COX-2 inhibition during periodontitis modulates myocardial infarction in rats

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Abstract

Aim: It has been shown that periodontitis increases systemic inflammation, which in turn up-regulates vascular cyclooxygenase-2 (COX-2). We hypothesised that an increase in vascular COX-2 expression plays a role in vascular homeostasis. Thus, we analysed the effect of COX-2 inhibition in an experimental infarction model in rats with periodontitis.

Materials and Methods: Wistar rats were subjected to ligature-induced experimental periodontitis or sham procedure. After 12 days of induction of periodontitis or sham procedure the animals were assigned to receive either etoricoxib (10 mg/kg/day,v.o.) or vehicle for four days. Infarction-like myocardial lesions were induced by injecting high doses of isoprenaline on days 13 and 14. The mortality associated with the induction of experimental infarction was measured. On day 15, blood samples were collected for the quantification of creatinine kinase N-acetylcysteine (CK-NAC) and lactate dehydrogenase (LDH); the hearts were collected for histological assessment.

Results: The periodontitis group that received etoricoxib presented significantly increased serum levels of CK-NAC ($p<0.05$) and LDH. The inflammatory infiltrate in the heart tissues were not significantly changed among the animals.

Conclusion: The data suggested that COX-2 modulates heart ischemia lesions during periodontitis. Selective COX-2 inhibitors must be used with caution especially during periodontitis, even for a short period.

Keywords: Periodontal diseases. Myocardial infarction. Vascular endothelium. Cyclooxygenase 2.

Introduction

Periodontitis is a chronic inflammatory disease that affects the tooth-supporting tissues (Cochran, 2008). As well as the local effects of periodontal disease, the literature shows that patients with severe periodontitis have a higher risk of developing cardiovascular disease, after adjustment for a large number of traditional risk factors (Demmer and Desvarieux, 2006).

The exact mechanisms underlying this association between cardiovascular disease and periodontitis are unclear. However, numerous studies have shown that patients with periodontitis also present higher serum levels of systemic inflammatory biomarkers, such as C-reactive protein (PCR) (Amar *et al*., 2003; Joshipura *et al*., 2004; Higashi *et al*., 2008), Interleukin-6 (IL-6) (Shi *et al*., 2015) and Tissue Plasminogen Activator (tPA) (Joshipura *et al*., 2004), which are related to vascular inflammation and endothelial dysfunction (Amar *et al*., 2003; Blum *et al*., 2007; Higashi *et al*., 2009; Mendes *et al*., 2016; Mercanoglu *et al*., 2004; Seinost *et al*., 2005; Tonetti *et al*., 2007).

Endothelial dysfunction is the first clinical sign that precedes atherogenesis and the development of morphological vascular changes (Flammer *et al*., 2012). In relation to endothelial dysfunction, much attention has been given to the effect of nitric oxide (Siragusa e Fleming, 2016); however, COX-derived products also play an

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important role (Félétou *et al.*, 2011). Interestingly, It has been shown that there is an increase in vascular expression of cyclooxygenase-2 (COX-2) during endothelium dysfunction (Chung *et al.*, 2010). It has been suggested that COX-2 expression in endothelial cells has the essential function of producing prostacyclin (Yu *et al.*, 2012), which has vasodilator and anti-thrombotic properties, and also that COX-2 inhibition could represent a cardiovascular risk (Mendes *et al.*, 2012). More recently, using a ligature-induced model of periodontitis in rats, our group (Mendes *et al.*, 2014) and others (Campi *et al.*, 2016) showed that the vascular inflammatory response during periodontitis up-regulated COX-2 expression in the vascular wall. Nevertheless, a complete understanding of the effect of up-regulation of vascular COX-2 during periodontitis and the effect of COX-2 inhibition still remains uncertain.

Therefore, in this study we aimed to elucidate the cardiac effect of acute COX-2 inhibition during periodontitis in rats submitted to ischemia lesions that were comparable to myocardial infarction.

Materials and Methods

Animals

Male Wistar rats of 10 weeks of age and weighing 200–250 g were used. The animals were housed in a temperature and light-controlled room with water and food ad libitum. All of the procedures were performed according to the European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes and the Ethical Principles of Animal Experimentation of the Brazilian Council for Animal Experiments. The experimental protocol was approved by the University's Institutional Ethics Committee (Protocol number 15521/2012).

Ligature-induced periodontitis

For periodontitis induction, the rats were anesthetized with intraperitoneal injections of ketamine and xylazine (90 and 15 mg/kg, respectively). We placed a cotton ligature (4/0) around the cervixes of the mandibular first molars and maxillary second molars of both sides (right and left) of each animal. Thereafter, four ligatures were placed on each animal (Brito *et al.*, 2013). The ligature was knotted on the buccal side so that it remained subgingival on the palatal side. The placement of ligatures induces periodontal disease by facilitating bacterial invasion of the gingiva (Rovin *et al.*, 1966). The group sham had the ligature removed promptly after the procedure.

Induction of myocardial infarction using isoprenaline

Myocardial infarction lesion was induced through the administration of isoprenaline (80 mg/kg, subcutaneous) for two consecutive days, in accordance with an established model (Rona *et al.*, 1959). At this level of dosage, isoprenaline promotes a decrease in blood pressure followed by an increase in heart rate. This results in a lack of oxygen, which leads to an ischemic lesion that is similar to a myocardial infarction.

Histological analysis

The hearts were collected and the samples placed in ALFAC solution (85% of 80% ethanol, 10% of 40% phormaldehyde and 5% of glacial acetic acid). Longitudinal sections in the left ventricle area were performed with scalpel blades. The samples were dehydrated and embedded in paraffin. The blocks were sectioned in 5 μm slices and stretched in histological slides. Sequentially, the slides were hydrated in xylol followed by a decreasing sequence of ethanol. Finally, the slides were stained with hematoxylin and eosin. The slides were analyzed using an optic microscope and scored in relation to the presence of inflammatory infiltrate and edema (0 = none; 1 = mild; 2 = moderate; 3 = severe). The analysis was performed by the same blinded and calibrated examiner.

Measurement of plasma levels of CK-NAC and LDH

The animals were anesthetized and the jugular was accessed. Blood samples were obtained using fresh vials containing heparin. The vials were centrifuged and the serum was stored at -80 °C for further analysis. The samples were analyzed for the levels of creatine kinase (CK-NAC; Labtest, Minas Gerais, Brazil) and lactate dehydrogenase (LDH; Labtest, Minas Gerais, Brazil).

Morphometrical analysis for quantification of alveolar bone loss

After the collection of blood samples and hearts, the mandible and maxilla were dissected. The specimens were stained with aqueous 1% methylene blue to identify the cementoenamel junction (CEJ). Standardized pictures were taken from the lingual and buccal sides of each specimen using an AXIOCAM ERc 5s ZEISS camera coupled with a Stemi 2000c magnifier. The measurement was performed using specific software (Image-Pro Plus, version 4.5, Media Cybernetics, Silver Spring, USA). To calculate the alveolar bone loss, the distance between the CEJ and the alveolar bone crest was measured. To evaluate the average alveolar bone height, five points were measured on the buccal and lingual parts of the lower molars, and three points for the upper molars. The average alveolar height was calculated for each molar. All the measurements were performed by the same previously calibrated and blinded examiner.

Experimental protocol

The rats were distributed into two groups and submitted to either the ligature or sham procedure. Twelve days after the procedure, the groups were randomly assigned to receive either etoricoxib (10 mg/kg/d, v.o.) or vehicle (water, 0.1 mL/kg/d, v.o.) until day 15. At days 13 and 14 all the animals, except the naive group, received a subcutaneous injection of isoprenalin (80 mg/kg, s.c.). At day 15 all the animals were euthanized and the blood samples, hearts and lower and upper maxillaries were collected for analysis (Figure 1A). We used 126 animals divided into the following groups: naïve (n=6), sham + vehicle (n=30), sham + etoricoxib (n=30), ligature + vehicle (n=30), ligature + etoricoxib (n=30). The sample size was calculated to detect a difference of 25% in mortality rate of the ligature groups with a power of 80% and α= 0.05. The Primer of Biostatistics™ (McGraw-Hill, New York) software was used to sample size calculation of survival experiments. For other analyses, the sample calculation was based on the standard deviation (SD) and the magnitude of difference between the groups obtained in the analysis of CK-NAC and LDH from previous studies (Ramos *et al*., 2012). Thus, considering 5 experimental groups, α= 0.05 and a power of 80%, 8 animals in each group are required for statistical significance. Therefore, 8 rats from each group were randomly selected to carry out the other analyses. The GPower 3.1.1 software was used to sample size calculation (Faul *et al*., 2007). The dose of etoricoxib was based on published studies (Holzhausen *et al*., 2005) and our own experience (Mendes *et al*., 2014).

Statistical analysis

Data are expressed as mean ± SEM. The number of animals (n) used in each experiment is indicated in Figure legends. Statistical significance was analyzed by one-way analysis of variance (ANOVA) followed by Tukey post-hoc test. All the data analyzed by ANOVA were submitted to Shapiro-Wilk and Bartlett test to prove normality and homogeneity of variances, respectively. The data of creatine kinase (CK) was transformed by logarithmic function to achieve Gaussian distribution and homoscedasticity. Kruskal-Wallis test followed by Dunn's post-hoc analysis was used to compare inflammatory infiltrate score, since these data were highly skewed. Differences in the survival study were determined with log rank test. A p value of less than 0.05 was considered significant. When the p value is less than 0.05, the exact value of p is shown in the figure. The graphs and the statistical analyses were performed using GraphPad Prism 9.0.1 version software (La Jolla, CA, USA).

Results

Effect of etoricoxib on bone loss induced by periodontitis

Each animal of the periodontitis groups had four ligatures (upper second molars and lower first molars – right and left). However, for the sake of clarity, the results were pooled from the right and left maxillae and mandibles (Figure 1B).

The ligatures promoted alveolar bone loss, which characterized periodontitis in the ligature groups (distance from alveolar bone crest to cementoenamel junction of 1.14 ± 0.07 mm and 1.08 ± 0.06 mm for ligature + vehicle and ligature + etoricoxib, respectively) compared to the sham groups (sham + vehicle 0.87 ± 0.07 mm and sham + etoricoxib 0.77 ± 0.04 mm) and naïve (0.69 ± 0.04 mm) ($p<0.05$). The etoricoxib treatment, which started 12 days after the ligature placement or sham procedure, had no effect on alveolar bone loss (Figure 1B).

Effect of etoricoxib and periodontits on the mortality rate of rats submitted to myocardial infarction induced by isoprenaline

In accordance with the literature (Ramos *et al*., 2012), approximately 20% of the animals died due to the infarction-like myocardial induced by isoprenaline injection. Sham + vehicle group: 23% of animals died; sham + etoricoxib: 25%; ligature + vehicle: 27%; ligature + etoricoxib: 21%. Interestingly, no animal died after the second injection of isoprenaline. No difference was observed regarding the mortality rate between the groups (Figure 1A).

Effect of periodontitis and etoricoxib treatment on plasma levels of CK-NAC and LDH in rats submitted to myocardial infarction induced by isoprenaline

The plasma analysis showed that the CK-NAC (Figure 2A) and LDH (Figure 2B) levels were significantly higher in the ligature plus etotricoxib group.

Naïve group presented a mean level of CK-NAC of 192.7±17.4 U/L. The sham groups levels were 1.18 and 2.12 fold higher compared to naïve (vehicle and etoricoxib respectively). Ligature groups levels were 2.8 and 4.8 folds higher than naive for vehicle and etoricoxib respectively ($p<0.05$).

The mean LDH level of naïve group was 125.7±12.1 U/L. Sham groups and ligature + vehicle presented no statistical difference compared to naïve. However, ligature + etoricoxib LDH levels were 1.5 fold higher than naïve ($p<0.05$).

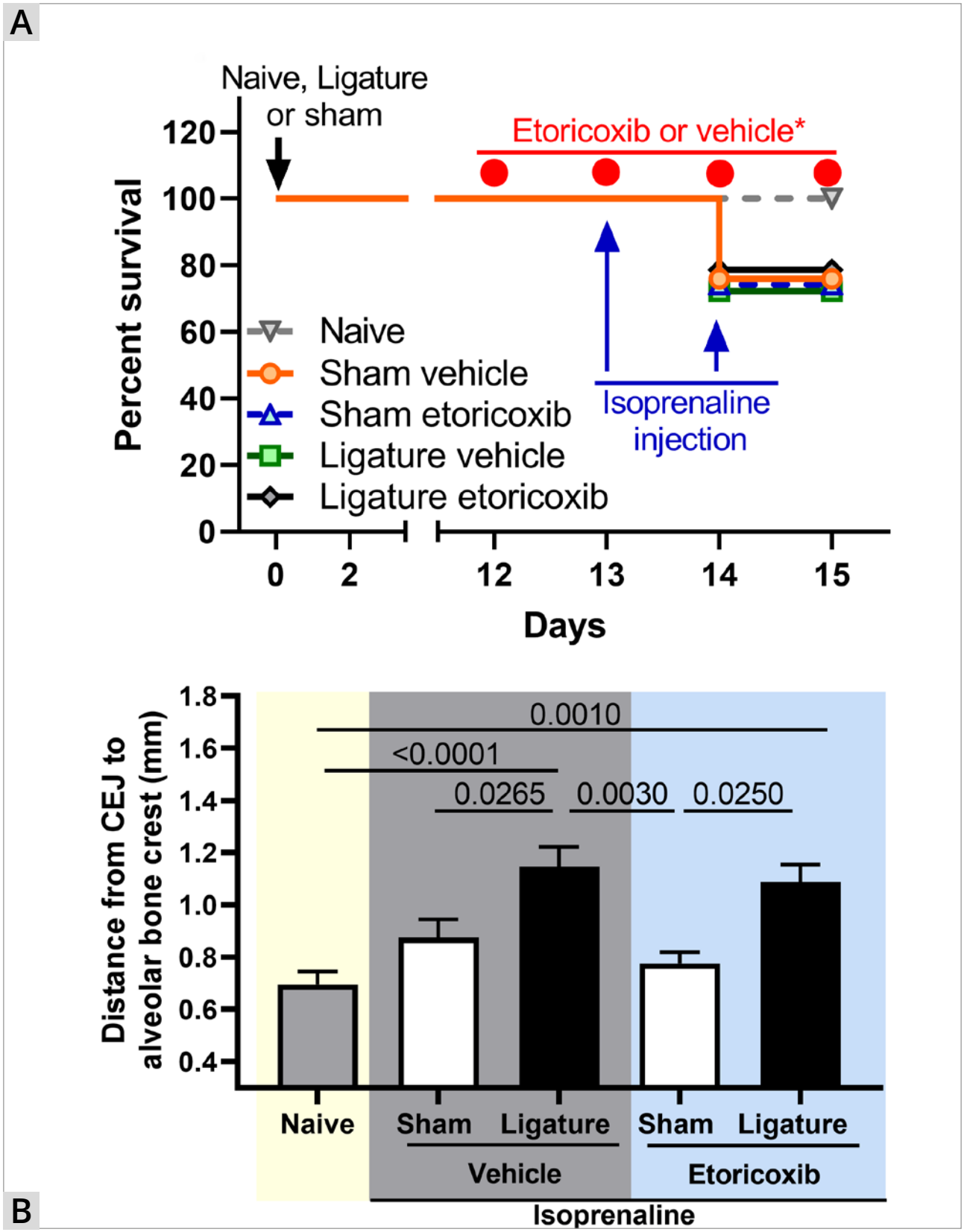

Figure 1. Survival rate and timeline of the protocol designed to study the role of selective cyclooxygenase-2 (COX-2) inhibition on heart ischemia in rats with periodontitis. A log rank test was used for the comparison of the survival curves (n=30 per group) (A). Effect of late treatment with etoricoxib on alveolar bone loss induced by periodontitis. Each bar represents the mean of eight animals and the vertical lines represent the SEM. We placed four ligatures around the upper second molars and first molars on both sides (right and left). However, for the sake of clarity, the results were pooled from the right and left maxillae and mandibles (B). Naïve group was composed by animals that did not go through any procedure (neither etoricoxib/vehicle nor ligature/sham procedure). The p values are provided in the figure. The absence of a p value means that there is no statistical significance between the groups (p>0.05).

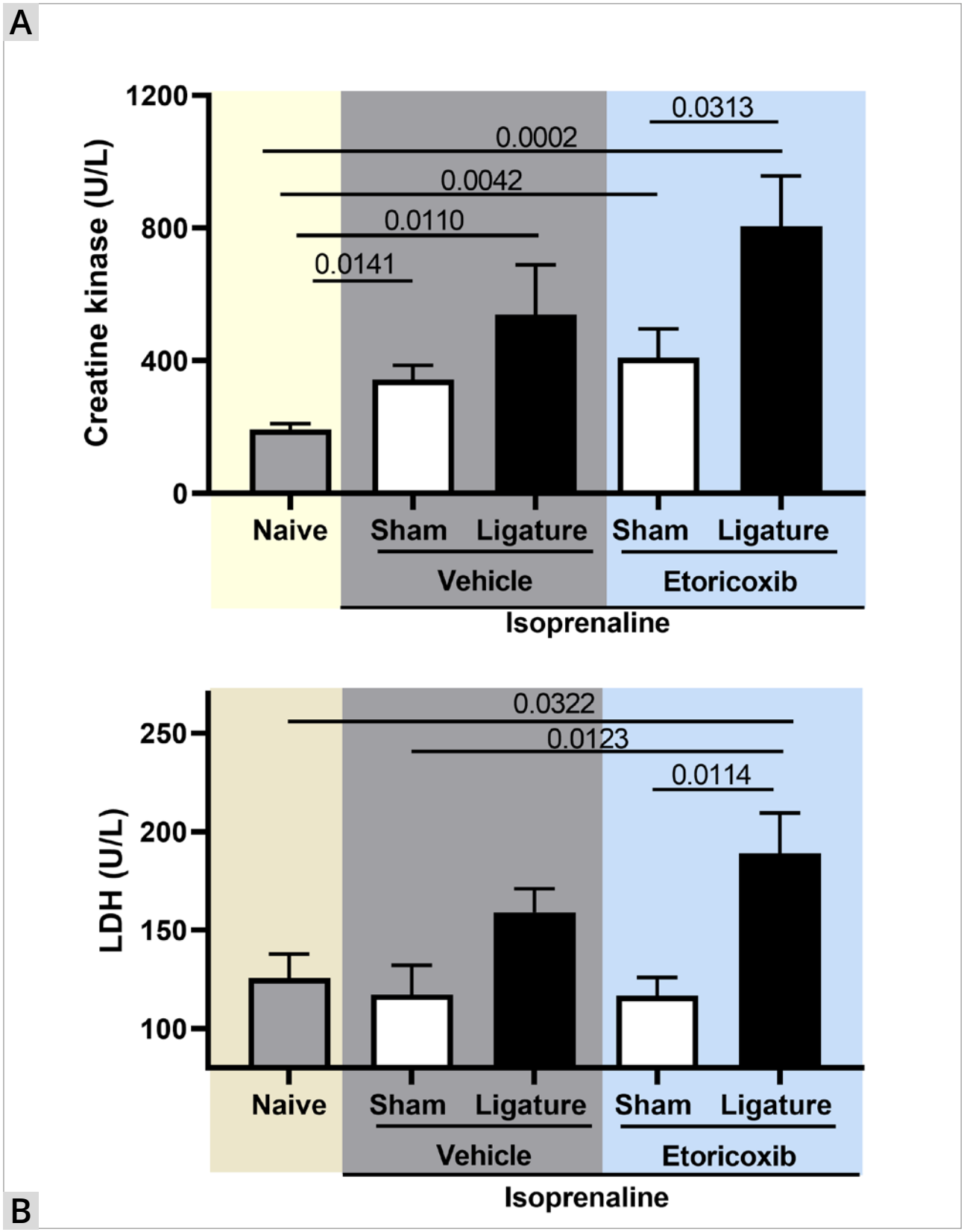

Figure 2. Effect of periodontitis and short-term COX-2 inhibition on plasmatic levels of creatine kinase (A) and LDH (B). Each bar represents the mean of eight animals and the vertical lines represent the SEM. The p values are provided in the figure. The absence of a p value means that there is no statistical significance between the groups (p>0.05).

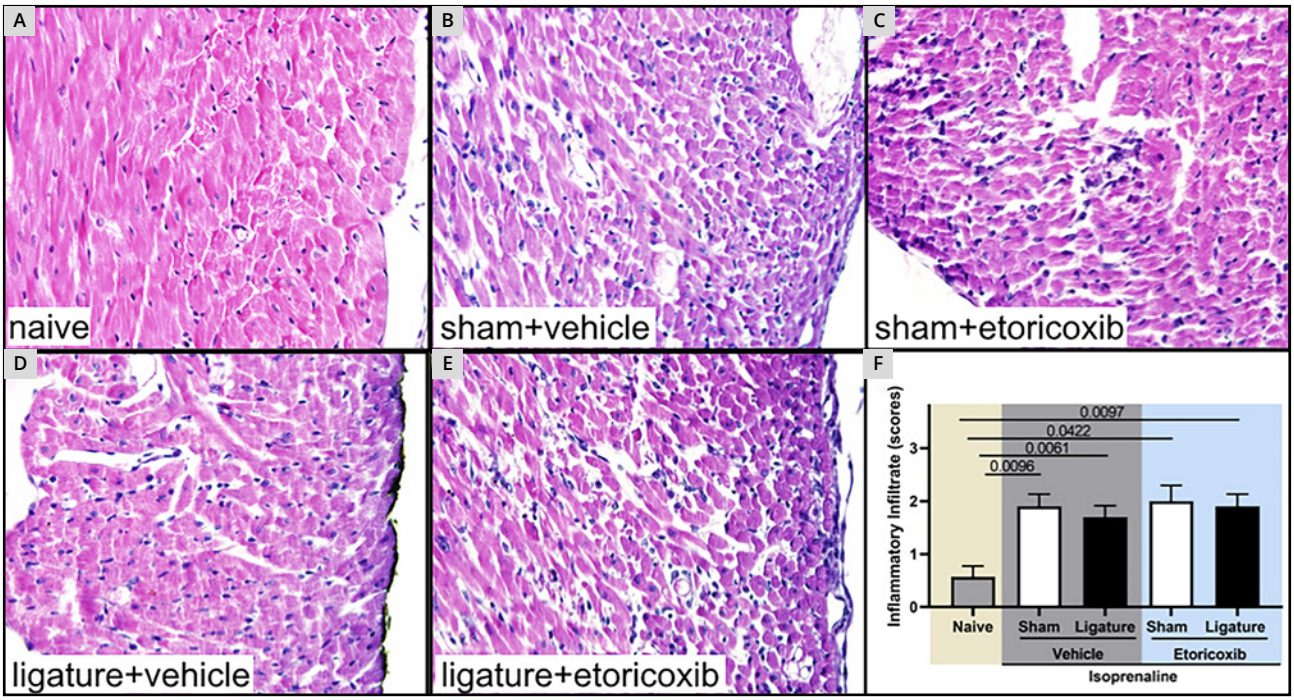

Figure 3. Effect of periodontitis and short term COX-2 inhibition on inflammatory infiltrate on heart tissue of isoproterenol-induced heart injury model. Light micrograph (hematoxylin and eosin; x100) of the left ventricle of the rat. (A) naïve; (B) sham + vehicle; (C) sham + etoricoxib; (D) ligature + vehicle; (E) ligature + etoricoxib. The samples were analysed and received scores regarding the inflammatory infiltrate as follows: 0 = no inflammatory infiltrate; 1= mild inflammatory infiltrate; 2 = moderate inflammatory infiltrate; 3 = severe inflammatory infiltrate. (F) Inflammatory infiltrate scores for the heart tissues. Each bar represents the mean of eight animals and the vertical lines represent the SEM. The p values are provided in the figure. The absence of a p value means that there is no statistical significance between the groups (p>0.05).

Histological analysis of the heart

The histological images of the infarcted hearts showed that all the groups presented an inflammatory infiltrate (Figure 3). The analysis of the inflammatory score showed no statistical difference among the experimental groups (sham + vehicle 1.9 ± 0.23; sham + etoricoxib 2.0 ± 0.29; ligature + vehicle: 1.7 ± 0.21; ligature + etoricoxib: 1.9 ± 0.23). All groups presented higher scores compared to naïve (Figure 3F). The mean score of naïve group was 0.5. p values are provided in the Figure 3F.

Discussion

The present study demonstrated that COX-2 inhibition modulated experimental ischemia lesions during periodontitis. COX-2 inhibition during periodontitis increased the serum levels of CK-NAC and LDH after heart ischemia. Therefore, these results suggest that COX-2 in periodontitis may be important for the proper maintenance of the vascular response and COX-2 inhibition may break up this balance.

Treatment with COX-2 inhibitors (coxibs) remains a very controversial issue. Clinical trials have shown that the use of coxibs is associated to an increased risk of cardiovascular complications (Chan *et al.*, 2009; Grosser *et al.*, 2006; Shi *et al.*, 2008), affecting nearly 1-2% patients per year who were included in randomized controlled trials. Such data drove to the withdrawal of rofecoxib in 2004, followed by valdecoxib in 2005 (Mendes *et al.*, 2012). However, all these side effects related to coxibs were due to the chronic use of the drugs. In cases of acute pain in patients with a history of gastrointestinal complications, coxibs remain a good option because its use for a short period of time is not associated to cardiovascular or gastrointestinal complications (Grosser *et al.*, 2006).

The pre-emptive inhibition of COX-2 prevents bone loss due to periodontitis either with celecoxib (Holzhausen *et al.*, 2002) or etoricoxib (Mendes *et al.*, 2014; Holzhausen *et al.*, 2005; Azoubel *et al.*, 2008). Nevertheless, since we aimed to analyze the effect of COX-2 inhibition on cardiovascular variables during

a chronic inflammation, etoricoxib administration was started on the 12th day, when alveolar bone loss was already established and a systemic inflammatory response was ongoing (Brito *et al*., 2013). However, in accordance with previous results (Mendes *et al*., 2014), when COX-2 inhibition is started later bone loss is no longer affected. Thus, these results suggest that the selective COX-2 inhibition has a limitation in the treatment of alveolar bone loss due to periodontitis.

As previously mentioned, the effect of COX-2 inhibition on periodontitis-induced bone loss is well described. However, the systemic effect of COX-2 inhibition during periodontitis is not well established. It is now accepted that periodontitis induces a low-grade systemic inflammatory response with consequent vascular dysfunction (Moura *et al*., 2017). In this sense, our group (Mendes *et al*., 2014) and others (Campi *et al*., 2016) have shown that vascular inflammatory response in periodontitis induces the upregulation of COX-2 in the vascular wall. The derivate products of vascular COX-2, mainly PGI2, may balance vascular function and homeostasis under pathological conditions (Zhu *et al*., 2020). Therefore, the use of COX-2 inhibitors when periodontitis is ongoing, can deprive the individual of the protection provided by COX-2 products.

Furthermore, the literature is controversial concerning the role of COX-2 in heart function. Genetically engineered mice deficient in COX-2 developed cardiac fibrosis, and COX-2 null hearts subjected to ischemia and reperfusion, demonstrated impaired recovery of left-ventricular function (Dinchuk *et al*., 1995; Camitta *et al*., 2001). Selective COX-2 inhibition in pigs resulted in increased mortality due to myocardial infarction (Timmers *et al*., 2007). On the other hand, some studies showed that the inhibition of COX-2 after acute myocardial infarction in mice improved heart function (Lapointe *et al*., 2004; Saito *et al*., 2000; Abbate *et al*., 2007; Delgado *et al*., 2004), and COX-2 inhibition attenuated infarct size (Lada-Moldovan *et al*., 2009).

In the present study, the data showed that the ischemia lesion model with isoprenaline increased the inflammatory infiltrate in heart tissues, as well as CK serum levels. No changes were observed in LDH levels, at least not after 24 hours of ischemia lesion. This agreed with previous data which showed an increase in LDH levels only at early times (6h) after isoprenaline injection, returning to normal levels thereafter (Ramos *et al*., 2012).

Experimental ischemia lesion with isoprenaline is related to a 20% ratio of death (Ramos *et al*., 2012), which was confirmed by our results. Our variables (periodontitis or COX-2 inhibition) did not change the mortality rate, at least not after 24 hours of myocardial infarct. However, further studies with a longer time frame should be conducted in order to evaluate the mortality rate of these animals over time.

Short-term COX-2 inhibition itself had no effect on the ischemia lesions. However, COX-2 inhibition worsened the heart lesions due to myocardial infarction during periodontitis. Nevertheless, we did not find any difference in the mortality rate and inflammatory infiltrate; the ligature plus etoricoxib group presented higher serum levels of CK-NAC and LDH, which are markers of muscle lesions. Consequently, our results suggest that COX-2 could play an important protective role in ischemic heart lesions of individuals with periodontitis. This agrees with previous studies that have shown that COX-2 inhibition was related to serious cardiovascular events by inducing an imbalance between thromboxane and prostacyclin production (Mendes *et al*., 2012; Grosser *et al*., 2006). Thus, in relation to COX-2 inhibition, during a chronic inflammation such as periodontitis a careful decision should be made regarding the use of certain therapeutic drugs, such as selective COX-2 inhibitors.

Our study has some limitations. First, the ligature-induced periodontitis did not reproduce all aspects of periodontal disease in humans. Second we used a limited number of animals and in a short time frame. Third, the serum markers used in this study are not specific for myocardial injury. Therefore, our results must be interpreted with caution and carefully extrapolated to a clinical situation.

Nevertheless, the data highlights the role of systemic inflammation due to periodontitis in cardiovascular diseases, which may be modulated by the short-term use of coxibs. There is consistent evidence that chronic COX-2 inhibition may be associated with harmful effects in the cardiovascular system (Chan *et al*., 2009; Grosser *et al*., 2006; Shi and Klotz, 2008; Huber and Terezhalmy, 2006). However, selective COX-2 inhibitors must be used with caution especially during periodontitis, even for a short period.

Conclusion

The results presented here suggested that COX-2 had a protective role against the heart ischemia during periodontitis, and even a short-term use of COX-2 inhibitors could disrupt this balance and increase the risk of heart damage.

Acknowledgments

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