Türk Biyokimya Dergisi [Turkish Journal of Biochemistry–Turk J Biochem] 2007; 32 (4) ; 160–164.

Research Article [Araştırma Makalesi]

Yayın tarihi 30 Aralık, 2007 © TurkJBiochem.com

[Published online 30 December, 2007]

# Effect of L-NAME Administration on Plasma, Heart Tissue NO and MDA Levels in Rabbit

## [Tavşanda L-NAME Uygulamasının Plazma, Kalp Dokusu NO ve MDA Seviyelerine Etkisi]

[1]Mahmut Selvi,
[2]Fehmi Karataş,
[3]Şule Coşkun,
[2]Deniz Erbaş

[1] Department of Biology Education, Gazi Faculty of Education, Gazi University, Teknikokullar, Ankara, 06500, Turkey.
[2] Department of Physiology, Faculty of Medicine, Gazi University, Ankara, Turkey.
[3] Departments of Biology, Science & Art Faculty, Gazi University, Ankara, Turkey.

**Yazışma Adresi**
[Correspondence Address]

Mahmut Selvi

Department of Biology Education, Gazi Faculty of Education, Gazi University, Teknikokullar, Ankara, 06500, Turkey.
E-mail: mselvi@gazi.edu.tr Tel.: (90) 312 2028096, Fax.: (90) 312 2228483.

Kayıt tarihi: 17 Eylül 2007, Kabul tarihi: 08 Kasım 2007
[Received: 17 September 2007, Accepted 08 November 2007]

**ABSTRACT**

Nitric oxide, produced in many mammalian tissues, is well known as an important mediator of many physiological and pathological responses. Nitric oxide synthesis plays an important role in many aspects of inflammation and wound healing. The purpose of this article is to comparatively investigate the effects of titanium, titanium + polyethylene glycol 4000, titanium + polyethylene glycol 4000 + N-nitro-L-arginine methyl ester and intraperitoneally injected N-nitro-L-arginine methyl ester, on plasma nitric oxide, malondialdehyde and, heart tissue nitric oxide and malondialdehyde levels. Plasma malondialdehyde, Nitrite and Nitrate and heart tissue malondialdehyde, nitrite and nitrate levels decreased after i.p. N-nitro-L-arginine methyl ester administration ($p < 0.05$). This leads to the conclusion that N-nitro-L-arginine methyl ester inhibits the deteriorating effects of free radicals without affecting wound healing. N-nitro-L-arginine methyl ester in polyethylene glycol 4000 and titanium also have no effect on tissue and plasma nitrite and nitrate and malondialdehyde levels meaning that nitric oxide generation is not affected from titanium and local nitric oxide synthase inhibitor.

**Key Words:** Nitric Oxide, N-nitro-L-arginine methyl ester, Titanium.

**ÖZET**

Birçok memelinin dokularında üretilen nitrik oksit fizyolojik ve patolojik cevapların önemli aracısıdır. Nitrik oksit sentezi birçok inflamasyon durumunda ve yara iyileşmesinde önemli rol oynar. Bu çalışmada Titanyum, titanyum + Polietilen glikol 4000, titanyum + Polietilen glikol 4000 + N-nitro-L-arjinin metil ester ve intraperitonal olarak uygulanan N-nitro-L-arjinin metil esterin, plazma nitrik oksit, plazma malondialdehit, kalp dokusu nitrik oksit ve malondialdehit düzeylerine etkilerinin araştırılması amaçlanmıştır. Plazma malondialdehit, nitrit ve nitrat ve kalp dokusu malondialdehit, nitrit ve nitrat düzeyleri i.p. N-nitro-L-arjinin metil ester uygulaması ile azalmıştır ($p<0.05$). Sonuçlar N-nitro-L-arjinin metil esterin iyileşme sürecini etkilemeksizin serbest radikallerin zararlı etkisini inhibe ettiğini düşündürtmektedir. Polietilen glikol 4000 içindeki N-nitro-L-arjinin metil ester ve titanyumun, doku ve plazma nitrit ve nitrat ve malondialdehit seviyeleri üzerine etkisi olmaması nitrik oksit üretiminin titanyum ve lokal nitrik oksit sentaz inhibitöründen etkilenmediğini düşündürtmektedir.

**Anahtar Kelimeler:** Nitrik Oksit, N-nitro-L-arjinin metil ester, Titanyum.

## INTRODUCTION

Nitric oxide (NO), derived both from the vascular endothelium and other cells in the cardiovascular system, plays a vital role in regulating vascular tone (1,2). Nitric oxide is known to play an important role in biological systems. NO is an endogenous gas that serves as biological messenger in many physiological processes including blood pressure control, neurotransmission, immune system ability to kill tumor cells and wound healing (3).

In addition, it is an important physiological and pathophysiological mediator in cardiovascular diseases. Nitric oxide is synthesized from L-arginine by three isoforms of nitric oxide synthase (NOS) (4). Two of the isoforms (neural NOS (nNOS) and endothelial NOS (eNOS)) are constitutive, and eNOS is found predominantly in endothelial cells. The third isoform, inducible NOS (iNOS), an inducible isoform, can be expressed in macrophages, vascular smooth muscle cells, and endothelial cells upon induction by cytokines and bacterial lipopolysaccharides. eNOS is a physiologically significant vasodilator and an inhibitor of vascular smooth muscle cell proliferation and platelet aggregation, and is generally considered to have cardiovascular protective and anti-atherosclerotic roles (5,6).

An increase in oxygen free radicals causes the deterioration of cell integrity and tissue necrosis (7). NO and superoxide ($O_2^-$) combine to form the highly cytotoxic peroxynitrite ($ONOO^-$) (8). This causes lipid peroxidation, whereas NO alone reacts with lipophilic peroxyl radicals preventing lipid peroxidation (9). As well known, the most damaging effect of free radicals is on lipid peroxidation. MDA is a relatively stable end product of lipid peroxidation. Cells or cellular fluid have reactive oxygen species (ROS) scavengers (10).

Polyethylene glycol (PEG) polymers are slow release vehicles for chemicals and are used for tissue adhesion. These polymers are used as a vehicle for the local delivery of exogenous substances (11). Titanium (Ti) has received a great deal of attention by dental researchers and clinicians. Titanium is considered the most ideal metal for in vivo applications because of its excellent biocompatibility. As a result, titanium and its alloys have been used extensively in the last several decades as materials for orthopedic implants, dental implant and medical devices (12). L-NAME is an isoform non-specific inhibitor of nitric oxide synthase (NOS) (10).

The purpose of the present study was to examine the systemic and local roles of L-NAME by investigation of plasma and heart tissue lipid peroxidation levels together with NO levels in rabbits.

## MATERIALS AND METHODS

### *Animals*

Female (n=30), New Zealand white rabbits, 5- months old, each weighing 2.5-3.0 kg were used. All animals were treated according to the guidelines laid down by the National Institutes of Health (NIH) in the USA regarding the care and use of animals for experimental procedures or with the European Communities Council Directive of 24 November 1986 (86/609/EEC). Animals were housed in clear plastic cages, fed with vegetables and tap water for 7 days prior to surgery.

### *Experimental protocol*

Anesthesia was administered intramuscularly using xylazine (5 mg/kg) + ketamine (35 mg/kg). Incisional wounds were made on right sides of mandibular mucosa between incisor and molar teeth of all rabbits under xylazine (5 mg/kg) + ketamine (35 mg/kg) anesthesia. After submucosal incisions animals were randomly assigned to one of 5 groups (13):

1- Untreated incisional group (n=6)
2- Titanium implanted group (n=6)
3- Titanium + PEG 4000 implanted group (n=6)
4- Titanium + PEG 4000 + L-NAME ($2x10^{-4}$ M) implanted group (n=6).
5- i.p. L-NAME administrated group (10 mg/kg) (n=6)

### *Materials*

The beads containing L-NAME (Sigma N 5751) were prepared under aseptic conditions. All glass materials and titanium molds were sterilized by dry heat. For the preparation of beads, polyethylene glycol 4000 was first melted and mixed with L-NAME solution. This mixture was delivered to molds with an automatic pipette. After solidification of the melted mass, the beads in titanium molds were used. The average weight of the beads together with titanium was 0.0725 ± 0.0025 g. Concentration of L-NAME in a bead was $2x10^{-4}$ M.

Control beads were prepared only with physiological saline instead of L-NAME solution. Intraperitoneal solution of L-NAME in the dose of 10 mg/kg was prepared in physiological saline. The solution was sterilized by filtration. Titanium rings were filled with L-NAME + PEG 4000 mixture to form bead shape forms and kept in closed ampules until assayed. Titanium rings outer diameter is 4.74 mm, inner diameter is 2.11 mm and height is 0.99 mm.

Wounds were closed with three silk sutures (3/0). Until the end of the experiments, all rabbits were fed with vegetables and tap water ad libitum. On day 7, rabbits were weighted and sacrified by an overdose of xylazine + ketamine. Plasma samples were obtained by cardiac puncture to investigate MDA and NOx levels. Heart-tissue samples were obtained and kept in a liquid nitrogen tank until analyses were performed.

Left ventricle samples were collected for tissue and plasma MDA level determinations. MDA levels were determined spectrophotometrically. NOx levels - stable end products of nitric oxide- in heart tissue and plasma were determined by the Griess reaction (14).

Tissue samples were homogenized in five volumes of

phosphate buffer (pH= 7.5) and centrifuged at 2000 x *g* for 5 min. To supernatants (0.5 ml) 0.25 ml of 0.3 M NaOH were added. After incubation for 5 min at room temperature, 0.25 ml of 10 % (w/v) $ZnSO_4$ was added for deproteinization. This mixture was then centrifuged at 14000 x *g* for 5 min and supernatants were used for the Griess assay.

Nitrate levels in tissue homogenates were determined spectrophotometrically, based on the reduction of nitrate to nitrite by $VCI_3$ (Vanadium (III) chlorid) (15). Nitrite levels were measured by the Griess reaction. Sodium nitrite and nitrate solutions (1, 10, 50, 100 μM) were used as standards.

Plasma samples were deproteinized prior to the assays. Plasma was added to 96 % cold ethanol (1/2 v/v) and then vortexed for 5 min. After incubation for 30 min at + 4 °C, the mixture was centrifuged at 14000 x *g* for 5 min and the supernatants were used for the Griess assay. Plasma MDA levels were assayed with spectrophotometric methods (16). Heparinized blood was obtained from rabbits and immediately centrifuged. The plasma was stored in liquid nitrogen for subsequent use.

Lipid peroxidation was quantified by measuring the formation of thiobarbituric acid reactive substances (TBARS). Briefly, 0.5 ml aliquots were removed from each tube after incubation with varying amounts of plasma or lymph in the presence of either AAPH or activated PMNs, centrifuged to remove cells and the supernatant added to 1 ml of a solution containing 15 % (w/v) TCA, 0.375 % (w/v) thiobarbituric acid, and 0.25 N HCl. Protein precipitant was removed by centrifugation, and the supernatants were transferred to glass test tubes containing 0.02 % (w/v) butylated hydroxytoluene to prevent further peroxidation of lipids during subsequent steps. The samples were then heated for 15 min at $100^0$C in a boiling water bath, cooled and centrifuged to remove precipitant. The absorbance of each sample was determined at 532 nm.

### *Statistical analysis*

Statistical analysis was carried out by using the statistical package SPSS for Windows, version 10.0 (SPSS Inc., Chicago, IL, USA). The results are presented as mean ± SD. Kruskal-Wallis test was used to compare the groups. Nonparametric Mann Whitney-U test was used to compare the two groups. P-values under 0.05 were considered as statistically significant.

## RESULTS

Plasma NOx levels were found to be decreased by i.p. L-NAME administration after oral mucosal incision. Plasma NOx levels were 79.25 ± 4.16 μM in the i.p. L-NAME given group. Differences between group 1, 2, 3, 4 compared to 5 were significant ($p < 0.05$) (Table 1). Heart tissue NOx levels were significantly lower when compared with the other four groups ($p < 0.05$) in i.p. L-NAME administered group (Table 1). This was expected; since L-NAME is a nonspecific NO synthase inhibitor. There were no statistically significant differences between other four groups when compared with each other ($p > 0.05$).

Plasma MDA levels were decreased compared with the other four groups in i.p. L-NAME administered group ($p < 0.05$) (Table 1). Heart tissue MDA levels were also decreased significantly in i.p. L-NAME administered group, compared with other four groups ($p < 0.05$) (Table 1). No significance was found ($p > 0.05$) when the other four groups were compared with each other.

## DISCUSSION

The results of the present study indicate that plasma NOx and MDA levels decreased when compared with other groups by i.p. L-NAME administration. Systemic L-NAME administration is more effective than the L-NAME implantation group in plasma NOx and MDA levels.

**Table 1.** The effect of L-NAME administration on plasma NOx, MDA levels and heart tissue NOx, MDA levels (mean ± SD)

| Groups | Plasma NOx levels (μM) | Heart tissue NOx levels (μmol/g) | Plasma MDA levels (nmol/ml) | Heart tissue MDA levels (nmol/g) |
|---|---|---|---|---|
| Group 1 (n=6) | 105 ± 14.2* | 13.78 ± 3.33* | 3.69 ± 0.35* | 6.18 ± 1.22* |
| Group 2 (n=6) | 96.8 ± 25.2* | 16.61 ± 8.97* | 3.35 ± 0.13* | 6.20 ± 1.87* |
| Group 3 (n=6) | 130 ± 5.83* | 11.39 ± 2.63* | 3.48 ± 0.13* | 4.40 ± 1.04* |
| Group 4 (n=6) | 115.4 ± 10.8* | 8.44 ± 1.50* | 3.39 ± 0.33* | 3.50 ± 0.38* |
| Group 5 (n=6) | 79.25 ± 4.16** | 5.65 ± 0.92** | 2.35 ± 0.16** | 2.50 ± 0.69** |

Group 1: Untreated wound

Group 2: Titanium (Ti) implanted wound.

Group 3: PEG within Ti implanted wound.

Group 4: L-NAME within PEG +Ti implanted wound

Group 5: i.p L-NAME administration (10 mg/kg)

(*-**) ($p < 0.05$)

The healing of deteriorated tissue is a critical biological response for surviving. Small and diffusible free radical NO acts as a important biological mediator on wound healing, immune system, dead of tumor cell and control of blood flowing. The beneficial effects of NO on wound healing may be attributed to its functional influences on angiogenesis, inflammation, cell proliferation, matrix deposition, and remodeling (17). Lee and coworkers reported maximal NOS activity on day 24 of wounding. This was followed by a steady decline over the next 5–7 days, with sustained production up to 10 days after wounding. The time course and magnitude of enzyme activity was paralleled by iNOS gene expression with an early burst of transcriptional activity at 24 h followed by continued low-level expression for up to 10 days. The initial peak of NO production is associated with the induction of iNOS in response to wounding (18).

Lohinai et al. (19), demonstrated that ligature induced periodontitis increased local NO production and mercaptoethylguanidine (MEG), a selective inhibitor of inducible nitric oxide synthase, treatment protected against the associated extravasations and bone destruction. Although exact function not clearly settled yet, salivary NO production may play a physiological role both as the natural antibacterial properties of salivary secretion and possibly in detoxification of oral carcinogens (20).

As well known, the most damaging effects of free radicals are lipid peroxidation. Malondialdehyde is a relatively stable end product of lipid peroxidation. An increase in free oxygen radicals causes the deterioration of cell integrity and tissue necrosis (21). In our study, heart tissue MDA levels were significantly decreased in the experimental group V (i.p. L-NAME administrated group; $p < 0.05$).

PEG is a water soluble polymer that is used in medical and pharmaceutical applications. PEG was used as a vector to carry and release the L-NAME from the titanium rings in this study. We have reconfirmed that PEG and titanium are inert materials for mouth.

Our findings confirmed that i.p. L-NAME is an inhibitor of plasma NOx and free radical levels. L-NAME in PEG and titanium also have no effect on tissue and plasma NOx levels meaning that NO generation will not be affected both from titanium and local NOS inhibitor.

Kim and Hwan Kim (22) suggested that L-NAME administration, deteriorated oxidative damage by ischemia reperfusion induced gastric mucosal injury in rats. In our study, a decrease on oxidative damage of plasma and heart tissue was also shown by administration of i.p L-NAME. L-NAME is a non-spesific NO synthase inhibitor. It inhibits all NOS enzymes in adrenal glands and brain (23,24). L-NAME has decreased oxidative stress by inhibiting NOS in this study.

The peripheral effects of a body applied on mouth tissue have been investigated in this study. The contribution and inhibition of NO on peripheral effects were investigated and no significant effect was found. However, synthesis of NO was inhibited by i.p application of L-NAME and oxidative deterioration on heart tissue and plasma in periphery was observed.

In conclusion, this is the first report on NO and lipid peroxide generation mechanism after titanium and systemic or local NOS inhibitor administration in the open literature. L-NAME inhibits the deteriorating effects of free radicals without affecting healing. L-NAME in PEG and titanium also have no effect on tissue and plasma NOx levels meaning that NO generation will not be affected either from titanium or local nitric oxide synthase (NOS) inhibitor. Similar studies on mouth are very scarce in the literature; are nearly absent, so this study will become certain in further studies on mouth wound healing and sustaining of mucosal homeostasis by investigating potential role of NO in salivary. This study is fundamental for the future research on mucosal healing.

## References


[1] Moncada S, Palmer RM, Higgs EA (1991) Nitric oxide: Physiology, pathophysiology and pharmacology. Pharmacol Rev. 43: 109-142.

[2] Nathan C (1992) Nitric oxide as a secretory product of mammalian cells. FASEB J. 6: 3051-3064.

[3] Howe LM, Boothe HW (2001) Nitric oxide. A review for veterinary surgeons. Vet Surg. 30 (1): 44-57.

[4] Forstermann U, Kleinert H (1995) Nitric oxide synthase: expression and expressional control of the three isoforms. Naunyn-Schmiedeberg's Arch Pharmacol.352: 351-364.

[5] Thiemermann C, Vane J (1990) Inhibition of nitric oxide synthesis reduces the hypotension induced by bacterial lipopolysaccharides in the rat in vivo. Eur J Pharmacol. 182: 591-595.

[6] Nava E, Palmer RM, Moncada S (1991) Inhibition of nitric oxide synthesis in septic shock: how much is beneficial? Lancet. 338: 1555-1557.

[7] Darley-Usmar V, Wiseman H, Halliwell B (1995) Nitric oxide and oxygen radicals. A question of balance (Review). FEBS Lett. 369: 131-135.

[8] Virag L, Szabo E, Bakondi E, Bai P, Gergely P, Hunyadi J, Szabo C (2002) Nitric oxide-peroxynitrite-poly(ADP-ribose) polymerase pathway in the skin. Exp Dermatol. 11: 189-202.

[9] Weller R (1997) Nitric oxide- a newly discovered chemical transmitter in human skin. Br J Dermatol. 137: 665-672.

[10] Coşkun Ş, Karataş F, Acartürk F, Olmuş H, Selvi M, Erbaş D (2005) The effect of L-NAME administrations after oral mucosal incision on wound NO level in rabbit. Mol Cell Biochem. 278: 65-69.

[11] Reece TB, Maxey TC, Kron IL. (2001) A prospectus on tissue adhesives. Am J Surg. 182: 40S-44S.

[12] Ustun Y, Esen E, Toroglu MS, Akova T (2004) Multidisciplinary approach for the rehabilitation of dentoalveolar trauma. Dent Traumatol. 5: 293-299.

[13] Camerman P, Fuller A (2000) Effects of nitric oxide synthase inhibitors on the febrile response to lipopolisaccharide and muramyl dipeptide in guinea pigs. Life Sci. 67: 2639-2645.

[14] Green LC, Wagner DA, Glogowski J, Skipper PL, Wishnok JS, Tannenbaum SR (1982) Analyses of nitrate, nitrite and [15N] nitrate in biological fluids. Anal Biochem. 126: 131-138.

[15] Miranda KM, Espey MG, Wink DA (2001) A rapid simple spectrophotometric method for simultaneous detection of nitrate and nitrite. Nitric Oxide. 5: 67-71.

[16] Kurtel H, Granger DN, Tso P, Grisham MB (1992) Vulnerability of intestinal fluid to oxidant stress. Am J Physiol Soc. 263: G: 573-578.

[17] Luo JD, Chen AF (2005) Nitric oxide: a newly discovered function on wound healing. Acta Pharmacol Sin. 26: 259-264.

[18] Lee RH, Efron D, Tantry U, Barbul A (2001) Nitric oxide in healing wound. A time course study. J Surg Res. 101: 104-110.

[19] Lohinai Z, Benedek P, Feher E, Gyorfi A, Rosivall L, Fazekas A, Salzman AL, Szabo C (1998) Protective effects of mercaptoethylguanidine, a selective inhibitor of inducible nitric oxide synthase, in ligature-induced periodontitis in the rat. Br J Pharm. 123: 353-360.

[20] Bodis S, Haregewoin A (1993) Evidence for the release and possible neural regulation of nitric oxide in human saliva. Biochem Biophys Res Com. 194: 347-350.

[21] Grace PA (1994) Ischaemia-reperfusion injury. Br J Surg. 81: 637-647.

[22] Kim H, Hwan Kim K (2001) Role of nitric oxide and mucus in ischaemia reperfusion induced gastric mucosal injury in rats. Pharmacology. 62: 200-207.

[23] Palacios M, Knowles RG, Palmer, RMJ, Moncada S (1989) Nitric oxide from L-arginine stimulates the soluble guanylate cyclase in adrenal glands. Biochem Biophys Res Commun. 165: 802-809.

[24] Knowles RG, Palacios M, Palmer RMJ, Moncada S (1990) Kinetic characteristics of nitric oxide synthase from rat brain. Biochem J. 269: 207-210.