Nitric oxide (NO) is both harmful and beneficial to the general pathophysiology of tissues. NO is synthesized from the amino acid L-arginine by NO synthase (NOS) and is a very reactive free radical (8, 17, 18). Three different isoforms of NOS have been identified (8, 18). Type I (neuronal NOS, nNOS or brain NOS, bNOS) and Type III (endothelial NOS: eNOS) are first found enzymes which are Ca²⁺/calmodulin-dependent. Ca²⁺/calmodulin-independent Type II (macrophage or inducible NOS, iNOS) is produced during the cytokine-expression phase of inflammatory processes (2, 24). It is known that NO produced by iNOS has an active role in the inflammatory process and activation of the epithelial proliferative potential (22). Once its expression is initiated, it lasts longer for hours or even for days compared to eNOS and nNOS expressions (12). Expression of iNOS occurs by stimulation of some proinflammatory mediators (2, 8). NOS and eNOS are also called constitutive NOS (cNOS). cNOS in constitutive form is effective on mediation of normal physiologic events, while elevated iNOS expression is reported to be observed in pathological processes (2, 3, 5, 12, 22, 24).

Dental follicles (DF) which contain odontogenic epithelial components may transform into chronic inflammatory tissues, cysts and other pathologies. DF provide stem cells and precursor cells for the supportive tissue surrounding the teeth called periodontium and are one of the three basic structures seen in the cap stage phase of teeth development. DF form the cementum, periodontal ligament (PDL) and adjacent alveolar bone (7, 25). Odontogenic epithelial components in DF are crucially important in odontogenic cyst and tumor development (4, 7, 13). On the other hand, proliferation potential of the epithelial components of pericoronal DF need to be further analyzed. There are not much literature data concerning the iNOS-iR in DFs. In the present study, periapical inflammatory lesions (PILs) were evaluated as they contain epithelial components and had been evaluated for iNOS-iR before. Periapical granuloma (PG) and radicular cyst (RC) are two different stages of PILs in chronic inflammatory process. It has been demonstrated that iNOS plays pivotal role in PILs progression; however, the mechanism by which iNOS influences PILs progression has not been entirely clarified. Ca²⁺/calmodulin-independent Type II NOS, also called macrophage NOS or iNOS isofrom, is responsible for NO production in development of PILs and is the most investigated type (2, 10, 11, 22, 23, 24). However, it is not clear whether iNOS expression in PG and RC groups differs between each other (11, 10, 23, 24). iNOS may have a pivotal role in activation and proliferation of odontogenic epithelial components in DFs like in PILs progression.

It is crucial to comprehend the harmful and protective amounts of iNOS expressions in order to clarify the relationship between iNOS and pathological processes. Evidently, only after this inspection it is possible to utilize iNOS as a marker and treatment instrument during the diagnosis and treatment of inflammatory process and malignant transformation. In order to understand the relationship between iNOS and pathological reactions clearly, the existence and amount of constitutive expression in healthy tissues should be determined. Thus, the aim of this research is to analyze and compare the existence of iNOS-iR in periapical granulomas (PGs), radicular cysts...
(RCs) and in asymptomatic unerupted third molars’ pericoronal dental follicles (DFs).

Materials and Methods
In this study, formalin-fixed and paraffin-embedded sections were investigated for iNOS-IR by the use of immunohistochemical (IHC) method. The tissue samples were obtained from asymptomatic unerupted third molars surgically removed and from tissues retained during apical resection/endodontic surgery, which are routinely examined histopathologically. Tissues which belong to patients with any systemic disease or patients who have been using antibiotics, antioxidants or any medication in the last six months were excluded. Additionally, DFs, which were obtained from patients having enlarged pericoronal tissue surrounding unerupted third molar, were also excluded from the study. The presence or absence of inflammation was elucidated by the density of the inflammatory cell infiltrate (especially lymphocytes and polymorphonuclear leukocytes). Histological appearance of the PGs and RCs was obscured by heavy infiltration of inflammatory cells. DFs associated with marked inflammation were also excluded from the study. Sample tissues for IHC processing were obtained from a total of 77 patients, aged between 14 and 55 (median 33). Gender distribution was 55% women (n=42) and 45% men (n=35). The study followed the tenets of the Declaration of Helsinki. Informed consent was obtained from all participant patients.

According to the histopathological characteristics, tissue samples were divided into three groups: PGs (n=20), RCs (n=33) and DFs (n=24). Tissue samples of PGs, RCs, and DFs were fixed in 10% buffered formalin solution for 18 hours.

After fixation of tissues, the specimens were rinsed with phosphate buffered saline (PBS) and were subsequently dehydrated in ethanol, cleared in xylene and embedded in paraffin. Sections (5 μm) were cut and mounted on glass slides. The sections were deparaffinized in xylene, rehydrated in decreasing concentrations of ethanol and covered with 10 mM sodium citrate buffer (pH 6.0), heated to 95°C and kept at that temperature for 5 minutes for antigen retrieval. Endogenous peroxidase activity was quenched by 5 min incubation in peroxidase blocking solution (DBS Universal Immunostaining Kit, CA).

Tissues were then incubated with a rabbit polyclonal antibody against iNOS (Chemicon, CA) for 2 h and then with a biotinylated secondary antibody for 10 minutes (DBS Universal Immunostaining Kit, CA). Negative controls were obtained by substitution of the primary antibody with nonimmune rabbit serum. Immunoreactivity was detected by means of horseradish-peroxidase(HRP)-streptavidin complex using diaminobenzidine (DAB) chromogen as a marker (DBS Universal Immunostaining Kit, CA). Sections were counterstained in Mayer’s hematoxylin for 30 seconds. Subsequently, the sections were rinsed and finally mounted (DBS CC/Mount, CA). All steps were carried out at room temperature in a humidified chamber.

Assessments, counts and photography were performed by a Laborlux K (Leitz, Germany) light microscope.

The results of IHC staining were evaluated semiquantitatively on the basis of a four-point scale: “−”: negative staining; “+”: low expression, less than 10% positive cells; “++”: moderate expression, 10-50% positive cells; “+++”: diffuse expression, more than 50% positive cells. Statistical analysis was performed using Pearson chi-square test and p values less than 0.05 were considered to be significant.

Results and Discussion
Different levels of iNOS-IR occurrence were detected in 3 different types of lesions, namely PGs, RCs and DFs. Sections serving as negative controls were all unstained. Cytoplasmic staining was observed in all immunoreactive cell types. As the results were evaluated, the investigation focused on whether there was an obvious difference in terms of immunoreactivity among epithelial cells, inflammatory cells and fibroblasts in the studied tissues. The determination of cells in which iNOS induction and production occurs, gives an idea about the constitutional expression or the relation of iNOS to the inflammatory process in these lesions or stimulation and proliferation of odontogenic epithelial components. It was observed that there was no detectable difference among lesion forming cell types in terms of immunoreactivity. Therefore, taking all cell types into account, immunoreactive (iNOS-IR-positive) cells in randomly selected microscopic areas were counted and the ratio of all cells in each area was calculated. Although the number of inflammatory cells infiltrating the tissues was increased, no significant level of iNOS-IR-positive cells was observed.

iNOS-IR in tissues was diffused in 20%, moderate in 75% and low in 5% of PGs (Fig. 1 and Fig. 2); diffuse in 24.25% and moderate in 75.75% of RCs (Fig. 3 and Fig. 4); diffuse in 50%, moderate in 45.83%, and low in 4.17% of DFs (Fig. 5 and Fig. 6) (Table 1).
When lining epithelium in RCs and PGs groups was compared, low iNOS expression was encountered only in PGs group. However, the ratios of moderate and diffuse expression were similar in both groups and there was no significant statistical difference in iNOS-IR between these two groups ($p>0.05$). Diffuse iNOS-IR was determined to be similar in both PILs groups and 50% of DFs group. The statistical analysis (Pearson chi-square test) showed that there were no significant differences between PGs, RCs and DFs groups regarding iNOS-IR ($p>0.05$).

In our study, 50% diffuse level of iNOS expression was detected in DFs group. Based on these results, iNOS-IR presence in DFs can be interpreted in two different and controversial ways. One of the interpretations is that iNOS-IR might exist in DF constitutively. Likewise, even in normal or neoplastic epithelial structures, iNOS constitutive expression may be observed (3, 14, 19). Another interpretation is that DFs may undergo an inflammatory process, causing chronic
Expression of iNOS in Periapical granuloma (PG), Radicular cyst (RC) and Dental follicle (DF) cells.

<table>
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<tr>
<th>Diagnosis</th>
<th>iNOS positive cells</th>
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<tr>
<td></td>
<td>- n (%)</td>
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<tr>
<td>PG</td>
<td>0</td>
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<td>DF</td>
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Comparison of inoS-IR levels in PGs, Rcs and DFs groups are not significantly different. According to the collected statistical data, DFs may have constitutive levels of iNOS. Therefore, it was determined that this condition may have a role in proliferation and activation of lining epithelium (22).

On the basis of our findings there was no significant statistical difference in iNOS-IR between PILs (PGs and Rcs) groups. Furthermore, no significant difference was observed of iNOS expression in DFs and PILs. These results indicate that iNOS might activate the proliferative potential of tissues that contain epithelial component in PILs as well as in DFs.

In the IHC research, it has been shown that iNOS-IR is encountered in pericoronal DFs and in PILs. The obtained results in this study are compatible with previously performed studies about iNOS-IR in PILs (10, 11, 22, 23, 24). One of the first studies revealing iNOS-IR presence in periapical tissues was performed by Takeichi et al. on radicular cysts. It is emphasized that NO, spontaneously produced in periapical tissues, plays an important role in regulation of chronically progressing infection (24). iNOS-IR was examined in another study (22) and it was detected in lining epithelium of Rcs. Therefore, it was determined that this condition may have a role in proliferation and activation of lining epithelium (22).

Conclusions
The results of this study showed that iNOS-IR levels in PGs, Rcs and DFs groups are not significantly different. According to the collected statistical data, DFs may have constitutive levels of iNOS. Therefore, on the basis of these results we support the prophylactic removal of asymptomatic unerupted third molars. It might be assumed that iNOS may be constitutively present in asymptomatic DFs, however further research is needed.

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