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Title: NF- κ B and COX-2 expression in radicular cysts: an immunohistochemical study

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Methods: Immunohistochemical staining was performed on 30 samples of radicular cysts. The immunoperoxidase reaction for NF- κ B and COX-2 was applied to the specimens, and the immunoexpression of both proteins was then semiquantitatively evaluated. The results were classified according to the following scores: 0 (no staining), 1 (1% to 50% stained cells), and 2 (>50% stained cells).

Results: The results show that in all cases there was exclusively cytoplasmic expression of COX-2 and NF- κ B at the epithelium and in inflammatory cells.

Conclusions: According to the immunohistochemical profile found in the current study on samples of radicular cysts, it is unlikely that NF- κ B mediates the activation of COX-2. However, the COX-2 immunohistochemical profile suggests that this protein may play a role in the pathogenesis of this lesion.

NF- κ B and COX-2 expression in radicular cysts: an immunohistochemical study

NF- κ B and COX-2 expression in radicular cysts

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Abstract

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Introduction: NF- κ B and COX-2 proteins are known to act directly in inflammatory processes. The objective of this study was to identify the immunohistochemical expression of these two proteins in the epithelium and inflammatory cells of radicular cysts.

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Introduction

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2 Radicular cysts are chronic periapical lesions of the jaw bones that originate
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4 from an inflammatory process in teeth with necrotic and infected pulp (1,2). The
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6 presence and action of inflammation products stimulates epithelial cells present in the
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8 periodontal ligament to proliferate, forming a cystic cavity which grows with the
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10 accumulation of fluid inside (3,4). Radicular cysts comprise about 68% of all cysts that
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12 affect the gnathic bones (5).
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18 Studies in the last few years have associated the transcription factor- κ B (NF- κ B)
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20 with development of many inflammatory and autoimmune diseases, such as ulcerative
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22 colitis, Crohn's disease, rheumatoid arthritis, systemic lupus erythematosus, diabetes
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24 mellitus type 1, multiple sclerosis, and celiac disease (6-8). NF- κ B is a group of
25
26 evolutionarily conserved DNA-binding proteins, involved in the regulation and
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28 expression of a wide variety of genes including mainly pro-inflammatory cytokines,
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30 angiogenic factors, cell adhesion molecules, enzymes, and antiapoptotic factors (9,10).
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32 The canonical pathway of NF- κ B is activated by a variety of stimuli including DNA
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34 damage, cytokines, physical stress, and free radicals. These stimuli cause the
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36 translocation of NF- κ B to the nucleus where it promotes the expression of genes that
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38 are important for the activation of immune responses (11). However, little is known
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40 about the role of NF- κ B in radicular cysts although it is known that the immune-
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42 inflammatory process is the basis of the formation of radicular cysts (3).
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52 One of the target genes of NF- κ B is the cyclooxygenase-2 (COX-2), a key enzyme
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54 in prostaglandin biosynthesis which is important in the pathogenesis of chronic
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56 inflammatory disorders. COX consists of two isozymes: constitutively expressed COX-1
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58 which is expressed in most cells, and inducible COX-2 which is usually undetectable
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1 under normal conditions (12) but is upregulated in inflamed tissues (5). Previous
2 studies have suggested that COX-2 may play an important role in the pathogenesis and
3 development of radicular cysts (5,13).
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7 To the best of our knowledge, no study has assessed both NF- κ B and COX-2
8 immunohistochemical expression in radicular cysts. Therefore, the aim of this study
9 was to identify the immunohistochemical expression of NF- κ B and COX-2 in the
10 epithelium of radicular cysts to improve our understanding of the molecular
11 mechanisms of this lesion.
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25 **Materials and methods**

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28 During a six-year period, from 2007 to 2012, 27 formalin-fixed, paraffin-
29 embedded tissue samples of radicular cysts were retrieved from the archives of the
30 Service of Oral Pathology of the João de Barros Barreto University Hospital (Belém,
31 Brazil). Sections were cut at a thickness of 5 μ m, dewaxed with xylene, and hydrated in
32 an ethanol series. The slides that received the *anti-NF- κ B* and *anti-COX-2* antibodies
33 were immersed in a solution containing citric acid (pH 6.0) and heated for 15 minutes
34 in a microwave oven at 95°C for antigen retrieval. Endogenous peroxidase quenching
35 was performed with a 6% hydrogen peroxide and methanol solution (v/v) in two baths
36 of 15 min each. After washing in Tris buffer (pH 7.4), slides were incubated with the
37 primary antibodies *anti-NF- κ B* (clone sc-8008; Santa Cruz Biotechnology, Santa Cruz,
38 CA, USA; 1:200, overnight) and *anti-COX-2* (Invitrogen, San Diego, CA, USA; 1:400, 60
39 min). All slides were subsequently exposed to the avidin-biotin complex (Advance™-kit
40 + HRP; Dako Cytomation, Carpinteria, CA, USA) and to diaminobenzidine chromogen
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1 (DAB+; Dako Cytomation, Carpinteria, CA, USA), and counterstained with Meyer's
2 hematoxylin. Slides were dehydrated in ethanol, cleared in xylene, and coverslipped.
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4 Prostate carcinoma fragments were used as positive control samples for NF- κ B and the
5 inflammatory cells present in the slides of radicular cysts were the positive control for
6
7 COX-2. We performed negative control staining by omitting the specific primary
8 antibody during the reaction.
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16 The reaction results were considered positive when brown stained cells were
17 observed, characterizing the presence of DAB in the immunohistochemistry reaction.
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19 The immunostained cells were observed by one investigator using light microscopy
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21 (40x) in the most representative areas, and the results were classified according to the
22 following scores: 0 (no staining), 1 (1% to 50% stained cells), and 2 (> 50% stained
23 cells). Scores of 1 and 2 were considered positive. This classification was based on a
24 study by Cairns *et al.* (14). Ethical permission was obtained from the ethical committee
25 of João de Barros Barreto University Hospital.
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41 **Results**

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45 After performing immunohistochemical staining, it was observed that all the
46 samples of radicular cysts had stained positive for both proteins. Cytoplasmic
47 immunostaining was detected in the lining epithelium and inflammatory cells for NF-
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60 A total of 30 samples were analyzed and the immunostaining profile of the
61 proteins is detailed in *Table 1*.

1 For COX-2, all the samples (100%) had a score of 2 in the epithelial lining. For
2 the inflammatory infiltrate, 28 samples (93.33%) had a score of 2 and two samples
3 (6.66%) had a score of 1. For NF- κ B, 29 samples (96.66%) had a score of 2 and one
4 sample (3.33%) had a score of 1, in both the epithelial lining and inflammatory
5 infiltrate.
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18 Discussion

19 This work aimed to evaluate the immunohistochemical expression of the
20 proteins NF- κ B and COX-2 in the epithelium and inflammatory cells of radicular cysts.
21 The results showed that, in all cases, there was exclusively cytoplasmic expression of
22 COX-2 and NF- κ B in the epithelium and in inflammatory cells.
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33 Tsai et al., in 2002, and Yang et al., in 2007, studied the role of COX-2 in the
34 pathogenesis of radicular cysts (5, 13). Tsai et al., using immunohistochemistry,
35 detected COX-2 expression in all the lining epithelia, subepithelial fibroblasts,
36 macrophages and endothelial cells of 30 radicular cyst specimens. Furthermore, an
37 association was shown between higher expression of COX-2 and higher levels of
38 inflammatory infiltrates (5). Yang et al., using the reverse transcription polymerase
39 chain reaction (RT-PCR) technique, reported a high level of COX-2 mRNA in both
40 epithelial and inflammatory cells of radicular cysts. However, in the same study, COX-2
41 mRNA was found to a greater extent in the group with moderate inflammation than in
42 the groups with low and high levels of inflammatory cells (13). Together, these two
43 studies corroborate our findings showing a relationship between the expression of
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COX-2, found in inflammatory cells, with the immunostaining observed in the epithelium of radicular cysts, suggesting that this protein may play an important role in the pathogenesis of radicular cysts.

The COX-2 enzyme has been reported to induce changes in the inflammatory process. It acts indirectly by increasing the levels of chemical mediators of inflammation, the prostaglandins (PGs). Prostaglandin E2 (PGE2) is known as the main product of the COX-2 pathway. Previous investigations have suggested that this mediator contributes to the bone destruction that occurs during cystic expansion (15, 16). In accordance with these results, a study using a cyclooxygenase inhibitor demonstrated a 43% reduction in periapical bone resorption in rat experimental models (17).

During periapical inflammation, the host cells of periapical tissues release many inflammatory mediators, proinflammatory cytokines, and growth factors through innate and adaptive immune responses (3) such as IL-1, TNF- α and bacterial LPS. These substances are strong activators of NF- κ B (18-20). Furthermore, COX-2 is one of the downstream nuclear targets of NF- κ B (21-23). Hence, we thought it was valid to assess NF- κ B immunoreactivity in the epithelium and inflammatory cells of radicular cysts.

When not stimulated, NF- κ B is linked to an inhibitory protein in the cytoplasm, I κ B. This complex prevents the translocation of NF- κ B to the nucleus. For its translocation, phosphorylation and degradation of I κ B are necessary. The action of specific protein kinases, such as the I κ B kinase complex (IKK), causes the phosphorylation of I κ B. Once phosphorylated, I κ B is recognized by the ubiquitin ligase

1 complex, bound to ubiquitin and degraded by the proteasome complex (20, 24). This
2 results in the liberation of NF- κ B to the nucleus where it promotes the expression of
3 target genes that encode proteins that perpetuate inflammatory responses (25).
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9 In the current study, it was observed that the immunoexpression of NF- κ B in
10 the epithelium and inflammatory cells of RCs was exclusively located in the cytoplasm,
11 which implies that NF- κ B was probably not mediating the activation of COX-2.
12 However, the COX-2 immunohistochemical profile suggests that this protein may play
13 a role in the pathogenesis of this lesion and its activation is probably induced by other
14 pathways. Future genetic and epigenetic investigations are necessary to add strength
15 to the results obtained in the current study.
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Figure Legends:

Immunoexpression of the proteins COX-2 (A, B) and NF- κ B (C, D). Positive staining of COX-2 in the cytoplasm 200X (A) and 400X (B). Positive staining of NF- κ B in the cytoplasm 200X (C) and 400X (D).

Figure
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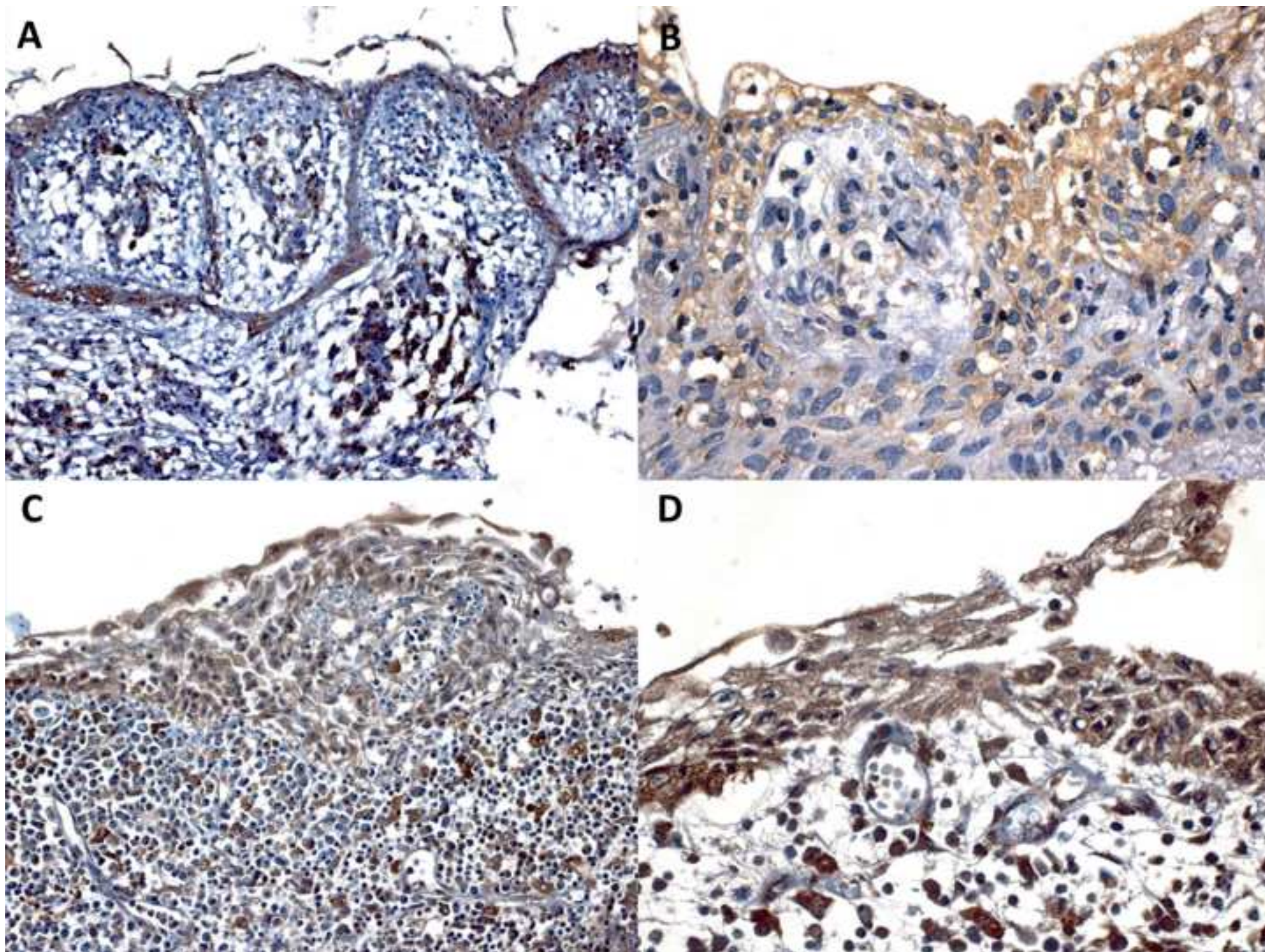


Table 1 –

	Scores	Epithelial cells			Inflammatory infiltrate		
		0	1	2	0	1	2
COX-2	n	0	0	30	0	2	28
NF-κB	n	0	1	29	0	1	29

Table 1. COX-2 and NF-κB immunoreactivity in radicular cysts. Score: 0 (no staining), 1 (1% to 50% of stained cells), 2 (>50% of stained cells).