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## Expression of COX -1 and -2 in a Clinical Model of Acute Inflammation

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### Abstract

Cyclooxygenase (COX) plays an important role in the induction of pain and inflammation as well as the analgesic actions of NSAIDs and coxibs. This study evaluates the expression of the two isoforms COX-1 and COX-2 in a clinical model in which the surgical removal of impacted third molars is used to evaluate the analgesic activity of anti-inflammatory drugs. A 3 mm punch biopsy was performed on the oral mucosa overlying one impacted third molar immediately before extraction of two impacted lower third molars. After the second tooth was extracted, a second biopsy was performed adjacent to the surgical site either immediately after surgery, 30, 60 or 120 minutes after surgery. RNA was extracted from the biopsies and RT-PCR was performed to assess mRNA levels of COX-1, COX-2 and glyceraldehyde-3-phosphate dehydrogenase (G3PDH). The RT-PCR products in the biopsies were normalized to G3PDH and compared to baseline. COX-2 mRNA was progressively increased at 30, 60, and 120 minutes after surgery ( $P < 0.05$ ); COX-1 mRNA was transiently decreased at 60 minutes during the post-surgical period ( $P < 0.05$ ). The results demonstrate peripheral elevation of COX-2 following tissue injury, which may contribute to increased prostaglandin E<sub>2</sub> at the site of injury, pain onset and the analgesic activity of both non-selective NSAIDs and selective COX-2 inhibitors.

**Perspective**—This clinical study uses a physiologically relevant model to determine the time course of expression of COX -1 and -2 in acute inflammation of the human oral mucosa. This study furthers our understanding of the contribution of the COX isoforms to acute pain.

### Introduction

Cyclooxygenase (COX), also known as prostaglandin H synthase, is the key enzyme in the synthesis of prostaglandins (PGs). Elucidation of the two COX isoforms gave rise to the concept that the constitutive enzyme COX-1 was responsible for the production of the PGs with homeostatic functions in tissues such as the stomach, kidney, and platelets, while COX-2, the inducible enzyme, was responsible for the production of the proinflammatory PGs.<sup>16, 27, 33</sup>

There is extensive evidence based on animal, as well as human studies supporting the role of COX-2 in the development of inflammation.<sup>27,32,34</sup> Animal models of inflammation have

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demonstrated that COX-2 mRNA and protein as well as PGs increase in a time-dependent manner that parallels the inflammatory process.<sup>1</sup> Inflammatory cytokines and endotoxins can induce a 10 to 80-fold increase in the level of COX-2 expression in monocytes, macrophages, chondrocytes, fibroblasts, and endothelial cells.<sup>1,2</sup> The contribution of COX-2 to inflammation is further supported by demonstration that the expression of COX-2 and production of PGs can be inhibited by anti-inflammatory cytokines and glucocorticoids.<sup>6, 25</sup>

The concept that COX-2 is the only COX isoform involved in inflammation has been challenged by a number of studies.<sup>10,21,35</sup> It is now believed that COX-1 is responsible for the initial prostanoid response to inflammatory stimuli, while COX-2 becomes the major contributor to prostanoid synthesis as inflammation progresses.<sup>9,16,31</sup> PGE<sub>2</sub> can be produced by PGE synthase from many different cell types including neurons, endothelial cells, and neutrophils. PGE<sub>2</sub> released in inflamed tissue sensitizes the terminals of afferent nerve fibers thereby enhancing nociceptive processing within the spinal cord and brain to evoke hyperalgesia.<sup>29</sup> COX-1 mRNA has a half-life of about 12-15 hours while COX-2 has a shorter half-life of less than 3.5 hours,<sup>19</sup> suggesting a close temporal link between tissue injury, COX-2 expression and elevated PGE<sub>2</sub> in comparison to constitutively expressed COX-1.

A previous study in the oral surgery model demonstrated differential production of products of COX-1 (thromboxane B<sub>2</sub>, the stable metabolite of thromboxane A<sub>2</sub>) and PGE<sub>2</sub> production mediated by both COX-1 and COX-2. The selective COX-2 inhibitor celecoxib did not have any detectable effect on thromboxane B<sub>2</sub> levels and only suppressed PGE<sub>2</sub> levels from 120-240 minutes following oral surgery.<sup>14</sup> A similar time course of action was also demonstrated for rofecoxib in the oral surgery model with the effects of the selective COX-2 inhibitor being seen at 60-240 minutes post-surgery.<sup>15</sup> These data suggest that the expression of COX-2 following tissue injury takes 1-2 hours to produce increased prostanoid levels that contribute to pain and the acute inflammatory process. We conducted a study to examine the *in vivo* expression of COX-1 and -2 in the human oral mucosa prior to and following post-surgical trauma and the onset of inflammation. Our results demonstrate that COX-2 mRNA rapidly increases in a time-dependent manner following surgery while the level of COX-1 mRNA transiently decreases, but otherwise shows no sustained alteration, during the post-surgical period.

## Materials and Methods

### Subjects and Study Design

The study was approved by the Institutional Review Board, National Institute of Dental and Craniofacial Research, National Institutes of Health. Informed consent was obtained from all subjects. Subjects, 16 years or older, were enrolled as outpatients and underwent surgical removal of impacted mandibular third molars. Inclusion criteria were the presence of two mandibular third molars, classified as partial or full bony impaction by clinical and radiographic examination. Exclusion criteria included the presence of infection or inflammation at either of the two extraction sites as determined by clinical examination. Subjects who were pregnant or nursing were excluded from the study as were those taking anti-depressants, diuretics, aspirin, coumadin or any other anticoagulants and any drugs such as steroids which might influence pain report or the synthesis and activity of COX.

Pre- and post-operative punch biopsies were obtained from each subject. The pre-operative biopsy was performed from the oral mucosa overlying one impacted third molar, immediately before the surgical extraction. Following this, both mandibular third molars were removed and the postoperative biopsy was obtained from the other extraction site. Subjects (n ≥ 10 per group) were randomly allocated with respect to the second biopsy into one of four groups: (1) time 0,

immediately after surgery (n=10); (2) 30 min post-surgery (n=13); (3) 60 min post-surgery (n=10); or (4) 120 min post-surgery (n=10).

### RNA extraction and RT-PCR Analysis

The samples were immediately frozen and maintained at  $-80^{\circ}\text{C}$ . Total RNA was extracted using the RNeasy RNA extraction kit (Qiagen, CA, USA). RNA yield was quantified using the RiboGreen RNA Quantitation Kit (Molecular Probes, Oregon, USA). The RNA yield was  $246 \pm 42$  ng/mg tissue weight. RT-PCR was carried out using the Access RT-PCR system (Promega, Madison, WI, USA). Primer sequences and assay parameters are given in Table 1.

The program used for RT-PCR on a thermocycler (Robocycler Gradient 96, Stratagene) consisted of  $48^{\circ}\text{C}$  for 45 minutes and  $94^{\circ}\text{C}$  for 2 minutes for the synthesis of first strand cDNA by reverse transcriptase; followed by 40 cycles of a denaturation step at  $94^{\circ}\text{C}$  for 30 seconds, an annealing step at  $55^{\circ}\text{C}$  for 1 minute, and an extension step at  $68^{\circ}\text{C}$  for 2 minutes for amplification of cDNA reverse transcribed from COX-2 mRNA. The final extension was done at  $72^{\circ}\text{C}$  for 7 minutes. The program for COX-1 was similar except that the extension was done at  $72^{\circ}\text{C}$ . For G3DPH, the extension and the final extension were done at  $68^{\circ}\text{C}$ . The amount of RNA template and number of cycles that would yield relative values in the linear range of amplification for each target transcript in the RT-PCR were determined by preliminary experiments. Each group of RT-PCR experiments included a negative control in which the extracted RNA was replaced by RNase-free water.

The PCR products were detected by electrophoresis in 2% agarose gels visualized with ethidium bromide staining. A fluorescence imaging system (AlphaImager, Alpha Innotech Corp., San Leandro, CA) was used to acquire the image of the fluorescent bands. Analysis of band intensity was performed on a Macintosh computer using the public domain NIH Image program (developed at the U.S. National Institutes of Health and available on the Internet at <http://rsb.info.nih.gov/nih-image>). The RT-PCR products were normalized to G3PDH and the results of post-surgical biopsies were compared to those of pre-surgical biopsies.

### Statistical Analysis

Changes in the ratio of COX-1/G3PDH and COX-2/G3PDH over time were compared to ratios in the pre-surgical biopsies. A permutation test for two related samples tested for change over time in levels of each COX isoforms was performed with post hoc testing to determine if any time points differed from baseline. Statistical analyses were performed with SPSS 6.1 (SPSS, Chicago, IL).

## Results

### Subjects

The study sample consisted of 43 usable subjects distributed among the treatment groups (Table 2). The mean age, 20 years, is characteristic of the young adult population undergoing the removal of impacted third molars. Subjects in the four groups did not differ in terms of demographic and surgical factors such as extraction difficulty, the dose of midazolam or the amount of lidocaine administered, that could affect the outcome of the study.

### COX-2 mRNA

Under the conditions used COX-2 expression in the preoperative biopsies was either not detectable or was seen as a faint band (Figure 1). Low levels of COX-2 message were detected in 51% of the pre-surgical biopsies. The ratio of COX-2 mRNA/G3PDH mRNA in biopsies obtained at time 0 immediately after surgery did not differ from that in the pre-surgical biopsies. Biopsies collected at 30, 60 and 120 minutes after surgery demonstrated a progressive

significant increases ( $P < 0.01$ ) in the level of COX-2 message over time as compared to preoperative levels (Figures 1 and 2). The presence of additional bands in Figure 1 is likely due to the large number of PCR cycles needed to detect COX-2.

### COX-1 mRNA

The COX-1 transcript was detected in all the pre-surgical and post-surgical biopsies (Figures 1 and 3). The ratio of COX-1 mRNA/G3PDH mRNA in the biopsies collected immediately after surgery did not differ significantly from that of the pre-operative biopsies (Figure 3). We detected a significant decrease in the ratio of COX-1 mRNA/G3PDH mRNA in samples collected at 60 min after surgery as compared to baseline ( $P < 0.05$ ). The level of COX-1 mRNA was numerically lower at 30 and 120 minutes after surgery as compared to baseline but was not statistically significant. No consistent change was observed with G3PDH.

### Discussion

It is widely accepted that COX-1 is responsible for the immediate prostanoid response to inflammatory stimuli while COX-2 becomes the primary contributor to prostanoid synthesis as inflammation progresses. The results of this study are supportive of this concept as we demonstrate COX-1 message at baseline and throughout the post-operative period in comparison to negligible COX-2 message prior to surgery with an increase in the level of COX-2 message during the post-surgical period.

This study demonstrates a significant decrease in the level of COX-1 mRNA in biopsies obtained 60 minutes after surgery ( $P < 0.05$ ) as compared to the level of COX-1 in preoperative biopsies. Prior studies have reported that the level of COX-1 mRNA and protein in the peripheral tissues do not change during acute inflammation.<sup>27,32</sup> It is conceivable that the discrepancy between these reports and our data is due to differences in the types of stimuli used and in the tissue examined. Liu et al. report a 2 to 5-fold decrease in the level of COX-1 mRNA in the myocardial and pleural tissues of rats following systemic administration of lipopolysaccharides (LPS).<sup>18</sup> A similar study reported a decrease in COX-1 mRNA in the rodent renal medulla one hour after injection of LPS.<sup>13</sup> While these data support our observations, the systemic administration of LPS is a model of sepsis and may not be reflective of the physiological changes of post-surgical trauma and acute inflammation. A subsequent study in the oral surgery model<sup>17</sup> using quantitative real time PCR in a larger sample replicated the observed decrease in COX-1 mRNA in the immediate postoperative period suggesting that acute tissue injury and inflammation in humans not only stimulates increased COX-2 mRNA but also transiently inhibits COX-1 mRNA transcription similar to immune stimuli such as LPS.<sup>13,18</sup>

The results from this study clearly demonstrate increased expression of COX-2 following the induction of inflammation. Previous studies examining the levels of PGE<sub>2</sub> in the extraction sites following oral surgery demonstrate a decrease in PGE<sub>2</sub> levels in the immediate post-operative period<sup>10,14</sup> followed by an increase which is coincident with report of moderate to severe pain.<sup>10,14,26</sup> While the relative contributions of COX-1 and COX-2 to PGE<sub>2</sub> production are not known, it is likely that COX-2 is primarily responsible for the increased levels of PGE<sub>2</sub> but with continued production of PGE<sub>2</sub> by COX-1.

COX-2 is induced in cultured gingival fibroblasts following application of proinflammatory agents such as interleukin-1 $\beta$  (IL-1 $\beta$ ), LPS and bradykinin.<sup>20,22,23,38</sup> The increased expression of COX-2 in fibroblasts results in enhanced synthesis of PGE<sub>2</sub>.<sup>23,36,37,38</sup> The same phenomenon can be observed in endothelial cells upon induction with IL- $\alpha$ .<sup>11</sup> Polymorphonuclear cells also up-regulate COX-2 expression in experimental systems following LPS stimulation.<sup>20</sup> Taken together with our results it appears that after tissue trauma,

mainly three types of cells contribute to COX-2 induction and subsequently to PG synthesis: the resident fibroblasts and endothelial cells as well as the invading PMNs. The increased levels of PGs may in turn, influence the maintenance of nociceptive processes in post-operative pain.

The oral surgery model is a useful and reproducible model of acute pain, widely used in analgesic research.<sup>3,4</sup> Adapting microdialysis to the oral surgery model has facilitated examination of the relationship between mediators of inflammation, pain report and analgesic activity in humans.<sup>5,12,26,30</sup> The results of previous studies using microdialysis and the oral surgery model demonstrated increased PGE<sub>2</sub> production at later time points during the postoperative period (120-240 minutes) suggestive of increased COX activity. The present study supports these observations by demonstrating increased COX-2 expression with negligible changes in COX-1 expression at the same time points. This increase in COX-2 expression also suggests a role of this isoform in the inflammatory response and subsequent resolution of tissue injury and repair, although the exact role needs clarification.<sup>7,28</sup> Taken together with previous demonstrations of a PGE<sub>2</sub> time course following surgery,<sup>10</sup> differential effects of coxibs on biomarkers for COX-1 and COX-2<sup>14,15</sup> and the ability to simultaneously measure pain and analgesia in humans, supports the utility and clinical relevance of the oral surgery model for mechanistic studies of inflammation.

To conclude, this study demonstrated the induction of COX-2 mRNA in acute inflammation in humans. The use of a physiologically relevant model of acute inflammation further enhances the generalizability of these findings, which are relevant to other types of acute inflammatory pain.

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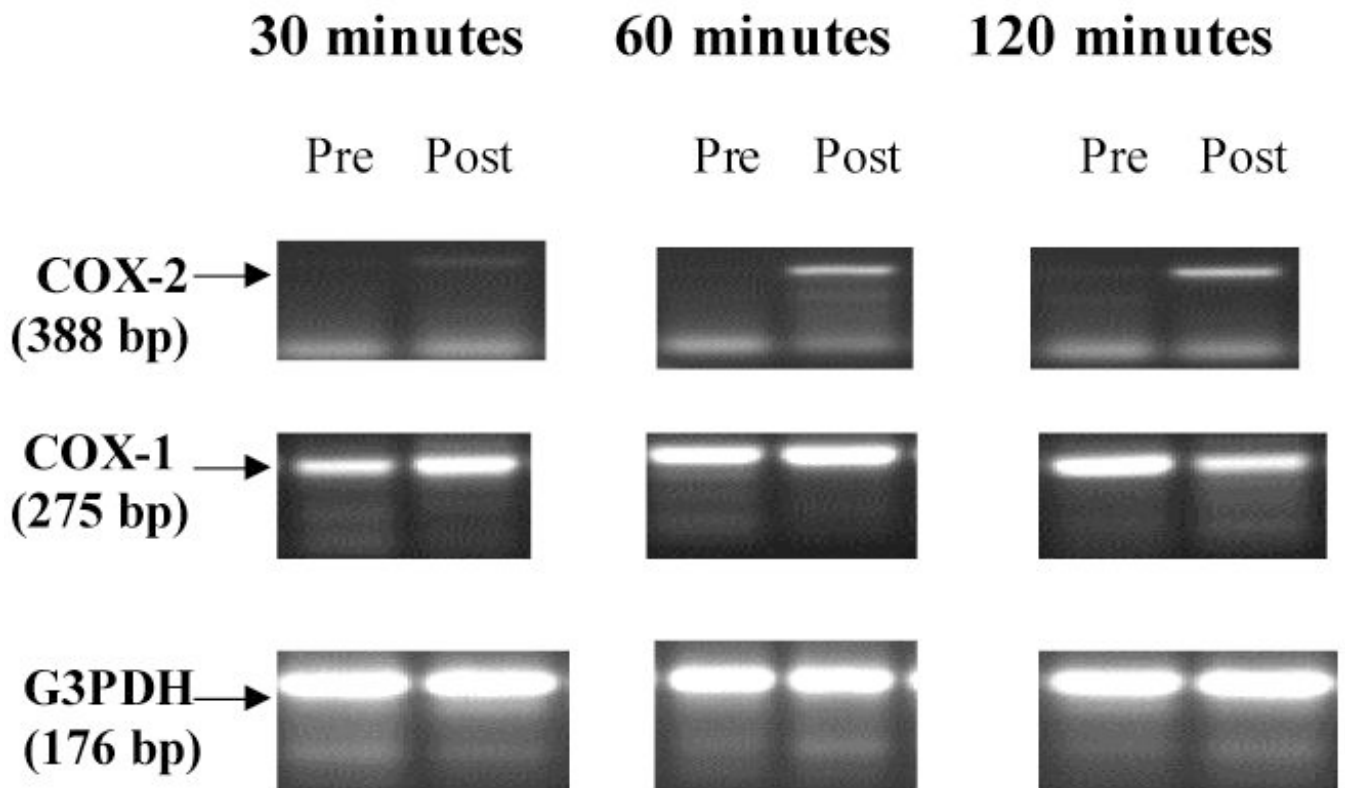
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### List of abbreviations used

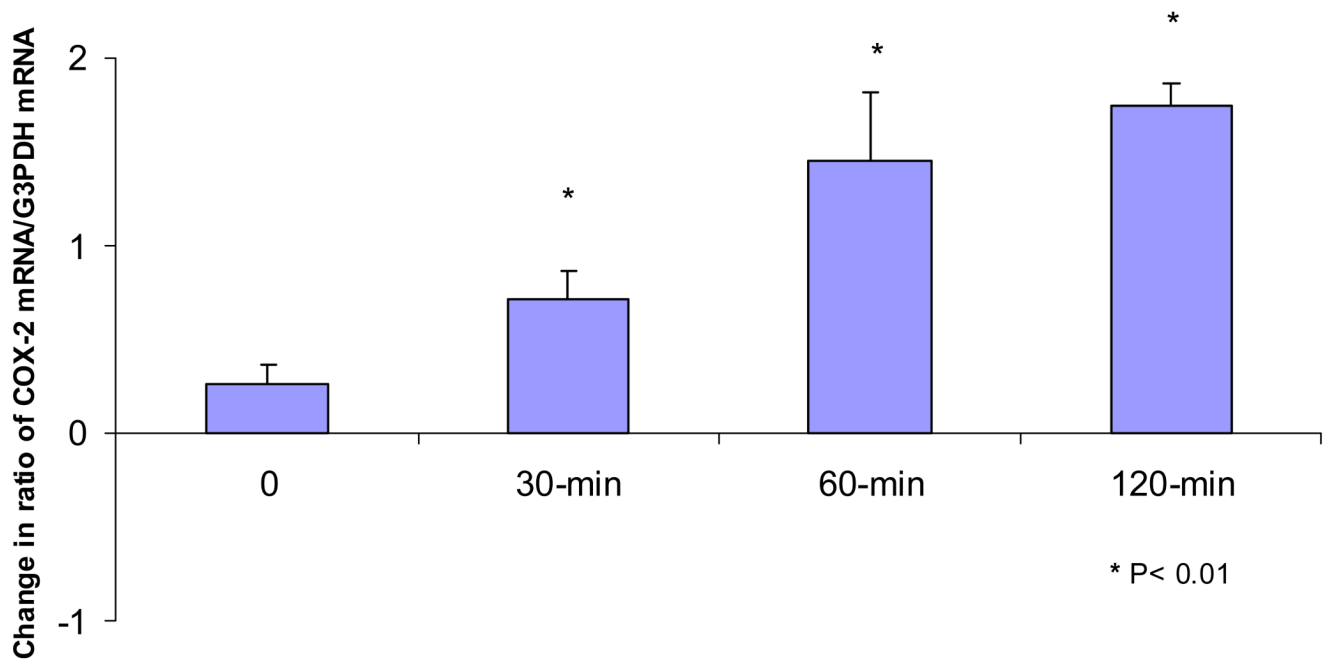
<b>COX</b>	Cyclooxygenase
<b>G3PDH</b>	Glyceraldehyde-3-phosphate dehydrogenase
<b>LPS</b>	Lipopolysaccharides
<b>NSAID</b>	Non-steroidal anti-inflammatory drug
<b>PG</b>	Prostaglandin
<b>PMN</b>	Polymorphonuclear



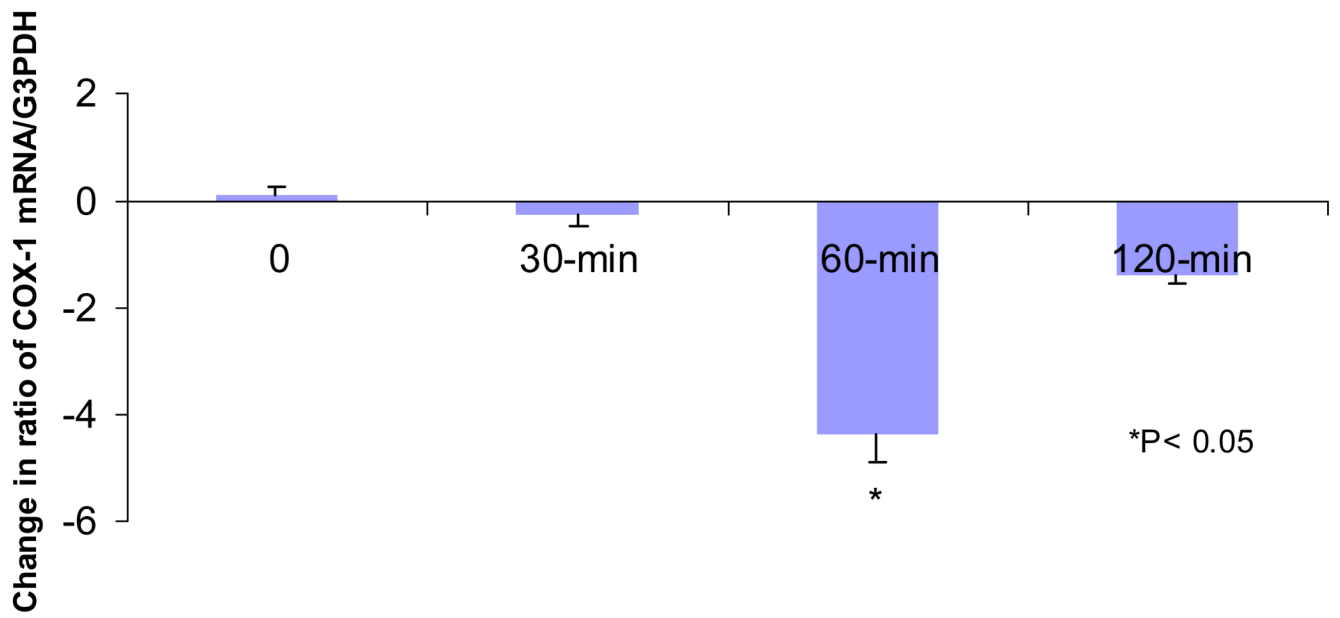
**Figure 1.**

Representative products of the RT-PCR for COX-2, COX-1 and G3PDH. Notice that COX-2 is either not detectable in the pre-surgical biopsies or is barely detected and is induced at 30, 60 and 120 minutes after surgery in these samples. In contrast, COX-1 was detected at all time points examined.





**Figure 2.** Change in the ratio of COX-2 mRNA/G3PDHmRNA in the postoperative biopsies as compared to the preoperative biopsies. “0” represents biopsies which were obtained immediately after surgery. A significant increase was detected in the biopsies obtained at 30, 60 and 120 minutes after surgery. \*P<0.05



**Figure 3.** Change in the ratio of COX-1 mRNA/G3PDH mRNA in the postsurgical biopsies as compared to the presurgical ones. "0" represents biopsies which were obtained immediately after surgery. A significant decrease in COX-1 mRNA was detected at 60 minutes after surgery. \*P<0.05

**Table 1**  
**Sequence of primers used and their product length**

Gene	Sequence of primers (5' to 3')	Product length (bp)	RNA Template (ng)	Number of cycles for PCR
COX-1	Forward-CAGACGACCCGCCTCATCCTCATAG	275	4	40
	Reverse-GCCTCAACCCCATAGTCCACCAACA			
COX-2	Forward-TGGGAAGCCTTCTTAACCTCTCCT	388	8	40
	Reverse-CTTTGACTGTGGGAGGATACATCTC			
G3DPH	Forward-GACCCCTTCATTGACCTCAACTAC	167	2	26
	Reverse- CATCGCCCCACTTGATTTTG			

Table 2

**Demographic and surgical features of the patient sample**

Time of biopsy from end of surgery (min)	N	Age (years)	Gender M/F	Extraction Difficulty*	Midazolam Dose (mg)	Lidocaine Dose (mg)
0	10	19.0 ± 3.9	6/4	6.4 ± 1.6	4.5 ± 0.7	155.6 ± 13
30	13	18.9 ± 3.5	7/6	6.5 ± 1.1	4.3 ± 0.7	144.8 ± 30
60	10	23.1 ± 5.9	3/7	7.0 ± 1.2	4.7 ± 0.5	164.0 ± 25
120	10	19.0 ± 3.3	4/6	7.0 ± 1.2	4.6 ± 0.6	162.0 ± 26

\* Extraction difficulty is the sum calculated by assigning a score of (2) for soft tissue impactions, (3) for partial bony impactions and (4) for full bony impactions.