

Original Article

Histological assessment of the local effect of different concentrations of aminoguanidine hydrochloride on bone healing in rats

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ABSTRACT

Background: Nitric oxide (NO) has several functions in bone healing and affects bone metabolism. Selective inducible NO synthase (iNOS) inhibitors can be used to assess the efficacy of NO for healing of bone defects. This study sought to assess the local effect of different concentrations of aminoguanidine hydrochloride (AG), a selective iNOS inhibitor, on bone healing in rats.

Materials and Methods: In this animal experimental study, 72 rats were divided into six groups of control, placebo, 5% AG, 10% AG, 15% AG, and 20% AG. A bone defect measuring 5 mm × 5 mm was created in the femur. The defect remained empty in the control group. In the placebo group, neutral gel was placed in the bone defect, and in the remaining four AG groups, different concentrations of AG were applied to the defects. Bone healing was assessed histologically. The healing score in the six groups was analyzed by the Kruskal–Wallis test. A $P < 0.05$ was considered statistically significant.

Results: The healing score in 20%, 15%, 10%, and 5% AG groups was significantly higher than that in the neutral gel and control groups ($P < 0.01$). Among the four groups of AG, 20% concentration showed better results, but the difference was not significant.

Conclusion: Four concentrations of AG caused greater bone healing compared to the other two groups. Selective iNOS inhibitors such as AG can be used to promote local bone healing.

Key Words: Aminoguanidine, bone, healing, nitric oxide, nitric oxide synthase

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INTRODUCTION

Bone healing following trauma is fast and efficient; however, this process may be compromised or even fail in some clinical situations.^[1] Healing of bone defects due to periodontal inflammation, bone surgery, bone fractures, or enucleation of the cysts or tumors involves signaling molecules.^[2] Bone healing is comprised of several overlapping phases, namely the inflammation phase, soft callus phase, callus

replacement with bone phase, and bone remodeling phase.^[1] Inflammation plays an important role in the healing process because inflammatory reaction is the first phase of healing in all tissues. After the primary inflammatory phase subsides, tissue components start to proliferate and the tissue response proceeds to the healing phase. Coordination of inflammatory and healing phases is a prerequisite to achieve bone

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regeneration.^[2] Several chemical mediators, such as nitric oxide (NO), are involved in the healing process; NO gas is a free radical that can serve as a proinflammatory or anti-inflammatory mediator depending on its production site, its concentration, and its potential to produce toxic derivatives, such as proxy nitrite, and has a wide range of biological effects.^[3] Through the oxidation of L-arginine, NO is synthesized by the NO synthase (NOS) enzymes in the presence of a large number of cofactors such as nicotine amide adenine di-nucleotide phosphate. Three different isoforms of NOS enzyme are available, namely neural NOS (nNOS), endothelial NOS (eNOS), and inducible NOS (iNOS); two of these isoenzymes are constitutive (nNOS and eNOS) and one isoenzyme is inducible (iNOS).

The nNOS and eNOS temporarily synthesize NO in low physiological amounts in the endothelial and neural tissues, and their activity is regulated by changes in the concentration of free intracellular calcium. However, in inflammatory and wound healing conditions, iNOS generates high levels of NO in macrophages and some other cells following stimulation by bacterial lipopolysaccharides and inflammatory cytokines such as interleukin (IL) 1 β , tumor necrosis factor alpha, and interferon-gamma, independent of calcium. Glucocorticoids and IL-4, IL-10 and transforming growth factor β (TGF- β) and anti-inflammatory cytokines inhibit the expression of iNOS in macrophages.^[4] The NO produced by iNOS plays an important role in cellular processes such as induction of apoptosis,^[5] inhibition of mitochondrial respiration,^[6] and regulating the balance between glycolysis and oxidative phosphorylation.^[7]

NO is a highly reactive molecule, and thus, it has numerous potential molecular targets. In normal conditions, in the endothelial smooth muscles and platelets, small amounts of NO produced by the vascular endothelium relax the adjacent smooth muscles and prevent the attachment of platelets to the endothelial walls.^[8] These processes play protective and anti-inflammatory roles. In contrast, in inflammatory conditions, high concentrations of NO are released in response to the stimulation by inflammatory cytokines and help the macrophages eliminate microbial and tumoral cells, following the reaction of NO with superoxide free radicals. However, these processes result in the destruction of host tissue as well.

NO significantly affects the function of osteoblasts and bone remodeling. Evidence shows that NO has a biphasic effect on the osteoclastic bone resorption and osteoblastic bone formation. Low concentrations of NO reinforce cytokines that cause bone loss and are necessary for normal function of osteoclasts. However, high concentrations of NO inhibit bone resorption by suppressing the production and activity of osteoclasts. Low concentrations of NO synthesized by eNOS may mediate growth and normal activity of osteoblasts, while high concentrations of NO, noticed after stimulation by proinflammatory cytokines, have an inhibitory effect on growth and differentiation of osteoblasts.^[8]

Aminoguanidine, as a selective inhibitor of iNOS enzyme, has been evaluated in several studies since 1992.^[9] It has two chemical groups namely guanidino nitrogen and hydrazine. The latter is probably responsible for the selective inhibition of iNOS by aminoguanidine hydrochloride (AG).^[10] Moreover, the inhibition of iNOS by AG is 10–100 times more efficient than the inhibition of eNOS and constitutive NOS.^[11]

NO is synthesized in wounds and plays an important role in successful wound healing.^[12] Soneja *et al.* showed that impaired wound healing in diabetics was due to decreased production of NO.^[13] Inhibiting the production of NO during wound healing delays re-epithelialization and collagen formation.^[13,14] In a study by Farhad *et al.*, the severity of induced periapical inflammation in the canine teeth of cats was significantly lower in the group who received systemic AG compared to the control group.^[15] In another study, Farhad *et al.* induced periapical lesions in the cats' canine teeth and then performed root canal therapy and administered AG systemically. They showed that healing of periapical lesions in the experimental group was significantly greater than that in the control group.^[16] The afore-mentioned studies evaluated the anti-inflammatory effects of systemic AG. Recently, Farhad *et al.* in their study showed that the healing of bone defects in the femur of rats following the local use of 20% AG was significantly higher than that of the control group.^[17]

Considering the possible variable effects of different concentrations of NO, the use of AG in different concentrations will probably have different effects on healing since the inhibition of NO synthesis and its

related effects depend on the concentration of AG. Therefore, this study aimed to assess the local effect of different concentrations of AG on the healing of bone defects in rats.

MATERIALS AND METHODS

In this animal experimental study, rats were used for the assessment of the rate of healing of bone defects. This study was approved by the Committee of Medical Ethics of Isfahan University of Medical Sciences (394442). The sample size was calculated to be a minimum of 12 samples in each group to find significant differences among the groups. Seventy-two adult male Wistar rats in the age range of 12–16 weeks and weight range of 300–400 g were selected. The rats were kept in the animal room of the Torabinejad Research Center of Isfahan University of Medical Sciences for 1 week before the surgery for the purpose of acclimation. The rats were kept in separate cages in an environment with natural lighting and standard temperature and humidity with *ad libitum* access to food and water.

The placebo and AG gel (Sigma-Aldrich, Saint Louis, USA) were prepared by a pharmacist in the School of Pharmacy of Isfahan University of Medical Sciences. For the preparation of gel with sustained release, carboxymethyl cellulose polymer was used as a base. The AG crystals are water soluble and have a pH of around 4. The AG gel was synthesized in 5%, 10%, 15%, and 20% concentrations. Due to the sensitivity of AG to light, air, and moisture, all phases of gel preparation were performed in neutral gas environment, and the synthesized gels were stored in a dark and cold environment at 4°C–8°C until the surgical procedure.

Surgical procedure

The rats were anesthetized by intramuscular injection of 10% ketamine (Alfasan, Woerden, Holland, the Netherlands) at a dose of 80 mg/kg and xylazine (Alfasan, Woerden, Holland, the Netherlands) at a dose of 0.5 mg/kg. After induction of anesthesia, the hair on the right femoral region was shaved and the skin was disinfected three times with 70% alcohol, betadine, and chlorhexidine. Through a direct lateral approach, the external surface of the condyle of the right lateral femur was exposed by a 1 cm incision made using a #15 scalpel (Kiato, Hannover, Germany) and #3 handle. The skin, the submucosal tissues, and the muscles

were retracted using a periosteal elevator, and access to the distal region of the femur was obtained between the muscles in the anterior and posterior compartments. To create a round defect measuring 5 mm × 5 mm in the distal condyle of the femur, a round bur (HM 141F 050, Meisinger, Dusseldorf, Germany) and a low-speed (15,000 rpm) surgical handpiece (S-11 model, W&H, Burmoos, Austria) were used. The defect was created at the center of femoral condyle while preserving the surrounding bony walls. During cavity preparation, frequent irrigation with 0.9% saline was performed at the interface of bur and bone to prevent overheating and eliminate bone chips and debris. During the procedure, the rats were randomly divided into the following six groups: control (Group 1), placebo (Group 2), 5% AG (Group 3), 10% AG (Group 4), 15% AG (Group 5), and 20% AG (Group 6). In the control group, bone defect remained empty. In the placebo group, neutral gel was placed in the bone defect, and in the AG groups, different concentrations of AG gel were applied to the defects. Surgical wound was then sutured in two layers (periosteum and muscle layer and skin layer). Suturing the periosteum–muscle layer enables maintaining the material in the defects. The periosteum–muscle layer was sutured with 4-0 absorbable vicryl suture (C.G Co., I.R, Iran), and the skin was sutured with 4-0 nylon suture (C.G Co., I.R, Iran). To prevent infection, chloramphenicol was sprayed on the site of sutures and cefazolin (Dana, Tabriz, Iran) was administered subcutaneously at a dose of 15 mg/kg every 12 h for the first 3 days postoperatively. Flunixin meglumine (Razak laboratories from active material supplied by Norbrook, Ireland) was administered subcutaneously at a dose of 2.5 mg/kg every 12 h during the first 3 days postoperatively for pain control. The rats were kept in separate cages (based on their group) and provided with water and food plate of laboratory animals (Behparvar, Tehran, Iran). The animals were then allowed to recover from anesthesia.

The rats were sacrificed at 8 weeks postoperatively for histological assessments under anesthesia via an overdose of halothane gas. The surgical site with 1 cm of safe margin was cut out. Soft tissue was removed, and the tissue specimens were placed in a coded dish containing 10% formalin for 1 day for fixation. The specimens were then demineralized in

7% nitric acid for 7 days. Then, the specimens were dehydrated and embedded in paraffin blocks. Three vertical sections were made of each defect, and the samples were prepared for staining. To obtain the best results, three specimens were randomly chosen as pilot. Each section was stained with Masson's trichrome staining or hematoxylin and eosin staining and evaluated by a pathologist. The slides of each specimen were compared, and since the hematoxylin and eosin staining yielded higher diagnostic value, the remaining specimens were stained with hematoxylin and eosin.

Histological assessment

The specimens were evaluated under a light microscope (Zeiss Carl, Oberkochen, Germany) by an experienced pathologist in a single-blind fashion. The specimens were inspected in terms of rate of healing while taking into account abscess formation, regeneration of tissues around the defect, necrosis, fibrous tissue formation, infiltration of neutrophils, acute and chronic infiltration of inflammatory cells, presence of granulation tissue, and bone formation. The specimens were classified into four groups in terms of the healing score [Table 1].^[17]

Statistical analysis

The Shapiro–Wilk test was applied to assess the normal distribution of histological data with regard to bone healing at 8 weeks. The Kruskal–Wallis test was used to analyze histological data regarding bone healing at 8 weeks in the six groups. The Mann–Whitney test was applied for pairwise comparisons. $P < 0.05$ was considered statistically significant.

RESULTS

Histological assessment

The Shapiro–Wilk test was applied to test the normal distribution of histological data regarding bone healing (the healing score) at 8 weeks, which showed

that the data did not have a normal distribution. Thus, the Kruskal–Wallis test was used to find the possible significant differences among the six groups. In the next step, the Mann–Whitney test was used for pairwise comparisons. Analysis of the histological data regarding bone healing at 8 weeks showed that the healing score in 20%, 15%, 10%, and 5% AG groups was significantly higher than that in the neutral gel and control groups ($P < 0.01$). No significant differences were noted between different concentrations of AG or between the neutral gel and control groups ($P = 0.213$). The frequency distribution of the healing scores in the six groups is presented in Table 2. Histological view of bone healing in the six groups is shown in Figure 1.

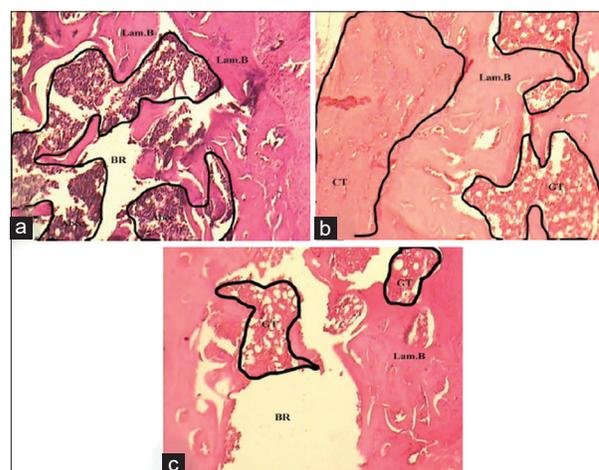


Figure 1: Histological view of healing scores; (a) Histological view of control and placebo groups showing abscess formation, infiltration of inflammatory cells, BR and lack of GT indicative of score 1 of healing (H and E: $\times 100$). (b) Histological view of 15% AG group showing infiltration of inflammatory cells, cell-rich GT, fibrous CT and BR indicative of score 2 of healing (H and E: $\times 100$). (c) Histological view of 20% AG group showing slight infiltration of inflammatory cells, fibrous CT and bone formation indicative of score 3 of healing (H and E: $\times 100$). Abce: Abscess, GT: Granulation tissue, CT: Connective tissue, BR: Bone resorption, Lam. B: Lamellar (mature) bone.

Table 1: Stages of healing

Stage A: Acute inflammatory stage (score 1)	Stage B: Organization stage (score 2)	Stage C: Advanced organization (score 3)	Stage D: Healing stage (score 4)
Abscess formation	Bone resorption	Presence of fibrous CT	Regeneration of tissues within defect
Necrosis	Acute/chronic inflammatory cells (30-60)	A few inflammatory cells (<30)	Lack of inflammation
Dens accumulation of PMNs	Cell-rich granulation tissue	Bone formation	Complete hard tissue formation
Lack of granulation tissue	Increased vascular buds		
	Presence of fibrosis around the granulation tissue		

PMN: Polymorphonuclear leukocyte, CT: Connective tissue

Table 2: Distribution of healing scores in the six groups

Group	Healing score, n (%)				Total
	A	B	C	D	
	Score 1	Score 2	Score 3	Score 4	
AG 20% ^a	0	5 (41.7)	7 (58.3)	0	12
AG 15% ^a	0	6 (50.0)	6 (50.0)	0	12
AG 10% ^a	0	7 (58.3)	5 (41.7)	0	12
AG 5% ^a	0	6 (50.0)	6 (50.0)	0	12
Placebo ^b	3 (25)	8 (66.66)	1 (8.33)	0	12
Control ^b	6 (50.0)	6 (50.0)	0	0	12

Different superscripts show significant differences between groups. AG: Aminoguanidine hydrochloride

DISCUSSION

This experimental study aimed to assess the effect of different concentrations of AG, an iNOS inhibitor, on bone healing in rats. The histological results showed that local application of 5%, 10%, 15%, and 20% concentrations of AG in bone defects significantly enhanced bone healing.

Basic scientists have extensively evaluated the role of NO in inflammatory diseases, such as rheumatoid arthritis and diabetes mellitus, and also in bone remodeling. Dental researchers have also investigated the role of NO in inflammatory processes of the oral mucosa,^[18] periodontal tissues,^[19] periapical areas,^[20] and dental pulp.^[21] Although reactive oxygen and nitrogen species such as NO are abundant at the site of inflammation and healing, their functions in the pulpal and periapical tissues have yet to be fully understood. Considering the fact that the function of NO affects different phases of healing, understanding its role in the pathophysiology of the healing process of bone defects can greatly help pharmacological interventions in the future.

NO serves as an inflammatory mediator and has several (sometimes, contradictory) functions. At some sites, NO shows an anti-inflammatory role via its antimicrobial activity. It also prevents leukocyte adhesion to the vessel walls, inhibits intravascular thrombosis, increases blood supply to the tissues, and regulates immunity. In some other areas, it plays a proinflammatory role by its cytotoxic activity and in damaging the host cells. Several studies have indicated the proinflammatory role of NO in the pulp and periapical tissues. Kawanishi *et al.*, in an experimental study, assessed the efficacy of a nonselective NOS inhibitor (1400 W) in rats with pulpitis of the upper incisors. They showed that NO probably played a role in infiltration of the inflammatory

cells and progression of the pulpitis, and 1400 W was introduced as a suitable modality to control the inflammatory pulp response.^[22] da Silva *et al.*, Fan *et al.*, and Law *et al.* showed that the concentration of NOS and its expression in the inflamed pulps were significantly higher than in normal dental pulps (free from inflammation).^[3,23,24] di Paola *et al.* in their study tied silk threads around the neck of the first molar teeth of rats and caused marginal periodontitis. Eight days later, level of activity and expression of iNOS in the gingival mucosal tissues increased by threefold compared to the control group. Further, neutrophil infiltration, lipid peroxidation, and alveolar bone loss significantly increased in the injured tissue. However, intraperitoneal injection of AG at a dose of 100 mg/kg for 8 days significantly decreased all inflammatory parameters and served a protective role against periodontitis by decreasing the synthesis of NO and oxidative stress.^[25] Farhad *et al.*, in their study on cats, exposed the pulp of the canine teeth to the oral environment in such a way that the root canal was exposed to the oral microflora for 7 days. The access cavity was then sealed with amalgam. In the test group, AG immersed in saline, and in the control group, saline alone was injected intraperitoneally every other day. Four weeks later, inflammatory response in the periapical region was histologically assessed. The results showed that the severity and degree of inflammation in the intervention group were significantly lower than those in the control group.^[15] A similar study confirmed the results of previous studies and showed that systemic administration of AG enhanced healing of periapical lesions.^[16] Since the first step of tissue healing is an inflammatory process, enhanced healing of periapical lesions might have been due to the inhibition of local inflammation, following the inhibition of NOS at the periapical region. The results of Fukada *et al.* were in contrast to those of Farhad *et al.* Fukada *et al.* assessed the role of NO in bone loss in animal models with iNOS insufficiency and induced apical periodontitis due to bacterial infection and stated that animal models with iNOS insufficiency had higher inflammatory cells and osteolytic lesions compared to normal animal models.^[26] The controversy in the results of studies may be due to the following: (i) AG is not only a selective iNOS inhibitor but also has antioxidant properties, which probably decreases free radicals and induces anti-inflammatory effects. (ii) Anatomical differences among rats, mice, and cats might have also affected the results.

Hama *et al.* stated that one mechanism explaining the role of NO in the development of periapical inflammatory lesions was the expression of specific receptors in the periapical granuloma by the inflammatory cells, such as macrophages entitled receptor for advanced glycation end products (RAGE) that infiltrated around the iNOS-producing cells. The attachment of RAGE to advanced glycation end products enhances blood supply and stimulates the release of proinflammatory cytokines.^[27] Another mechanism suggested in previous studies is the role of microbial flora of the mouth. The role of microorganisms in the pathogenesis of the pulp and periapical diseases has been extensively discussed in the scientific literature.^[28] Trauma to the host tissue due to microbial infection of endodontically treated teeth can be due to the direct effect of proteolytic toxins and destructive microbial products or the inflammatory–immunity interactions, causing tissue destruction as in the periradicular bone.^[29] Although the expression of iNOS in the early phases of wound healing is high, no data are available on the decreased activity of iNOS in the next phases of healing. The activity of iNOS probably decreases by the elimination of inflammatory responses or cytokine signals. High levels of NO are probably produced in infected wounds with continuous inflammatory response. TGF β 1 is one among the most potent iNOS inhibitors during the process of wound healing.^[30] It is not yet known whether NO is a nonspecific mediator of host defense during the primary phase of healing or is a more specific controlling signal for successful completion of the healing process.^[31]

In addition to the inflammatory phase, the effect of NO and AG on other components of the healing process must be taken into account. Fibroblasts are the main cells involved in the healing phase. It has been suggested that enhanced healing of the periapical lesions may be due to the antioxidative effects of AG on fibroblasts. Wang *et al.* stated that AG enhanced the growth and proliferation of fibroblasts. It seems that during the cell cycle, AG prevents cells from entering from M or S phase into the G0 phase and increases mitosis. In other words, by maintaining the cells in the proliferating state, it prevents the initiation of aging phenotype.^[32] Our findings are in line with those of Wang *et al.*

One important factor in wound healing is collagen production by fibroblasts. Its first step is hydroxylation of proline by the prolyl hydroxylase enzyme. This

enzyme needs iron, oxygen, and ascorbic acid for its activity.^[33] Shukla *et al.* evaluated the possible role of NO in the formation of collagen during wound healing. The results showed that NO had an inhibitory effect on the hydroxyproline content of wounds. Regarding its mechanism of action, it was stated that NO, due to inhibition of prolyl hydroxylase, decreases the collagen content of wound because prolyl hydroxylase requires iron and oxygen for its activity,^[34] while NO inhibits iron-containing enzymes by the formation of iron nitrosyl complexes.^[35] Another important point is that NOS needs oxygen for its function as well. Increased expression of NOS in wounds during the inflammatory phase can decrease the oxygen available for prolyl hydroxylase. Thus, in the presence of L-NAME, the inhibition of NOS provides more oxygen for prolyl hydroxylase and results in increased synthesis of hydroxyproline. However, regarding the effect of NO on collagen metabolism in fibroblasts, the results contrary to those of Shukla *et al.* have also been reported.^[14,36] Schäffer *et al.*, in their study on mice, showed that when S-methyl isothiuronium, which is a competitive inhibitor of NOS, was applied on wounds for 10 days during the healing process, the content of hydroxyproline significantly decreased.^[14] Such a controversy in the results of studies may be due to differences in animals used, type and dose of drugs used as NOS inhibitors, the methodology of studies, and particularly the duration of administration of NOS inhibitors.

The role of NO is also important in angiogenesis, which is another important aspect of wound healing. NO can stimulate the proliferation of endothelial cells and prevent their apoptosis. In addition, it can mediate the production of vascular endothelial growth factor (VEGF). It seems that NO is necessary for the activity of proangiogenic cytokines. For instance, VEGF, which is a potent angiogenic factor, regulates the production of NO. In fact, VEGF increases the production of NO by increasing the content of eNOS. Further, angiogenic effect of VEGF is NO dependent because the pharmacological block of NOS inhibits the proliferation of endothelial cells due to the effect of VEGF and mitogen-activated protein kinase.^[37] Moreover, it has been shown that NO plays a role in conversion of VEGF from the neutral state to angiogenesis.^[38] The NO produced by eNOS has both proinflammatory and anti-inflammatory properties. Under physiological conditions, NO released from the endothelium regulates vascular tone and maintains the

vessels open by preventing platelet aggregation and decreasing the expression of adhesion molecules.^[39] Corbett *et al.* showed that in the primary phase of fracture healing, expression of eNOS is highest in the cortical blood vessels and osteocytes, and as the healing advances, it returns to its baseline level.^[40] High level of NO and maximum intensity of vascular reactions in the primary phase of healing are probably responsible for increased blood flow over time.^[40,41] Pipili-Synetos *et al.* and Hatjikondi *et al.* stated that decreased level of NO stimulated angiogenesis,^[42,43] while Ziche *et al.* in their study showed that NO promoted angiogenesis.^[44] Different effects of NO on angiogenesis are probably due to its versatile function in different concentrations. The correlation of angiogenesis and wound healing has yet to be clearly understood because increased vascularization following angiogenesis in the inflammatory phase of healing brings more inflammatory mediators to the site, which delays the healing process. However, vascularization is a critical step in the proliferative phase of wound healing.^[45]

The role of NO in bone healing is complex and multifactorial; NO is a signaling molecule with multiple functions and serves as an intracellular signaling regulator in bone with definite effects on proliferation and longevity of osteoblasts, osteoclast function, and bone remodeling.^[46-50] Baldik *et al.* showed that local administration of a single dose of bovine serum albumin containing NO along with demineralized bone matrix induced bone formation by 62% higher than that by bone matrix alone in bone defects in the femur of rats at 10 weeks postoperatively. They also showed that oral administration of AG enhanced defect fill. Radiographic and histological results in their study showed osteoinductive effects of local NO and its systemic inhibition by AG.^[51] Giardino *et al.* showed that AG as an antioxidant prevented the formation of reactive oxygen species and lipid peroxidation *in vivo*. Their findings showed that in the AG group, the positive role in bone healing could be due to protection against the adverse effects of excessive production of NO by iNOS.^[52] Paul-Clark *et al.*, in their study on rats, evaluated the effect of direct and indirect administration of specific and nonspecific NOS inhibitors on the severity of inflammation. Their results showed that NOS inhibitors had variable effects on the severity of inflammation depending on their method of administration.^[53]

It should be noted that although AG is a selective iNOS inhibitor, in the presence of calcium, calmodulin, and other cofactors, it can inhibit eNOS and nNOS, which are responsible for prevention of bone loss,^[54] and it is a confounder in such studies.

It appears that in addition to the method of administration of NO inhibitors, their dosage also affects the severity of inflammation and the healing score, and many of the adverse effects seen due to the inhibition of NOS may be due to the administration of improper drug dosage. Leitão *et al.* evaluated the effect of NOS inhibitors on the alveolar bone loss in rats with induced periodontitis lesions and showed that daily administration of 5 and 10 mg/kg doses of AG and L-NAME significantly decreased alveolar bone loss by approximately 50%. However, these effects were dose dependent and daily administration of AG at a dose of 100 mg/kg could not prevent alveolar bone resorption or local inflammatory changes.^[55] This finding may be explained by the fact that high concentrations of AG can inhibit physiologic NOS. It seems that to achieve the desired therapeutic effects, continuous administration of low doses of NOS inhibitors is preferred over the administration of high doses.^[16] Based on the results of the current study, it seems that 5% AG can be the preferred dosage for enhanced bone healing.

Nonspecific NOS inhibitors such as L-NAME interfere with eNOS and nNOS, which play important physiologic functions.^[56] Thus, selective iNOS inhibitors are ideal for the elimination of inflammatory responses without adverse effects on physiological reactions. Further, NO plays an important role in maintaining homeostasis via its physiological functions. However, if its production significantly increases, it may cause adverse effects due to the induction of inflammation. Considering the fact that NO has a wide range of biological functions, its inhibition can have many systemic effects. Thus, in clinical application of NOS inhibitors, local administration must be preferred over systemic use. Farhad *et al.* in their study showed that healing of bone defects in the rat femurs with local use of 20% AG was significantly higher than that of the control group.^[17] Thus, this study was designed to assess the process of healing with local use of different concentrations of AG. AG is commercially available in the form of crystal. Thus, its application to the bone defect must be via a carrier for easy handling. On the other hand, if this material is applied alone in bone defects, it will be quickly washed out of

the area due to the presence of tissue fluids and will have a short-term effect. Thus, a gel base is required to enable its slow release. This material is soluble in carboxymethyl cellulose polymer gel. Considering its physicochemical properties, it seems that maximum concentration of AG gel that can be loaded onto this gel base is 20%. To ensure that the gel is neutral and has no effect on healing, gel base without AG was applied in bone defects in the placebo group. The AG gel used in this study enables sustained release of AG within 2 weeks. The expression of iNOS has the highest level in the primary phase of acute inflammation,^[57] and over time, the activity of iNOS can decrease due to the elimination of inflammatory responses or cytokine signals.^[30] Thus, it can be stated that the gel used in this study releases AG for adequate duration of time.

CONCLUSION

Based on the histological results, different concentrations of AG yielded superior bone healing compared to the control group in rats. Since, in pharmaceutical interventions, minimum dose with maximum effect is always favored, 5% concentration of AG is recommended for use in future studies.

Considering the fact that AG has yet to have clinical applications, further studies are required to assess the possibility of using AG to enhance healing of bone defects in the periapical surgeries.

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Conflicts of interest

The authors of this manuscript declare that they have no conflicts of interest, real or perceived, financial or non-financial in this article.

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