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FACULDADE DE FARMÁCIA, ODONTOLOGIA E ENFERMAGEM
PROGRAMA DE PÓS-GRADUAÇÃO EM ODONTOLOGIA

ASSIS FILIPE MEDEIROS ALBUQUERQUE

AVALIAÇÃO DA EXPRESSÃO GÊNICA DAS CICLOOXIGENASES 1 E 2 EM MODELO CLINICO DE ANALGESIA PREEMPTIVA SOB USO DE DIFERENTES ANTIINFLAMATÓRIOS NÃO ESTEROIDAIS EM CIRURGIAS PARA REMOÇÃO DE TERCEIROS MOLARES MANDIBULARES

FORTALEZA – CE

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Área de Concentração: Clínica Odontológica

Orientador: Prof. Dr. Fábio Wildson Gurgel Costa.

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RESUMO

As ciclooxigenases (COXs) desempenham um papel importante no desenvolvimento de eventos inflamatórios relacionados a cirurgias de terceiros molares. Embora o uso pré-operatório de antiinflamatórios não esteroidais (AINEs) seja empregado rotineiramente em tais procedimentos, são escassos estudos que os correlacionem com a expressão gênica das COXs. Assim, a presente tese é composta por dois capítulos que têm como objetivo, respectivamente: 1) realizar uma revisão sistemática sobre a expressão gênica das COXs em cirurgias de terceiros molares; 2) avaliar a expressão das COXs 1 e 2 em modelo clínico de analgesia preemptiva sob uso de diferentes AINEs. No capítulo 1, uma revisão sistemática cadastrada na plataforma PROSPERO sob o número 42017060455 foi realizada de acordo com as recomendações do guia PRISMA**.** Os resultados deste estudo mostraram uma variabilidade de metodologias acerca do tipo de material coletado, medicações utilizadas e genes avaliados, o que dificulta a obtenção de dados consistentes sobre a relação entre a real eficácia clinica das medicações e a expressão das isoformas das COX. No capítulo 2, foi realizado um ensaio clínico, randomizado, triplo cego, placebo-controlado para avaliar o efeito de dois AINEs sobre a expressão gênica das COXs 1 e 2 em modelo clínico de analgesia preemptiva envolvendo a remoção cirúrgica de terceiros molares mandibulares. Os pacientes elegíveis foram aqueles que necessitavam realizar a remoção dos dois terceiros molares inferiores, com semelhança do padrão de inclusão, na faixa etária de 18 e 35 anos. Eles foram randomicamente alocados em 3 grupos para receber 1 hora antes do procedimento uma dose única de ibuprofeno 400mg, etoricoxibe 120 mg ou placebo. Uma amostra de tecido gengival foi obtida logo após a anestesia e com 30 minutos do início do ato cirúrgico para avaliar o curso temporal da expressão do RNAm para as COXs por meio de reação em cadeia de polimerase quantitativa em tempo real (qRT-PCR). Em relação a expressão gênica o grupo ibuprofeno e etoricoxibe tiveram um aumento significativo de COX-1 de T0 para t30 coparado ao grupo placebo ($p=0,020$). Todos os grupos tiveram um aumento da expressão de COX-2, com menor aumento no grupo etoricoxibe ($p=0.023$). Os grupos experimentais mostraram uma correlação significativa entre os níveis de COX-1 e COX-2 e os parâmetros clínicos de dor, e o grupo ibuprofeno mostrou uma correlação oposta entre a expressão de COX-1 e a abertura máxima da boca ($p<0,05$). Em conclusão, a indução do RNAm da COX-2 esteve diretamente relacionada à inflamação tecidual em cirurgias de terceiros molares, bem como a relação entre os níveis de COX-1 e COX-2 foi inversamente proporcional à seletividade do AINE administrado, o que corroborou com os achados clínicos de dor encontrados.

Palavras-chave: Analgesia preemptiva. Dente serotino. Ciclooxigenases. Expressão gênica.

Abstract

Cyclooxygenases (COXs) play an important role in the development of inflammatory events related to third molar surgeries. Although the preoperative use of nonsteroidal anti-inflammatory drugs (NSAIDs) has been considered in such procedures, studies correlating the gene expression of COXs are scarce. In this context, the present thesis is composed by two chapters that aim, respectively: 1) to carry out a systematic review on the gene expression in surgeries of third molars; 2) to evaluate the gene expression of COXs 1 and 2 in an oral model of preemptive analgesia under the use of different NSAIDs. In Chapter 1, a systematic review registered on the PROSPERO platform under number 42017060455 was carried out according to the recommendations of the PRISMA guide. The results of this study showed the variability of methodologies about the type of material collected, medications used and genes evaluated, which makes it difficult to obtain consistent data on the relationship between the actual clinical efficacy of the medications and the expression of COX isoforms. In Chapter 2, a randomized, double-blind, placebo-controlled clinical trial was conducted to evaluate the effect of two NSAIDs and their relationship with COXs 1 and 2 gene expression in an oral model of preemptive analgesia involving the surgical removal of mandibular third molars. Eligible patients were those who needed to perform the removal of the two lower third molars with similar inclusion pattern, being between 18 and 35 years old. They were randomly allocated into three groups to receive 1 hour before the procedure a single dose of ibuprofen 400mg, etoricoxib 120mg or placebo. A sample of gingival tissue was obtained shortly after anesthesia and 30 minutes after the beginning of the surgical procedure to evaluate the temporal course of mRNA expression for the COXs by quantitative real-time polymerase chain reaction (qRT-PCR). All groups had a significant decrease in COX-2:COX-1 from T0 to T30 (placebo, $p=0.013$, ibuprofen, $p<0.001$, etoricoxib, $p=0.047$). Experimental groups showed a significant correlation between COX-1 and COX-2 levels and clinical pain parameters, and the ibuprofen group showed an opposite correlation between COX-1 expression and maximum mouth opening ($p <0,05$). In conclusion, induction of COX-2 mRNA was directly related to tissue inflammation triggered in third molar surgeries, as well as the relationship between COX-1 and COX-2 levels was inversely proportional to preoperative NSAID selectivity, which corroborated with the clinical findings of pain found.

Keywords: Preemptive analgesia. Third molars. Cyclooxygenases. Gene expression.

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AINEs Drogas antiinflamatórias não esteroidais

AP Analgesia Preemptiva

ASA *America Society of Anesthesiologists* (Sociedade Americada de Anestesiologia)

ANXA3 Anexina A3

CCL2 Quimiocina (motivo C-C) ligante 2

COX Ciclooxigenase

COX-1 Enzima ciclooxigenase tipo 1

COX-2 Enzima ciclooxigenase tipo 2

COX-3 Enzima ciclooxigenase tipo 3

CONSORT *Consolidated standards of reporting trials (*Normas consolidadas de ensaios clínicos)

FDA *Food and Drug Administration* (Administração de alimentos e drogas)

IL Interleucina

MMP *Matrix metalloproteinase* (Metaloproteinase de matriz)

NSAIDs *Nonsteroidal anti-inflammatory drugs* (Drogas antiiflamatórias não esteroidais)

PGE Prostaglandina E

PGE_2 Prostaglandina E_2

pg Picograma

PLA/PLA2 Fosfolipase/Fosfolipase A2

PRISMA *Preferred Reporting Items for Systematic Review and Meta-Analysis* (Itens de Relatório Preferidos para Revisão Sistemática e Meta-Análise)

PROSPERO *Prospective International Registry of Systematic Reviews* (Registro Internacional Prospectivo de Revisões Sistemáticas)

RNAm RNA mensageiro

qRT-PCR Cadeia de polimerase quantitativa em tempo real

SOCS3 Supressor 3 da sinalização de citocina

SOD2 Superoxido dismutase 2

TNF- α Fator de necrose tumoral alfa

VAS *Visual Analogue Scale* (Escala visual analógica - EVA)

SUMARIO

1. INTRODUÇÃO GERAL

Dentre os procedimentos cirúrgicos realizados pelo cirurgião-dentista, a cirurgia para remoção de terceiros molares destaca-se pela sua frequência e procura no consultório odontológico (MARTIN, KANATAS, HARDY, 2005). Por ser um procedimento considerado invasivo, comumente associa-se a variados níveis de dor, podendo afetar significativamente a qualidade de vida dos pacientes particularmente durante os três primeiros dias do período pós-operatório quando os eventos clínicos inflamatórios se encontram mais intensos (BENEDIKTSDOTTI et al., 2004; MOLLER et al., 2005; ALBUQUERQUE et al., 2017). Em virtude disso, a exodontia dos terceiros molares tem sido amplamente utilizada como modelo de estudo em ensaios clínicos de analgesia para avaliação de dor aguda. Diversas pesquisas envolvendo novas drogas a serem submetidas à avaliação pela Food and Drug Administration (FDA) utilizam esse tipo de modelo de dor devido a sua adequada e bem-estabelecida reprodutibilidade (AVERBUCH e KATZPER 2003; COSTA et al., 2015-A).

A sinalização nociceptiva da dor fisiológica inflamatória é iniciada pela ativação dos receptores especializados na dor (nociceptores), que são fibras sensoriais polimodais dos neurônios sensoriais, podendo ser polimodais a depender do tipo de estÍmulo. Embora a dor fisiológica tenha uma função protetora de alertar o corpo de estímulos potencialmente prejudiciais, a dor inflamatória associada a qualquer tipo de dano tecidual tem um caráter patológico, que se manifesta clinicamente através da hiperalgesia (BURIAN e GEISSLINGER, 2005). Dessa maneira, os AINES apresentam efeito antinociceptivo, devido sua propriedade de inibir a formação das ciclooxigenases (COX), as quais afetam diretamente a cascata do ácido araquidônico. Este é formado após a ativação celular devido a um trauma ou lesão local, através dos fosfolipídios da membrana celular pela ação da enzima fosfolipase A2, liberando o ácido araquidônico, que por sua vez é instável sendo metabolizado por duas vias, (1) lipoxigenase, que tem como produto final os leucotrienos e (2) ciclooxigenase, tendo

como produtos finais as prostaglandinas (PG) e o tromboxano (TXB) (KAHN et al. 2002, LEE et al. 2007). Estes últimos são responsáveis por atividades homeostáticas do organismo e por ação inflamatória local como: ação na musculatura lisa, potencialização do edema, aumento da temperatura corporal, hiperalgesia, vasodilatação, dentre outros (BURIAN e GEISSLINGER, 2005). Além dessas duas vias, outras substâncias também estão ligadas a essa cascata, como a histamina, prostaciclina e algumas citocinas pró-inflamatórias, as quais destacam-se o fator de necrote tumoral (TNF-α) e a interleucina 1β (IL-1β) que estão diretamente ligadas a inflamação local (ALBUQUERQUE et al., 2017).

Além da dor, as complicações pós-operatórias mais comumente relacionadas à remoção de terceiros molares inferiores são o trismo (limitação de abertura bucal) e o edema, decorrentes do processo inflamatório local, com a expressão das isoformas da COX e prostaglandinas desempenhando um importante papel em seu desenvolvimento (VAN GOOL, TEN BOSCH, BOERING, 1977, COSTA et al., 2015-B).

De fato, a resposta inflamatória é parcialmente mediada por prostaglandinas e a síntese destas é iniciada pela liberação de ácido araquidônico a partir dos fosfolipídios da membrana celular. A conversão subsequente de ácido araquidônico em prostaglandinas é catalisada pelas ciclooxigenases (BURIAN e GEISSLINGER, 2005). Atualmente, reconhecem-se três isoformas desta enzima: COX-1, COX-2 e COX-3. A COX-1 é expressa constitutivamente em muitos tecidos (vasos sanguíneos, plaquetas, estômago, intestino e rins), desempenhando funções homeostáticas. Um gatilho de estímulo, incluindo a inflamação, injúria e estresse mecânico, desencadeiam a síntese da COX-2, a qual, em consequência, induz a produção de prostaglandinas (PGE) e citocinas pró-inflamatórias como o TNF-α e interleucinas (IL-1β, IL-6) (LIPSKY, 1999; KAHN, GUTIÉRREZ, AQVIST, 2018). A COX-3 foi descoberta recentemente, sendo uma isoforma genética semelhante à COX-1, identificada em cérebros de cães (CHANDRASEKHARAN et al., 2002). Em adição, duas variantes do RNAm da COX-2

também foram descobertas, sendo designadas como COX-2a e COX-2b (OLSEN et al., 2012).

O conceito de que a COX-2 é a única isoforma da COX envolvida na inflamação tem sido questionado (LEE, RODRIGUEZ, DIONNE, 2005; GORDON et al., 2002). Acredita-se que a COX-1 seja responsável pela resposta prostanóide inicial ao estímulo inflamatório, enquanto que a COX-2 torna-se o principal participante na síntese de prostanóides durante o progresso do processo inflamatório (KAHN et al., 2002). Com a descoberta da COX-3, observou-se que esta participa dos eventos finais do processo inflamatório não produzindo prostanóides pró-inflamatórios, diferentemente das outras isoformas de COX (WILLOUGHBY, MOORE, COLVILLE-NASH, 2000). Com a metabolização do acido araquidônico pelas COX, ocorre a liberação de prostaglandina E_2 (PGE_2) em tecidos inflamados, sensibilizando os terminais das fibras nervosas aferentes e incrementando o processo nociceptivo para evocar hiperalgesia (SVENSSON e YAKISH 2002; EHRICH et al., 1999). O RNA mensageiro (RNAm) proveniente da expressão de COX-1 apresenta uma meia vida de cerca de 12-15 horas, enquanto COX-2 apresenta uma meia vida mais curta de menos de 3.5 horas (LUKIW, BAZAN 1997; KAHN, GUTIÉRREZ, AQVIST, 2018), sugerindo uma intrínseca ligação temporal entre a injúria tecidual, a expressão de COX-2 e o aumento dos níveis de PGE_2 em comparação à COX-1 expressada constitutivamente.

Um estudo prévio em modelo de cirurgia oral demonstrou uma produção distinta de produtos oriundos da COX-1 (tromboxano B_2, um metabólito estável do tromboxano A2) e produção de PGE_2 mediada tanto por COX-1 como por COX-2 (KHAN et al., 2007). Considerando o alto nível de atividade inflamatória e levando em consideração o benefício máximo ao paciente submetido a uma cirurgia para remoção de terceiros molares mandibulares, insere-se a analgesia preemptiva como estratégia farmacológica amplamente pesquisada nas últimas décadas. O interesse por tal terapêutica, baseia-se na hipótese de que a

administração pré-operatória de um determinado medicamento possa reduzir a severidade da dor decorrente do procedimento cirúrgico para remoção de terceiros molares ou mesmo prevenir/minimizar o estabelecimento de dor pós-operatória decorrente do trauma estabelecido (COSTA et al., 2015-A; AU et al., 2015).

Considerando-se o conceito e objetivo da analgesia preemptiva, alguns recursos farmacológicos utilizando outras drogas que não apenas com finalidade analgésica vêm sendo extensivamente pesquisados em grandes centros (COSTA et al., 2015-A). Nesse contexto, as drogas antiinflamatórias não-esteroidais (AINEs) inibem a síntese de prostaglandinas e são comumente prescritas para alívio da dor e controle do edema após cirurgias realizadas na cavidade oral. Destaca-se o ibuprofeno (inibidor não seletivo das COX) como um dos AINEs mais comumente utilizados para alívio da dor de origem dentária e sua eficácia no tratamento desse tipo de dor tem sido avaliada em diversos ensaios clínicos (EHRICH et al. 1999, GORDON et al 2002, LEE et al 2005, AU et al., 2015; COSTA et al., 2015-A e B, ALBUQUERQUE et al. 2017). Por outro lado, estudos recentes têm mostrado a eficácia do etoricoxibe, um inibidor seletivo COX-2 com poucos efeitos gastrointestinais, no tratamento de dor aguda oriunda de cirurgia de terceiros molares (COSTA et al., 2015-B).

Nesse cenário, faz-se necessário o desenvolvimento de pesquisas que possam avaliar não apenas os efeitos clínicos da administração pré-operatória de AINEs seletivos e não seletivos COX-2 relacionados as cirurgias de terceiros molares, mas também realizar uma correlação desses achados clínicos (dor inflamatória e abertura bucal) com mediadores inflamatórios por meio da avaliação laboratorial da expressão gênica tanto das isoformas das COXs (COX-1 e COX-2) quanto de seus subprodutos (por exemplo, PGE_2 e TBX_2), dentre outros.

2. OBJETIVOS

OBJETIVO GERAL:

Avaliar a expressão gênica das ciclooxigenases 1 e 2 após a remoção de terceiros molares em modelo clínico de analgesia preemptiva utilizando AINES seletivos e não selevivos.

OBJETIVOS ESPECÍFICOS

1. Revisar revisão sistemática acerca de achados laboratoriais oriundos de estudos que tenham avaliado a expressão gênica, de forma direta ou indireta, das COXs em cirurgias para remoção de terceiros molares;
2. Avaliar o curso temporal da expressão do RNAm para COX-1 e COX-2 a partir de modelo clínico de analgesia preemptiva envolvendo a remoção cirúrgica de terceiros molares mandibulares sob administração pré-operatória de diferentes AINEs.
3. Correlacionar parâmetros clínicos (dor inflamatória e abertura bucal) com os níveis teciduais de COX-1 e COX-2 em modelo oral de analgesia preemptiva envolvendo a remoção cirúrgica de terceiros molares mandibulares sob administração pré-operatória de diferentes AINEs.

3. HIPÓTESES

3.1 Hipótese nula

A analgesia preemptiva, através da administração pré-operatória de AINEs, não altera a expressão gênica da COX-1 e COX-2 e consequentemente não se relaciona com repercussões clínicas em cirurgias para remoção de terceiros molares mandibulares.

3.2 Hipótese alternativa

A analgesia preemptiva, através da administração pré-operatória de AINEs, altera a expressão gênica da COX-1 e COX-2 e consequentemente relaciona-se diretamente com repercussões clínicas em cirurgias para remoção de terceiros molares mandibulares.

4. DESFECHOS

4.1 Desfecho primário

Ocorrência de alteração na expressão gênica da COX-1 e COX-2 de acordo com o grupo experimental.

4.2 Desfecho secundário

Ocorrência de evento inflamatório pós-operatórios (dor) no grupo experimental, devido à expressão (aumentada/reduzida) das COX-1 e COX-2.

5. CAPÍTULO

Esta Tese está baseada no Artigo 46, do Regimento Interno do Programa de Pós-Graduação da Universidade Federal do Ceará, que regulamenta o formato alternativo para trabalhos de conclusão de mestrado e doutorado (dissertações e teses) e permite a inserção de artigos científicos de autoria ou co-autoria do candidato.

Por se tratar de pesquisa envolvendo seres humanos, os protocolos utilizados neste trabalho foram submetidos à apreciação e foram devidamente aprovados pelo Comitê de Ética em Pesquisa em Seres Humanos do Hospital Universitário Walter Cantídio, tendo sido aprovado e protocolado sob o no. CAAE 44058715.4.0000.5045

Desta forma, a presente tese é composta por dois artigo científico redigido de acordo com a revista científica escolhida.

5.1 Capítulo 1

"**Preemptive analgesia-related gene and protein expressions in third molar surgery under non-steroidal anti-inflammatory drugs protocols: a registered systematic review of clinical studies."**

Este artigo seguiu as normas de publicação do periódico:

- Medicina Oral Patologia Oral y Cirugia Bucal (ISSN 1698-4447)
  Qualis CAPES (B1); Fator de impacto: 1,156

5.2 Capítulo 2

"**Cyclooxygenases 1 and 2 in oral surgical model comparing the effect of single-dose preemptive ibuprofen and etoricoxib: RT-qPCR study with postoperative inflammatory events correlation**."

Este artigo seguiu as normas de publicação do periódico:

- Clinical Oral Investigations (ISSN 1436-3771)
  Qualis CAPES: A1; Fator de impacto: 2,308

Title Page

Preemptive analgesia-related gene and protein expressions in third molar surgery under non-steroidal anti-inflammatory drugs protocols: a registered systematic review of clinical studies

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Running title: Gene expression in third molar sugeries.
Key words: third molar, gene expression, preemptive analgesia, systematic review.

Abstract

Background: This study aimed to analysis translational studies focusing on the third molar removal through a systematic review approach.

Materials and Methods: A PROSPERO-registered systematic review (CRD42017060455) was conducted following the PRISMA statements to summarize current knowledge on the gene expression in third molar surgeries. A search was performed in PubMed's Medline and Scopus databases, without date or language restrictions, using the {[(Third molar) OR (preemptive) OR (cyclooxygenase inhibitors) OR (acute inflammation) + (gene expression)]}.

Results: All included studies evaluated the gene expression in third molar extraction model, adopting the preemptive analgesia methodology in 6 investigations. The sample analyzed was obtained from gingival tissue biopsy (n=4), blood (n=1), transudate (n=1) and gingival tissue biopsy/trasudate (n=1). There was a heterogeneity regarding evaluated genes, drug protocol, sample studied, and method for gene expression.

Conclusion: In summary, third molar surgeries were associated with different COX-related gene expression patterns. Although inflammatory events following the surgical procedure are associated with COX isoforms, data from preemptive analgesia studies are scarce, especially correlating gene expression and clinical parameters.

Introduction

The evaluation of specific gene expression has been widely useful as an important tool in several studies focused on the field of dentistry, including its use in translational investigations in order to carry out experimental designs for diagnosing purposes, as well as to evaluate pharmacological-based drug protocols commonly indicated in clinical situations (1,2). Also, the direct gene analysis by observing its Messenger RNA (mRNA) expression or even through the measurement of specific mediators such as cytokines has identified important findings that support a relationship between tissue damage, degree of inflammatory process, and onset of clinical related events such as pain and edema in surgical procedures for removal of third molars (3-5).

In fact, removal of third molars is an invasive procedure capable of triggering various levels of pain and other related inflammatory events, which can significantly affect the patient quality of life (6). These findings have contributed to the routinely use of third molar surgery as a useful clinical model to analysis the efficacy of conventional prescribed analgesics and anti-inflammatory drugs in order to minimize the effects from the established inflammation since the surgical intervention until the postoperative period (7,8). In addition, pain and edema are expected following the surgical procedure; thus, it is supposed that these events elicited by inflammation are correlated with gene-related increase of key pro-inflammatory cytokines released at the site of the injury, e.g. interleukin 1 beta (IL-1β) and tumor necrosis factor alpha (TNF-α), which have showed a significant increase after the extraction of the third molars (3) and posses a direct link with cyclooxygenase isoforms gene expression during the inflammatory process (9).

Cyclooxygenase gene evaluation has obtained substantial interest over the time in experimental investigations due to its value in laboratory setting and clinical situations since the routine use of selective and non-selective COX-2 non-steroidal anti-inflammatory drugs (NSAIDs) may be associated with different levels of COX expression in studies methodologies testing these medicines for relief of third molar-related inflammatory symptoms (3,10). The use of methods allowing to quantify the COX expression following clinical procedures under pharmacological analgesic and anti-inflammatory protocols, and to explain the influence of the aforementioned drugs on pain variables are of great significance (10-12).

Besides COX isoforms have been commonly studied as target genes during the inflammatory process, other genes have been investigated in the field of the third molar surgery (2). Recently, some authors have performed a quantitative analysis of gene expression in translational researches evaluating the effect of preoperative administration of different NSAIDs on the severity of clinical events related to the inflammation in patients underwent to the surgical removal of third molars (12,13). Although the quantitative real-time polymerase chain reaction (RT-qPCR) is considered a tool widely used in experimental investigations (14), other methodologies have been proposed for evaluating gene expression in third molar surgeries and its correlation with postoperative clinical symptoms, such as pain and edema (11,13). However, an overview of these studies by means of a systematic analysis was not published to date. Systematic reviews are important approaches designed for investigating specific issues of scientific interest using clear, well defined, and rigorous methods (15). These studies characteristically involve a meticulous and comprehensive plan and search strategy consequent a priori, aiming to reduce bias by identifying, appraising, and synthesizing all pertinent studies on a certain topic (16,17).

The scientific significance of systematic reviews depends on several factors. Although there is a standardized way of being carried out, some articles have conducted methodological failures in relation to the structuring of the research. In this context, both the Preferred Reporting Items for Systematic Review and Meta-Analysis (PRISMA), available since 2009 (18), as well as the register of these studies in a platform called "prospective international registry of systematic reviews" (PROSPERO), accessible since 2011, have been adopted in order to minimize inconsistences during the review process (15,19). Thus, the importance of obtaining adequate data regarding COX gene expression in translational studies focusing on the third molar removal justifies the present PROSPERO registered systematic review, which was designed based on standardized methodology and following the PRISMA guide recommendations.

Materials and methods

Protocol and Registration

A systematic review was conducted to summarize current knowledge on data from gene expression recorded in clinical studies analyzing the preemptive use of NSAIDs in third molar surgeries. In addition, this systematic review was registered in the PROSPERO

database and conducted following the PRISMA statements (#42017060455).

Information Sources and Search Strategy

The PICO strategy (Patient/Population: patients; Intervention: preemptive analgesia for third molar removal; Comparison: gene expression; Outcome: studied variables) was used to establish the starting question to be answered by this systematic review: "Is there variation of the gene expression in patients underwent to preemptive analgesia with NSAIDs in third molar surgeries?"

In order to perform the search strategy, PubMed's Medline, Scopus, and SciencDirect were used as electronic databases to retrieve articles without date or language limits. The present systematic review was conducted on April 10, 2017, and Federal University of Ceará (Brazil), School of Dentistry, computer network was used to perform the electronic data search. The algorithm used was: {[(Third molar) OR (preemptive) OR (cyclooxygenase inhibitors) OR (acute inflammation) + (gene expression)]}.

Other sources were also used to include additional articles. A manual search of related journals, including Medicina Oral Patologia Oral y Cirugia Bucal; British Journal of Oral and Maxillofacial Surgery; International Journal of Oral and Maxillofacial Surgery; Journal of Craniofacial Surgery; Journal of Cranio-Maxillofacial Surgery; Journal of Maxillofacial and Oral Surgery; Journal of Oral and Maxillofacial Surgery; Anesthesia and analgesia, Prostaglandins, Leukotrienes and Essential Fatty Acids, Oral Surgery, Oral Medicine, Oral Pathology, Oral Radiology and oral endodontic, Clinical Pharmacology & Therapeutics, British Journal of Pharmacology, The Clinica Journal of Pain, British Journal of Rheumatology, European Journal of Pharmacology, Pain, Inflammation Research, Journal of Pain, Anesthesiology, was performed. Also, reference lists obtained from the identified articles and relevant reviews on the subject also were checked for possible additional studies.

Elegibility criteria

The inclusion criteria adopted in this review were: articles without language or year of publication restrictions; clinical studies involving gene expression in oral surgery; and studies involving human beings. As exclusion criteria, it was not considered eligible: case reports, case series, literature reviews, and editor's notes.

Study selection and data collection process

A two-phase selection of the articles was conducted. In Phase 1, two independent researchers (AFMA and CMSP) determined eligibility by reading titles and abstracts of each identified study; subsequently, same articles found in different database were excluded (duplicated). In Phase 2, the full text of those which were eligible was assessed following the inclusion criteria. Any reviewers disagreements were resolved by consensus with a third researcher (FWGC).

The researchers independently extracted the data using previously established criteria. Each selected study was analyzed, and the following variables adopted for the present systematic review were summarized when available: study origin, number of patients, sex, age, use of preemptive analgesia therapy, studied drugs, type of material collected for gene expression analysis, evaluation time, and quantitative data of the studied gene.

Risk of bias in individual studies

The methodological validity of selected studies was assessed by two independent reviewers (AFMA and CMSP) using the Joanna Briggs Institute Meta Analysis of Statistics Assessment and Review Instrument (JBI-MAStARI) as previously reported (20). The reviewers independently scored each data item as "yes", "no", "unclear" or "not applicable" and assessed the quality of each included study. The third author (FWGC) resolved through discussion any disagreement between the authors. Risk of bias was categorized as high (up to 49% score "yes"), moderate (50-69% score "yes"), and low (more than 70% score "yes").

Synthesis of results

Data were imported into an Excel (Microsoft Corporation, Redmond, WA) spreadsheet aiming to obtain relative and absolute frequencies.

Results

Study selection

The selection process of the articles can be observed in Figure 1. The search strategy rendered an initial amount of 7,177 articles, of which 86 studies were identified in more than one database (duplicated articles) and, then, they were removed. From the remaining articles, 7,076 were excluded because they did not discuss the investigated topic adopted in the present study. Manual searches in related journals did not result in addition studies, and the 15

identified articles were completely read. From this total, 9 studies were excluded because they did not meet the eligibility criteria since these articles did not evaluate gene expression in third molar surgeries. Additional search in reference lists from the selected studies rendered 2 new articles. Therefore, 7 articles were evaluated in this systematic review.

Study characteristics

All the selected studies were originated from North America (USA), and they rendered 929 patients, showing a predominance among male individuals (69%) in comparison with the female sex (31%). The volunteers were adults in most of the analyzed investigations, with age ranging from 16 to 66 years and an approximately mean age of 25 years (Table 1).

Regarding the methodological aspects, all articles performed gene expression in patients undergoing to third molar surgery. The sample evaluated for this purpose was the gingival tissue removed during the surgical procedure in five studies (n=722 patients) (2,4,5,20,21), followed by blood analysis in one investigation (n=104 patients) (22), and gingival exudate analysis in another study (n=103 patients) (13).

Risk of bias within studies

According to Figure 2, the mean percentage of score "yes"was 76,18±11,88, ranging from 77,77% to 88,88%. The risk of bias within studies was considered moderate in two studies and low in five studies.

Results of individual studies

There was a prevalence of COX-2 selective NSAIDs among the articles published in the field of the preemptive analgesia. The following drugs were found among the investigations: rofecoxib (n=5 articles) (2,4,21-23), ibuprofen (n=5 articles) (2,13,21-23), ketorolac (n=1 article) (4), acetaminophen (n=1 article) (4), indomethacin (n=1 article) (23), celecoxib (n=1 article) (13) and placebo (n=6 articles) (2,4,13,21-23). In addition, all methodologies were planned in order to obtain data from COX gene expression. However, a variability of methods was observed. According to Table 1, three studies quantified the gene expression by using RT-qPCR analysis and the remaining studies performed an indirect evaluation of the COX expression from the analysis of related genes: thromboxane B_2 (TXB_2) (4,13,23), prostaglandin E_2 (PGE_2) (4,13,23), matrix metalloproteinase (MMP) (21), phospholipase A2 (PLA), suppressors of cytokine signaling 3 (SOCS), and interleukins 1 and

6 (IL-1 and IL-6, respectively) (2,22).

The pain was the unique clinical parameter evaluated as a primary outcome regarding the inflammatory events following surgical removal of third molars. Among these studies, pain was measured by means of visual analog scale (VAS) in four investigations (2,4,13,23), and the other ones only included a laboratory analysis without a proper clinical observation. These articles evaluated pain at different study period, such as 0-6h (n=1), 0-180min (n=1), 2-4h and 48h (n=1), and 24h (n=1) postoperatively. In addition, VAS was used in two articles and scores ranging from 1 to 4 (mild, moderate, grave, and severe respectively) was employed in the other studies.

When the pharmacological class of the studied NSAIDs was evaluated regarding COX gene expression (2,4,21,22), it was observed that coxibs (rofecoxib and celecoxib) presented a significant selectivity related to COX-2, ranging from 5 to 500 times more than other non-selective NSAIDs (ibuprofen, acetaminophen, and indomethacin) and placebo, being evaluated by RT-qPCR or analysing genes that display influence on COX-1 and COX-2 gene expressions, such as TXB_2, PGE_2, and MMP. Table 2 describes the main results found in the studies.

Discussion

Third molar surgery is a routine clinical procedure performed at the dental office, and it has been involved in studies evaluating the use of preoperative medications (NSAIDs) to minimize the inflammatory events observed during the postoperative period (7). In fact, this clinical model has been widely reproduced in translational researches aiming to evaluate protocols of medicines since 1976, when its validation in pharmacological studies was provided (24). Thus, the present systematic review based on the PRISMA methodology reported relevant data in the context of oral surgery by evaluating gene expression following removal of maxillary and mandibular third molars.

It was observed a considerable variability of methodologies used for clinical and laboratory purposes, including the gene studied, the preemptive analgesic medication, the type of material collected for gene expression analysis, the evaluated study periods, and the method for analyzing the gene-related mRNA. Since varied methods to evaluate the gene expression of COX or related genes was performed among the critically reviewed articles, some difficult to obtain a standardized and reliable analysis was presently found. From the

eligible studies, there were three investigations that directly evaluated the COX gene (2,4,5), and the remaining studies used an indirect way to provide this evaluation. These articles analyzed the TXB_2 and PGE_2 (4,13,23), MMP (20), or a set of genes including PLA, SOCS3, and IL-6 (21,22).

These genes are involved in the inflammatory response, which is mediated by prostaglandins produced after the cell membrane phospholipids-related arachidonic acid metabolism. It can be performed by two COX pathways. COX-1 is the isoform constitutively expressed, acting during the regulation and homeostasis physiological processes, although it is already seen in the onset of acute inflammation. COX-2 is an inducible isoform produced in inflammatory and infectious processes, showing a direct relation with the increased production of pro-inflammatory prostaglandins and cytokines such as PGE_2, TNF-α, and IL-1β (3,9,22). PGE2 is released into inflamed tissues in order to sensitize afferent nerve fibers terminals, increasing the nociceptive process to evoke hyperalgesia (13,23), while the TNF-α exerts remarkable effects, including activating lymphocytes, stimulating the synthesis of other proinflammatory cytokines such as IL-1β and IL-6, and triggering the production of prostaglandins. IL-1β sensitizes nociceptors and causes hyperalgesia, therefore working actively in the pain pathophysiology (3). Messenger RNA from COX-1 expression has a half-life of about 12-15 hours while COX-2 has a shorter half-life of less of 3.5 hours, suggesting an intrinsic temporal link between tissue injury, COX-2 expression, and increased PGE_2 levels compared to COX-1 expression, which is responsible for coagulation and directly linked to the COX-1 activity (4,13,23). In addition, metalloproteinase (MMP) matrix plays an important role in the inflammation, and it is regulated by PGE_2, serving as a reference for COX-2 (21). Other genes that are also related to inflammation and scarcely studied are IL-6, SOCS3 and PLA, which may be increased when inflammation increases, serving as regulatory parameter for COX levels locally (22).

Ehrich *et al.* (23) evaluating indirectly COX isoforms expression (TXB_2 for COX-1 and PGE_2 for COX-2) through blood samples showed that rofecoxib was a potent COX-2 selective NSAID, exhibiting about 800 times more selectivity for COX-2 than COX-1, and it exhibited about 1000 times more COX-2 selectivity in comparison with a non-selective NSAID (indomethacin). Furthermore, as previously confirmed by Lee *et al.* (4) in a study with gingival tissue samples, it was observed that TXB2 levels did not statistically alter when compared the placebo group and testing groups (rofecoxib and acetaminophen), demonstrating no COX-1 related interference. Also, the preoperative administration of these

NSAIDs in individuals underwent to third molar surgeries resulted in a suppression of PGE_2 levels when compared to the placebo group, which highlights the COX-2 selectivity pattern.

According to Kahn *et al.* (13), TXB_2 gene expression was directly related only to the COX-1 isoform, and PGE_2 gene expression was both highly associated with COX-1 levels in a 60-minute period following the onset of the inflammatory process and COX-2 levels when recorded after 60 minutes. In this aforementioned study, the COX-2 selectivity provided by celecoxib preemptively administrated resulted in PGE_2 gene suppression without alteration of the TXB_2 levels during the postoperative period over than 60 minutes. Also, the ibuprofen suppressed both PGE_2 and TXB_2 at both evaluated study periods, which is consistent with its COX-1 / COX-2 inhibitory effect.

Clinically, several clinical trials have shown that NSAIDs ameliorate the symptomatology associated with third molar surgeries (3,6,7,10). On the basis of the inflammation, MMP family-related genes have been involved with a decrease of the inflammatory process severity when COX-2 selective drugs are prescribed to patients that underwent surgical removal of these teeth. Wang *et al.* (21) showed in samples of gingival tissue that MMPs play an essential role in acute inflammatory injuries and their activity is regulated by the action of the COX-2 mediated PGE_2 release. These reported a significant increase of MMPs expression in a clinical study using rofecoxib in comparison to ibuprofen and placebo, which may contribute to the rofecoxib-associated adverse effects, which may interfere with the resolution of inflammation and onset of these undesirable effects. In addition to the MMP study, other genes mediated by COX-2 expression and associated with the occurrence of inflammatory events, the arachidonic acid pathway, apoptosis/angiogenesis process, cell adhesion, and signal transduction were previously analyzed (21). Wang *et al*. (22) observed that gene expression. of ANXA3 (annexin 3; involved in the regulation of inflammatory responses, cell differentiation and cytoskeletal protein interactions and is associated with multiple human diseases), SOD2 (superoxide dismutase 2; expressed in the central nervous system under several inflammatory conditions), SOCS3 (supressor of cytokine signalin 3; regulates the signaling of cytokines or hormones, modulating the outcome of autoimmune infections and diseases, as well as the underlying mechanisms), and IL1RN (IL1 receptor antagonist; associated with several markers of systemic inflammation) were increased in the group treated with rofecoxib, which was a plausible result since these genes are related with the inhibition of phospholipase A2 action after the establishment of a local trauma and decrease in cytokine signaling pathway. Also, both groups treated with

rofecoxib and ibuprofen in that study showed an increase in gene expression of the inflammatory mediators IL-6 (a cytokine involved in the inflammation and infection responses, and in the regulation of metabolic, regenerative, and neural processes), and CCL2 (a chemokine C-C motif ligand 2, which is involved in neuroinflammatory processes and present at the sites of tooth eruption and bone degradation) after surgical trauma when compared to placebo. These results emphasize that COX selectivity is involved not only in anti-inflammatory effects but also in the increase of pro-inflammatory cytokines, which may play an additional role during the inflammatory process in local injuries, such as the third molar removal (22).

Regarding the gene expression over the time, Kahn *et al.* (5) performed a quantitative analysis of COX-1 and COX-2 isoforms in a clinical model of third molar extraction. These authors observed that all samples used to evaluate gene expression presented in preoperative samples destined for COX-2 study, a very weak detected band with low value in the baseline assessment (51%). However, there was a significant and progressive increase in COX-2 expression at 30, 60, and 120 minutes after surgery. When COX-1 expression was recorded, it was detected a slight decrease of its levels at 30 minutes, a considerable reduction of its concentration at 60, and a significant reduction of its levels at 120 minutes, which were lower than the preoperative period. In fact, the finding related to the expression of COX-2 was already experienced, since no preemptive NSAIDs drugs were used and considering the inflammatory insensitivity triggered by surgical extraction of a third molar. About COX-1 results, other studies that indirectly evaluated its expression support that finding (4,13,21,22). Another study that quantitatively described the COX-1 and COX-2 gene expression was performed by Lee *et al.* (2), in which an increase of COX-2 and a decrease in COX-1 were observed between 2-4 hours postoperatively, returning to pre-surgical values at 48-h after surgery. These findings suggest that acute injury related to inflammatory process stimulate increased gene expression of COX-2 and transient inhibition of the COX-1 expression. There was also a slight increase in the expression of IL-1β (2-4h), PLA2 (2-4h and 48h), and a decrease in PTGH levels (enzyme encoded to degradation prostaglandins) over the times (2).

The postoperative pain was assessed in four translational studies that provided a laboratory analysis in order to asses the COX gene expression following third molar surgery (2,4,13,23). In the study performed by Ehrich *et al.* (23), the analgesic efficacy of rofecoxib and ibuprofen was postoperatively evaluated, and significant reduction of painful perception of the two groups was found in comparison to the placebo group; however, there was no

statistically significant difference between both NSAIDs. The time requered for pain ameriloates did not differ between groups, but it was statistically significant in comparison to placebo. The use of rescue analgesia at 2-hour postoperative period was reported by 75% of patients treated with placebo, whilst testing groups (rofecoxib and ibuprofen) showed 25% of volunteers that received rescue drugs 6 hours after the surgical removal of the third molar, highlighting the efficacy of these NSAIDs in relieving pain postoperatively.

In the study of Lee *et al.* (4) that evaluated the preemptive analgesic efficacy of rofecoxib, acetaminophen, ketorolac, and placebo there was a gradual increase in pain scores until the first 3 hours, showing no statistically significant difference between rofecoxib and acetaminophen groups in comparison with the placebo group; however, preoperative ketorolac use obtained the best results regarding pain relief at 2-hour studied period, rescue medication intake, as well as the cumulative effect of pain scores over the time in comparison with placebo. In contrast with acetaminophen, rofecoxib showed a statistically significant reduction of pain scores after 2 hours of the surgical procedure. Also, the authors of this study also pointed that the found results were reasonable in relation to gene expression of TXB_2 and PGE_2. Lee *et al.* (2) showed that gene expression of COX isoforms was not the only factor supporting the observed analgesic efficacy of rofecoxib and ibuprofen preemptively used. These authors investigated the COX gene polymorphism variability and showed that in patients with the homozygous allele for COX-2 (G/G) there was a significant reduction of pain 48 hours after administration of rofecoxib in comparison with ibuprofen. In individuals with reduced expressivity of alleles (homozygous and heterozygous, C/C and G/C respectively) for COX-2, it was observed an opposite effect on pain relief at 48-hour postoperative period, since improvement of pain scores was observed by using ibuprofen. Thus, these findings reinforce the role of gene variability polymorphisms enhancing the efficacy of the preemptive analgesic medication in third molar surgeries.

In another investigation performed by Kahn *et al.* (13), it was shown that celecoxib and ibuprofen rendered better results in relieving pain postoperatively than the placebo group, which presented an increasing pain intensity over the studied periods. However, celecoxib did not differ from placebo regarding pain relief at certain periods (120, 180, and 240 minutes). Otherwise, ibuprofen group resulted in pain scores reduction over the time.

Conclusion

In summary, third molar surgeries were associated with different COX-related gene expression patterns. Although inflammatory events following the surgical procedure are associated with COX isoforms, data from preemptive analgesia studies are scarce, specially correlating gene expression and clinical parameters. In addition, the present findings were controversial in relation to selective and non-selective NSAIDs administered preoperatively in third molar surgeries aiming to control the postoperative pain level.

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Figures and legends

Figure 1. Flowchart showing the elegibility criteria adopted in the present study.

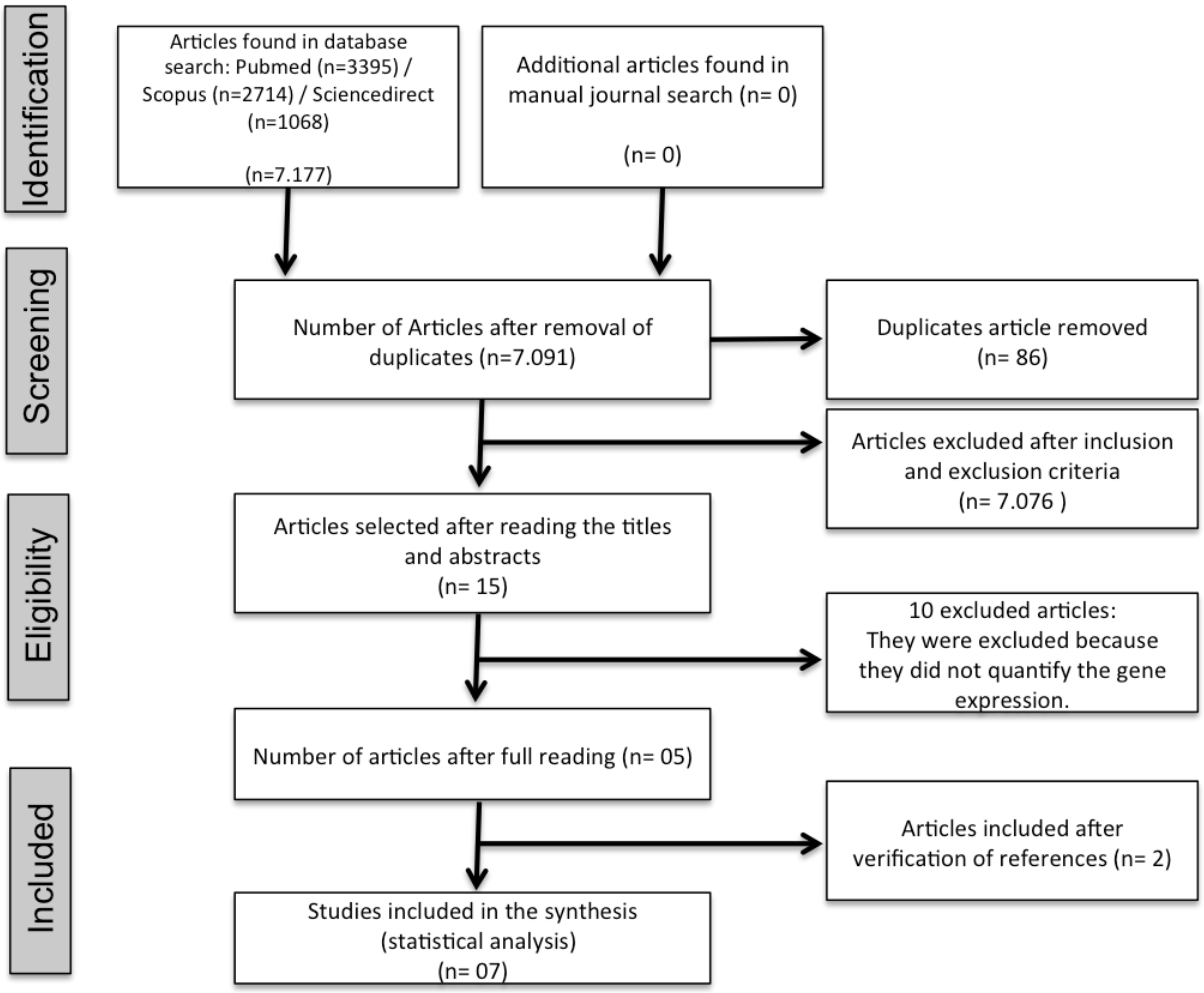
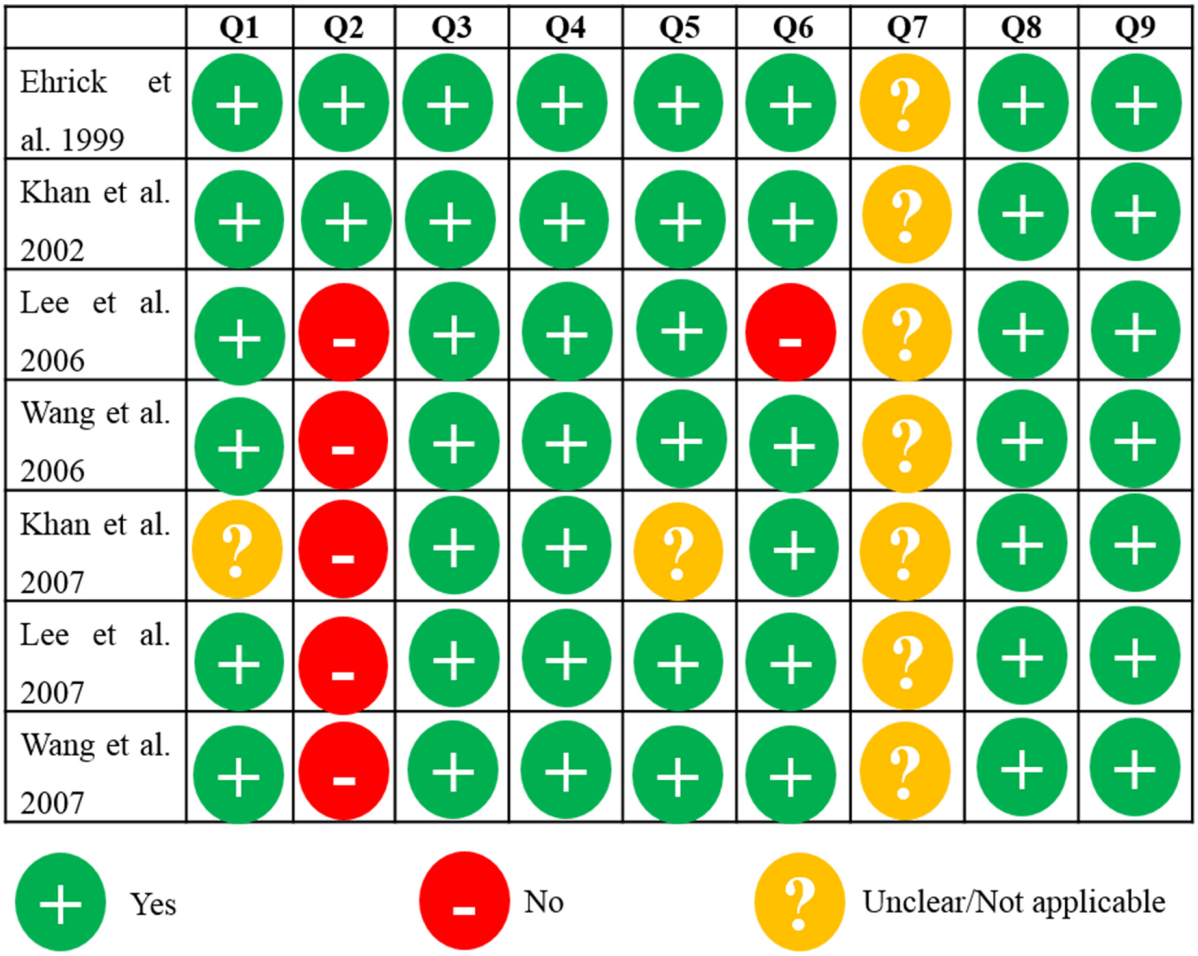


Figure 2. Critical appraisal checklist summary for randomised control/pseudo-randomised trials (JBI-MAStARI).



Q1 Was the study based on a random or pseudorandom sample?

Q2 Were the criteria for inclusion in the sample clearly defined?

Q3 Were confounding factors identified and strategies to deal with them stated?

Q4 Were outcomes assessed using objective criteria?

Q5 If comparisons are being made, was there sufficient description of the groups?

Q6 Was the follow-up carried out over a suficiente time period?

Q7 Were the outcomes of people who withdrew described and included in the analysis?

Q8 Were the outcomes measured in a reliable way?

Q9 Was na appropriate statistical analysis used?

Tables and legends

Table 1. Characterization of the selected studies regarding year of publication, origin, evaluated genes, drug protocol, sample studied, and performed test.

Author	Origin	Evaluated genes	Drug protocol	Sample studied	Method
Ehrich et al. 1999	USA	1) TXB_2 2) PGE_2	1) Rofecoxib 2) Indomethacin 3) Placebo	Blood	Radioimmunoassay (PGE_2) and enzyme immunoassay (TXB_2)
Khan et al. 2007	USA	1) COX-1 2) COX- 2	None	Gingival tissue	RT-qPCR
Lee et al. 2007	USA	1) COX-1 2) COX-2 3) TXB_2 4) PGE_2	1) Ketorolac 2) Rofecoxib 3) Acetominophen 4) Placebo	Gingival tissue and surgical site transudate	RT-qPCR
Wang et al. 2006	USA	1) MMP	1) Rofecoxib 2) Ibuprofen 3) Placebo	Gingival tissue	Microarray and RT-qPCR
Wang et al. 2007	USA	1) PLA 2) SOCS3 3) IL6 4) IL1	1) Rofecoxib 2) Ibuprofen 3) Placebo	Gingival tissue	Microarray and RT-qPCR
Lee et al. 2006	USA	1) COX-1 2) COX-2 3) IL1 4) PLA2 5) P23 6) PTGES 7) PGDH	1) Rofecoxib 2) Ibuprofen 3) Placebo	Gingival tissue	RT-qPCR
Kahn et al. 2002	USA	1) TXB_2 2) PGE_2	1) Celecoxib 2) Ibuprofen 3) Placebo	Surgical site transudate	Radioimmunoassay (PGE_2) and enzyme immunoassay (TXB_2)

Table 2. Characterization of the selected studies according to the sample number, study design, outcomes, and conclusion.

Authors	Participants	Study design	Outcomes
Ehrich et al. 1999	n=104 *Male (n=97)* *Female (n=7)*	- Parallel group; - Double blind; - Randomized; - Placebo-controlled.	- Rofecoxib showed a selectivity greater than 800-fold for COX-2 with the use of CHO cells expressing human COX-1 and COX-2; - LPS-stimulated prostaglandin E_2 dose-concentration-dependent inhibition was observed with both rofecoxib and indomethacin; - Indomethacin inhibited TXB2, which did not occur with rofecoxib even at concentrations of 1000 mg. - Total pain relief over 6 hours after NSAID administration was similar between rofecoxib 50 mg and 500 mg doses, and 400 mg ibuprofen ($p > 0.20$). All drugs tested showed positive results higher than placebo ($p < 0.001$).
Khan et al. 2007	n=43 *Male (n=20)* *Female (n=23)*	- Clinical trial.	- The expression of COX-2 at 30, 60, and 120min ($p < 0.05$), and the COX-1 rate at 60 min presented a reduction ($p < 0.05$).
Lee et al. 2007	n=119 *Male (n=57)* *Female (n=62)*	- Clinical trial; - Randomized; - Placebo-controlled.	- Release of PGE_2 was suppressed by ketorolac, rofecoxib and acetaminophen compared to placebo at 3h, coincident with increased gene expression of COX-2; - The release of TXB2 was suppressed only by ketorolac; - COX-2 gene expression remained elevated within 24 hours with continuous treatment with ketorolac and paracetamol; - COX-1 gene expression was significantly down-regulated at 24h by ketorolac, rofecoxib, and acetaminophen.
Wang et al. 2006	n=51 *Male (NI)* *Female (NI)*	- Clinical trial; - Randomized; - Placebo-controlled.	- Rofecoxib showed increased MMP expression compared to ibuprofen and placebo. - ANXA3, SOD2, SOCS3, and IL2 expresseion was increased in the rofecoxib group; - IL6 and CCL2 expression was increased with the use of NSAIDs.
Wang et al. 2007	n=79 *Male (NI)* *Female (NI)*	- Clinical trial; - Randomized; - Placebo-controlled.	- Rofecoxib increased ANXA3, SOD2, SOCS3, and IL1RN expression, and suppressed cytokine signaling cascades in comparison with placebo; - Rofecoxib and ibuprofen increased IL6 and CCL2 gene expression in comparison with placebo.
Lee et al. 2006	n=430 *Male (NI)* *Female (NI)*	- Clinical trial; - Blind; - Randomized; - Placebo-controlled.	- PTGS1 expression slightly decreased ($p<0.001$) and PTGS2 expression markedly increased ($p<0.001$) at 2 to 4h after surgery; - Ibuprofen and rofecoxib significantly increased COX-2 expression at 48-hour period ($p <0.001$ and

			<0.049, respectively). - G/G allele at the 765G>C nucleotide position in PTGS2 showed significant increase of PTGS2 expression (p=0.012) at 2 and 4h period; - Rofecoxib relief pain intensity in patients with G/G allele 48h after surgery compared with ibuprofen (p=0.008).
Kahn et al. 2002	n=103 *Male (n=42)* *Female (n=61)*	- Clinical trial; - Randomized; - Placebo-controlled.	- Celecoxib and ibuprofen showed a significant analgesic effect in comparison with placebo (p<0.01), and celecoxib efficacy was intermediate between ibuprofen and placebo; - A similar ratio was observed for suppression of prostaglandin E_2 at specific time points consistent with COX-2 expression (p <0.001); - Ibuprofen consistently suppressed TBX2 levels at all study periods (p<0.05), while the effect of celecoxib did not differ from placebo.

Cyclooxygenases 1 and 2 in oral surgical model comparing the effect of single-dose preemptive ibuprofen and etoricoxib: RT-qPCR study with postoperative inflammatory events correlation

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ABSTRACT

Objective: This study aimed to evaluate the gene expression of cyclooxygenases (COX) in an oral model of preemptive analgesia.

Materials and methods: Gingival tissue was collected during extraction of lower third molars from a randomized, triple-blind, split-mouth and placebo-controlled study. The eligible patients were randomly sorted to receive a single dose either of ibuprofen 400mg, or etoricoxib 120 mg or a placebo, one hour prior to surgery. The temporal course of RNAm was evaluated for COX 1 and 2 by means of a quantitative polymerase chain reaction in real time (RT-qPCR) at time zero and 30 minutes after the surgical procedure began, and it was correlated with clinical parameters (pain and maximum mouth opening).

Results: There was a significant increase in COX-1 expression between T0 and T30 in ibuprofen ($p = 0.004$) and etoricoxib ($p = 0.010$) groups. As regards COX-2, there were increases from T0 to T30 in all groups (placebo, $p = 0.012$; ibuprofen, $p < 0.001$; etoricoxib, $p < 0.001$). All groups showed a significant decrease in COX-2:COX-1 ratio from T0 to T30 (placebo, $p = 0.013$; ibuprofen, $p < 0.001$; etoricoxib, $p = 0.047$). Experimental groups showed a significant correlation between COX-1 and COX-2 levels and clinical pain parameters.

Conclusions: The present preemptive analgesia study concludes that COX-2 RNAm induction was directly linked to third molar-related tissue inflammation and that the relation between COX-1 and COX-2 levels were inversely proportional to the preemptively administered nonsteroidal anti-inflammatory drugs COX-2 selectivity.

Clinical relevance: There was a correlation between gene expression of cyclooxygenases and inflammatory process-related clinical events.

Keywords: preemptive analgesia; dental extraction; cyclooxygenases; real-time polymerase chain reaction.

INTRODUCTION

Cyclooxygenase (COX) catalyzes the initial steps in the synthesis of prostaglandins (PGs) and other eicosanoids from arachidonic acid. PGE2, one of the many arachidonic acid metabolites derived from COX, is released in inflamed tissues, sensitizing afferent nerve fibers, and increasing nociception to evoke a hyperalgesic state [1]. At least two different COX isoforms have been previously described in the literature [2]. COX-1 is constitutively expressed, whereas COX-2 expression is induced secondary to inflammation [3]. In spite of this paradigm, inducible expression of COX-1 has been reported during inflammatory response and cellular differentiation, and constitutive expression of COX-2 occurs mainly in the parenchymal cells of many tissues, including brain, kidney, and female reproductive system [2].

COX-1 and -2 are derived from different genes and constitute the main targets of non-steroidal anti-inflammatory drugs (NSAIDs). A novel COX-1 splice variant termed COX-3, sensitive to acetaminophen, was discovered, and is considered to play a key role in the biosynthesis of prostanoids known to be important mediators in pain and fever [4]. The messenger RNA (mRNA) originated from COX-1 expression presents a half-life of approximately 12-15 hours, whereas COX-2 gives rise to mRNA with a shorter half-life of less than 3.5 hours [5]. These findings suggest an intrinsic temporal connection between tissue injury, COX-2 expression and the observed increase in PGE2 levels during inflammation. This connection is not observed in association with the constitutively expressed COX-1. COX inhibition provided by NSAIDs confers relief of pain and inflammation that follows oral surgery procedures, justifying clinical interest on COX isoforms [6].

Third molar surgeries are highly invasive procedures capable of triggering various levels of inflammatory pain that may potentially impact the quality of life of patients with short and medium-term repercussions; hence, these procedures have been historically established models to study the efficacy of various centrally and non-centrally acting analgesics and anti-inflammatory drugs [7-9]. A previous study demonstrated a distinct synthesis of COX-1 metabolites and PGE2 production mediated by COX-1 and -2 following oral surgery procedures, in the absence of medications to control pain and inflammation [10].

Preemptive analgesia aims to prevent or diminish postoperative pain and inflammation, reducing the need for medication in the days immediately following surgery [6,11]. Studies have demonstrated etoricoxib's efficacy as a selective COX-2 inhibitor with few gastro-intestinal effects when used to treat acute pain associated with oral-dental surgery [6], and 120 mg was described as the minimum dose of etoricoxib that demonstrates maximum analgesic effect [12]. In addition, ibuprofen is one of the most commonly used drug to control dental pain, and its efficacy in treating pain associated with dental surgery in the postoperative period has been widely demonstrated [11,13]. The present study aimed to evaluate the COX-1 and COX 2 gene expression in gingival tissue collected from patients exposed to the preemptive administration of placebo, ibuprofen, and etoricoxib, and to evaluate its influence on clinical inflammatory events (pain scores, rescue medication intake, and maximum mouth opening).

MATERIAL AND METHODS

Study Design

This study had an analytical design. Gingival tissue was collected during extraction of impacted lower third molars from patients, during the course of a previous clinical trial that had a randomized, triple-blind, split-mouth and placebo-controlled study design [14]. The

following inclusion criteria were adopted to standardize the level of traumatic injury generated by surgery: (1) patients with third molars requiring ostectomy, with or without associated tooth sectioning; (2) patients with third molars that showed similar patterns of root formation, position, and degree of impaction. In addition, the following exclusion criteria were adopted: smokers, pregnant or breast feeding, users of medications that could interact with the drugs used in this study, patients with orthodontic bands on the mandibular second molars, confirmed history of allergy to NSAIDs, signs of any preoperative inflammatory or infectious condition, systemic chronic disease, use of NSAIDs within the past 21 days, or the presence of periodontal disease, swelling, fever, or trismus prior to surgery [14].

During that study, patients donated tissue for the present investigation by signing an informed consent form. Patients had been subjected to preemptive analgesia by taking ibuprofen 400mg, or etoricoxib 120mg or a placebo with no active pharmaceutical principal. In addition, the previously recorded pain scores by using the visual analog scale (VAS) (at 0, 2, 4, 6, 8, 10 and 12 hours, and 1, 5 and 7 days postoperatively), rescue medication intake, maximum mouth opening (at baseline and 7 days postoperatively), and the surgical period duration were evaluated in the present research.

Sample size calculation

Previously, a study by Costa et al. (2015) [6] observed VAS of 2.7±1.6 and 0.2±0.1 for two different groups preemptively treated with placebo and etoricoxib, respectively [6]. Third molar extractions were performed in these patients by following the same surgical protocol adopted in the present study. The data obtained established that a minimum sample size of 5 surgical sites per group yields a power of 90%, and alpha=0.05 in order to accept or reject the null hypotheses. Thus, in order to obtain the minimum sample size of 5 surgical sites per group from the original study carried out by Albuquerque et al. [14], a second randomization

was performed using a method to generate the random allocation sequence ("randomization per block" function of the Microsoft Excel®).

Sample acquisition

This study sample consisted of 30 fragments of pericoronal tissue evenly distributed according to treatment received (study group – ibuprofen, n=10; etoricoxib, n=10; placebo, n=10), and time of collection per group (T0, n=5 per group and T30, n=5 per group).

Gingival fragments of pericoronal tissue (close to the tooth being removed) were collected in two separate moments (T0= at the beginning of surgery and T30= 30 minutes later). The fragments were stored in an microtube ou cryotube an Eppendorf microcentrifuge tube and kept at -80^{0}C in a freezer for posterior analysis. Samples were identified by a number so that the investigator would not know which group gingival samples belonged to.

Study of the time-course of COX-1 and COX-2 mRNA expressions

To study the time-course of the COX-1 and COX-2 gene expression, gingival specimens collected at T0 and T30 were analyzed in triplicates. Ten samples were analysed per group rendering a total of 30 samples – ibuprofen 400 mg (T0, n=5 + T30, n=5), etoricoxib 120 mg (T0, n=5 + T30, n=5) and placebo groups (T0, n=5 + T30, n=5).

Construction of the primers for the GAPDH, COX-1, and COX-2 genes

The primers were designed on the basis of data obtained from the NCBI gene bank using the PrimerBlast program with exclusive specificity for *Homo sapiens* (Table 1). The primers were produced by Invitrogen technology. GAPDH was used as the endogenous control (housekeeping) gene because it is a gene that is not affected by the inflammatory condition that is being analyzed in the present study and also to normalize samples for

possible differences in cDNA quantities added in each reaction. The primers used for the target genes (COX-1 and COX-2) were developed by exon-exon ligating, thereby making genomic DNA amplification unfeasible (Table 1).

Spectrophotometric Quantification

To test the efficacy of extraction and total RNA purity the concentration of total RNA in the samples was determined by RNA dilution (known dilution factor) together with a spectrophotometric reading in quartz cuvettes, using wavelengths of de 260 nm (A_{260}) and 260/280 nm (A_{260}/A_{280}).

RNA extraction and cDNA synthesis

Isolation of total RNA was performed using the PureLink® RNA Mini Kit (Life Technologies, New York, USA). According to the manufacturer's instructions, 800 µL of Trizol solution was added to each frozen samples, and the lysate was aspirated and centrifugation at 10,000 g for 3 min at room temperature. Thereafter, all lysates were diluted 1:1 with 70% ethanol and subjected to a mini-column. After binding of the RNA to the column, DNA digestion was performed using RNAse-free DNAse (340 Kunitz units/mL) for 15 min at room temperature. After washing the column three times, the RNA was eluted with 30 µL RNAse-free water. The RNA concentration was estimated by reading the absorbance at 260 nm and was checked for purity at 280 nm in a spectrophotometer (Amersham Biosciences, Cambridge, England). For each sample, RNA concentrations were adjusted and used to synthesize cDNA with 1 µL. Before the reverse transcription reaction, samples of RNA were incubated for 5 min at 70 ºC and then cooled in ice. The reverse transcription was performed in a total volume of 20 µL composed of 10 µL of sample RNA, 4 µL reverse transcriptase buffer (Invitrogen, São Paulo, Brazil), 8 units RNase out, 150 units of reverse

transcriptase Superscript III, 0036 U random primers, 10 mM DTT and 0.5mM of each dNTP (Invitrogen, São Paulo, Brazil). The mixture was incubated at 42 ºC for 1 h, subsequently at 80 ºC for 5 min, and finally stored at –20 ºC. The negative control was prepared under the same conditions, but without the addition of reverse transcriptase.

Quantitative reverse transcription (qRT)-PCR evaluation

Quantification of mRNA was performed using SYBR GreenMaster Mix (PE Applied Biosystems, Foster City, CA). PCR reactions were composed of 1 µL cDNA as a template in 7.5 µL of GoTaq® qPCR Master Mix (Promega Corporation, Madison, WI, USA), 5.5 µL of ultra-pure water, and 0.5 µM of each primer. The primers were designed by using the PrimerQuestSM program (http://www.idtdna.com), and GAPDH was used as the normalizing gene. The specificity of each primer pair was confirmed by melting curve analysis of PCR products. The thermal cycling profile for the first round of PCR was: initial denaturation and activation of the polymerase for 10 min at 95 ^{o}C, followed by 40 cycles of 15 sec at 95 ºC, 30 sec at 58 ºC, and 30 sec at 72 ºC. The final extension was for 10 min at 72 ºC. All reactions were performed in StepOne Real-Time PCR (Applied Biosystems, Foster, CA, USA). Relative quantifications of mRNA were carried out using the comparative threshold cycle (^{Ct}) (C_t) method according to Wang et al. [15,16].

Statistical Analysis

Normality of the data was verified through Kolmogorov-Smirnov test, and data were expressed in mean and standard error of the mean for comparison with pared t-test or ANOVA (1-way or 2-way) followed by Bonferroni post hoc test. Chi-square and Fisher Exact tests were used to evaluate associations between categorical variables (n, %) (GraphPad Prism

5.0, $p < 0.05$). In addition, Pearson correlation was used in order to correlate COX-1 and COX-2 levels with the reported clinical parameters.

RESULTS

Sample characterization

The average age of the patients was 22 years. Patients did not differ regarding demographic or surgical factors, such as the eventual extraction difficulties, dental position, or quantity of anesthetic used (Table 2).

mRNA expression of COX-1 and COX-2

RT-PCR showed no difference in COX-1 expression in the placebo group from T0 (9.3 ± 0.4) to T30 (9.4 ± 0.2); however, in the groups treated with ibuprofen (T0, 8.3±0.2; T30, 9.3±0.2, $p = 0.004$) and etoricoxib (T0, 8.7 ± 0.2; T30, 9.3 ± 0.2, $p = 0.010$) showed a significant increase in the COX-1 expression from the first (T0) to the second moment (T30) (Figure 1 A). The increase in the COX-1 expression was significantly greater in the groups treated with ibuprofen (0.9 ± 0.3) and etoricoxib (1.1 ± 0.2) than in the placebo group (0.1 ± 0.2) ($p = 0.020$).

All three groups showed an increase in COX-2 expression from T0 (placebo, 7.6 ± 0.6; ibuprofen, 7.9 ± 0.6; etoricoxib, 8.0 ± 0.8) to T30 (placebo, 9.6 ± 0.5, $p = 0.012$; ibuprofen, 10.7 ± 0.6, $p < 0.001$; etoricoxib, 10.3 ± 0.7, $p < 0.001$) (Figure 1 B). Only the group treated with etoricoxib (0.9 ± 0.7) showed a modest increase in COX-2 expression compared to the placebo group (3.1 ± 0.4) ($p = 0.023$); however, there was no difference between the placebo group and the group treated with ibuprofen (2.7 ± 0.5) (Figure 2).

The three groups showed a significant reduction in the ratio of COX-2 to COX-1 expressions from T0 (placebo, 1.0 ± 0.1; ibuprofen, 1.3 ± 0.1; etoricoxib, 1.1 ± 0.1) to T30

(placebo, 0.8 ± 0.1, $p = 0.013$; ibuprofen, 0.9 ± 0.1, $p < 0.001$; etoricoxib, 0.9 ± 0.1, $p = 0.047$).

Relationship between COX-1 and COX-2 expressions and clinical parameters

Clinically, pain scores of the ibuprofen group were significantly lower than the placebo group from 8h to 24h after the surgical procedure ($p < 0.001$). The pain scores of the etoricoxib group were significantly lower in comparison with the placebo group from 4h to 24h after the surgical procedure ($p < 0.001$), and pain scores of the etoricoxib group were significantly lower in comparison with the ibuprofen group 4h after the surgical procedure ($p = 0.047$). The area under the postoperative pain experience curve of the placebo group (31.2) was 2.6 times higher than the ibuprofen group (11.8) and 5.2 times higher than the etoricoxib group (6) (Figure 3). In relation to the maximum mouth opening 7 days after surgery, there was a statistical difference ($p = 0.001$) between placebo (11.5 ± 1.9 mm), ibuprofen (4.4 ± 0.7 mm), and etoricoxib (2.4 ± 0.6 mm) groups.

According to Tables 3-5, group treated with ibuprofen showed an inverse correlation between COX-1 level at T0 and pain peak after 4h ($p = 0.034$, $r = -0.905$), between COX-1 level at T30 and baseline mouth opening ($p = 0.044$, $r = -0.889$) and after 7 days ($p = 0.013$, $r = -0.915$). There was also a significant inverse correlation between COX-2 level at T0 and pain peak after 10h ($p = 0.001$, $r = -0.990$) and 12h ($p = 0.001$, $r = -0.990$), and T30 and pain peak after 24h ($p = 0.001$, $r = -0.993$). In addition, COX-2 level and the consumption of rescue medication were directly correlated ($p = 0.001$, $r = 0.990$).

In the group treated with etoricoxib, there was a significant inverse correlation between COX-1 level at T0 and pain peak after 2h ($p = 0.015$, $r = -0.947$), as well as direct correlation between COX-1 and pain peak after 6h ($p = 0.032$, $r = 0.910$). COX-2 level showed a significant direct correlation with pain peak after 24h in both T0 ($p = 0.006$, $r = 0.969$) and T30 ($p = 0.027$, $r = 0.919$) evaluated periods.

DISCUSSION

Third molar surgery was selected to validate the clinical model used in the research because it has been widely practiced and validated in pharmacological trials since 1976 by Cooper and Beaver [17], as well as being a common dental procedure in which postoperative pain is usually short-lasting reaching its height in the initial stage immediately after the surgical trauma affecting the surrounding tissues [18]. That model has been considered highly important in clinical investigations to distinguish the analgesic effects of various drugs, as was the case in the present research, or to investigate the effects of different dosages of a single drug [19,20].

This study investigated the effect of preemptive oral administration of ibuprofen and etoricoxib on COX-1 and COX-2 levels in gingival tissue. These two drugs are commonly administered in lower third molar removal procedures as a means of controlling postoperative pain. In fact, the area under the curve, correlating the pain scores over the time, showed that both experimental groups reduced the pain scores in comparison with the placebo group, and the etoricoxib was the drug who significantly reduced the pain scores. Cyclooxygenase, also known as prostaglandin H synthase is the key enzyme in prostaglandin synthesis. The original elucidation of the two COX isoforms gave rise to the concept that the constitutive enzyme COX-1 was responsible for the production of prostaglandins with homeostatic functions in stomach and kidney tissues and in platelet aggregation, whereas COX-2 is induced and responsible for the production of pro-inflammatory substances, especially PGE2 [21]. The contribution of COX-2 to inflammation is further supported by the fact that COX-2 expression can become from ten to 80 times greater in the presence of pro-inflammatory cytokines such as IL-6, IL-1, and prostaglandin production can be inhibited by anti-inflammatory cytokines [22]. However, most studies have focused on measuring tissue

cytokine levels instead of evaluating the impact of the use of COX-2 selective NSAIDs on tissue levels of these enzymes. To our knowledge this is the first investigation evaluating gene expression of COXs in human tissues following third molar surgery from a split-mouth study, concomitantly evaluating the preemptive analgesic effect of etoricoxib and ibuprofen.

Khan et al. [10] conducted a similar clinical study that used the same surgical procedures and collected gingival specimen in patients underwent third molar surgery without preoperative administration of NSAIDs aiming to evaluate COX expression in oral tissues without the use of medication. The aforementioned study showed a gradual increase in COX-2 expression at 30, 60, and 120 minutes after surgery, which is expected for those patients that did not intake any NSAIDs. For COX-1, however, there was a slight drop at 30 minutes and a significant reduction at 60 minutes, but by 120 minutes, the COX-1 expression returned to initial levels. However, it is difficult to make any comparisons between their study and the present one because each temporal analysis was carried out with a different patient. In other words, no single patient was subsequently analyzed at three times so that there is no way of knowing whether the data would have maintained the same pattern had it been registered for a single patient on all occasions. In comparison with the present study design, there were no medications investigated in the gene expression study conducted by Khan et al. [10]. If there is a potential change in COX expression-related parameters following an inflammatory process such as dentoalveolar surgeries, these data could be properly evaluated in third molar studies involving NSAIDs as presently performed. In the present investigation, COX-1 levels in the placebo group did not differ between T0 and T30, differing from Kahn et al. [10] that observed a slight COX-1 level decrease in the studied groups. In the ibuprofen and etoricoxib groups, however, there was a slight increase that is believed by the use of those drugs as the reduction in COX-2 could lead to compensatory expression of COX-1.

No significant differences were detected in the COX-2/COX-1 ratio among the three studied groups. That can be explained by cascade compensations and the selectivity of the medications. A high level of COX-1 and COX-2 was observed in the placebo group, whereas in the etoricoxib and ibuprofen groups due to the reduction in COX-2 expression and the increase in COX-1 expression, there were no observable differences in the COX2/COX1 ratios. That compensatory behavior shows that even non-selective drugs can have satisfactory analgesic and anti-inflammatory effects [23], which is supported by our findings since the Pearson correlation showed a statistically significant difference in the experimental groups. Ibuprofen group showed an opposite correlation between specific pain peaks and gene expression of both COXs, an opposite relationship between maximum mouth opening and COX-1 expression, and a direct correlation between COX-2 expression and rescue medication intake. Etoricoxib group showed an opposite correlation between the COX-1 gingival level and specific pain peaks and a direct correlation between COX-2 level and determined pain scores.

In third molar studies evaluating gene expression of COXs along with the preemptive use of NSAIDs [24,25], it is possible to observe results that corroborate with the present findings regarding the temporal expression of COXs. Lee et al. 2006 [24] showed a COX-1 expression decrease (36%, $p < 0.001$) after 2 and 4h postoperatively, and a significant COX-2 expression increase (300%, $p < 0.001$). The test groups (ibuprofen and rofecoxib) had a significant increase in COX-2 when compared to the placebo group ($p < 0.001$ and < 0.049, respectively). In addition, these authors showed a significant relationship between gene polymorphism variability and patient pain relief after the use of NSAIDs. In the study performed by Lee et al. 2007 [25], ketorolac decreased COX-1 gene expression at the 24-h postoperative evaluation and suppressed TBX_2. Also, PGE_2-related COX-2 expression remained high even in the 3-h and 24-h periods. These findings showed that the effect of

COX-selective inhibitors on PGE_2 levels contribute to inflammatory pain relief after third molar surgery, which may support the present results.

In conclusion, the present preemptive analgesia study concludes that that COX-2 RNAm induction was directly linked to third molar-related tissue inflammation and that the relation between COX-1 and COX-2 levels were inversely proportional to the preemptively administered NSAID COX-2selectivity. Clinically, COX-1 and COX-2 gene expressions were correlated with third molar-related inflammatory events, notably the pain parameters. In addition, it should be highlighted that no similar gene expression-related laboratory studies have been performed testing the preoperative administration of etoricoxib or ibuprofen in a split-mouth study with third molar surgeries to date. Considering the methodology adopted in the present study, further experimental studies could be designed using the third molar as a clinical model in the field of the preemptive analgesia.

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All authors have viewed and agreed to the submission.

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FIGURE LEGENDS

Figure 1. COX-1 and COX-2 tissue level between the studied groups at 0 and 30 minutes after the surgical procedure. *p<0.05 in relation to the experimental period 0 minutes of the same group (paired t-test).

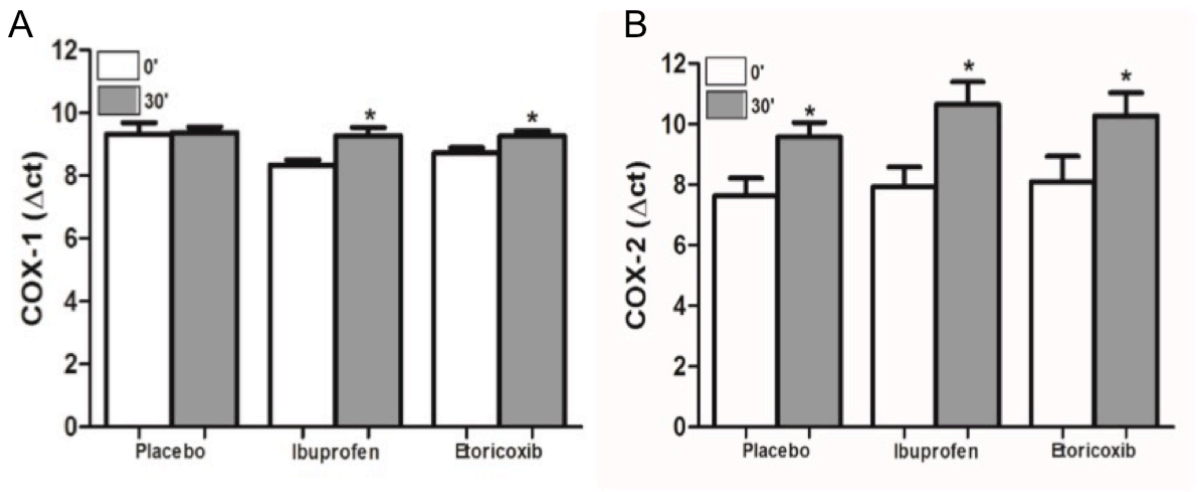


Figure 2. Variation of the COX-1 and COX-2 tissue level between the studied groups after the surgical procedure. *p<0.05 in relation to the experimental groups versus the placebo group (one-way ANOVA test).

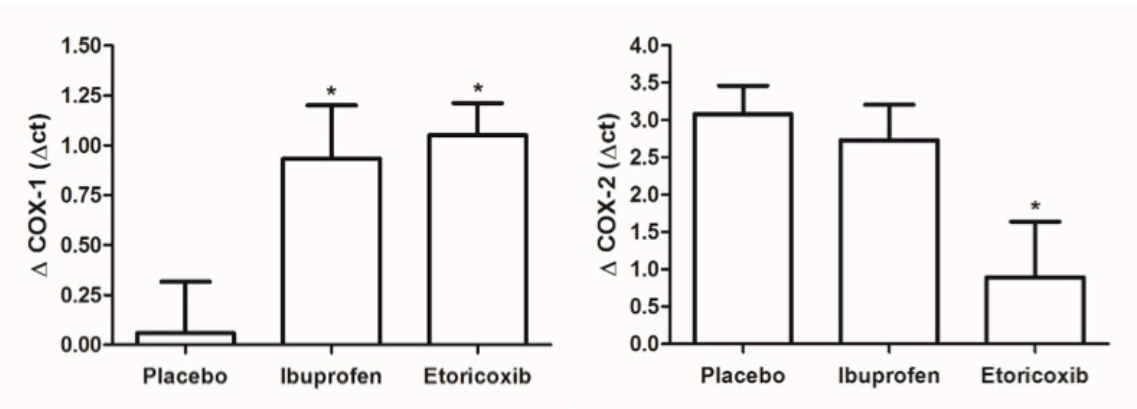
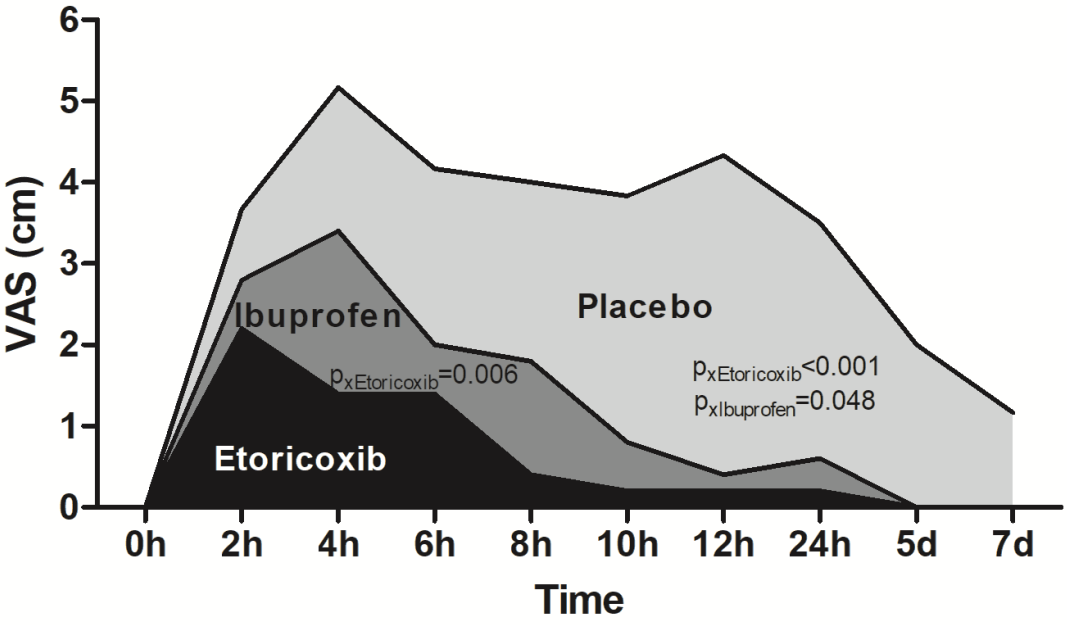


Figure 3. Graphical representation of the pain scores over the studied periods. The area under the curve shows pain experience significantly reduced in etoricoxib ($p < 0.0001$) and ibuprofen ($p = 0.006$) groups in comparison with placebo, and significantly reduced in etoricoxib group in comparison with ibuprofen group ($p = 0.0488$). P, placebo; two-way ANOVA test.



TABLES AND LEGENDS

Table 1. Genes and related primer sequence used in the present study.

Genes	Symbol	Primer sequence
Glyceraldehyde-3-phosphate dehydrogenase	GAPDH (24bp)	F5'TGGTATCGTGGAAGGACTC3' R5'TAGAGGCAGGGATGATGTT3'
Ciclooxygenase-1	COX-1 (25bp)	F5'CTGCCCTCCTCAAGACTTTAGCTT3' R5'TCCAACTGATTTAAGCAAAAGAGGAAT3'
Ciclooxygenase-2	COX-2 (25bp)	F5'CCTTCGAAATGCAATTATGAGTT3' R5'CACAGGAGGAAGGGCTCTAGT3'

F, forward; R, reverse. GAPDH was used as a housekeeping gene.

Table 2. Sample characterization

	Groups			
	Placebo	**Ibuprofen**	**Etoricoxib**	**p-Value**
Gender				
Male	0 (0%)	2 (40%)	2 (40%)	0.256
Female	5 (100%)	3 (60%)	3 (60%)	
Age (years)				
≤20	0 (0%)	2 (40%)	0 (0%)	0.136
21-30	3 (60%)	1 (20%)	4 (80%)	
31-40	2 (40%)	2 (40%)	0 (0%)	
>40	0 (0%)	0 (0%)	1 (20%)	
Degree of tooth eruption				
Total bone inclusion	0 (0%)	1 (20%)	1 (20%)	0.525
Partial bone inclusion	3 (60%)	3 (60%)	4 (80%)	
Erupted partially	2 (40%)	1 (20%)	0 (0%)	
Relation with mandibular ramus				
Class I	3 (60%)	1 (20%)	2 (40%)	0.406
Class II	2 (40%)	4 (80%)	2 (40%)	
Class III	0 (0%)	0 (0%)	1 (20%)	
Relation with second molar				
Position A	1 (20%)	1 (20%)	0 (0%)	0.558
Position B	4 (80%)	4 (80%)	4 (80%)	
Position C	0 (0%)	0 (0%)	1 (20%)	
Relation with inferior alveolar canal				
Interruption of the radiopaque line	1 (20%)	0 (0%)	1 (20%)	0.167
Darkening of the third molar root	0 (0%)	2 (40%)	0 (0%)	
Deflection of the root	0 (0%)	2 (40%)	0 (0%)	
Absent	3 (60%)	1 (20%)	3 (60%)	
Interruption of the radiopaque line + darkening of the third molar root	0 (0%)	0 (0%)	1 (20%)	
Interruption of the radiopaque line + deflection of the root	1 (20%)	0 (0%)	0 (0%)	
Surgery period (minutes)				
≤10	0 (0%)	0 (0%)	1 (20%)	0.558
11-15	4 (80%)	4 (80%)	4 (80%)	
>15	1 (20%)	1 (20%)	0 (0%)	
Tooth sectioning during surgery				
Yes	3 (60%)	3 (60%)	2 (40%)	0.765
No	2 (40%)	2 (40%)	3 (60%)	
Tooth position				0.741
Vertical	1 (20%)	2 (40%)	2 (40%)	
Mesioangular	4 (80%)	3 (60%)	3 (60%)	

*$p<0.05$, Chi-square or Fisher exact tests.

Table 3. Pearson correlation between COX-1 and COX-2 gene expressions and clinical parameters (placebo group).

		COX-1			COX-2		
		T0	**T30**	**T30-T0**	**T0**	**T30**	**T30-T0**
Placebo							
Surgery period	r	-0.240	0.107	0.426	0.605	0.121	-0.388
	p-Value	0.698	0.864	0.474	0.280	0.847	0.519
Maximum mouth openning (baseline)	r	-0.463	-0.427	0.423	-0.552	-0.833	-0.137
	p-Value	0.433	0.473	0.478	0.334	0.080	0.826
Maximum mouth openning (7 days)	r	0.261	-0.109	-0.460	-0.578	-0.099	0.381
	p-Value	0.671	0.862	0.436	0.308	0.874	0.526
Maximum mouth openning (**Δ)**	r	0.561	0.101	-0.777	-0.391	0.330	0.529
	p-Value	0.325	0.872	0.122	0.515	0.587	0.359
Rescue medication intake	r	-0.483	0.037	0.757	0.406	-0.700	-0.792
	p-Value	0.409	0.953	0.138	0.497	0.188	0.110
VAS 0h	r	0.000	0.000	0.000	0.000	0.000	0.000
	p-Value	1.000	1.000	1.000	1.000	1.000	1.000
VAS 2h	r	-0.252	0.239	0.547	0.374	-0.775	-0.818
	p-Value	0.683	0.698	0.340	0.535	0.123	0.090
VAS 4h	r	0.535	0.873	-0.217	0.820	-0.362	-0.884
	p-Value	0.353	0.053	0.726	0.089	0.549	0,094
VAS 6h	r	-0.071	0.432	0.402	0.569	-0.711	-0.926
	p-Value	0.910	0.468	0.502	0.317	0.178	0.052
VAS 8h	r	-0.336	0.178	0.632	0.395	-0.771	-0.832
	p-Value	0.581	0.774	0.253	0.510	0.127	0.081
VAS 10h	r	-0.574	-0.127	0.788	0.148	-0.905	-0.731
	p-Value	0.311	0.839	0.114	0.812	0.070	0.161
VAS 12h	r	-0.522	-0.255	0.621	-0.212	-0.453	-0.143
	p-Value	0.367	0.679	0.264	0.732	0.443	0.818
VAS 24h	r	-0.315	-0.147	0.379	-0.191	-0.159	0.040
	p-Value	0.605	0.813	0.529	0.758	0.798	0.949
VAS 5d	r	-0.558	-0.089	0.788	0.135	-0.787	-0.641
	p-Value	0.329	0.887	0.114	0.828	0.114	0.244
VAS 7d	r	-0.252	0.239	0.547	0.374	-0.775	-0.818
	p-Value	0.683	0.698	0.340	0.535	0.123	0.090

*p<0,05, correlação de Pearson; VAS, visual analogue scale.

Δ= T30 – T0

Table 4. Pearson correlation between COX-1 and COX-2 gene expression and clinical parameters (ibuprofen group)

		COX-1			COX-2		
		T0	**T30**	**T30-T0**	**T0**	**T30**	**T30-T0**
Ibuprofen							
Surgery period	r	-0.191	0.634	0.608	0.496	0.293	-0.212
	p-Valor	0.758	0.250	0.276	0.396	0.632	0.733
Maximum mouth openning (baseline)	r	-0.049	**-0.889***	-0.694	-0.493	0.054	0.633
	p-Valor	0.938	**0.044**	0.194	0.399	0.931	0.252
Maximum mouth openning (7 days)	r	-0.012	**-0.951***	-0.762	-0.569	-0.030	0.608
	p-Valor	0.984	**0.013**	0.134	0.317	0.962	0.277
Maximum mouth openning (**Δ**)	r	0.143	-0.213	-0.244	-0.278	-0.328	-0.118
	p-Valor	0.819	0.730	0.693	0.651	0.590	0.850
Rescue medication intake	r	-0.468	0.716	0.813	**0.990***	0.875	0.403
	p-Valor	0.426	0.173	0.094	**0.001**	0.052	0.501
VAS 0h	r	0.000	0.000	0.000	0.000	0.000	0.000
	p-Valor	1.000	1.000	1.000	1.000	1.000	1.000
VAS 2h	r	-0.206	0.001	0.104	-0.280	-0.253	-0.437
	p-Valor	0.740	0.998	0.867	0.648	0.681	0.462
VAS 4h	r	**-0.905***	-0.219	0.277	0.192	0.618	0.596
	p-Valor	**0.034**	0.724	0.651	0.757	0.267	0.289
VAS 6h	r	-0.609	-0.306	0.058	-0.427	-0.178	-0.220
	p-Valor	0.275	0.617	0.926	0.473	0.775	0.722
VAS 8h	r	0.240	-0.359	-0.410	-0.769	-0.843	-0.644
	p-Valor	0.697	0.553	0.492	0.129	0.073	0.240
VAS 10h	r	0.468	-0.716	-0.813	**-0.990***	-0.875	-0.403
	p-Valor	0.426	0.173	0.094	**0.001**	0.052	0.501
VAS 12h	r	0.468	-0.716	-0.813	**-0.990***	-0.875	-0.403
	p-Valor	0.426	0.173	0.094	**0.001**	0.052	0.501
VAS 24h	r	0.616	-0.427	-0.654	-0.875	**-0.993***	-0.677
	p-Valor	0.269	0.473	0.231	0.052	**0.001**	0.210
VAS 5d	r	0.000	0.000	0.000	0.000	0.000	0.000
	p-Valor	1.000	1.000	1.000	1.000	1.000	1.000
VAS 7d	r	0.000	0.000	0.000	0.000	0.000	0.000
	p-Valor	1.000	1.000	1.000	1.000	1.000	1.000

*p<0,05, correlação de Pearson; VAS, visual analogue scale.

Δ= T30 – T0

Table 5. Pearson correlation between COX-1 and COX-2 gene expression and clinical parameters (etoricoxib group)

		COX-1			COX-2		
		T0	**T30**	**T30-T0**	**T0**	**T30**	**T30-T0**
Etoricoxib							
Surgery period	r	0.797	0.377	-0.707	0.212	0.179	-0.461
	p-Valor	0.106	0.532	0.182	0.732	0.773	0.434
Maximum mouth openning (baseline)	r	-0.452	-0.361	0.335	0.683	0.798	0.053
	p-Valor	0.445	0.551	0.582	0.203	0.106	0.932
Maximum mouth openning (7 days)	r	-0.397	-0.278	0.316	0.710	0.807	0.010
	p-Valor	0.508	0.650	0.604	0.179	0.099	0.988
Maximum mouth openning (**Δ**)	r	0.690	0.780	-0.380	-0.392	-0.590	-0.301
	p-Valor	0.197	0.120	0.527	0.514	0.295	0.623
Rescue medication intake	r	0.639	0.709	-0.344	0.502	0.299	-0.113
	p-Valor	0.246	0.180	0.570	0.389	0.625	0.856
VAS 0h	r	0.000	0.000	0.000	0.000	0.000	0.000
	p-Valor	1.000	1.000	1.000	1.000	1.000	1.000
VAS 2h	r	**-0.947***	-0.732	0.693	0.037	0.197	0.405
	p-Valor	**0.015**	0.160	0.195	0.953	0.751	0.499
VAS 4h	r	-0.525	-0.441	0.377	0.619	0.604	0.841
	p-Valor	0.364	0.457	0.531	0.266	0.281	0.075
VAS 6h	r	-0.767	0.073	**0.910***	-0.013	0.044	-0.385
	p-Valor	0.130	0.907	**0.032**	0.984	0.944	0.522
VAS 8h	r	-0.865	-0.651	0.636	-0.265	-0.067	0.062
	p-Valor	0.059	0.234	0.249	0.666	0.914	0.921
VAS 10h	r	-0.797	-0.377	0.707	-0.212	-0.179	0.461
	p-Valor	0.106	0.532	0.182	0.732	0.773	0.434
VAS 12h	r	-0.797	-0.377	0.707	-0.212	-0.179	0.461
	p-Valor	0.106	0.532	0.182	0.732	0.773	0.434
VAS 24h	r	0.155	-0.163	-0.245	**0.969***	**0.919***	0.568
	p-Valor	0.804	0.794	0.692	**0.006**	**0.027**	0.318
VAS 5d	r	0.000	0.000	0.000	0.000	0.000	0.000
	p-Valor	1.000	1.000	1.000	1.000	1.000	1.000
VAS 7d	r	0.000	0.000	0.000	0.000	0.000	0.000
	p-Valor	1.000	1.000	1.000	1.000	1.000	1.000

*p<0,05, correlação de Pearson; VAS, visual analogue scale.

Δ= T30 – T0

CONCLUSÕES GERAIS

1. Análise sistemática da literatura revisada evidenciou que as cirurgias para remoção de terceiros molares foram associadas a diferentes padrões de expressão gênica relacionados à COX. Embora os eventos inflamatórios após tais procedimentos estejam associados às isoformas da COX, os dados obtidos desses estudos e que envolviam analgesia preemptiva são escassos, especialmente correlacionando expressão gênica e parâmetros clínicos.

2. O emprego pré-operatório de AINEs alterou a expressão gênica das COX em cirurgias de terceiros molares inferiores quando da realização do estudo clinico/laboratorial. Observou-se também que a indução do RNAm da COX-2 esteve diretamente relacionada ao trauma tecidual associado, e que a relação entre os níveis de COX-1 e COX-2 foi inversamente proporcional à seletividade do AINE utilizado.

3. Os níveis teciduais relativos à expressão gênica de COX-1 e COX-2 exibiram, clinicamente, correlação com eventos inflamatórios no período pós-operatório de cirurgias de terceiros molares realizadas sob a ótica da analgesia preemptiva, com destaque para os parâmetros relacionados a dor.

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ANEXO 1

UNIVERSIDADE FEDERAL DO CEARÁ/ PROPESQ

PARECER CONSUBSTANCIADO DO CEP

Elaborado pela Instituição Coparticipante

DADOS DO PROJETO DE PESQUISA

Título da Pesquisa: AVALIAÇÃO DO EFEITO DA ANALGESIA PREEMPTIVA SOB OS NÍVEIS DE CICLOOXIGENASES E CITOCINAS PRÓ-INFLAMATÓRIAS EM CIRURGIAS DE TERCEIROS MOLARES

Pesquisador: ASSIS FILIPE MEDEIROS ALBUQUERQUE

Área Temática:

Versão: 1

CAAE: 44058715.4.3001.5054

Instituição Proponente: Universidade Federal do Ceará/HOSPITAL UNIVERSITARIO WALTER

Patrocinador Principal: Financiamento Próprio

DADOS DO PARECER

Número do Parecer: 1.243.234

Apresentação do Projeto:

Projeto de pesquisa para fins de mestrando em odontologia de Assis Filipe Medeiros Albuquerque, sob orientação do Prof. Dr. Fábio Wildson Gurgel Costa pautado na avaliação do efeito da analgesia preemptiva sob os níveis de mediadores químicos inflamatórios em cirurgias de terceiros molares. Trata-se de um ensaio clínico duplo cego,placebo controlado, cruzado. A amostra será de 18 pacientes (36 sítios cirúrgicos), os quais serão divididos igualmente entre 3 grupos, oriundos da demanda espontânea do Serviço de Cirurgia e Traumatologia. Os pacientes do grupo experimental receberão, 1 hora antes do procedimento cirúrgico a depender da randomização, ibuprofeno 400mg ou etoricoxibe 120mg e os do grupo controle receberão placebo 1 hora antes do procedimento cirúrgico. Além disso, também será realizada a dosagem de TNF e IL-1. Os dados, após coletados, serão tabulados em tabela do software Microsoft Excel® versão 2010, e analisados por meio do programa Statistical Package for the Social Sciences (SPSS), versão 17.0 para Windows® sendo considerado o valor de < 0,05.

Objetivo da Pesquisa:

OBJETIVO GERAL: Avaliar o efeito da analgesia preemptiva sob os níveis de mediadores químicos inflamatórios em cirurgias de terceiros molares.

OBJETIVOS ESPECÍFICOS:

- Quantificar a expressão gênica das ciclooxigenases 1 e 2 e níveis de TNF e IL-1 em cirurgias para remoção de terceiros molares utilizando modelo de analgesia preemptiva;
- Correlacionar os parâmetros laboratoriais com parâmetros clínicos de avaliação da analgesia preemptiva em cirurgias para remoção de terceiros molares.

Avaliação dos Riscos e Benefícios:

A pesquisa apresenta os riscos inerentes às cirurgias de terceiros molares, no entanto, tratam-se de procedimentos odontológicos já bem estabelecidos. Todas as possíveis complicações são previstas no TCLE como também as orientações necessárias.

Enfatiza-se que a realização dessa pesquisa pode contribuir para o desenvolvimento de protocolos medicamentosos eficazes no controle da dor pós-operatória deste tipo de cirurgia.

Comentários e Considerações sobre a Pesquisa:

A Pesquisa é exequível sob o ponto de vista técnico e apresenta relevância pela frequência das cirurgias de terceiros molares e por tratar da dor decorrente do tratamento cirúrgico, seus mecanismos e forma de alívio.

Considerações sobre os Termos de apresentação obrigatória:

O pesquisador apresentou ao comitê: Carta de encaminhamento, Folha de Rosto, Projeto detalhado, Termo de fiel depositário, Autorizações dos locais(Divisão de Gestão de Cuidados; Serviço de Cirurgia e Traumatologia Bucomaxilofacial;Chefe do Departamento de Clínica odontológica e Núcleo de Biotecnologia de Sobral), Orçamento e origem dos recursos (no valor total de R$ 13.061,64. Recursos provenientes do pesquisador

principal), Termo de ciência dos setores, Curriculum vitae dos pesquisadores, TCLE, Cronograma.

Recomendações:

Não se aplica.

Conclusões ou Pendências e Lista de Inadequações:

Não há pendências éticas nem documentais.

Considerações Finais a critério do CEP:

Este parecer foi elaborado baseado nos documentos abaixo relacionados:

Tipo Documento	Arquivo	Postagem	Autor	Situação
Outros	MODELO DE ORÇAMENTO DE	06/03/2015		Aceito

UNIVERSIDADE FEDERAL DO CEARÁ/ PROPESQ

Continuação do Parecer: 1.243.234

Outros	PROJETO DE PESQUISA.pdf	16:51:39		Aceito
Outros	Currículo do Sistema de Currículos Lattes (Assis Filipe Medeiros Albuquerque).pdf	06/03/2015 16:55:19		Aceito
Outros	Currículo do Sistema de Currículos Lattes (Fábio Wildson Gurgel Costa).pdf	06/03/2015 16:56:24		Aceito
Outros	CRONOGRAMA EM FORMA DE TABELA.pdf	06/03/2015 16:57:42		Aceito
Outros	Termo de ciência do responavel pelo setor.pdf	27/03/2015 09:49:30		Aceito
Outros	Termo de Ciencia do setor da pesquisa 4.pdf	27/03/2015 09:51:10		Aceito
Folha de Rosto	Folha de Rosto.pdf	27/03/2015 09:49:01		Aceito
Outros	Termo de ciência do setor da pesquisa 2.jpeg.pdf	27/03/2015 11:28:42		Aceito
Outros	Termo de ciência do setor da pesquisa 3.jpeg.pdf	27/03/2015 11:28:58		Aceito
Outros	Declaração de ConcordânciA (Encaminhamento do CEP).pdf	27/03/2015 11:31:29		Aceito
Outros	Carta de apresentação do projeto ao CEP 1.pdf	27/03/2015 11:32:34		Aceito
Outros	Carta de apresentação do projeto ao CEP 2.pdf	27/03/2015 11:33:10		Aceito
Outros	Declaração de Origem de recursos financeiros.pdf	27/03/2015 11:34:40		Aceito
Outros	Declaração de compromisso para autorização de dados.pdf	27/03/2015 11:35:30		Aceito
Informações Básicas do Projeto	PB_INFORMAÇÕES_BÁSICAS_DO_PROJETO_477460.pdf	27/03/2015 11:37:17		Aceito
Outros	TERMO FIEL DEPOSITÁRIO.pdf	14/04/2015 10:05:43		Aceito
Informações Básicas do Projeto	PB_INFORMAÇÕES_BÁSICAS_DO_PROJETO_477460.pdf	14/04/2015 10:07:35		Aceito
Projeto Detalhado / Brochura Investigador	Projeto Detalhado Plataforma brasil.pdf	05/06/2015 08:11:14		Aceito
TCLE / Termos de Assentimento / Justificativa de Ausência	Termo de concentimento.pdf	05/06/2015 08:13:25		Aceito
Informações Básicas do Projeto	PB_INFORMAÇÕES_BÁSICAS_DO_PROJETO_477460.pdf	05/06/2015 08:14:52		Aceito

Situação do Parecer:
Aprovado

Endereço: Rua Cel. Nunes de Melo, 1000
Bairro: Rodolfo Teófilo **CEP:** 60.430-275
UF: CE **Município:** FORTALEZA
Telefone: (85)3366-8344 **Fax:** (85)3223-2903 **E-mail:** comepe@ufc.br

UNIVERSIDADE FEDERAL DO CEARÁ/ PROPESQ

Necessita Apreciação da CONEP:
Não

FORTALEZA, 24 de Setembro de 2015

Assinado por:
FERNANDO ANTONIO FROTA BEZERRA
(Coordenador)

Endereço: Rua Cel. Nunes de Melo, 1000
Bairro: Rodolfo Teófilo **CEP:** 60.430-275
UF: CE **Município:** FORTALEZA
Telefone: (85)3366-8344 **Fax:** (85)3223-2903 **E-mail:** comepe@ufc.br

ANEXO 2

NORMAS REVISTA: Medicina Oral Patología Oral y Cirugia Bucal - eISSN: 1698-6946

CAPITULO I

INSTRUCTIONS FOR AUTHORS - *Medicina Oral Patología Oral y Cirugia Bucal - eISSN: 1698-6946*

JOURNAL SECTIONS

1. Oral Medicine and Pathology
Clinicopathological as well as medical or surgical management aspects of diseases affecting oral mucosa, salivary glands, maxillary bones, and temporomandibular joints, as well as orofacial neurological disorders, Craneomandibular disorders and Orofacial pain neck and facial pathology, and systemic conditions with an impact on the oral cavity. Gerodontology.

2. Oral Surgery
Surgical management aspects of diseases affecting oral mucosa, salivary glands, maxillary bones, teeth, temporomandibular joints, oral surgical procedures. Surgical management of diseases affecting head and neck areas.
Laser in Dentistry. IMPLANTOLOGY.

3. Medically compromised patients in Dentistry
Articles discussing medical problems in Odontology will also be included, with a special focus on the clinico-odontological management of medically compromised patients, and considerations regarding high-risk or disabled patients.

Medicina Oral Patología Oral y Cirugia Bucal no longer ADMITS CASE REPORTS.

ARTICLE SUBMISSION

Articles may only be submitted through our web site and in ENGLISH.
Log on our web site and we will send you an USER NAME and PASSWORD to submit the article.

http://www.medoral.es

For submitting NEW OR MODIFIED MANUSCRIPTS the description of the process is:

1. Log in to http://www.medoral.es
2. Click on "Submit a manuscript" for submitting a NEW articles. Click on "Submissions needing revision" for submitting a MODIFIED article.
3. Delete ALL previously uploaded documents, including all the figures in the case of submitting a MODIFIED article.
4. Upload a word document entitled: "LETTER TO THE EDITOR".
If this is a modification of a previously submitted article, this letter should include the answers to ALL the reviewer´s comments.
5. Include a separate word document entitled: "MANUSCRIPT".
The manuscript must include the following items:
• Title of the article
• Authors (First and last name)
• Contact address for the corresponding author
• Running title
• Key words
• Abstract
• Text of the article
• References
• Table legends
• Figure legends
If you are resubmitting a modified document in response to the reviewers' comments, all changes MUST be highlighted in RED.

6. Upload TABLES, one at a time. Do not include tables in the manuscript document. Each table should be in a separate word document.
Please note that tables must have portrait orientation; we do not accept tables with landscape orientation.

7. Upload FIGURES, one at a time. Do not include figures in the manuscript document. Figures must be at least 900 X 600 pixels in size and in JPEG (.jpg) or TIFF (.tif, .tiff) format; file size must be less than 5 MB. Please transform your figures to JPEG or TIFF format without compression. All figures that do not correspond to these requirements will be rejected.

All accepted articles of this ONLINE VERSION will be published in ENGLISH and included in the SCIENCE CITATION INDEX EXPANDED *(since 2008)*, JOURNAL CITATION REPORTS *(since 2008)*, INDEX MEDICUS, MEDLINE, PUBMED, SCOPUS, EMCARE, EMBASE, INDICE MEDICO ESPAÑOL.

Articles will normally be included in one of the different journal sections. Authors should indicate the section in which they wish their article to be included, although the Editor may change this upon advice from reviewers. Articles received will always undergo revision by a committee of experts *(peer review process)*. Only original articles will be accepted, authors being responsible for the meeting of this regulation. Authors are also ***RESPONSIBLE*** for all opinions, results and conclusions contained in articles, which will not necessarily be shared by the journal's Editor and reviewers. All accepted articles become the property of Medicina Oral S.L., and their date of reception and acceptance will be reflected; thus, their subsequent publication in other media is not allowed without written permission by the Editor. Authors will transfer IN WRITING the copyright of their contributions to Medicina Oral S.L.

TYPES OF ARTICLES

1. Research articles: Analytical investigations such as cross-sectional surveys, case-control studies, cohort studies and controlled clinical trials will be recommended for publication. For clinical trials, authors must specify legal permissions obtained. Articles should not exceed 12 pages (including references) in DIN A-4 format, 30 lines per page. Not more than three figures and four tables should be included; up to 30 references.

2. Review articles: Articles of special interest and those entailing an update on any of the topics identified as subjects for this journal will be accepted. They should not exceed 14 pages (references included) in DIN A-4 format, with 30 lines per page. We recommend systematic reviews and meta-analysis. They should contain a maximum of three figures and four tables per article; up to 40 references.

ARTICLE STRUCTURE

Articles should include the following:
1. First page: *This should include the title of the article, as well as a running* title, the authors' full name and academic post, and an address for correspondence, including telephone and fax numbers, and e-mail address.
2. Following pages: *These in turn will include the following headings, according* to the type of contribution (research articles, review articles):

Research articles
— Summary, containing 150-300 words ALWAYS structured as: objectives, study design, results and conclusions.- Key words.- Introduction.- Material and methods: specifying statistical procedures used.- Results.- Discussion.- References.

Review articles
— Summary: containing 150-300 words.- Key words. -Introduction. - Material and methods: specifying how the search was made (date base selected, search strategy, screening and selection of the papers and statistical analysis). - Results and Discussion. - References.

REFERENCES

1. We do NOT accept book references.
2. We only admit references of articles INDEXED in PubMed-Medline.
3. The references should be numbered consecutively in order of appearance, being quoted in parentheses in the text. Unpublished observations and personal communications should not be included as references. The Uniform Requirements for Manuscripts Submitted to Biomedical Journals format is required throughout.
http://www.nlm.nih.gov/bsd/uniform_requirements.html
Example: Authors numbering six or less should all be quoted; when more authors are present, first six names will be quoted, followed by et al.

> Halpern SD, Ubel PA, Caplan AL. Solid-organ transplantation in HIV-infected patients. N Engl J Med. 2002;347:284-7.

Ethical requirements regarding human and animal experimentation

This journal adheres to the ethical guidelines
- Ethical requirements regarding human experimentation
http://www.wma.net/en/30publications/10policies/b3/index.html

- Ethical requirements regarding animal experimentation
http://www.wma.net/en/30publications/10policies/a18/

Conflict of interest requirements

A conflicts of interest exists if authors or their institutions have financial or personal relationships with other people or organisations that could inappropriately influence (bias) their actions. Financial relationships are easily identifiable, but conflicts can also occur because of personal relationships, academic competition, or intellectual passion.

(http://www.wma.net/en/30publications/10policies/i3/)
Adopted by the 60th WMA General Assembly, New Delhi, India, October 2009.
2012 World Medical Association, Inc.

All submissions to *Medicina Oral Patología Oral y Cirugía Bucal* must include disclosure of all relationships that could be viewed as presenting a potential conflict of interest.

- At the end of the text, under a subheading "Conflicts of interest", all authors must disclose any financial and personal relationships with other people or organisations that could inappropriately influence (bias) their work. Examples of financial conflicts include employment, consultancies, stock ownership, honoraria, paid expert testimony, patents or patent applications, and travel grants, all within 3 years of beginning the work submitted. If there are no conflicts of interest, authors should state that.
- All authors are required to provide a signed statement of their conflicts of interest as part of the author statement form.

Information
E-mail: medicina@medicinaoral.com

Indexed in:

- *Science Citation Index Expanded*
- *Journal Citation Reports*
- *Index Medicus, MEDLINE, PubMed*
- *Emcare, Embase, SCOPUS*
- *Índice Médico Español*

Free full-text at PMC (US National Library of Medicine, National Institute of Health, NIH/NLM, USA) since 1012
http://www.ncbi.nlm.nih.gov/pmc/journals/1898/

ANEXO 3

NORMAS REVISTA: Clinical Oral Investigations
(ARTIGO CAPITULO II)

ISSN: 1432-6981 (print version); ISSN: 1436-3771 (electronic version)

Instructions for Authors

TYPES OF PAPERS

Papers may be submitted for the following sections:

- Original articles
- Invited reviews
- Short communications – with up to 2000 words and up to two figures and/or tables
- Letters to the editor

It is the general policy of this journal not to accept case reports and pilot studies.

EDITORIAL PROCEDURE

If you have any questions please contact:

Professor Dr. M. Hannig
University Hospital of Saarland
Department of Parodontology and Conservative Dentistry
Building 73
66421 Homburg/Saar
Germany
Email: eic.hannig@uks.eu

MANUSCRIPT SUBMISSION

Manuscript Submission

Submission of a manuscript implies: that the work described has not been published before; that it is not under consideration for publication anywhere else; that its publication has been approved by all co-authors, if any, as well as by the responsible authorities – tacitly or explicitly – at the institute where the work has been carried out. The publisher will not be held legally responsible should there be any claims for compensation.

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Please follow the hyperlink “Submit online” on the right and upload all of your manuscript files following the instructions given on the screen.

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The Springer Author Academy is a set of comprehensive online training pages mainly geared towards first-time authors. At this point, more than 50 pages offer advice to authors on how to write and publish a journal article.

- Springer Author Academy

TITLE PAGE

The title page should include:

- The name(s) of the author(s)
- A concise and informative title
- The affiliation(s) and address(es) of the author(s)
- The e-mail address, telephone and fax numbers of the corresponding author

Abstract

Please provide a structured abstract of 150 to 250 words which should be divided into the following sections:

- Objectives (stating the main purposes and research question)
- Materials and Methods
- Results
- Conclusions
- Clinical Relevance

These headings must appear in the abstract.

Keywords

Please provide 4 to 6 keywords which can be used for indexing purposes.

TEXT

Text Formatting

Manuscripts should be submitted in Word.

- Use a normal, plain font (e.g., 10-point Times Roman) for text.
- Use italics for emphasis.
- Use the automatic page numbering function to number the pages.
- Do not use field functions.
- Use tab stops or other commands for indents, not the space bar.
- Use the table function, not spreadsheets, to make tables.
- Use the equation editor or MathType for equations.
- Save your file in docx format (Word 2007 or higher) or doc format (older Word versions).

Manuscripts with mathematical content can also be submitted in LaTeX.

- LaTeX macro package (zip, 181 kB)

Headings

Please use no more than three levels of displayed headings.

Abbreviations

Abbreviations should be defined at first mention and used consistently thereafter.

Footnotes

Footnotes can be used to give additional information, which may include the citation of a reference included in the reference list. They should not consist solely of a reference citation, and they should never include the bibliographic details of a reference. They should also not contain any figures or tables.

Footnotes to the text are numbered consecutively; those to tables should be indicated by superscript lower-case letters (or asterisks for significance values and other statistical data). Footnotes to the title or the authors of the article are not given reference symbols.

Always use footnotes instead of endnotes.

Acknowledgments

Acknowledgments of people, grants, funds, etc. should be placed in a separate section on the title page. The names of funding organizations should be written in full.

REFERENCES

Citation

Reference citations in the text should be identified by numbers in square brackets. Some examples:

1. Negotiation research spans many disciplines [3].
2. This result was later contradicted by Becker and Seligman [5].
3. This effect has been widely studied [1-3, 7].

Reference list

The list of references should only include works that are cited in the text and that have been published or accepted for publication. Personal communications and unpublished works should only be mentioned in the text. Do not use footnotes or endnotes as a substitute for a reference list.

The entries in the list should be numbered consecutively.

- Journal article

  Gamelin FX, Baquet G, Berthoin S, Thevenet D, Nourry C, Nottin S, Bosquet L (2009) Effect of high intensity intermittent training on heart rate variability in prepubescent children. Eur J Appl Physiol 105:731-738. https://doi.org/10.1007/s00421-008-0955-8

  Ideally, the names of all authors should be provided, but the usage of “et al” in long author lists will also be accepted:

  Smith J, Jones M Jr, Houghton L et al (1999) Future of health insurance. N Engl J Med 965:325–329

- Article by DOI

  Slifka MK, Whitton JL (2000) Clinical implications of dysregulated cytokine production. J Mol Med. https://doi.org/10.1007/s001090000086

- Book

  South J, Blass B (2001) The future of modern genomics. Blackwell, London

- Book chapter

  Brown B, Aaron M (2001) The politics of nature. In: Smith J (ed) The rise of modern genomics, 3rd edn. Wiley, New York, pp 230-257

- Online document

  Cartwright J (2007) Big stars have weather too. IOP Publishing PhysicsWeb. http://physicsweb.org/articles/news/11/6/16/1. Accessed 26 June 2007

- Dissertation

  Trent JW (1975) Experimental acute renal failure. Dissertation, University of California

Always use the standard abbreviation of a journal's name according to the ISSN List of Title Word Abbreviations, see

- ISSN.org LTWA

If you are unsure, please use the full journal title.

For authors using EndNote, Springer provides an output style that supports the formatting of in-text citations and reference list.

- EndNote style (zip, 2 kB)

Authors preparing their manuscript in LaTeX can use the bibtex file spbasic.bst which is included in Springer's LaTeX macro package.

TABLES

- All tables are to be numbered using Arabic numerals.
- Tables should always be cited in text in consecutive numerical order.
- For each table, please supply a table caption (title) explaining the components of the table.
- Identify any previously published material by giving the original source in the form of a reference at the end of the table caption.
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APÊNDICE 1

Apêndice X - METODOLOGIA LABORATORIAL PARA ANÁLISE DA EXPRESSÃO GÊNICA DAS CICLOOXIGENASE (COX-1 E COX-2) EM ANALISE DE RTq-PCR

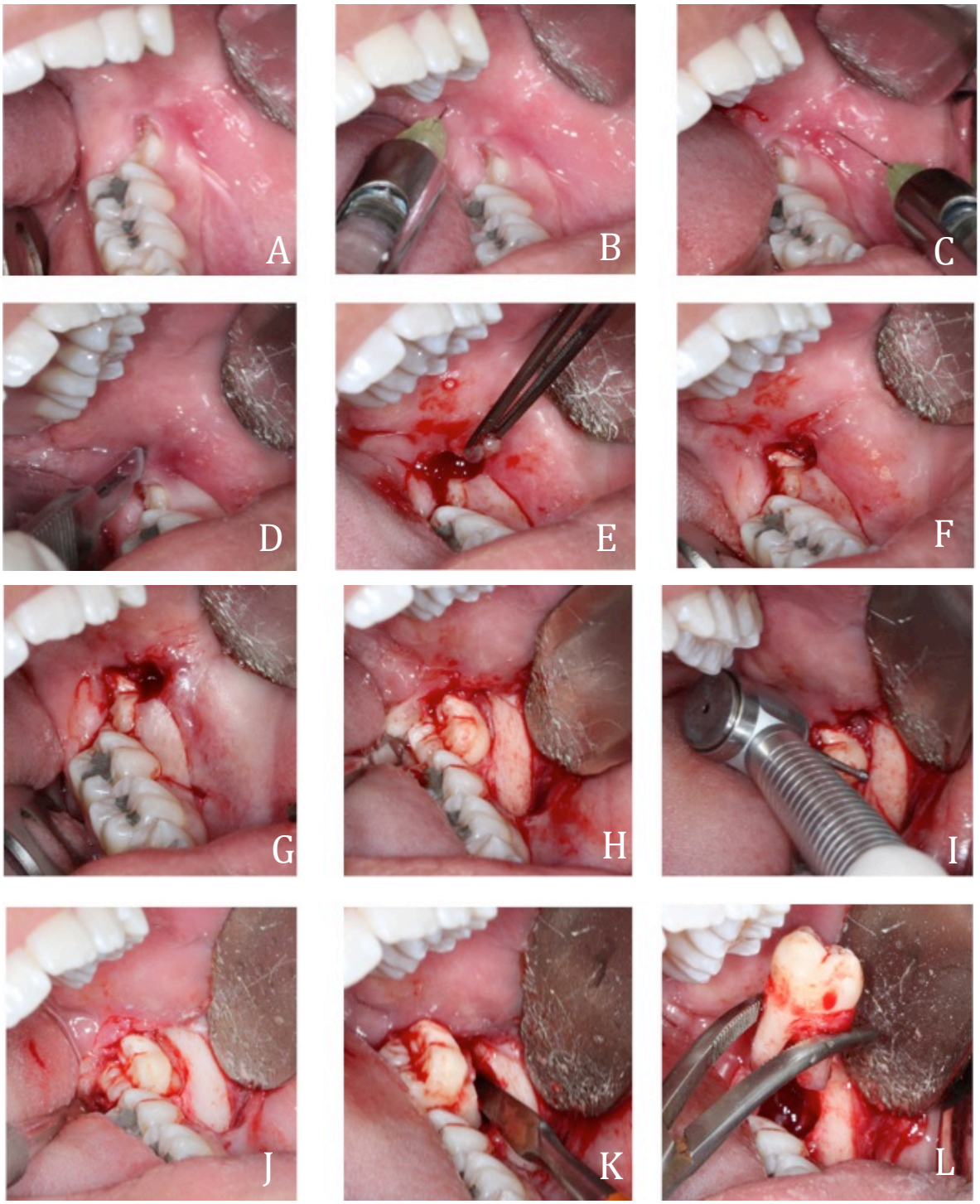
1. **Aquisição das Amostras** (ALBUQUERQUE et al. 2017)

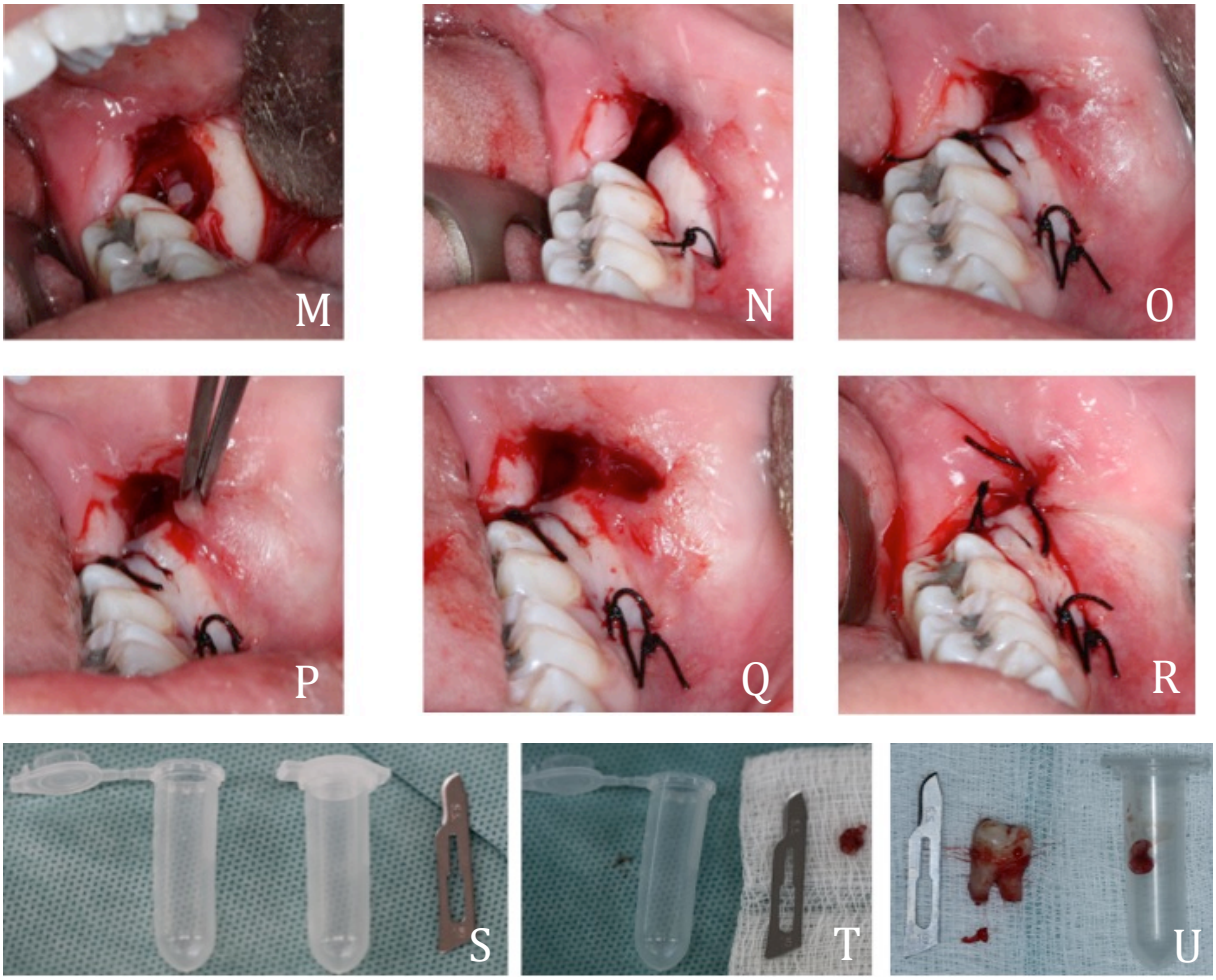
Os pacientes foram submetidos a dois procedimentos cirúrgicos para remoção dos terceiros molares inferiores, intercalados por um período mínimo de 28 dias. O protocolo cirúrgico já padronizado e a técnica cirúrgica utilizada foi a que é comumente realizada para remoção de terceiros molares como publicado no estudo de BEZERRA et al. (2011).

Em ordem cirúrgica, a anestesia local foi realizada a partir do bloqueio do nervo alveolar inferior (NAI), nervo lingual e nervo bucal (Figura A-C), obtida utilizando-se o anestésico cloridrato de mepivacaína a 2% com adrenalina 1:100.000 (Mepiadre®, DFL, Brasil). Depois de constatada a anestesia, foi realizada a remoção de um fragmento de tecido pericoronário de 3mm de extensão (Figura D-F), sendo o material colhido acondicionado em microtubo estéril (eppendorf) de 300µL em solução de TRIZOL. Em seguida, foi realizado um retalho em envelope de espessura total e o osso que esteja impedindo a remoção do dente foi removido com uma broca odontológica carbide cirúrgica nº 702 (FGXL, Brasil) montada em uma peça de mão cirúrgica de alta rotação sob irrigação profusa e constante soro fisiológico 0,9% (Figura G-J). Quando necessário, foi realizada odontosecção nesta mesma fase com a broca previamente utilizada. O dente foi então removido com um elevador do tipo reto ou Seldin (Figura K e L). Decorridos 30 minutos do inicio do procedimento cirúrgico, certificando-se que o paciente esteja sobre efeito analgésico do anestésico local satisfatório, foi realizada a remoção de um segundo tecido pericoronário similarmente ao que foi descrito anteriormente, e as bordas da ferida cirúrgica foram cuidadosamente suturadas utilizando-se fio de seda 4.0 (Shalon®) (Figura M-R). Os fragmentos removidos foram acondicionados em eppendorfs estéreis e colocados solução de TRIZOL (Figura S-U). Os pontos foram removidos após 7 dias. Além disso, cada participante foi devidamente informado sobre as recomendações pós-operatórias necessárias. A duração do procedimento cirúrgico, quantidade de anestésico local, realização de osteotomia e/ou odontosecção, e intercorrências foram anotadas em um formulário padrão.

Etapa 1: Fase Clínica

*** Protocolo Cirúrgico**





➢ **Armazenamento das amostras**

- As amostras coletadas foram colocadas em um freezer a - 80^{0}C

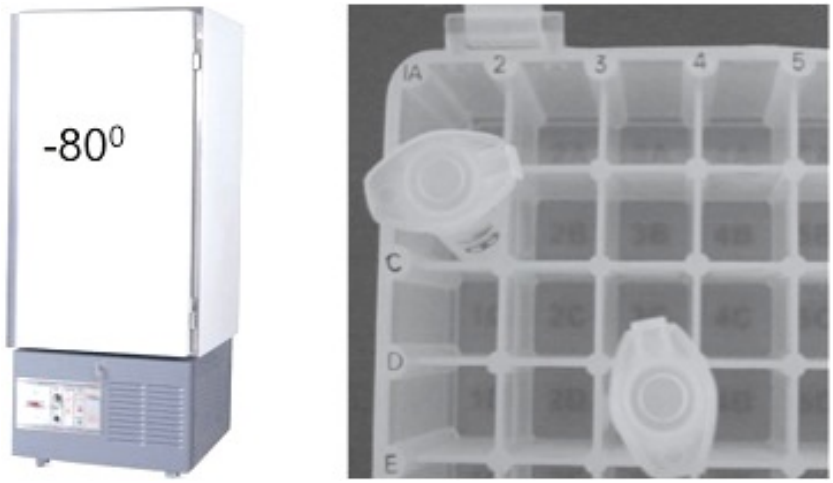
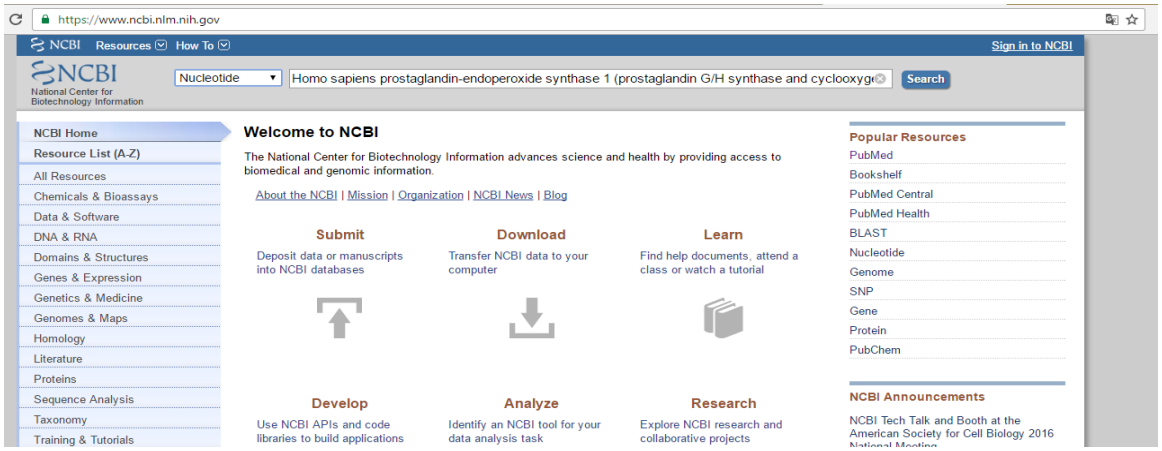


Figura 1: Foto ilustrativa do freezer a -80^0 e da caixa com os eppendorfs alocados.

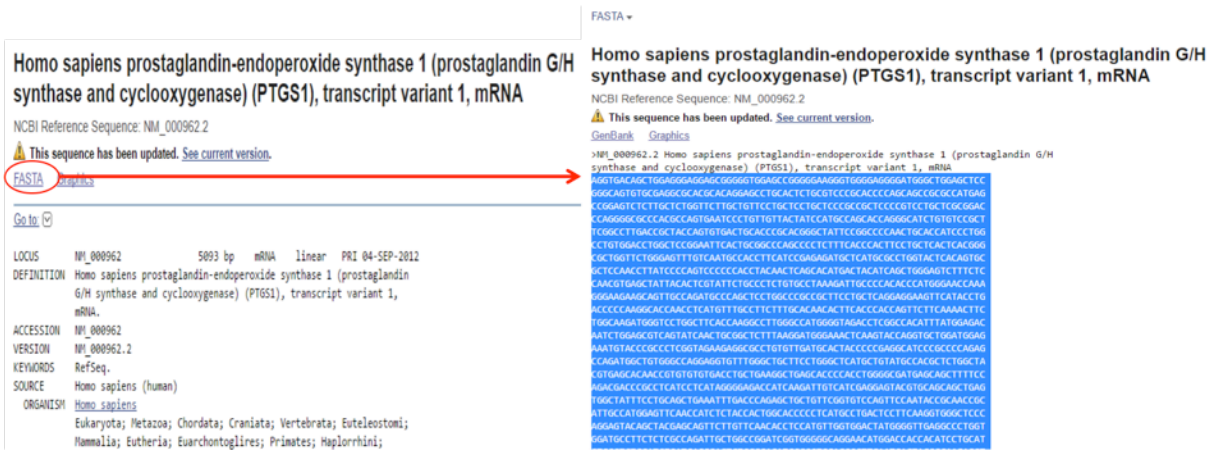
Etapa 2: Fase laboratorial

Fase 01: Construção dos Primers

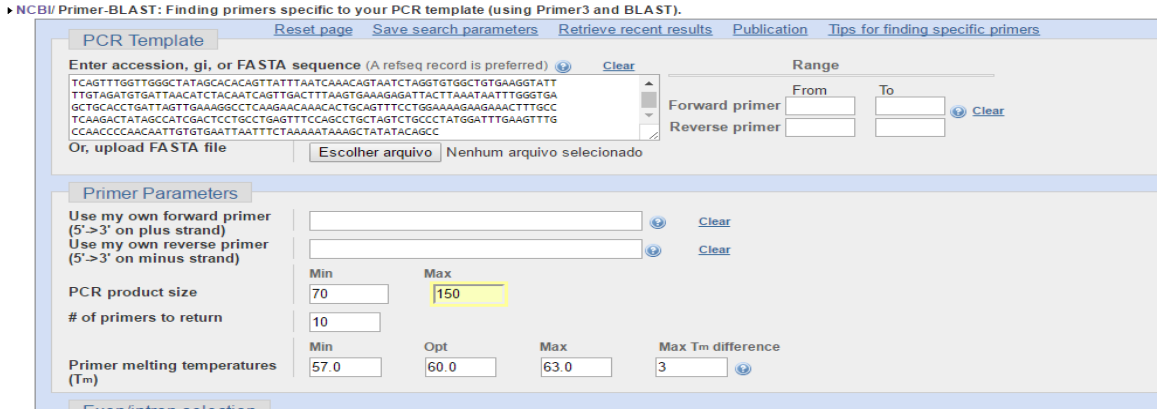
Site: NCBI - National Center for Biotechnology Information
Procurar a sequência do gene



- Procurar a sequencia do gene (COX-1, COX-2 e GAPDH)



- Site: - Primeblast



Obs: 70-150pb (tamanho do fragmento de DNA que será amplificado no qPCR)

Sequencia de nucleotídeos para formação de cada primer

- **COX-1**

Primer pair 1

	Sequence (5'->3')	Length	Tm	GC%
Forward primer	CAGACGACCCGCCTCATCCTCATAG	25	60.23	60.00%
Reverse primer	GCCTCAACCCCATAGTCCACCAACA	25	60.40	56.00%

Products on target templates

>NM_000962.2 Homo sapiens prostaglandin-endoperoxide synthase 1 (prostaglandin G/H synthase and cyclooxygenase) (PTGS1), transcript variant 1, mRNA

```
product length = 275
Forward primer  1     CAGACGACCCGCCTCATCCTCATAG  25
Template        1120  .........................  1144

Reverse primer  1     GCCTCAACCCCATAGTCCACCAACA  25
Template        1394  .........................  1370
```

- **COX-2**

Primer pair 1

	Sequence (5'->3')	Length	Tm	GC%
Forward primer	TGGGAAGCCTTCTCTAACCTCTCCT	25	58.12	52.00%
Reverse primer	CTTTGACTGTGGGAGGATACATCTC	25	54.73	48.00%

Products on target templates

>NM_000963.2 Homo sapiens prostaglandin-endoperoxide synthase 2 (prostaglandin G/H synthase and cyclooxygenase) (PTGS2), mRNA

```
product length = 388
Forward primer  1    TGGGAAGCCTTCTCTAACCTCTCCT  25
Template        510  ........................  534

Reverse primer  1    CTTTGACTGTGGGAGGATACATCTC  25
Template        897  ........................  873
```

- **GAPDH**

Primer pair 1

	Sequence (5'->3')	Length	Tm	GC%
Forward primer	GACCCCTTCATTGACCTCAACTAC	24	54.92	50.00%
Reverse primer	CATCGCCCCACTTGATTTTG	20	52.02	50.00%

Products on target templates

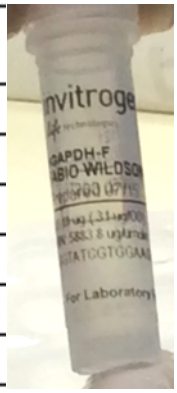
>NM_002046.3 Homo sapiens glyceraldehyde-3-phosphate dehydrogenase (GAPDH), mRNA

```
product length = 166
Forward primer  1    GACCCCTTCATTGACCTCAACTAC  24
Template        205  .......................  228

Reverse primer  1    CATCGCCCCACTTGATTTTG  20
Template        370  ....................  351
```

- Sequencia enviada para a empresa Invitrogen para confecção dos primers, para ser usada na RTq-PCR.

Nome do oligonucleotídeo	Sequência
hGAPDH-F	TGGTATCGTGGAAGGACTC
hGAPDH-R	TAGAGGCAGGGATGATGTT
hCOX1-F	CTGCCCTCCTCAAGACTTTAGCTT
hCOX1-R	TCCAACTGATTTAAGCAAAAGAGGAAT
hCOX2-F	CCTTCGAAATGCAATTATGAGTT
hCOX2-R	CACAGGAGGAAGGGCTCTAGT

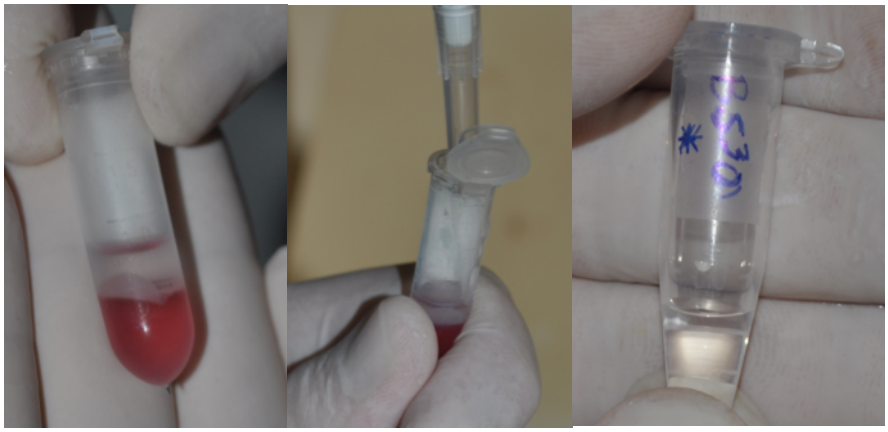


Fase 2: Extração do RNA (de acordo com as normas do fabricante)

- **Fase de separação**

- O isolamento do RNA total foi realizado utilizando o kit de purificação Trizol® Plus (Invitrogen, São Paulo, Brasil). De acordo com as instruções do fabricante, foram adicionados 800 µL de solução de Trizol a cada amostra congelada e o lisado foi aspirado e a centrifugação a 10 000 g durante 3 min à temperatura ambiente.

- Após a centrifugação, foi realizado a remoção do sobrenadante (fase clara) e colocação em outro tubo e a fase rosa foi descartada.



- Posteriormente, todos os lisados foram diluídos 1: 1 com etanol a 70% e submetidos a uma mini-coluna. Após a ligação do RNA à coluna, a digestão de DNA foi realizada utilizando DNAse livre de RNAase (340 unidades de Kunitz / mL) durante 15 min à temperatura ambiente. Após lavar a coluna três vezes, o RNA foi eluído com 30 µL de água isenta de RNAse (Descrito abaixo).

- **Fase de ligação**

- Transferir 500 µL da amostra para o tubo SPIN, e centrigugar por 15 segundos à 12000x g, à temperatura de 4^0C.

- **Fase de lavagem**

- Adicionar 350 µL de Wash Buffer I (reagente do KIT) e centrifugar por 12000x g por 15 segundos à temperatura ambiente.

- Passar a coluna do tubo SPIN, para o tubo WASH TUBE e adicionar 80 µL de DNase PureLink (preparo de acordo com as normas do fabricante.

Componente	Volume
10x DNase I Rraction Buffer	8 µL
DNase Resuspensão (sol de estoque)	10 µL
RNase Free Water	62 µL
Volume total	80 µL por amostra

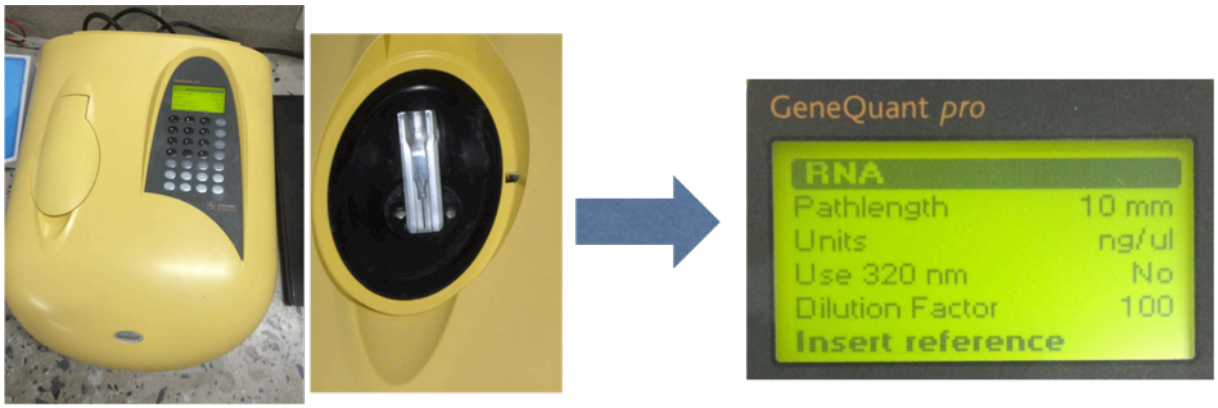
- Adicionar 350 µL de Wash Buffer I (reagente do KIT) e centrifugar por 12000x g por 15 segundos à temperatura ambiente.
- Adicionar 500 µL de Wash Buffer II, e centrifugar por 12000x g, por 1 minuto, à temperatura ambiente.
- Transferir a coluna do WASH TUBE para o RECOVERY TUBO.

- **Fase de Eluição para aquisição do RNA**

- Adicionar 30 µL de RNase-free water (reagente do KIT) e incubar à temperatura ambiente por 1 minuto. Após esse período centrifugar por 2 minutos à temperatura ambiente e descartar a coluna e coleta do tubo RECOVERY TUBE.

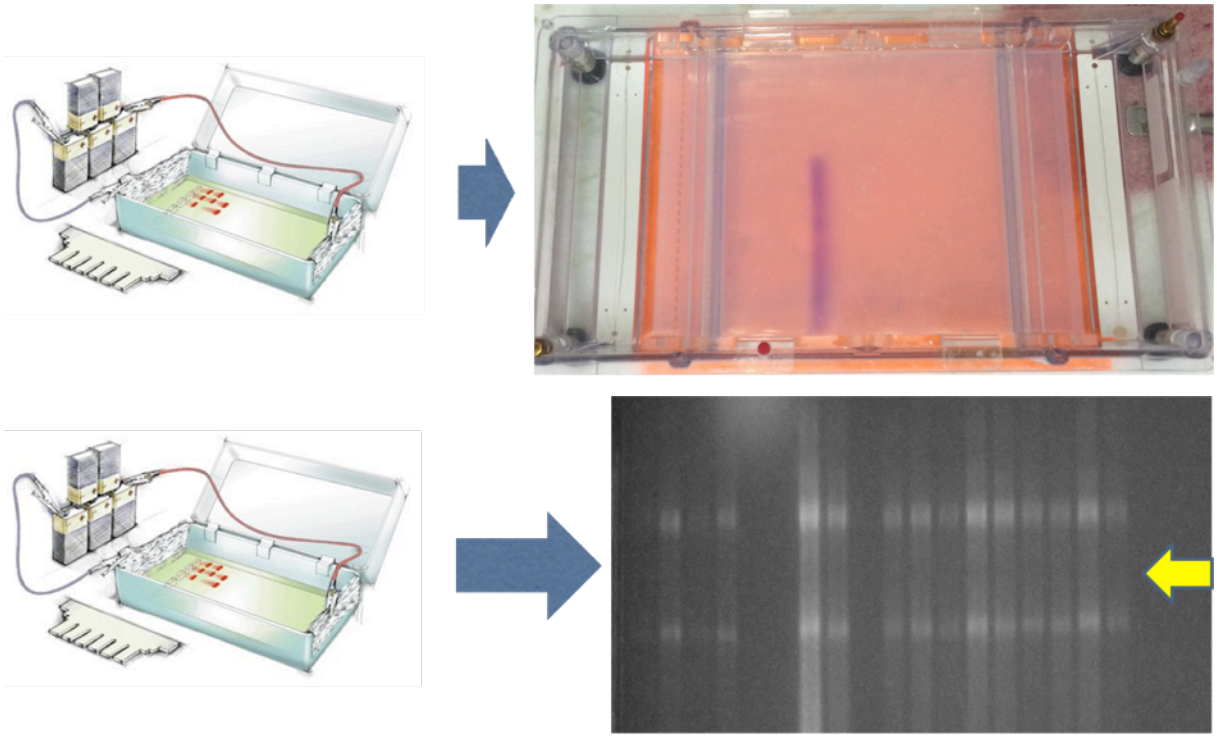
- **Teste da eficácia da extração e pureza do RNAm**

- Para testar a eficácia da extração e pureza do RNA total, a concentração de RNA total das amostras foi determinada por diluição de RNA (fator de diluição conhecido) juntamente com uma leitura no espectrofotômetro em cubetas de quartzo, usando comprimentos de onda de 260 nm (A260) e 260/280 nm (A260 / A280), (Amersham Biosciences, Cambridge, Inglaterra).



- ELETROFORESE

Gel de agarose, para avaliação da integridade da extração do RNA



- **Reação de Transcrição Reversa (cDNA)**

Transformação do RNAm em DNA complementar para a realização do RTq-PCR

- Antes da reação de transcrição reversa, amostras de RNA foram incubadas por 5 min a 70 ºC e depois resfriadas em gelo.

- A transcrição reversa foi realizada em um volume total de 20 µL composto por 10 µL de amostra de RNA, 4 µL de tampão de transcriptase reversa (Invitrogen, São Paulo, Brasil), 8 unidades de RNase out, 150 unidades de transcriptase reversa Superscript III, 0036 U random primers, 10 mM DTT e 0,5 mM de cada dNTP.

- Reação de Transcrição para 1 amostra 15 µL de cDNA

Solução	Quantidade
Amostra de RNA	10,0 µL
Tampão de transcriptase reversa (Invitrogen, São Paulo, Brasil)	4,0 µL
RNAse out	8 unidades
transcriptase reversa Superscript III	150 unidades
Random primer	0036 U
DDT	10 mM
dNTP (Invitrogen, São Paulo, Brasil)	0,5 mM

Total	15 µL + 15 µL de RNAm = 30 µL de cDNA

Fase 07: Reação de Cadeira da Polimerase em Tempo Real (RTqPCR)

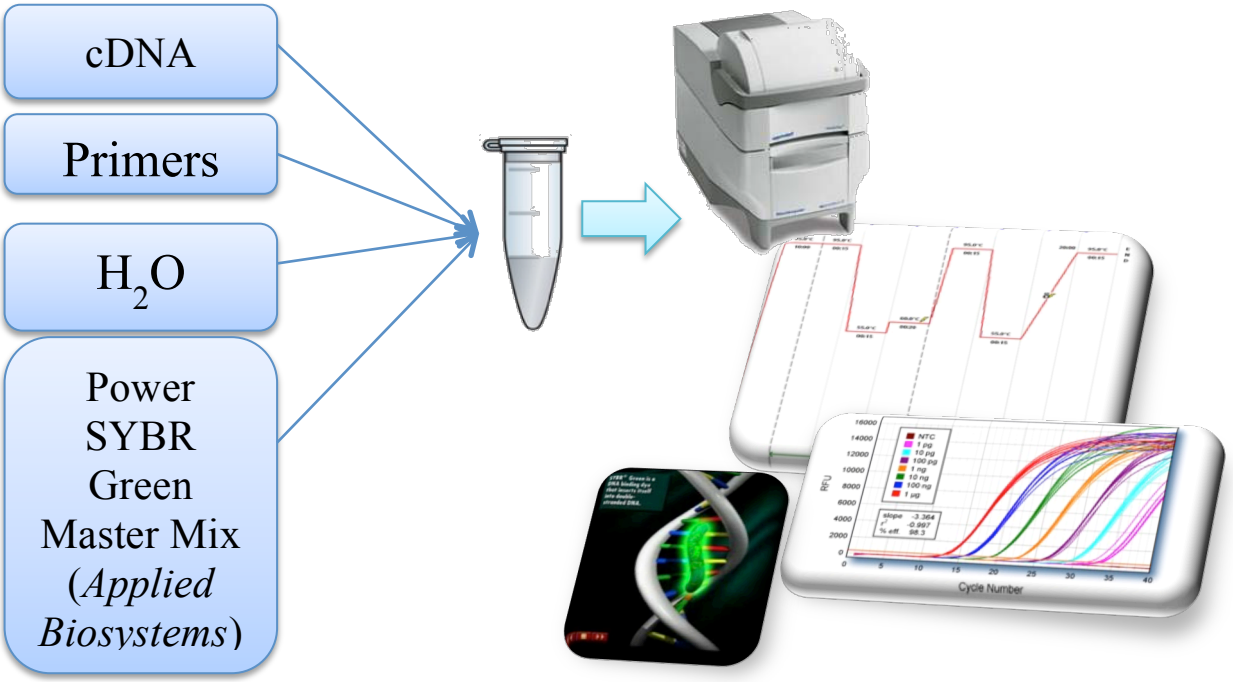
- Desenho esquemático da placa para a realização do PCR

	1	2	3	4	5	6	7	8
A	COX2 NEGATIVO	GAPDH NEGATIVO	COX2 PLACEBO – T0	COX2 PLACEBO – T0	COX2 PLACEBO – T0	GAPDH PLACEBO – T0	GAPDH PLACEBO – T0	GAPDH PLACEBO – T0
B	COX2 IBUPROFENO – T0	COX2 IBUPROFENO – T0	COX2 IBUPROFENO – T0	GAPDH IBUPROFENO – T0	GAPDH IBUPROFENO – T0	GAPDH IBUPROFENO – T0	COX2 ETORICOXIBE – T0	COX2 ETORICOXIBE – T0
C	COX2 ETORICOXIBE – T0	GAPDH ETORICOXIBE – T0	GAPDH ETORICOXIBE – T0	GAPDH ETORICOXIBE – T0	COX2 PLACEBO – T30	COX2 PLACEBO – T30	COX2 PLACEBO – T30	GAPDH PLACEBO – T30
D	GAPDH PLACEBO – T30	GAPDH PLACEBO – T30	COX2 IBUPROFENO – T30	COX2 IBUPROFENO – T30	COX2 IBUPROFENO – T30	GAPDH IBUPROFENO – T30	GAPDH IBUPROFENO – T30	GAPDH IBUPROFENO – T30
E	COX2 ETORICOXIBE – T30	COX2 ETORICOXIBE – T30	COX2 ETORICOXIBE – T30	COX2 ETORICOXIBE – T30	GAPDH ETORICOXIBE – T30	GAPDH ETORICOXIBE – T30		

- Na placa de PCR em cada poço é colocado as substâncias de cDNA, o Primer, água e Syber Green para a realização em triplicada do PCR.

- A quantifição de RNAm foi realizada utilizando SYBR GreenMaster Mix (PE Applied Biosystems, Foster City, CA). As reações de PCR foram compostas por 1 µL de cDNA como modelo em 7,5 µL de mistura principal GoPCq ® (Promega Corporation, Madison, WI, EUA), 5,5 µL de água ultrapura e 0,5 µM de cada primer.

- O perfil do ciclo térmico para o primeiro ciclo de PCR foi: desnaturação inicial e ativação da polimerase por 10 minutos a 95^{o}C, seguido de 40 ciclos de 15 segundos a 95 ºC, 30 segundos a 58 ºC e 30 segundos a 72 ºC. A extensão final foi por 10 min a 72 ºC. Todas as reações foram realizadas em PCR StepOne Real-Time (Applied Biosystems, Foster, CA, EUA).



- Termociclador utilizado na pesquisa.

- Sequencia do RTq-PCR