

## Use of aminoguanidine, a selective inducible nitric oxide synthase inhibitor, to evaluate the role of nitric oxide in periapical inflammation

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**Abstract:** The purpose of this study was to evaluate the effects of aminoguanidine (AG) as a selective inhibitor of inducible nitric oxide synthase (iNOS) on the degree of inflammatory response in periapical lesions in the canine teeth of cats. Root canals from 52 cat canine teeth were exposed to the oral cavity and sealed after 7 days. One day before pulp exposure, cats were administered either AG (experimental group) or normal saline (control group), which was continued on a daily basis until the day of sacrifice. Animals were sacrificed at 28 days after pulp exposure. Inflammatory response in the periapical zones was analyzed histologically. The degree of periapical inflammation in the AG group was significantly lower than that in the control group ( $P < 0.05$ ). Selective iNOS inhibitors such as AG thus reduce the intensity of inflammatory responses in periapical lesions. (J Oral Sci 53, 225-230, 2011)

**Keywords:** apical periodontitis; aminoguanidine; inflammation; nitric oxide; nitric oxide synthase.

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### Introduction

Apical periodontitis is a sequela to endodontic infection that manifests itself as the host defense response to microbial challenge emanating from the root canal system. It is viewed as a dynamic encounter between microbial factors and host defenses at the interface between infected radicular pulp and the periodontal ligament, which results in local inflammation and resorption of periapical hard tissues. Pulpal infection, which mainly involves gram-negative bacteria, may occur as a result of caries, restorations or trauma (1). Such infection first elicits an immune response in the dental pulp, which stimulates a secondary immune response to bacteria in the periapical region after total pulpal necrosis. This reaction represents a protective response to bacteria in the necrotic pulp and root canal system. Pulpal and periapical immune responses initially involve innate immunity, and adaptive immune elements are activated as responses become more chronic. Under these conditions, complex immunologic mechanisms that protect the pulp and periapical region, while causing host tissue destruction and mediating periapical bone resorption, are activated.

Free radicals are highly reactive molecules of either host or bacterial origin that can damage nearby cells. Nitric oxide (NO) may act as a pro-inflammatory or anti-inflammatory mediator in human tissues. NO is a soluble gas, and depending on the site of production and the concentration produced, exerts several different biologic effects. It is synthesized by three distinct isoforms of nitric oxide synthase (NOS) (2). Neuronal and endothelial

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isoforms of NOS are constantly produced in low amounts in the body. Inducible NOS (iNOS) generates high concentrations of NO upon stimulation by bacterial lipopolysaccharides and inflammatory cytokines such as IFN- $\gamma$ , IL-1 $\beta$  or TNF- $\alpha$  (3). NO synthesized by iNOS has a distinct role in cellular processes, including the induction of apoptosis (4), inhibition of mitochondrial respiration (5) and regulation of oxidative phosphorylation, in addition to its cytotoxic effects on target cells (6).

Aminoguanidine (AG) is a bifunctional molecule comprising the guanido group from L-arginine linked to hydrazine, and was first described by Corbett et al. (7) as a selective inhibitor of iNOS. Misko et al. (8) showed that AG selectively inhibits iNOS without increasing blood pressure; AG was over 50-fold more effective at inhibiting the enzymatic activity of iNOS than endothelial or neuronal isoforms of NOS (9).

The aim of this study was to evaluate the effects of AG as a selective inhibitor of iNOS on the degree of inflammation in periapical lesions of canine teeth from cats.

## Materials and Methods

This study was approved by the Ethics Committee of the Isfahan University of Medical Sciences. For this study, the cat model was selected because of its availability, ease of handling and anesthetizing, and well-understood pulpal and periapical anatomy (10). Fifty-two canine teeth from 1-year-old male cats ( $n = 13$ ) were used in this interventional experimental study, with all teeth having intact crowns and healthy periapical and periodontal tissues on clinical examination and pretreatment radiographs. Necessary vaccinations were performed for all experimental animals. General anesthesia was induced with 0.02 mg/kg intramuscular 2% acepromazine (Alfasan, Woerden, Holland) and 10 mg/kg intramuscular ketamine hydrochloride (Parke Davis, Courbevoie, France). Buccal infiltration of 2% lidocaine containing 1:100,000 epinephrine (Xylocaine, Dentsply Pharmaceutical, York, PA, USA) was injected at the apex. Maxillary and mandibular canines were isolated with rubber dams and pulp exposure was performed by removal of the 1/3 coronal portions of the crown. The coronal areas of the root canals were enlarged using a #1/4 round bur to the depth of the bur diameter. Working length was determined by inserting a #15 file (Mani, Utsunomiya, Japan) and taking a radiograph. Pulp was removed using a barbed broach (Zipperer, Munich, Germany) with minimal damage to periapical tissues. Root canals were left exposed to the oral cavity for 7 days in order to allow microbial contamination, and access openings were sealed with amalgam (Kerr, Romulus, MI, USA).

## Drug treatments

There were six animals in the control group and seven in the experimental group. Four canine teeth from each cat were used. The required amount (25 mg/kg) of AG hydrochloride (Sigma-Aldrich, St Louis, MO, USA) for each cat was dissolved in 2 ml of sterile normal saline. In a preliminary study, it was confirmed that this concentration of AG was sufficient to block the synthesis of NO in serum without any harmful systemic effects. One day before pulp exposure, cats were given an intraperitoneal injection of either AG or normal saline (control group), and injections were continued on a daily basis until the day of sacrifice. Animals were sacrificed at 28 days after pulp exposure. Vital perfusion was performed so that periapical tissues became fixed before degeneration. Specimens were fixed in 10% neutral buffered formalin and demineralized in 7% nitric acid, and were subsequently dehydrated, embedded in paraffin and sectioned along the canines in the buccolingual plane for hematoxylin and eosin staining. Five 5- $\mu$ m sections (separated by 50  $\mu$ m), including the apex of canines and periapical bone, were prepared for each specimen. Histology of the periapical zones was analyzed by light microscopy (Carl Zeiss 452904-9901, Oberkachen, Germany) by an expert pathologist using a single-blind protocol. Stages of inflammation from 1 to 4, as defined in Table 1, were used to score specimens. Inflammation scores of 3.00 and 4.00 were characterized by the presence of more inflammatory cells (indicating advanced inflammatory process), fewer fibroblasts (indicating limited repair process), bone and cementum resorption with granulation tissue formation, PDL degeneration, and focal areas of necrosis with abscess formation. Inflammation scores 1.00 and 2.00 were characterized by fewer inflammatory cells (indicating limited inflammatory process), more fibroblasts (indicating advanced repair process), intact bone and cementum, intact or widened PDL, and fibrous tissue formation. Mean inflammation scores in the two groups were compared by Mann-Whitney *U*-test. A probability value of  $P < 0.05$  was considered to indicate a significant difference between the groups.

## Results

All cats tolerated the surgical procedures throughout the study period. Treated teeth did not show any evidence of swelling or sinus tract. Mean scores for inflammation in the AG and control groups were 2.6786 ( $\pm 0.6118$ ) and 3.1250 ( $\pm 0.7974$ ), respectively. Distribution of inflammation scores within the AG and control groups is shown in Table 2. The majority (83.3%) of control samples were distributed between inflammation scores 3.00 and 4.00, while the majority (92.9%) of AG samples were distributed

Table 1 Stages of inflammation

Stage I (score 1)	Stage II (score 2)	Stage III (score 3)	Stage IV (score 4)
No inflammatory response	<10 Macrophages & plasma cells	10-25 Macrophages & plasma cells	>25 Macrophages & plasma cells
>30 Fibroblasts	10-30 Fibroblasts	5-9 Fibroblasts	1-4 Fibroblasts
Mature fibrous tissue with a lot of collagen	Immature fibrous tissue with little collagen	Granulation tissue	Focal areas of necrosis
PDL intact	PDL widening	PDL degeneration	PDL degeneration
Cementum intact	Cementum intact	Cementum resorption	Abscess
Bone intact	Bone intact	Bone resorption	Abscess

between inflammation scores 2.00 and 3.00. The difference between the experimental and control groups was statistically significant ( $P = 0.017$ ). Thus, AG as a selective iNOS inhibitor significantly reduced the degree of periapical inflammation in cats.

## Discussion

Basic science researchers have studied the role of NO in inflammatory diseases such as rheumatoid arthritis (6), diabetes (7) and bone remodeling (11). In addition, researchers in dentistry have evaluated the function of NO in the inflammatory processes of the oral mucosa (12), periodontal tissues (13,14), pulp (15) and periapical areas (16). Cytokines and reactive intermediates of oxygen and nitrogen are frequently found at inflammatory sites, but their function in pulp and periapical tissues is not yet fully understood. Specifically, NO exhibits a dual function in inflammatory conditions; under some circumstances, it inhibits inflammation, and in others, it is an effective pro-inflammatory factor (17).

As this dual function of NO can exist in apical periodontitis, a better understanding of the role of NO in the pathophysiology of inflammation in periapical lesions may be helpful in future pharmacological interventions. The bulk of previous studies have only addressed the presence or absence of NO in inflamed pulp, granulomas and periapical cysts; however, as the presence or absence of a substance in a specific tissue does not necessarily indicate its role, the exact function of NO in inflammatory processes of teeth and surrounding tissues is poorly understood and remains controversial. Thus, to obtain a clearer picture of the complex inflammatory processes in the periapical area, the present animal model was designed to investigate the role of NO in periapical lesions.

Kawanishi et al. (15) evaluated the anti-inflammatory effects of an iNOS-specific inhibitor (1400 W) on experimentally induced rat pulpitis in a similarly designed experiment. Their results, which were consistent with our

Table 2 Distribution of inflammation scores (count and percentage) in Aminoguanidine (AG) and control groups

Inflammation Score	Groups	
	Control	AG
1.00	1 4.2%	1 3.6%
2.00	3 12.5%	8 28.6%
3.00	12 50.0%	18 64.3%
4.00	8 33.3%	1 3.6%
Total	24 100.0%	28 100.0%

findings, suggested that NO is responsible for the infiltration of immunocompetent cells in the development of pulpitis. Investigations by da Silva et al. (18), Law et al. (19), Di Nardo Di Maio et al. (20) and Fan et al. (21) all showed greater concentrations of NOS enzymes in inflamed zones in comparison to noninflamed areas of the pulp. The results of the present study are similar to the observations in these experiments. Yasuhara et al. (22) suggested that NO is involved in the regulation of pulp cell growth, apoptosis and mineralization. According to Min et al. (23), NO is able to induce IL-8 expression via the mitogen-activated protein kinase (MAPK) and nuclear factor -  $\kappa\beta$  (NF- $\kappa\beta$ ) pathways, which play important roles in the inflammatory responses of pulpal and periapical lesions.

Expression of iNOS has been investigated in periapical granulomas and cysts by numerous investigators (24-27). Hama et al. (28) proposed a mechanism regarding the role of NO in the progression of periapical inflammation; they showed that inflammatory cells such as macrophages express receptor for advanced glycation endproduct (RAGE) in periapical granulomas, which infiltrates around

cells producing iNOS. Binding of advanced glycation endproduct (AGE) with RAGE accelerates vascular disease and induces synthesis and secretion of pro-inflammatory cytokines. The results of the present study differ from the findings of Fukada et al. (29). They evaluated the role of NO in bone loss in bacterially induced apical periodontitis using iNOS-deficient mice. They reported that iNOS-deficient mice exhibited greater inflammatory cell recruitment and osteolytic lesions than normal mice. The difference in these results may be due to: 1) AG, which, in addition to being an iNOS selective inhibitor, has antioxidant properties, and this may have reduced free radicals and induced anti-inflammatory effects (11); 2) in our study, iNOS concentrations were reduced pharmacologically, but in the Fukada et al. study, iNOS was completely blocked; and 3) mouse teeth and surrounding tissues are structurally different from cat teeth.

Most of the studies investigating the role of NO in

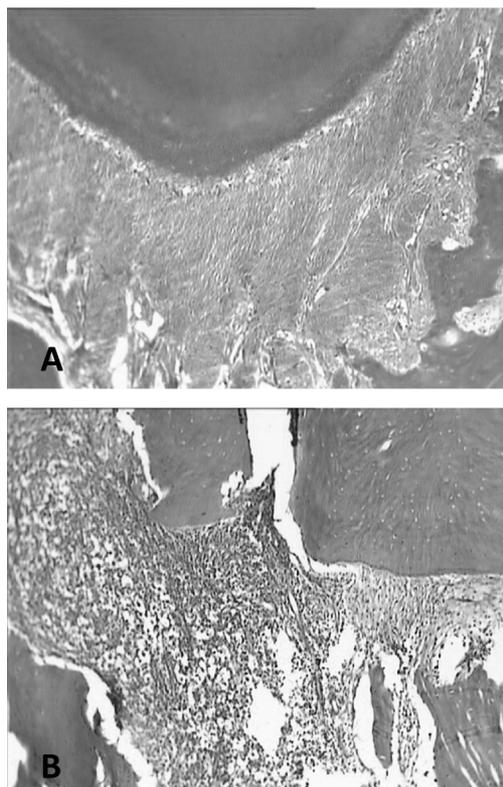


Fig. 1 (A) Histological view of the AG group after 4 weeks showing minimal inflammation corresponding to an inflammation score of 2.00 (H&E;  $\times 100$ ). (B) Histological view of the control group after 4 weeks showing severe inflammation corresponding to an inflammation score of 4.00 (H&E;  $\times 100$ ).

inflammatory processes of the periodontium have shown that NO is a pro-inflammatory mediator that can cause alveolar bone resorption (12-14). Di Paola et al. (30) reported that when AG was used to reduce the NO concentration in rats with induced marginal periodontitis, the density of inflammatory cells and alveolar bone resorption decreased. The results of these studies support the findings of our research.

In this study, the root canals of both the control and AG groups were contaminated with oral microbial flora, and the only difference between the two groups was the AG drug intervention in the experimental group. Kakehashi et al. (31) established the role of microbes in pulp and periapical pathosis. The damage to host tissues from microbial infection of the root canal system is the result of: i) direct damage from proteolytic and noxious waste products of microorganisms; and ii) host inflammatory/immune response that leads to tissue destruction such as periradicular bone. As the inflammatory process advances from stage I to stage IV, typical forms of chronic apical periodontitis develop, accompanied by periapical granuloma and possible micro-abscess formation. Humoral and cellular immunity are activated to limit further inflammation and to initiate tissue repair (32). The histopathological feature of periapical lesions is a dense infiltration of immunocompetent cells such as neutrophils, macrophages, lymphocytes, plasma cells, giant cells, NK cells and mast cells. Neutrophils and macrophages are the first line of defense in periapical lesions. These defensive cell types are involved in cell-mediated innate immunity that phagocytose microorganisms, dead cells and debris. T and B cells play an important role in antigen-specific immune response and are the predominant cell type in human periapical lesions. In addition, at the lesion periphery, plasma cells and lymphocytes are observed (32). Inflammatory mediators including cytokines, antibodies, growth factors and arachidonic acid metabolites are involved in the initiation and maintenance of inflammation. These inflammatory mediators have specific and overlapping functions and are directly or indirectly involved in periapical lesion formation and establishment (32).

In conclusion, the present study provides *in vivo* evidence regarding the role of NO in the progression of the inflammatory process in the periapical area. The results of this study support the use of iNOS-selective inhibitors such as AG to reduce the inflammatory intensity in periapical lesions (Fig. 1). As NO has other biological functions, it is likely that AG has other systemic effects, and there have been few dentistry studies regarding the processes mediated by AG and NO; further research is

therefore required to comprehensively evaluate the role of AG on inflammatory processes. Future research can focus on different concentrations of AG, the role of AG on the healing of periapical lesions and its mechanism of action. In addition, the effects of iNOS-selective promoters and other iNOS-selective inhibitors on the inflammatory process should be evaluated.

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