiperalgesia and neurogenic inflammation, resulting in nociceptive behaviors (Hong & Wiley, 2005; Nilius, Owsianik, Voets, & Peters, 2007; Tominaga & Tominaga, 2005).

Stimulation of the nociceptive C fiber results in both orthodromic conduction to the spinal cord and antidromic conduction to other axon branches, i.e. the axon reflex that can stimulate the release of peptides, such as substance P (SP) and calcitonin gene-related peptide (CGRP), resulting in vasodilation and increased permeability. Studies have shown that this neurovascular response mediated by the nerve axon reflex is reduced in diabetic neuropathic patients (Malik et al., 2011; Marche et al., 2017).

Neural changes in the peripheral portion of the trigeminal system could be confirmed by the evaluation of neuronal antidromic activity in the peripheral nociceptive neurons on periarticular tissue, measuring by release of both neuropeptides SP and the CGRP, after intra-articular injection of 1.5% capsaicin, which it was in significantly

lower in periarticular tissue of diabetic rats. These results are in agreement with other studies reporting that the nerve axon reflex is reduced in diabetic neuropathic patients and neurotransmitter systems are also affected, with decreased expression of substance P and CGRP detected at the peripheral nerves of diabetic rats (Malik et al., 2011; Pradhan, Nabzdyk, Andersen, LoGerfo, & Veves, 2009; Uehara, Yamagishi, Otsuki, Chin, & Yagihashi, 2004).

Na^+/K^+ ATPase, a component of the sodium / potassium pump involved in contractility, cell growth and differentiation, has been shown to be decreased in vascular and neuronal tissues of diabetic patients and animal models (Das Evcimen & King, 2007; Greene, Lattimer, & Sima, 1988; Meier & King, 2000). Several pathways apparently influence Na^+/K^+ ATPase activity in diabetic neuropathy, such as: increased polyol pathway, diacylglycerol (DAG) / protein kinase C (PKC) pathway and advantage glycation end products (AGE), oxidative stress, among others (Feldman, Nave, Jensen, & Bennett, 2017; Yagihashi, Mizukami, & Sugimoto, 2011; Zenker, Ziegler, & Chrast, 2013).

Some studies have shown that axon injury is associated with reduced Na^+/K^+ ATPase, with the influx of Na^+ exceeding Na^+ export capacity. Subsequent increases in Na + intra-axonal may cause reversal of the Na^+ / Ca^{++} exchanger, so that instead of removing the intracellular Ca^{++}, it will import it, resulting in intra-axonal Ca^{++}, overload and at the beginning of Ca^{++} cascades, (Lehning, Doshi, Isaksson, Stys, & LoPachin, 1996; Li, Jiang, & Stys, 2000; Stys, Waxman, & Ransom, 1992).

According to Persson and collaborators (2013), the activity of the sodium channels and sodium / calcium exchanger may contribute to axonal injury of peripheral axons, a result of reduced mitochondrial ATP production and impairment of Na^+/K^+ ATPase activity. Based on this information, we can suggest that the increase of intracellular Ca^{++} will activate PKC pathway (Donnelly, 2008; Waxman, Black, Kocsis, & Ritchie, 1989), which, at least in part, may explain our results.

Changes in PKC activation and/or PKC isoform expression can modulate the activity of Na^+/K^+–ATPase leading to reduced Na^+/K^+–ATPase activity, resulting in decreased nerve conduction and nerve regeneration (Geraldes & King, 2010; Sima, 2003; Skundric & Lisak, 2003). In addition, in sciatic nerve Na^+/K^+–ATPase activity also ameliorated by non-specific and β-specific PKC inhibitors, respectively, and both reduce hyperalgesia and C-fiber hyperexcitability in the STZ rat (Geraldes & King, 2010; Sima, 2003).

It was suggested that diabetes results in a peripheral nerve alteration, was assessed the involvement of the central nervous system (CNS) in diabetes-induced hyponociception in TMJ. Following peripheral nerve/tissue injury, one important contributor to increased nociceptive transmission are microglial cells in the dorsal horn of the spinal cord and medullary dorsal horn of the trigeminal system (Chiang, Dostrovsky, Iwata, & Sessle, 2011; Clark & Malcangio, 2012; Gao & Ji, 2010; Piao et al., 2006; Wodarski, Clark, Grist, Marchand, & Malcangio, 2009; Y. F. Xie et al., 2007).

Microglia are the most susceptible sensors of brain pathology. Upon any detection of signs for brain lesions or nervous system dysfunction, microglia are rapidly activated (Kettenmann, Hanisch, Noda, & Verkhratsky, 2011). This activation must be initiated by the biochemical changes in the peripheral tissues. In this study, diabetic rats showed significantly increased of protein level of CX3CR1 (microglial marker) in the trigeminal subnucleus caudalis after 7 and 28 days of diabetes induction associated with p38 mitogen-activated protein (MAPK) and fraktalkine (FKN) release.

CX3CR1 upregulation is essential for the activation of p38 in spinal microglia after neuronal injury. Therefore, it is likely that activation of p38 is an underlying mechanism for CX3CR1 to regulate neuropathic pain (Y. F. Xie et al., 2007). Our results showed a statistically significant increase in the expression of p-p38MAPK and CX3CR1 receptor observed in the medullary dorsal horn of diabetic rats when compared to normoglycemic at 7 and 28 days.

In addition, diabetic animals demonstrated significantly protein level of FKN in the trigeminal subnucleus caudalis after 14 and 28 days, than that observed at normoglycemic animals. It has been demonstrated that high level of endogenous FNK expressed on neurons in the adult CNS acts as a neuronal off signal maintaining microglia in a quiescent state, mediating a neuroprotective role for FNK/CX3CR1 signaling (Lauro, Catalano, Trettel, & Limatola, 2015).

Formalin is a noxious stimulus commonly used in animal behavioral experiments. The mechanism underlying formalin-induced nociception depends on two factors: (1) activation of primary afferent sensory neurons through a direct action on transient receptor potential cation channel, subfamily A, member 1 (Fischer, Tambeli, & Parada, 2008; Macpherson et al., 2007; McNamara et al., 2007); and (2) the release of inflammatory mediators from mast cells, which synergistically stimulate nociceptive primary afferent neurons (Fischer et al., 2008; Parada, Tambeli, Cunha, & Ferreira, 2001; Ting et al., 2007). Thus, considering that formalin-induced inflammatory

response, it was evaluated the effect of diabetes in the release of pro-inflammatory cytokines induced by the carrageenan.

In this study, an increase in the inflammatory response in diabetic rats characterized by significantly higher protein level of keratinocyte chemoattractant (KC) in the periarticular tissues of diabetic rats. CXCL1, also known as KC, were increased in diabetic animals, it was also reported that, in patients with diabetic neuropathy, the serum level of CXCL1 is higher than in healthy people (Zychowska, Rojewska, Pilat, & Mika, 2015). In addition, serum CXCL1 levels are higher in patients with demyelinating forms of diabetic neuropathy (Zhou & Zhou, 2014). Otherwise, diabetes significantly reduced formalin-induced nociception in the TMJ of rats. These results confirm previous data of this study.

On the other hand, in diabetic animals it was observed a tendency to increase interleukine-1β (IL-1β) in periarticular tissues. IL-1β is a cytokine that is primarily released by immune cells but is also secreted by resident monocytes, macrophages, adipocytes, and other cells at sites of diabetic complications. Indeed, in diabetic neuropathy, IL-1β has been postulated to contribute to nerve damage and miscommunication between Schwann cells and axons during the early stages of diabetic neuropathy (Forbes & Cooper, 2013; Kiasalari, Rahmani, Mahmoudi, Baluchnejadmojarad, & Roghani, 2017).

Overall, the results suggest that regardless of the increased inflammatory response, diabetes-induced hyponociception in the TMJ of rats as a result of a damage in the peripheral neurons of the trigeminal system mediated by the inhibition of the Na^+/K^+-ATPase.

Author's contributions

All authors listed above have contributed sufficiently to the project to be included as authors: Luiz Marques Rocha-Neto has directly participated in the execution of the experimental work and has contributed to the critical discussion of the results and to the elaboration of the manuscript. Jaime Rodolfo Gamarra-Suárez, Fabiana Furtado Freitas, Augusto Muzilli Jr[1], Cristina Gomes Macedo has been responsible for the behavioural assays. Marcelo Henrique Napimoga participated in the initial planning of this study. Juliana Trindade Clemente-Napimoga makes substantial contributions to

the conception and design of this study, and to the analysis and interpretation of data. All the authors have revised the manuscript critically.

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3 CONCLUSÃO

Este estudo sugere que a hiponocicepção induzida pelo diabetes na ATM de ratos é resultado de alterações neuronais periféricas dependentes da sinalização das isoformas da PKC α e β e ativação da Na^+/K^+ ATPase.

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ANEXOS

Anexo 1 - Certificado de aprovação do comitê de ética

UNICAMP

CEUA/UNICAMP

CERTIFICADO

Certificamos que o projeto intitulado **"AVALIAÇÃO DA RESPOSTA VASCULAR INFLAMATÓRIA NA ARTICULAÇÃO TEMPOROMANDIBULAR DE RATOS NA FASE INICIAL DO DIABETES TIPO 1"**, protocolo nº **4151-1**, sob a responsabilidade de **Profa. Dra. Juliana Clemente Trindade Napimoga / Luiz Marques Da Rocha Neto**, que envolve a produção, manutenção e/ou utilização de animais pertencentes ao filo *Chordata*, subfilo *Vertebrata* (exceto o homem) para fins de pesquisa científica ou ensino, encontra-se de acordo com os preceitos da **LEI Nº 11.794, DE 8 DE OUTUBRO DE 2008**, que estabelece procedimentos para o uso científico de animais, do **DECRETO Nº 6.899, DE 15 DE JULHO DE 2009**, e com as normas editadas pelo **Conselho Nacional de Controle da Experimentação Animal - CONCEA**, tendo sido aprovado pela **Comissão de Ética no Uso de Animais** da **Universidade Estadual de Campinas - CEUA/UNICAMP,** em **04 de abril de 2016**.

Vigência do projeto: 04/2016-02/2020
Início do experimento envolvendo manipulação animal: ABRIL/2016
Espécie/Linhagem: Rato heterogênico / HanUnib: WH (WISTAR)
No. de animais: 144
Idade/Peso: 02 meses / 300g
Sexo: machos
Origem: CEMIB/UNICAMP

A aprovação pela CEUA/UNICAMP não dispensa autorização prévia junto ao **IBAMA**, **SISBIO** ou **CIBio**.

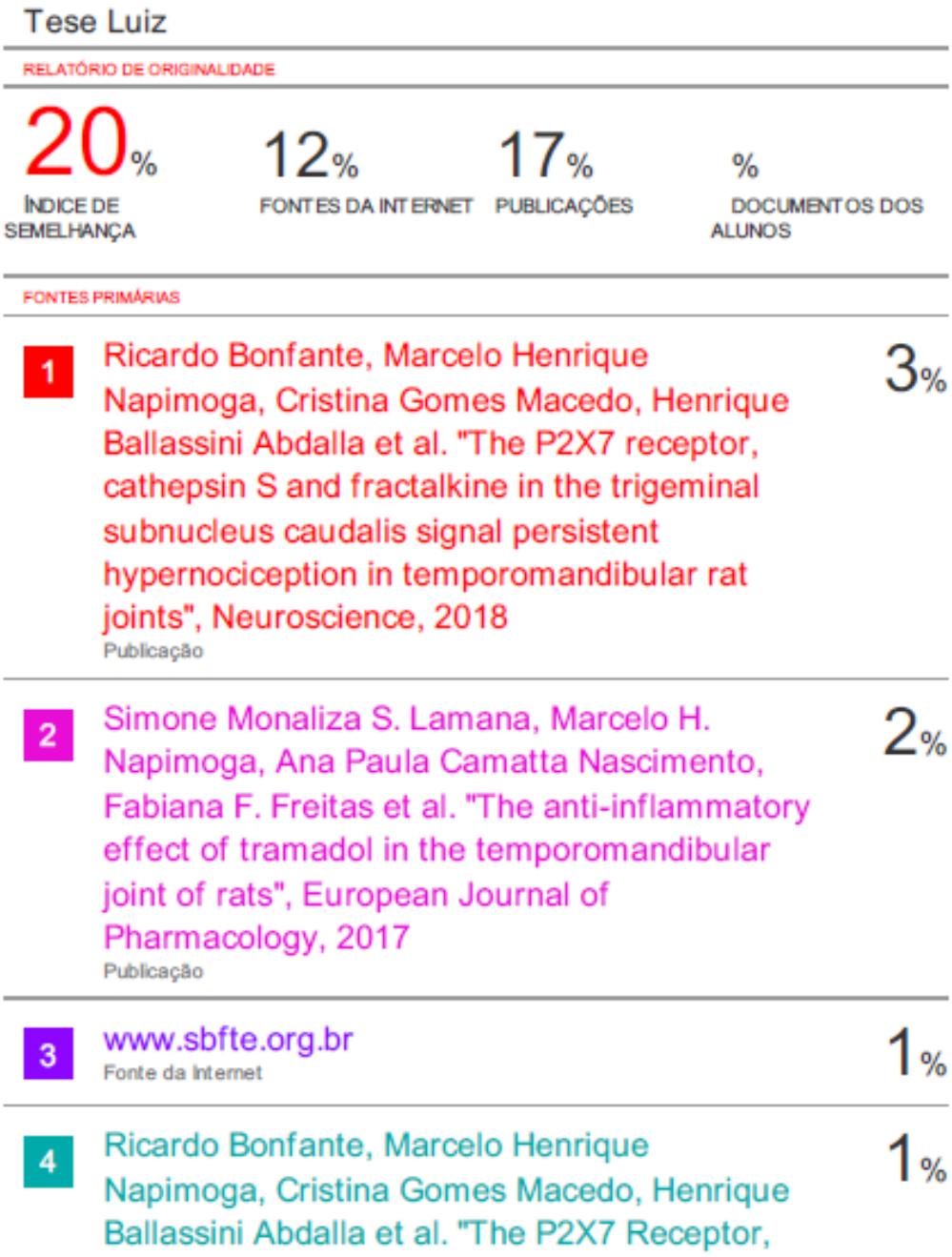
Campinas, 04 de abril de 2016.

Profa. Dra. Liana Maria Cardoso Verinaud
Presidente

Fátima Alonso
Secretária Executiva

IMPORTANTE: Pedimos atenção ao prazo para envio do relatório final de atividades referente a este protocolo: até 30 dias após o encerramento de sua vigência. O formulário encontra-se disponível na página da CEUA/UNICAMP, área do pesquisador responsável. A não apresentação de relatório no prazo estabelecido impedirá que novos protocolos sejam submetidos.

Anexo 2 - Relatório final de similaridade



Tese Luiz

RELATÓRIO DE ORIGINALIDADE

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Anexo 3 - Submissão para publicação em revista científica

Subject: Submission Confirmation

*** Automated email sent by the system ***

Early phase of type 1 diabetes decreases the responsiveness of C-fiber nociceptors in the temporomandibular joint of rats.
Research Paper
Corresponding Author: Dr. Juliana Trindade Clemente-Napimoga

Dear Dr. Clemente-Napimoga,

Your submission entitled "Early phase of type 1 diabetes decreases the responsiveness of C-fiber nociceptors in the temporomandibular joint of rats." has been received for consideration in Neuroscience.

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