Influence of L-NAME and L-Arg on ischaemia-reperfusion induced gastric mucosa damage

Hayrettin Öztürk[1], Ismail H. Kara[2], Selçuk Otçu[1], Nihal Kilinc[3], Yusuf Yagmur[4]

Dicle University, Faculty of Medicine, (1) Department of Paediatric Surgery ; (2) Department of Family Practice ; (3) Department of Pathology ; (4) Department of General Surgery Diyarbakır, Turkey.

Abstract

Objective : **The aim of this study was to investigate effects of L-NAME and L-Arginine on gastric mucosal injury induced by ischaemia-reperfusion.**

Methods : **In the experiment, 20 New Zealand rabbits were used (2700-3000 g). Celiac artery was clamped for 30 min for ischaemia and then 60 min of reperfusion followed this after all rabbits were anaesthetized. In the Sham-control group (G 1, n = 5), laparotomy was performed, and the celiac artery was prepared without clipping. Group 2 (Untreated, n = 5) rabbits were only subjected to ischaemia-reperfusion. Group 3 (n = 5) rabbits had L-Arginine Methyl Ester (L-Arg) 3 mg/kg/min as IV infusion during the first 15 min of the reperfusion. Group 4 (n = 5) rabbits had a nitric oxide inhibitor NG-nitro-L-arginine methyl ester (L-NAME) 100 µg/kg/min IV during the first 15 min of the reperfusion. After 60 min of reperfusion, the rabbits were killed, and their stomachs were removed for histopathologic evaluation and determination of malondialdehyde (MDA) level.**

Results : **After ischaemia-reperfusion, Untreated group had macroscopic necrosis involving 50 ± 6% of total gastric mucosa area and deep mucosal necrosis involving 10 ± 5% of mucosal strips. In the group treated with L-NAME, macroscopic mucosal necrosis involved 52 ± 6% of total gastric mucosa area and deep mucosal necrosis involved 11 ± 3% of mucosal strips (both $p > 0.05$ versus Untreated group). L-Arg treatment significantly reduced macroscopic mucosal necrosis area to 20 ± 6% and deep mucosal necrosis to 3 ± 1% (both $p < 0.05$ versus Untreated group and L-NAME group). MDA level in the L-Arg group was significantly lower when compared to control and L-NAME group MDA level ($p < 0.05$).**

Conclusion : **These results suggest that NO increase induced by L-Arginine injection is involved in the protection of gastric mucosa after ischaemia-reperfusion.** (Acta gastroenterol. belg., **2002**, 65, **150-154**).

Key words : gastric mucosal injury, ischaemia-reperfusion, nitric oxide, L-Arginine Methyl Ester, NG-nitro-L-arginine methyl ester.

Introduction

The involvement of nitric oxide (NO) is a mutual in increasing variety of physiological process in many tissues including the gastrointestinal tract. NO accounts for the biological activity of endothelium-derived relaxing factor (1) and modulate gastrointestinal blood flow as well as vascular and mucosal integrity (2-4). NO also acts as a neurotransmitter involved with non-adrenergic non-cholinergic relaxation of gastric and intestinal smooth muscle and sphincter tone (5). In all of these situations, NO is produced from L-Arginine by a constitutive Ca^{++} dependent form of the enzyme nitric oxide synthase (NOS). NG-nitro-L-arginine methyl ester (L-NAME) would dose-dependently inhibit gastric mucosal NO synthesis and decrease mucosal blood flow (6). However, it is not clear if NO is cytotoxic or cytoprotective toward gastric mucosal lesions in ischaemia-reperfusion (7).

The aim of the present study was to evaluate whether NO protects gastric mucosa against ischaemia-reperfusion injury in rabbits. For investigation of the role of NO, we examined the effects of the L-Arginine injection induced NO increase and NO synthase inhibitor L-NAME on the formation of acute gastric mucosal lesions induced by ischemia-reperfusion in the rabbits.

Materials and methods

Experimental procedure

This experimental study was done in the Dicle University Animal Research Laboratory. 20 New Zealand female rabbit (2700-3000 g) were fasted overnight but allowed free access to water. Anesthesia was induced with ketamine hydrochlor 100 mg/kg and xylocain 3 mg/kg IV. A catheter (24 Gauge) was also placed in a femoral vein for drug administration. The stomach was exposed through a ventral midline abdominal incision. Acute gastric mucosal injury was produced by ischaemia-reperfusion. For this purposes, we adapted Wada K. *et al*. (8) method, which was done on rats to our rabbits study. Celiac artery was clamped for 30 min for ischaemia and then 60 min of reperfusion was done. In group 1 rabbits (Sham-control, n = 5), laparotomy was performed and the celiac artery was prepared without clipping. In the group 2 rabbits (Untreated, n = 5), celiac artery was clamped 30 minutes for making ischaemia, clamp was removed and 60 minutes reperfusion was performed. In group 3 rabbits (L-Arg, n = 5), 30 minutes ischaemia and 60 minutes reperfusion was performed and L-Arginine Methyl Ester (3 $mg^{-1}kg^{-1}min^{-1}$, Sigma Chemical Co., St. Louis, USA) infused for 15 minutes in the beginning of reperfusion. In group 4 rabbits (L-NAME, n = 5), after 30 minutes ischaemia, a nitric oxide inhibitor, NG-nitro-L-arginine methyl ester (100 $\mu g^{-1}kg^{-1}min^{-1}$, Sigma Chemical Co., St. Louis, USA)

Correspondent address : Hayrettin Öztürk, M.D., Dicle University, Medical School, Department of Pediatric Surgery, 21280 Diyarbakır, Turkey. E-mail : ozturkhayrettin@ hotmail.com.

Table 1. — **Mean values of MDA and macroscopic and microscopic gastric mucosal damage of the all groups**

Groups	MDA (nmol/mg wet tissue)	Macroscopic necrosis (% of total mucosal area)	Microscopic necrosis (% of mucosal strip length)
Sham-control	5.17 ± 1.58	0	0
Untreated	38.04 ± 4.42	50.6 ± 6	10 ± 5
L-Arg	15.75 ± 3.41*	20 ± 6*	3 ± 1*
L-NAME	42.55 ± 3.78	52 ± 6	11 ± 3

Note. Values expressed as mean ± SD.
**P < . 05* compared with Untreated and L-NAME groups *(Mann-Whitney U test).*

was infused for 15 minutes at the beginning of 60-minute reperfusion. After sixty minutes of reperfusion, the rabbits were killed, and their stomachs were removed for histopathological evaluation and determination of malondialdehyde (MDA) level.

Gross gastric mucosal analysis

All rabbits underwent stomach excision after ischaemia-reperfusion and drug administration. The stomach was opened along the greater curvature and photographed in standard fashion. The extent of macroscopic necrosis was measured by computerized image analysis as previously described (9,10).

Histologic analysis

Standardized specimens of the gastric wall (0.5 cm width, 1.5 cm length) were used. Specimens were cut obliquely from the corpus mucosa. The specimens were stained with hematoxylin and eosin after fixation in 10% buffered formalin and paraffin embedding. They were evaluated qualitatively and quantitatively and the extent of histologic deep mucosal necrosis, defined as necrotic lesions penetrating into the mucosa deeper than 100 µm, was measured with an objective mounted micrometer (200× magnification, Olympus Eyepiece Micrometer®). The data were expressed as a percentage of the total length of the evaluated mucosal strip (11). Both macroscopic and histologic evaluations were performed in blinded fashion on coded mucosal specimens.

Determination of Malondialdehyde

The degree of lipid peroxidation in tissue homogenates was assessed by the method of Ohkawa *et al*., (12) measuring MDA levels. The principle of the method is based on measuring the concentration of the pink chromogen compound that forms when MDA couples to thiobarbituric acid (TBA). MDA was used as the standard, and the appreciation was performed using a standard curve obtained from MDA-TBA reaction, as described in the method (12). The protein content of homogenates was determined according to the procedure of Lowry *et al*., (13) and values were expressed as nanomoles of MDA per milligram of protein (nmol MDA/mg protein). All analyses were performed in duplicate.

Statistical Analyses

Statistical analyses were performed with SPSS 7.5 computer software. Multiple non-parametric comparative analyses were done with Kruskal-Wallis One Way ANOVA and then Mann-Whitney U test was used. A *P* value less than 0.05 were considered to be statistically significant.

Results

Histopathology

No morphological damage was observed in any of the rabbit in the Sham-control group (Fig. 1A). After ischaemia-reperfusion, untreated group had macroscopic necrosis involving 50 ± 6% of total gastric mucosa area. Histology revealed desquamation of the surface epithelial cells, with many areas of hemorrhagic necrosis extending from the surface to the midmucosa or even to the basal mucosa (Table 1) (Fig. 1B).

In the group treated with L-NAME, macroscopic mucosal necrosis involved 52 ± 6% of total gastric mucosa area and deep mucosal necrosis involved 11 ± 3% of mucosal strips (both $p > 0.05$ versus Untreated group) (Table 1) (Fig. 1C).

L-Arg treatment significantly reduced macroscopic mucosal necrosis area to 20 ± 6% and deep mucosal necrosis to 3 ± 1% (both $p < 0.05$ versus Untreated group and L-NAME group) (Table 1) (Fig. 1D).

Malondialdehyde

Mean MDA levels in the Sham-control, Untreated, L-Arg and L-NAME group were 5.17 ± 1.58, 38.04 ± 4.42, 15.75 ± 3.41, and 42.55 ± 3.78 nmol/mg wet tissue respectively. Mean MDA values were again significantly increased in the Untreated, L-NAME and L-Arg groups in comparison with the Sham-control group ($p < 0.0001$, $p < 0.001$, $p < 0.0001$ respectively). On other hand, MDA level in the L-Arg group was significantly lower when compared to Untreated and L-NAME group MDA level ($p < 0.05$). No significant difference was found between MDA levels of L-NAME group and Untreated group (Table 1).

Discussion

Several studies have demonstrated the phenomenon of reperfusion injury (14,15). Parks and Granger have shown that relatively little injury to the intestinal mucosa occurs during the ischaemic period, the majority occurring during reperfusion (16). The injury observed after 3 hours of ischaemia (blood flow reduced to 20% of normal) and 1 hour of reperfusion is more severe than that observed after 4 hours of ischaemia (14). From this

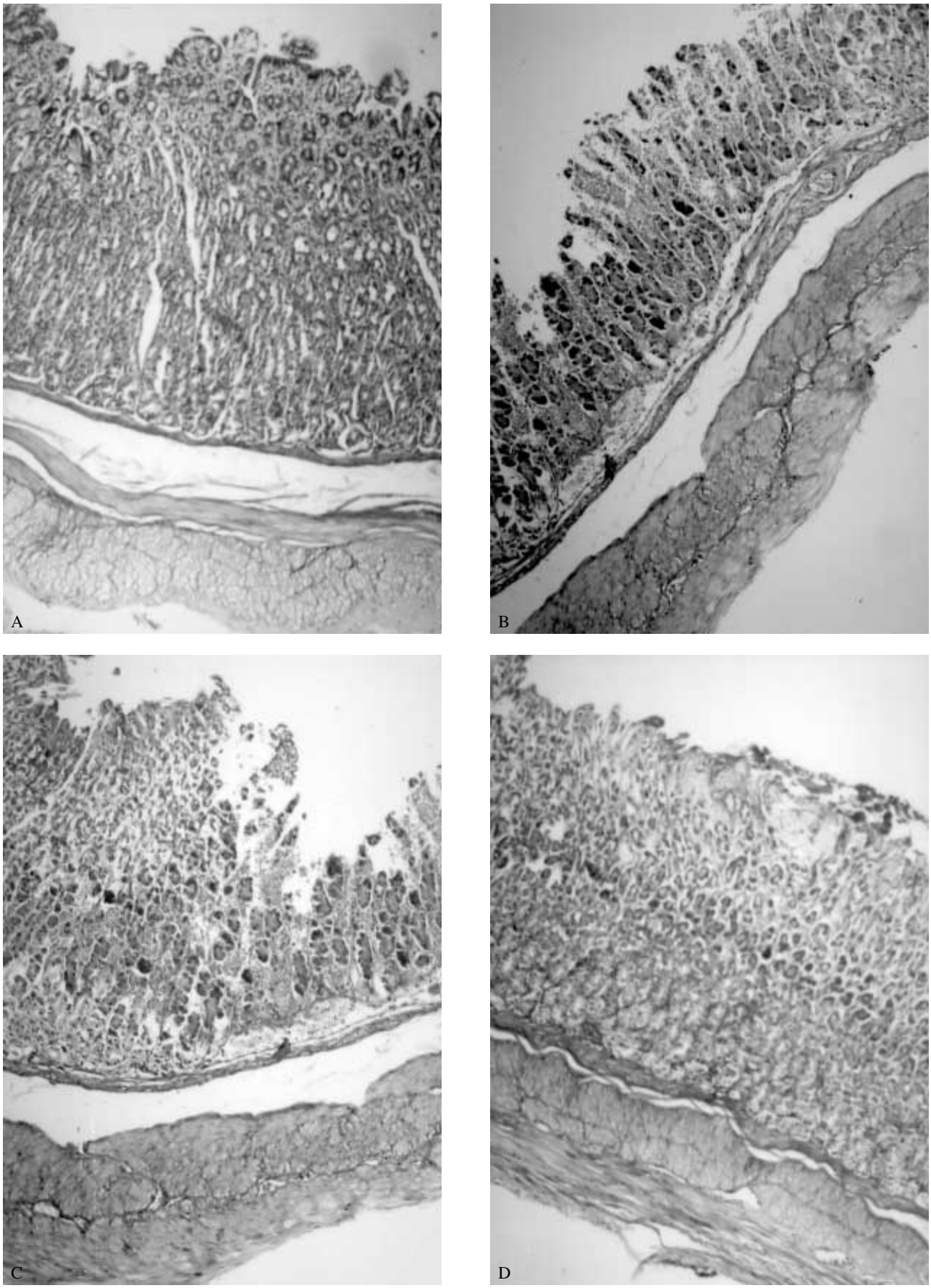

Fig. 1. — (A) There are no histological abnormalities in Sham-control group, (B-C) gross gastric mucosal necrosis with ischaemia-reperfusion Untreated and L-NAME groups, (D) In L-Arg treated group, mucosal necrosis was reduced (Hematoxylin-Eosin X 100).

point, we also chose a 60-min reperfusion period with aim of seeing complete effects of reperfusion injury phenomenon.

NO may protect the gastrointestinal mucosa from a variety of stimuli (caustic ingestion, ischaemia, ischaemia/reperfusion injury, early endotoxic shock) by maintaining mucosal perfusion, inhibiting neutrophil adhesion to mesenteric endothelium, blocking platelet adhesion, and preventing mast cell activation (17).

NO is produced from L-arginine by a constitutive Ca^{++} dependent form of the enzyme nitric oxide synthase (NOS). The distribution of gastric mucosal NOS activity between cells separated by counter flow centrifugation into fractions of different size indicated its presence in mucus-containing epithelial cells. It was suggested that NO could be involved in both the function and integrity of gastric epithelial cells (18).

We noticed that NO increase induced by L-Arg injection significantly reduced ischaemia-reperfusion induced gastric microscopic and macroscopic mucosal necrosis. Our result was similar to those results printed before (17,18). This may be explained by NO tonic vasodilator effect modulating gastric blood flow and oxygen uptake through influence on the gastric microcirculatory structures responsible for vascular resistance and the nutrient circulation (19,20). On the other hand, gastric mucosal lesions in the L-NAME group were not significantly increased when compared to untreated group. This may be explained in two ways. The first was previously explained as that inhibition of NO with L-NAME may be ineffective because of low level of NOS however L-Arg induce NOS and shows protective effects (21). The second may be explained under view of Wada study. Our use of L-NAME at the beginning of reperfusion may be ineffective because of Rapid decrease in NO level the baseline level just after the removal of the clamp on the celiac artery. In Wada *et al*. study (22), effect of L-NAME on NO levels during ischemia was studied and *w*ith the clamping of the celiac artery, a continuous increase in NO level was observed during ischemia. However, just after the removal of the clamp, the NO level rapidly decreased to the baseline level. Until ischemia, administration of L-NAME (30 mg/kg i.p) did not show a significant reduction in the NO level compared with the baseline level. The clamping of the celiac artery caused only a slight increase in NO level compared with the baseline value.

Gastrointestinal ischaemia-reperfusion injury is characterized by vasoconstriction, microcirculatory deficit, and cellular inflammation and lipid mediator synthesis (23). Lipid peroxidation measurement is a more practical and safer method to evaluate the factors causing cellular injury and activation common pathway. The ability of hydroxyl radicals to initiate lipid peroxidation can result in the formation of lipid-derived free radicals such as conjugated dienes, lipid hydroperoxide radicals, and lipid hydroperoxides. MDA is the end product of lipid peroxidation and is a well-known parameter for determining the increased free radical formation in intestinal tissue (24,25). In the present study, our results showed an increase in MDA concentrations in gastric mucosa by the ischaemia-reperfusion. However, MDA level in the L-Arg group was significantly lower when compared to untreated and L-NAME groups.

These results indicate that NO increase induced by L-Arg injection treatment significantly reduces both macroscopic and microscopic gastric mucosal damage induced by ischaemia-reperfusion injury. These protective effects of NO may be due to its increasing effect of gastric mucosal microcirculation and acting as a free radical scavenger.

References

1. MONCADA S.R., PALMER M.J., HIGGS E.A. Nitric Oxide. Physiology, Pathophysiology, and Pharmacology. *Pharmacol Rev.*, 1991, **43** : 109-42.
2. BOUGHTON-SMITH N.K., HUTCHESON I.R., DEAKIN A.M., WHITTLE B.J., MONCADA S. Protective effect of S-nitrosa N-acetyl-penicilamine in endotoxin-induced acute intestinal damage in the rat. *Eur. J. Pharmacol*., 1990, **191** : 485-8.
3. PIQUE J.M., ESPLUGUES J.V., WHITTLE B.J.R. Endogenous nitric oxide as a mediator of gastric mucosal vasodilatation during acid secretion. *Gastroentrology*, 1992, **102** : 168-74..
4. WHITTLE B.J., LOPEZ-BELMONTE J., MONCADA S. Regulation of gastric mucosal integrity by endogenous nitric oxide : interactions with prostanoids and sensory neopeptides in the rat. *Br. J. Pharmacol*., 1990, **99** : 607-11.
5. SANDERS K.M., WARD S.M. Nitric oxide as a mediator of nonadrenergic noncholinergic neurotransmission. *Am. J. Physiol*., 1992, **262** : 379-92.
6. LIPPE I.T., HOLZER P. Participation of endothelium derived nitric oxide but not prostocyclin in the gastric mucosal hyperaemia due to acid back-diffusion. *Br. J. Pharmacol*., 1992, **105** : 708-14.
7. ANDREWS F. J., MALCONTENTI-WILSON C., O'BRIEN P.E. Protection against gastric ischemia-reperfusion injury by nitric oxide generators. *Dig. Dis. Sci*., 1994, **39** : 366-373 .
8. WADA K., KAMISAKI Y., KITANO M., NAKAMOTO K., ITOH T. Protective effect of cystathionine on acute gastric mucosal injury induced by ischemia-reperfusion in rats. *Eur. J. Pharmacol*., 1995, **294** : 377-382 .
9. SARFEH I.J., TARNAWSKI A., MALKI A., MASON G.R., MACH T., IVEY K.J. Portal hypertension and gastric mucosal injury in rats ; effects of alcohol. *Gastroenterology*, 1983, **84** : 987-93.
10. MAEDA R., GUILMETTE E., TARNAWSKI A., SARFEH I.J. Bile acid induced gastric mucosal injury ; significance of portal hypertension and capillary mucosal permeability. *J. Surg. Res*., 1984, **36** : 312-4.
11. ISHIKAWA T., TARNAWSKI A., SARFEH I.J. Epidermal growth factor protects gastric mucosa against ischemia-reperfusion injury. *J. Clin. Gastroenterol*., 1993, **17** : 104-8.
12. OHKAWA H., OHRISHI N., YAGI K. Assay for lipid peroxide for animal tissues by thiobarbituric reactions. *Anal. Biochem*., 1979, **95**: 351-358.
13. LOWRY O.H., ROSEBROUGH N.J., FAIR A.L. *et al*. Protein measurement with the folin phenol reagent. *J. Biol. Chem*., 1951, **193** : 265-275.
14. ZIMMERMAN B.J., GRANGER D.N. Reperfusion injury. *Surg. Clin. North Am*., 1992, **72** : 65-83.
15. AKGUR F.M., OLGUNER M., YENICI O., GOKDEN M., AKTUG T., YILMAZ M., ATAC G. The effect of alloprinol pretreatment on intestinal hypoperfusion encountered after correction of intestinal volvulus. *J. Pediatr. Surg*., 1996, **31** : 1205-1207.
16. PARKS D.A., GRANGER D.N. Contributions of ischemia and reperfusion to mucosal lesion formation. *Am. J. Physiol*., 1986, **250** : G749-53 .
17. SALZMAN A.L. Nitric oxide in the gut. *New Horiz*, 1995, **3** : 352-64.
18. BROWN J.F., TEPPERMAN B.L., HANSON P.J., WHITTLE B.J., MONCADA S. Differential distribution of nitric oxide synthase between cell fractions isolated from the gastric mucosa. *Biochem. Biophys. Res. Commun*., 1992, **184** : 680-85.
19. GUSTAW P., PAWLIK W.W., CZARNOBILSKI K., SENDUR R., KONTUREK S.J. Nitric oxide is involved in the mediation of gastric blood flow and tissue oxygenation. *J. Physiol. Pharmacol*., 1994, **45** : 361-8.

20. DURAKBASA C.U., DAGLI T.E., MOUNI H., HAKLAR G., BILSEL A.S., YUKSEL M., AKTAN A.O. Nitric oxide and endothelin relationship in intestinal ischemia/reperfusion injury. *Prostaglandins Leukot Essent Fatty Acids*, 1998, **59** : 379-83 .
21. MHANNA M.J., FERKOL T., MARTIN R.J., DRESHAJ I.A., VAN HEECKEREN A.M., KELLEY T.J., HAXHIU M.A. Nitric oxide deficiency contributes to impairment of airway relaxation in cystic fibrosis mice. *Am. Journal of Respir Cell Mol. Biol*., 2001, **24** : 621-626 .
22. WADA K., KAMISAKI Y., OHKURA T., KANDA G., NAKAMOTO K., KISHIMOTO Y., ASHIDA K., ITOH T. Direct measurement of nitric oxide release in gastric mucosa during ischemia-reperfusion in rats. *Am. J. Physiol*., 1998, **274** : G465-71 .
23. MANGINO J.E., KOTADIA B., MANGINO M.J. Characterization of hypothermic intestinal ischemia-reperfusion injury in dogs. Effects of glycine. *Transplantation*, 1996, **62** : 173-178 .
24. CAPLAN M.S., SUN X.M., HSUEH W. Hypoxia causes ischemic bowel necrosis in rats : The role of platelet-activating factor (PAF-Acheter). *Gastroenterology*, 1990, **99** : 979-86 .
25. CZYRKO C., STEIGMAN C., TURLEY D.L., DROTT H.R., ZIEGLER M.M. The role of reperfusion injury in occlusive intestinal ischemia of the neonate : Malonaldehyde derived fluorescent products and correlation of histology. *J. Surg. Res*., 1991, **51** : 1-4.