Selective nitric oxide synthase inhibitor promotes bone healing

Ali Reza Farhad1, Sayed Mohammad Razavi2, Ali Reza Rozati3, Neda Shekarchizade4, Maziar Manshaei5

1Department of Endodontics, Dental Research Center, School of Dentistry, Isfahan University of Medical Sciences, 2Department of Oral Pathology, Implant Research Center, School of Dentistry, Isfahan University of Medical Sciences, 3Orthopaedic Surgeon, Private Practice, 4Department of Endodontics, Dental Materials Research Center, School of Dentistry, Isfahan University of Medical Sciences, 5Dental Research Center, School of Dentistry, Isfahan University of Medical Sciences, Isfahan, Iran

ABSTRACT

Background: Nitric oxide (NO) has many functions in wound healing and bone metabolism. This study sought to assess the local effect of aminoguanidine (AG), a selective inducible NO synthase (iNOS) inhibitor, on the rate of bone healing.

Materials and Methods: This experimental interventional study was conducted on 36 rats, which were randomly divided into three groups of control, placebo, and AG. Bone defects measuring 5 mm × 5 mm were created in the femur. In control group, bone defects remained empty. A placebo gel was applied to defects in the placebo group. AG gel was placed in bone defects in AG group. New bone formation and healing were assessed using histological and histomorphometric analyses. The healing score and the percentage of new bone formation (total bone mass, immature bone, and mature bone) were compared among the three groups using the Kruskal–Wallis test and analysis of variance, respectively. A \( P < 0.05 \) was statistically significant.

Results: The mean healing score in AG group (3.17 ± 0.577) was significantly higher than that in control (2.67 ± 0.49) and the placebo (2.58 ± 0.515) groups (\( P = 0.036 \)). The percentage of new mature (lamellar) bone in AG group (22.06 ± 1.90) was significantly higher than that in control (20.94 ± 2.03) and the placebo (20.53 ± 1.20) groups (\( P = 0.008 \)).

Conclusion: The rate of bone healing was faster in the AG compared to the other two groups. Local application of selective iNOS inhibitors like AG may be efficient as an adjunct in the clinical setting where local bone formation is required.

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

INTRODUCTION

Wound healing is a dynamic process requiring the involvement of soluble mediators, blood cells, extracellular matrix, and parenchymal cells. Wound healing has three phases of inflammation, tissue formation and remodeling, which overlap one another.[1] Many chemical mediators are involved in the phases of inflammation and healing; nitric oxide (NO) is among these mediators. NO can show pro- or anti-inflammatory properties depending on its concentration, the potential to produce toxic derivatives such as proxy nitrite and the location of the pathological process.[2]

NO is a free gas radical. NO synthase (NOS) affects the L-arginine and results in the production of nitric oxide (NO). This is an open access article distributed under the terms of the Creative Commons Attribution-NonCommercial-ShareAlike 3.0 License, which allows others to remix, tweak, and build upon the work non-commercially, as long as the author is credited and the new creations are licensed under the identical terms.

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NO. Three isoforms of NOS are available namely endothelial and neural constitutive NOS (cNOS), and inducible NOS (iNOS). Cells containing cNOS produce small amounts of NO immediately and transiently in response to factors that increase the intracellular level of free calcium; whereas, cells expressing iNOS produce large amounts of NO over a longer period after a several-hour lag phase as long as the inducing enzyme is present.[3] NO is an important signaling molecule in bone, which is produced in response to different stimuli such as the pro-inflammatory cytokines, mechanical tension, and sexual hormones.[4] NO mediates the function of bone cells and the process of bone remodeling.[4,5]

NOS inhibitors decrease the bone mass via creating an imbalance between bone resorption (BR) and bone formation.[6] Evidence shows that N-Nitroarginine methyl ester (L-NAME), a NOS inhibitor, inhibits the process of bone formation, which is induced by mechanical factors. This indicates that NO is a primary mediator in increasing the bone mass via mechanical stimulation.[7,8] High concentrations of NO inhibit BR by suppressing the production of osteoclasts and their activity; whereas, lower concentrations of NO reinforce bone loss cytokines and are necessary for normal function of osteoclasts. Moreover, the growth and differentiation of osteoblasts are inhibited by higher concentrations of NO. Lower concentrations of NO produced by cNOS enzyme may mediate the proliferation and normal activity of osteoblasts.[4] Inhibiting the synthesis of NO in the wound decreases the accumulation of collagen and subsequently, the mechanical strength of the wound.[9,10] Moreover, it was demonstrated that local application of NO-releasing polymers enhanced wound healing.[11]

Aminoguanidine (AG) was introduced in 1992 as one of the first selective iNOS inhibitors.[12] AG is fifty times more effective in inhibiting the enzymatic activity of iNOS than inhibiting endothelial and neural isoforms.[13] Farhad et al.[14] in their study on the role of NO in the progression of the inflammatory process in the periapical tissue in a cat model demonstrated that systemic administration of a selective iNOS inhibitor (AG) significantly decreased the severity of inflammation in the periapical lesions induced in the periapical region of canine teeth. Moreover, Farhad et al.[15] reported that systemic administration of AG significantly increased the healing score of induced periapical lesions in canine teeth in a cat model.

Bone defects may develop under different clinical conditions such as endodontic periapical and maxillofacial surgeries. In such cases, bone tissue must be regenerated as soon as possible. Numerous studies have evaluated the effect of NO or systemic inhibition of its synthesis on inflammation and wound healing. However, the obtained results are conflicting, and since no previous study has assessed the local effect of the selective iNOS inhibitor on the bone healing score, the current experiment was designed. This study aimed to assess the local effect of selective inhibition of iNOS by AG on bone healing score in rats.

MATERIALS AND METHODS

Animal model

A rat model was used to assess the bone healing rate. The sample size was calculated to be 12 in each group considering the minimum number of animals required for obtaining a statistically significant difference among groups. The study protocol was approved by the Ethics Committee of Isfahan University of Medical Sciences (393029). Thirty-six male Wistar rats aged between 12 and 16 weeks and weighing between 300 and 400 g were selected. The study was conducted in the animal lab of Torabinejad Research Center at Isfahan University of Medical Sciences Isfahan, Iran. The rats were kept in separate cages under standard controlled temperature, and humidity conditions and they had free access to food and water.

Aminoguanidine and placebo gel preparation

To provide gradual and controlled release of gel, carboxymethyl cellulose polymer was used as a base. To prepare the gel, 70 g of 4% carboxymethyl cellulose polymer was dissolved in pure water heated up to 60°C to obtain a homogenous gel; 40 g of this gel was used as the placebo gel.

AG crystals are water soluble. To prepare 20% gel, 6 g of AG powder was dissolved in 2 mL of pure water and added to 30 g of gel. A homogeneous 20% gel was prepared as such. Since AG is sensitive to air and moisture, all the preparation steps of the AG gel were performed under neutral gas environment.

Surgical procedure

Anesthesia was induced by administration of 10% ketamine (Alfasan, Woerden, Holland) at a dose of 50 mg/kg and 2% acepromazine (Alfasan, Woerden, Holland) at a dose of 0.5 mg/kg. After the induction of anesthesia, distal of the left femur was shaved and
scrubbed with alcohol, betadine, and chlorhexidine three times. A 1 cm incision was made in a direct lateral approach to expose the external surface of the lateral condyle of the left femur. Fascia was retracted, and the distal femur was approached through the anterior and posterior compartment muscles. A 5 mm × 5 mm defect was created in the distal condyle of the femur using an HM 141F 050 round bur (Meisinge, Dusseldorf, Germany) and S 11 model low-speed handpiece (W and H, Burmoos, Austria). The defect was created at the center of the femoral condyle in such a way that the bony walls around the defect were preserved. The surgical site was constantly irrigated with sterile saline during drilling to prevent over-heating. Hemorrhage from the defect site was controlled by constant local pressure [Figure 1]. Rats were randomly divided into three groups of 12 namely AG, placebo and control. Bone defects remained empty in the control group. In the placebo group, the placebo gel was applied to defects and in AG group, bone defects were filled with AG gel. Surgical wound was closed in two layers (periosteum-muscle and cutaneous layers). Closure of the muscle helps maintain the material in place. The muscle layer was sutured using 4-0 vicryl sutures (C.G Co.I.R, Iran) while skin closure was carried out using 4-0 nylon sutures (C.G Co.I.R, Iran). Chloramphenicol antibiotic was sprayed on the suture sites. Animals recovered from anesthesia and 2.5 mg/kg flunixin meglumine (Razak Laboratories from active material supplied by Nobrook, Ireland) was injected subcutaneously every 12 h postoperatively for 3 days for pain relief. To prevent infection, 15 mg/kg cefazolin (Dana, Tabriz, Iran) was injected subcutaneously every 12 h for 3 days. At 8 weeks, the rats were sacrificed using Halothane for histological and histomorphometric analyses. Bone and soft tissue were detached from the defect site. Specimens were fixed by immersing in 10% neutral buffered formalin for 1 day at room temperature and then demineralized by 1 week immersion in 7% nitric acid. Specimens were dehydrated and embedded in paraffin blocks. Sections were made perpendicular to the long axis of the defects, and the specimens were stained with hematoxylin and eosin according to the standard protocol.

**Histological assessment**

The specimens were histologically assessed under a light microscope (Carl Zeiss, Oberkachen, Germany) by an expert pathologist in a single-blind fashion. Histological parameters of the healing stage [Table 1] were evaluated.

**Histomorphometric assessment**

In histomorphometric assessment, the percentages of total bone, woven bone, and lamellar bone (LB) were separately evaluated in the defects. For this purpose, microscopic slides were prepared, and micrographs were obtained of each slice under a light microscope (100e Nikon YSLight, Tokyo, Japan) equipped with a digital camera (Nikon DP R camera, Tokyo, Japan) at 40× and 100× magnifications. Three micrographs were obtained of each specimen (superior border, middle part, and inferior border). Micrographs were coded (based on the slide code), saved in a computer and assessed using Adobe Photoshop software (Adobe, San Jose, CA, USA). For this purpose, the saved images were opened in the software. Using the “image” feature, calculations were made to determine the percentage of osteogenesis in the defect and the percentage of immature (woven) and mature (lamellar) bone. Finally, the mean value of osteogenesis for each coded micrograph was recorded as the final value of new bone formation (total bone).

**Statistical analysis**

Kolmogorov–Smirnov test was used to assess normal distribution of data. Histological data related to the healing score at 8 weeks were analyzed in the three groups using the Kruskal–Wallis test. Histomorphometric data of woven bone, LB, and total bone were analyzed using analysis of variance and then Tukey test. A $P < 0.05$ was statistically significant.
RESULTS

Histological assessment
Analysis of histological data for bone healing at 8 weeks [Table 2] revealed that the healing score in AG group was significantly higher than that in the control and placebo groups ($P = 0.036$). No significant difference was noted between control and placebo groups ($P = 0.680$), but there were significant differences between control and AG groups ($P = 0.038$) and placebo and AG groups ($P = 0.021$) [Figure 2]. The mean healing score was $2.67 \pm 0.49$ in the control group, $2.58 \pm 0.515$ in the placebo group, and $3.17 \pm 0.577$ in the AG group. The histological pattern of healing scores is shown in Figure 3.

Histomorphometric assessment
The analysis of histomorphometric data at 8 weeks revealed that LB formation in defects in the AG group was significantly higher than that in the control and placebo groups ($P = 0.008$). No significant difference was noted between control and placebo groups ($P = 0.954$), but there were significant differences between control and AG groups ($P = 0.026$) and placebo and AG groups ($P = 0.013$) [Figure 4]. The mean percentage of LB formation in the three groups of control, placebo, and AG is shown in Table 3. No significant difference was noted among the three groups in terms of woven ($P = 0.417$) and total bone ($P = 0.090$) formation. However, total bone formation in the AG group was higher than that in the other two groups, this difference was not statistically significant. The pattern of mature bone formation in the three groups is shown in Figure 5.

DISCUSSION

This study showed that local application of AG into the bone defects accelerated bone healing. It appears that inhibition of NO synthesis at the initial phases of inflammation accelerates the process of healing in the following phases. A number of studies have evaluated the function of NO in the inflammatory process of the oral mucosa,\textsuperscript{[16]} periodontal tissue,\textsuperscript{[17,18]} dental pulp\textsuperscript{[19]} and periapical region.\textsuperscript{[20]} Reactive oxygen and nitrogen species such as NO are abundantly found at the sites of inflammation and healing, but their function has yet to be fully understood. Considering the fact that the function of NO can affect different phases of healing, better understanding of the role of NO in pathophysiology of the healing process of bone defects can be useful for future pharmacological interventions. Thus, to better understand, the complex process of bone healing, the current animal study was conducted aiming to assess the role of NO in the process of bone healing.

The iNOS is not normally expressed in healthy noninflamed tissues. Some previous studies have demonstrated the pro-inflammatory role of NO in
the pulp and periapical tissues. Kawanishi et al.\textsuperscript{19} stated that NO might be responsible for infiltration of immune cells in pulpitis. da Silva et al., Law et al., and Fan et al. indicated higher concentrations of NOS enzyme in inflamed tissues compared to noninflamed sites.\textsuperscript{2,21,22} In an induced periapical lesion in a rat model, the NOS released from the macrophages, which had been stimulated by lipopolysaccharides, induced apoptosis of macrophages and osteoblasts and enhanced the progression of periapical lesions.\textsuperscript{20} It is not known whether NO is a nonspecific host defence mediator in the primary phase of healing or is a more specific controlling signal for successful completion of the healing process.\textsuperscript{23}

In addition to the inflammatory phase, the effect of NO and AG on other components of the healing phases must be taken into account. Fibroblasts are the main cells in the healing phase. Fibroblasts present in the wound produce more NO than normal tissue fibroblasts.\textsuperscript{24} NO enhances the migration and proliferation of fibroblasts.\textsuperscript{24} Wang et al.\textsuperscript{25} showed that AG can be beneficial for growth and proliferation of fibroblasts. It appears that AG stops the cell division or prevents the entry of cells from the M or S phase into the G0 phase of cell cycle. The results of Wang et al. support our findings.

Blood supply is a critical factor influencing the healing of fractures\textsuperscript{26} and considering the role of NO in angiogenesis and vasodilation; it may be specifically important in bone healing. NO stimulates the proliferation of endothelial cells, protects them

| Table 2: Distribution of healing scores in the control, placebo, and aminoguanidine groups |
|------------------|------------------|------------------|------------------|------------------|
| Groups           | Stage A (Score 1) | Stage B (Score 2) | Stage C (Score 3) | Stage D (Score 4) |
| Control, n (%)   | 0                | 4 (33.3)         | 8 (66.7)         | 0                | 12 (100.0)       |
| Placebo, n (%)   | 0                | 5 (41.7)         | 7 (58.3)         | 0                | 12 (100.0)       |
| AG, n (%)        | 0                | 1 (8.3)          | 8 (66.7)         | 3 (25.0)         | 12 (100.0)       |

P value between control and placebo groups - 0.680. P value between control and AG groups - 0.038. P value between placebo and AG groups - 0.021.

AG: Aminoguanidine

| Table 3: The mean percentage of lamellar, woven and total bone formation at 8 weeks in the control, placebo, and aminoguanidine groups |
|------------------|------------------|------------------|
| Group            | Lamellar         | Woven            |
| Control          | 20.94±2.03       | 20.22±2.22       |
| Placebo          | 20.53±1.20       | 20.72±3.12       |
| AG               | 22.06±1.90       | 20.03±2.12       |

P value between control and placebo groups - 0.954. P value between control and AG groups - 0.026. P value between placebo and AG groups - 0.013 for lamellar bone formation. AG: Aminoguanidine
from apoptosis and mediates the production of vascular endothelial growth factor.\[^{27}\] NO produced by endothelial NOS (eNOS) has both pro- and anti-inflammatory properties. Under physiological conditions, NO released from the endothelium regulates vascular tonicity and maintains the vessels’ patency through preventing platelet accumulation and decreasing the expression of adhesion molecules.\[^{28}\]

Corbett\[^{29}\] et al. demonstrated that in the primary phase of fracture healing, the expression of eNOS in cortical blood vessels and osteoblasts reaches its maximum level. As the process of healing progresses, this expression returns to its baseline value. High levels of NO and maximum intensity of vascular reaction in the primary phase of healing are probably responsible for the increased blood flow during this period.\[^{29,30}\]

The correlation of angiogenesis and wound healing has yet to be confirmed. Increased blood flow in the inflammatory phase of healing results in delivery of higher levels of inflammatory mediators to the site which delay the process of healing. In contrast, angiogenesis seems necessary in the proliferative phase of healing.\[^{31}\]

The role of NO in wound healing is complex and multifactorial. Baldik\[^{32}\] et al. showed that local administration of single dose bovine serum albumin containing NO along with demineralized bone matrix-induced new bone formation by 62% higher than that by bone matrix alone in femoral bone defects in rats at 10 weeks postoperatively. This finding was in contrast to our obtained results; which may be due to the different functions of NO at different concentrations. They also demonstrated that oral administration of AG enhanced defect filling. Giardino\[^{33}\] et al. explained that the positive role of AG in bone healing may be attributed to the protection against adverse and destructive effects of excessive NO production. Paul-Clark\[^{34}\] et al. in their study on a rat model evaluated the effect of direct and indirect administration of NOS inhibitors on the intensity of inflammation. In acute inflammation, delivery of NOS inhibitors directly into the site of inflammation aggravated the inflammatory response and prolonged the process of healing. Several mechanisms may explain the more severe inflammatory response. The inhibition of NO production increases the level of histamine, leukotriene B4, cytokine-induced neutrophil chemoattractant and free oxygen radicals, which indicates the protective role of local production of NO in the process of inflammation. On the other hand, systemic administration of NO inhibitors decreases inflammation.\[^{34}\] It appears that NOS inhibitors have different effects on the intensity of inflammation, depending on their method of administration. In the absence of acute inflammation, the use of local NO inhibitors did not increase the infiltration of inflammatory cells. This result was in line with our findings. In addition to the method of the administration of NO inhibitors, the prescribed dosage can also affect the intensity of inflammation and rate of healing. Leitão\[^{18}\] et al. reported that 5 and 10 mg/kg doses of AG significantly decreased BR in rat experimental models with artificially induced periodontal lesions; whereas, 100 mg/kg dose of AG did not inhibit alveolar bone loss or local inflammatory changes. These results may be attributed to the fact that high concentrations of AG inhibit physiologic NOS. To achieve optimal effects, administration of NOS inhibitors at low doses may be more efficacious than the use of high doses.\[^{35}\]

Farhad\[^{14}\] et al. in their study on a cat model evaluated the effect of systemic administration of AG on the severity of inflammation and demonstrated that AG significantly decreased the severity of inflammation in periapical lesions. In another study, Farhad\[^{15}\] et al. assessed the effect of systemic administration of AG on healing rate of periapical tissues and showed that selective NO inhibitor enhanced the healing of periapical lesions. The first phase of healing is based on an inflammatory process. Thus, enhanced recovery of periapical lesions in their study may be attributed to the suppression of local inflammation due to the inhibition of NOS in the periapical region. The results

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**Figure 5**: Histological view of LB formation; (a) control group; (b) placebo group; (c): Aminoguanidine group (H and E, x100). F: Fibrous connective tissue; LB: Lamellar bone and BR: Bone resorption.
of the afore-mentioned two studies\cite{14,15} were in accordance with our findings. This indicates that the local application of AG may have the same desired effects as systemic administration of iNOS inhibitors without the unwanted systemic side effects.

Nonspecific NOS inhibitors such as L-NAME interfere with eNOS and neuronal NOS, which have important physiological functions.\cite{36} Thus, selective iNOS inhibitors are ideal for the alleviation of inflammatory responses with no adverse effects on physiological reactions. Considering the wide spectrum of biological functions of NO, its inhibition may bring about numerous systemic effects. Thus, in the clinical application of NOS inhibitors, local administration must be preferred to systemic administration. For this reason, the current study was designed to assess the process of healing with the local application of AG.

Commercially, available AG is supplied in the form of crystals. Thus, for application into bone defects, it requires a carrier for easy handling. On the other hand, if this material is applied to the bone defect alone, it is quickly washed out of the area by the tissue fluids and would only exert a short-term effect. To maintain the material in the desired location for a longer period, a slow-release gel base is required. AG is soluble in carboxymethyl cellulose polymer gel. To ensure the neutral state of this gel and that it has no effect on healing, the base gel alone (without AG) was applied to bone defects in the placebo group. The AG gel used in the current study gradually releases AG within 2 weeks. The expression of iNOS is the highest in the primary phase of acute inflammation\cite{37} and over time, the activity of iNOS probably decreases due to the elimination of inflammatory responses or cytokine signals.\cite{38} Thus, it can be assumed that the gel used in this study releases AG at the target site for a sufficient period.

**CONCLUSION**

This study presented evidence about the role of NO in the process of bone healing. The obtained results supported the local application of selective iNOS inhibitors such as AG for enhancement of bone healing. However, AG has yet to be used in the clinical setting and requires further investigations. AG may be suitable for future use in periapical surgeries involving bone defects. Future studies are required to investigate the local application of AG at lower concentrations to determine the minimum concentration of AG gel capable of significantly enhancing bone healing.

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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 nonfinancial in this article.

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