

## BASIC RESEARCH

## SODIUM NITROPRUSSIDE AS A NITRIC OXIDE DONOR IN A RAT INTESTINAL ISCHEMIA-REPERFUSION MODEL

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**AIM:** The aim of this study was to investigate the efficacy of sodium nitroprusside in the reduction of the intestinal ischemia-reperfusion injury as a nitric oxide donor after intraperitoneal administration.

**METHODS:** The histopathological examinations and tissue malonyldialdehyde levels of 35 Wistar albino rats that were subjected to ischemia-reperfusion, were performed in 5 groups. The groups include Control, Ischemia -reperfusion, Sodium nitroprusside, NG-Nitro-L-Arginine Methyl Ester (L-NAME) and Sodium nitroprusside+L-NAME. Each rat was subjected to ischemia for 40 minutes and reperfusion for 30 minutes, except the control group. The medications were done intraperitoneally as saline 4 ml/kg, Sodium nitroprusside 5 mg/kg, L-NAME 10 mg/kg just before reperfusions.

**RESULTS:** Significant tissue injury in histological sections and an increase in tissue levels of Malonyldialdehyde was detected in the I/R group. The efficacy of intraperitoneal administration of Sodium nitroprusside in both Sodium nitroprusside alone and Sodium nitroprusside+L-NAME groups was found statistically significant for the reducing of injury scores ( $p<0.05$ ). The difference between the Ischemia/reperfusion and Sodium nitroprusside groups was found statistically significant as in the Ischemia/reperfusion and Sodium nitroprusside+L-NAME groups due to the tissue Malonyldialdehyde levels ( $p<0.05$ ). There was no statistical difference between Ischemia/reperfusion and L-NAME groups.

**CONCLUSION:** Ischemia/reperfusion induced injury might be reduced by the intraperitoneal administration of Sodium nitroprusside, even in the presence of L-NAME, in the rat intestinal model.

**KEYWORDS:** Ischemia. Reperfusion. Intestine. Sodium nitroprusside. Rat.

## INTRODUCTION

It is a well known phenomenon that reperfusion is as dangerous for the tissues as ischemia, particularly in the heart and the intestines.<sup>1</sup> Intestinal ischemia is a result of systemic factors (hypovolemia, hypotension, hypoxia, sepsis) or local factors (mechanical obstruction of the intestine or the intestinal vessels). The restored circulation results in the formation of free oxygen radicals and other acute phase reactants. Resulting cellular death occurs via

the lipid peroxydation of the cell wall.<sup>2,3</sup>

The role of endogenous endothelin peptides in the intestinal ischemia-reperfusion (I/R) model has been well studied.<sup>4</sup> Although the exact mechanism is still not fully understood, investigations on nitric oxide (NO), an endothelial-derived relaxation factor, revealed its importance in the management of I/R injury.<sup>5,6</sup> NO inhibits lipid peroxidation via the lipophilic free radical effect.<sup>7</sup> There are many studies concerning NO agonists and antagonists, but the main concern in recent years has been NO donors releasing exogenous NO. 3-morpholinosydnonimine (SIN-1), spermine NO, diethanolamine nitric oxide (DEA/NO), S-nitroso-N-acetylpenicillamine (SNAP), FK409, S-nitrosoglutathione (GSNO), NOC5, NOC12, CAS 754, and Gliseryl trinitrate

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are the major products used.<sup>1,7</sup>

Sodium Nitroprusside (SNP) is another nitric oxide donor investigated for its intravascular and intraluminal effects on ischemia reperfusion injury. We investigated the effects of NO in I/R injury with the commercially available and inexpensive product sodium nitroprusside (SNP) administered intraperitoneally in a rat intestinal model.

## MATERIALS AND METHODS

### Ethics

This study was performed in the investigation laboratories of Gazi University with the permission of the Gazi University Ethical Committee.

### Study design

The study was conducted with 35 male Wistar albino rats weighing between 200-230 grams, divided into 5 groups. Rats were fed standard food until the last 12 hours of the study and were given water ad libitum. A midline incision was performed and the superior mesenteric artery was clamped for 40 minutes to create ischemia. Intestinal segments were taken into the abdominal cavity and a light source was used to avoid hypothermia. All medications were administered intraperitoneally (i.p) immediately before removal of the superior mesenteric artery clamp, and the intestines were allowed to reperfuse for 30 minutes after the abdominal wall was closed.

The groups are designed as:

Group 1 (Control)	Laparatomy		
Group 2 (I/R)	Ischemia 40 min.	Saline (4 ml/kg i.p.)	Reperfusion 30 min
Group 3 (SNP)	Ischemia 40 min.	SNP (5 mg/kg i.p.)	Reperfusion 30 min
Group 4 (L-NAME)	Ischemia 40 min.	L-NAME (10 mg/kg i.p.)	Reperfusion 30 min
Group 5 (SNP + L-NAME)	Ischemia 40 min.	SNP (5 mg/kg i.p.) +L-NAME (10 mg/kg i.p.)	Reperfusion 30 min

In the SNP group, Sodium Nitroprusside (Nipruss, ADEKA) was given i.p. at 5 mg/kg; in the N<sup>G</sup>-Nitro-L-Arginin Methyl Ester (L-NAME) group, L-NAME was given i.p. at 10 mg/kg (Sigma Chemical Co). In the SNP+L-NAME group, SNP and L-NAME were given i.p. at 5 mg/kg and 10 mg/kg, respectively

Intestinal tissue samples taken from the jejunum 30 minutes after the reperfusion were preserved in 10%

formaline solution for histopathological examinations and snap frozen in liquid nitrogen and then stored at -80°C for tissue MDA level determination.

### Pathological examination

Tissue samples were examined under the light microscope after haemotoxylen-eosine (H&E) staining using the criteria reported by Park and Chiu.<sup>8</sup> Briefly, [0] was described as normal mucosa, [1] as subepithelial space at villus tips, [2] as extension of subepithelial space with moderate lifting, [3] as massive lifting down sides of villi, with some denuded tips, [4] as denuded villi, dilated capillaries, [5] as disintegration of lamina propria, [6] as crypt layer injury, [7] as transmucosal infarction and [8] as transmural infarction.

### Tissue malonyldialdehyde (MDA) level

Tissue MDA levels were studied as reported by Uchiyama et al.<sup>9</sup> Tissue samples were homogenized with 1.15% cold KCL, and then 1% phosphoric acid and 0.6% thiobarbituric acid were added sequentially. After boiling in a water bath for 45 minutes, n-buthanol was added. The material was centrifuged at 2500 rpm for 5 minutes and spectrophotometric determinations were performed at 535 and 520 nm. The results are given as nmol/gr of tissue.

### Statistics

Tissue MDA levels and the results of the pathological examinations were subjected to the Kruskal-Wallis test. Post-hoc multiple comparison test (Bonferroni) was also performed in order to find significant differences between groups. Values of p<0.05 were accepted as significant.

## RESULTS

There is a significant difference between groups according to Kruskal-Wallis variance analysis (p<0.001). The histopathological scores are displayed in Table 1

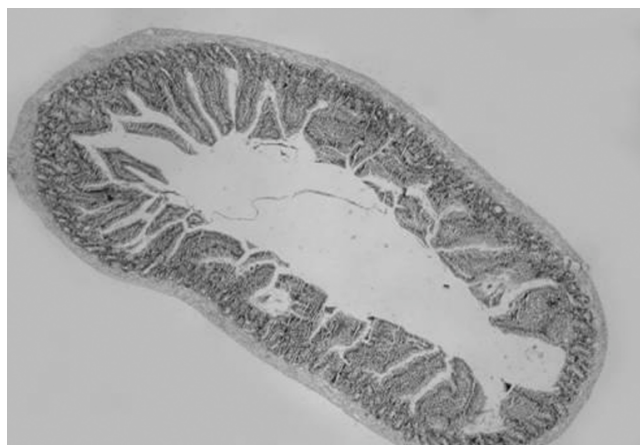
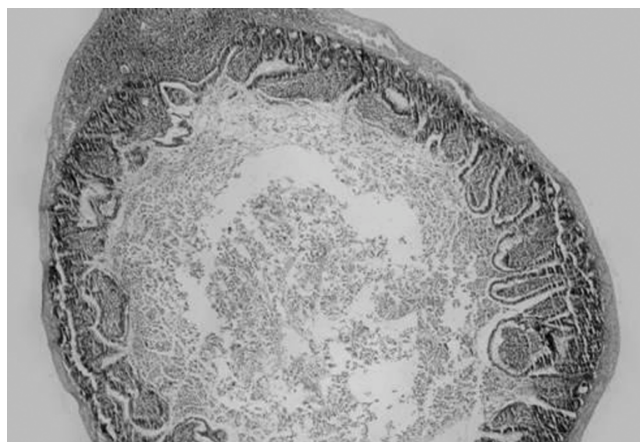
**Control Group.** The mean histopathological injury score in the control group is 0. The histological appearance of the control group is shown in Figure 1.

**I/R Group.** In the samples of the I/R group, the mean score of injury is 3.28 ± 0.48. Histopathological examination reveals significant villous epithelial separation, luminal epithelial seeding and naked subepithelial areas (Figure 2).

**SNP Group.** In the SNP group, the mean injury score is 1.43 ± 0.53, and a contact epithelial lining, except with

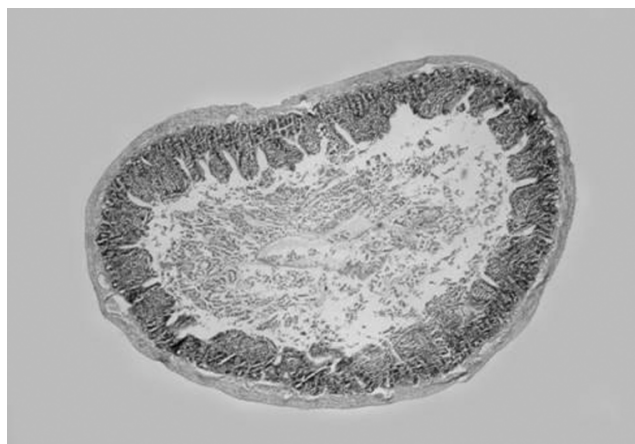
**Table 1** - The histological injury scores according to the criteria of Park and Chiu.

	Control group	I/R group**	SNP group*	L-NAME group**	SNP+L-NAME group*
1.Rat	0	3	2	2	3
2.Rat	0	3	2	4	3
3.Rat	0	3	1	2	2
4.Rat	0	4	1	4	1
5.Rat	0	4	1	3	2
6.Rat	0	3	1	4	2
7.Rat	0	3	2	4	1
Total Score	0	23	10	23	14
Mean $\pm$ SD	0 $\pm$ 0.0	3.28 $\pm$ 0.48	1.42 $\pm$ 0.53	3.28 $\pm$ 0.95	2.0 $\pm$ 0.81

**Figure 1** - Histological appearance of the control group (H&E x 40).**Figure 3** - Histological appearance of the SNP group. A contact epithelial lining except for some subepithelial spacings (H&E x 40).**Figure 2** - Histological appearance of the I/R group. Villous epithelial separation, luminal epithelial seeding and naked subepithelial areas (H&E x 100).

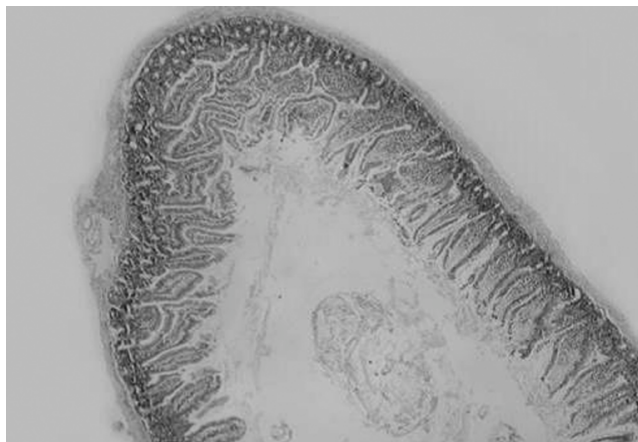
some subepithelial spacings, is observed upon histopathological examination (Figure 3).

**L-NAME Group.** In the L-NAME group, where endogenous NO synthesis is thought to be inhibited, the mean score of injury is  $3.28 \pm 0.95$ , as in the I/R group. Similar to the I/R group, free mucosal cell particles can be seen in the lumen and epithelial layer separations are obvious (Figure 4).

**Figure 4** - Histological appearance of the L-NAME group. Epithelial separations and luminal epithelial seeding is obvious (H&E x 40).

**SNP+L-NAME Group.** In the SNP+L-NAME group, the mean score of injury is  $2.0 \pm 0.81$ . Pathological examination revealed an increase in the amount of subepithelial spaces and epithelial separations in this group (Figure 5).

Statistical analysis of the data with the Mann-Whitney U test shows that the I/R group is statistically significantly different than the SNP and the SNP+L-NAME groups



**Figure 5** - Histological appearance of the SNP+L-NAME group. An increase in the amount of subepithelial spaces and epithelial separations (H&E x100).

( $p < 0.05$ ). The difference is not significant between either the I/R group and the L-NAME group or the SNP and the SNP+L-NAME group. There is also a statistically significant difference between the L-NAME group and the SNP group, and between the L-NAME group and the SNP+L-NAME group ( $p < 0.05$ ).

Tissue MDA levels are displayed in Table 2

**Table 2** - Tissue Malonyl dialdehyde levels of the groups (nmol/gr tissue).

	n	Mean	SD	Minimum	Maximum
Control group	7	3.71	1.38	2	6
I/R group**	7	14.42	2.50	11.00	18.00
SNP group*	7	8.85	1.06	7.00	10.00
L-NAME group**	7	12.85	3.53	8.00	17.00
SNP+L-NAME group*	7	9.42	1.27	7.00	11.00

**In the Sham Group,** the tissue MDA level is  $3.71 \pm 1.38$  nmol/gr of tissue.

**In the I/R Group,** I/R injury to the intestinal tissues results in a significant increase in MDA levels. The mean MDA level in this group is  $14.42 \pm 2.50$  nmol/gr of tissue.

**In the SNP Group,** the mean MDA level is significantly decreased to  $8.85 \pm 1.06$  nmol/gr of tissue. There is no difference between the SNP and SNP+L-NAME groups, although the difference between the MDA levels of the SNP and L-NAME groups are again statistically significant ( $p < 0.05$ ).

**In the L-NAME Group,** the mean tissue MDA level is  $12.85 \pm 3.53$  nmol/gr of tissue. There is no difference between the L-NAME and I/R groups.

**In the SNP+L-NAME Group,** the mean MDA level is  $9.42 \pm 1.27$  nmol/gr of tissue and the difference compared with the L-NAME group is statistically significant ( $p < 0.05$ ).

## DISCUSSION

The deleterious effect of reperfusion on intestines and its exacerbation of the ischemic event is strongly suggested.<sup>10</sup> The mechanism responsible for the injury caused by reperfusion is the formation of free oxygen radicals and the oxidative stress generated by these reactive oxygen species. In fact, the mechanism of injury is not fully understood, as there are other factors involved such as activation of phospholipase A2 and alterations of calcium flux. Chan et al. reported that third degree injury begins after only 15 mins of ischemia in the jejunum and after 60 mins in the ileum.<sup>11</sup> Ischemia lasting more than 40 minutes can cause irreversible damage in the small intestine. Kurose et al. reported that ischemia lasting over 30 minutes does not cause a change in reperfusion.<sup>12</sup> In order to avoid such a scenario, we used jejunal segments after 40 minutes of ischemia. As 40 minutes of ischemia is sufficient for creating ischemic injury and is less than the time needed for irreversible transmural infarction, the maximum grade of injury was found to be 4 in all groups. In the I/R group, the mean level of injury was  $3.28 \pm 0.95$ , which is similar to the results reported by Kazez et al. with the same scoring system.<sup>13</sup> Schoenberg et al. showed that ischemia itself causes less injury within the same time period.<sup>3</sup> Most of the systems used for grading the injury were qualitative or semi-quantitative.<sup>8</sup> Quadeckers et al. studied the different grading methods and reported that the Park and Chiu method for grading injury has advantages over the others because it minimizes examiner-based differences in pathological examination and detailed structural aspects.<sup>8</sup> Park's classification alone is not able to take the villous and crypt injury into account.<sup>8</sup> Apoptosis is also a valuable area of study for estimating tissue injury, and has become more popular in recent years.<sup>14</sup>

The histopathological results of the study are also supported by the increase in the tissue MDA levels. The MDA levels of normal intestinal tissue in the sham group were found to be  $3.71 \pm 1.38$  nmol/gr of tissue, which increased to  $14.42 \pm 2.50$  nmol/gr of tissue in the I/R group. These results demonstrate the correlation of histological and biochemical markers of the tissue injury. Lipid peroxidation results from the reaction of reactive oxygen metabolites, especially the hydroxyl and hydroperoxyl radicals with the membrane bound polyunsaturated fatty acids with a loss of a carbon radical and its rearrangement for formation of a conjugated diene. This conjugated form reacts immediately with oxygen to form peroxide radicals. Peroxide radicals initiate a chain reaction by removing a hydrogen atom from the other fatty acids. The end product of this reaction is tissue MDA and hydroperoxide.<sup>17</sup>

The spectrophotometric analysis of this tissue MDA is an important marker of *in vivo* lipid peroxidation. The other lipid peroxidation products used for the same purpose are conjugated dienes, endoperoxidases and hydrogen peroxidase. Many investigators used tissue MDA as a marker of lipid peroxidation in ischemic tissue,<sup>7,15,16</sup> although there are some limitations of the procedure. There are more specific assay methods including C11-BODIPY that may be studied where available. Estimation of the tissue MDA reacting with amine groups on the phospholipids by photometric analysis of the fluorescent effect is the most frequently used method, as performed here.<sup>18,19</sup>

Increasing the level of NO production is thought to decrease the reperfusion injury of the post-ischemic intestine via the increase in the tissue perfusion or by protecting the mucosa from the high levels of the cytotoxic agent superoxide.<sup>20</sup> Increasing the perfusion is an important factor, because dilution provides buffering of the acidic lamina propria of reperfused intestine and cleansing of the media from the toxins passing through the epithelium. Investigations into the mechanism of the I/R effect on NO production on the post-ischemic intestine revealed that reperfusion following 20 minutes of occlusion of the superior mesenteric artery causes a decrease in nitrite/nitrate levels, which is explained by the inhibition or inactivation of the NOS.<sup>12</sup> Increased production of superoxide in post-ischemic tissue is also responsible for minimizing the effect of endothelium-derived NO. The half-life of NO is very short (about 6 seconds) and the basic mechanism of this short half-life is thought to be the reaction with superoxide, as the half-life of NO increases in samples treated with superoxide dismutase.<sup>21</sup>

As the endogenous production and the use of nitric oxide decreases after I/R, the importance of exogenous NO is better understood. Mason et al. reported the increase in free oxygen radicals after the decrease of tissue NO levels.<sup>22</sup> Endogenous NO can inhibit or delay the injury caused by the free oxygen radicals in the early periods of I/R. The results of the previous studies revealed that nitric oxide donors might cause vasodilatation and scavenge the free oxygen radicals, the major cause of injury in reperfusion injury. However, the function of NO is still not fully understood as NO reacts with superoxide anion to form peroxynitrite anion (ONOO<sup>-</sup>), a highly reactive oxidizing agent capable of causing tissue damage.

Payne and Kubes studied the permeability of intestines with I/R injury treated with SIN 1, CAS-754 and nitroprusside, and reported a significant decrease in permeability

even in the presence of the NOS inhibitor L-NAME.<sup>20</sup> NO released by the donor compounds not only inactivates the superoxide radicals, the product of hypoxanthine-xanthine pathway, but also has a direct inhibitory effect on inflammatory cells. The inhibitory effect of NO on neutrophils and mast cells occurs via the inhibition of neutrophil adhesion, migration and aggregation and blocking the histamine release from the mast cells.

SNP is a cost effective and easily accessible compound. In our study, intraperitoneal use of the compound resulted in a significant decrease in the level of tissue injury, as seen both histopathologically and by the changes in tissue MDA levels with respect to the control group. This significant decrease in both SNP and SNP+L-NAME groups convinced us that SNP administered intraperitoneally can supply enough NO concentration in the tissues. SNP acts not only by inactivating the superoxide radicals and suppressing the inflammatory response, but can also cause vasodilatation of the constricted vessels after ischemia. On the other hand, it should also be noted that SNP is a disodium salt of nitroprusside ( $\text{Na}_2[\text{Fe}(\text{CN})_5\text{NO}] \cdot 2\text{H}_2\text{O}$ ) with a central iron molecule surrounded by one nitric oxide and five cyanide ligands. Wang et al. presented evidence that SNP donates iron to cells. The increase in intracellular iron is another cause of oxidative injury.

L-NAME is a NOS inhibitor. It inhibits both  $\text{Ca}^{2+}$  dependent neuronal nitric oxide synthase (nNOS) and  $\text{Ca}^{2+}$  independent inducible nitric oxide synthase (iNOS).<sup>23</sup> Luo et al. reported that L-NAME reduces the I/R injury by inhibiting the endogenous NO.<sup>24</sup> Kurose et al. showed that L-NAME causes the formation of platelet-leukocyte aggregates, and reported that decreased NO production caused an increase in aggregate formation.<sup>11</sup> P-selectin specific monoclonal antibodies were thought to be the main factors of this aggregate formation. NO donors hinder platelet-leukocyte aggregate formation by blocking P-selectin function on the I/R activated platelet surface.

We studied the L-NAME and L-NAME+SNP groups separately to compare the effects of exogenous NO. We did not find any difference neither between the I/R and L-NAME groups nor between SNP and SNP+L-NAME groups. This can be explained by a significant decrease in NO levels due to NOS inactivation and superoxide related NO inactivation after 40 minutes of ischemia followed by reperfusion.

In summary, this experimental I/R model showed us that intraperitoneal administration of SNP may be beneficial in reducing I/R injury.

## REFERENCES

- Barie PS. Schemes against ischemia; solutions for reperfusion (injury)? *Crit Care Med.* 1999;27:684-685.
- Horton JW, Walker PB. Oxygen radicals, lipid peroxidation, and permeability changes after intestinal ischemia and reperfusion. *J Appl Physiol.* 1993;74:1515-1520.
- Schoenberg MH, Beger HG. Reperfusion injury after intestinal ischemia. *Crit Care Med.* 1993;21:1376-1386.
- Anadol AZ, Bayram O, Dursun A, Ercan S. Role of endogenous endothelin peptides in intestinal ischemia-reperfusion injury in rats. *Prostaglandins Leucot Essent Fatty Acids.* 1998;59:279-283.
- Nijkamp FP, Folkerts G. Nitric oxide: initiator and modulator. *Clin Exp Allergy.* 1997;27:347-350.
- Vallance P, Hingorani A. Endothelial nitric oxide in humans in health and disease. *Int J Exp Pathol.* 1999;80:291-303.
- Van Ye TM, Roza AM, Pieper GM, Henderson J Jr, Johnson CP, Adams MB. Inhibition of intestinal lipid peroxidation does not minimize morphologic damage. *J Surg Res.* 1993;55:553-558.
- Quaedackers JS, Beuk RJ, Bennet L, Charlton A, oude Egbrink MG, Gunn AJ, et al. An evaluation of methods for grading histologic injury following ischemia/ reperfusion of the small bowel. *Transplant Proc.* 2000;32:1307-1310.
- Uchiyama M, Mihara M. Determination of malonaldehyde precursor in tissues by thiobarbituric acid test. *Anal Biochem.* 1978;86:271-8.
- Sies H, de Groot H. Role of reactive oxygen species in cell toxicity. *Toxicol Lett.* 1992;64-65 Spec No:547-551.
- Chan KL, Zhang XH, Fung PC, Guo WH, Tam PK. Role of nitric oxide in intestinal ischemia-reperfusion injury studied using electron paramagnetic resonance. *Br J Surg.* 1999;86:1427-1432.
- Kurose I, Wolf R, Grisham MB, Granger DN. Modulation of ischemia/reperfusion- induced microvascular dysfunction by nitric oxide. *Circ Res.* 1994;74:376-382.
- Kazem A, Demirbag M, Ustundag B, Ozercan IH, Saglam M. The role of melatonin in prevention of intestinal ischemia-reperfusion injury in rats. *J Pediatr Surg.* 2000;35:1444-1448.
- Shah KA, Shurey S, Green C. Apoptosis after intestinal ischemia-reperfusion injury: a morphological study. *Transplantation.* 1997;27;64:1393-1397.
- Lai HS, Chen WJ, Chiang LY. Free radical scavenging activity of fullerene on the ischemia-reperfusion intestine in dogs. *World J Surg.* 2000;24:450-454.
- Lai HS, Chen Y, Chen WJ, Chang J, Chiang LY. Free radical scavenging activity of fullerene on grafts after small bowel transplantation in dogs. *Transplant Proc.* 2000;32:1272-1274.
- Nalini S, Mathan MM, Balasubramanian KA. Oxygen free radical induced damage during intestinal ischemia/reperfusion in normal and xanthine oxidase deficient rats. *Mol Cell Biochem.* 1993;124:59-66.
- Czyrko C, Steigman C, Turley DL, Drott HR, Ziegler MM. 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.
- Otamiri TA. Influence of quinacrine on plasma malondialdehyde after small intestinal ischemia and reperfusion. *Circ Shock.* 1988;24:63-69.
- Payne D, Kubes P. Nitric oxide donors reduce the rise in reperfusion-induced intestinal mucosal permeability. *Am J Physiol.* 1993;265 (1 pt 1):G189-95.
- Wallace JL, Miller MJ. Nitric oxide in mucosal defence: a little goes a long way. *Gastroenterology.* 2000;119:512-520.
- Mason RB, Pluta RM, Walbridge S, Wink DA, Oldfield EH, Boock RJ. Production of reactive oxygen species after reperfusion in vitro and in vivo: protective effect of nitric oxide. *J Neurosurg.* 2000;93:99-107.
- Luo CC, Chen HM, Chiu CH, Lin JN, Chen JC. Effect of N(G)-nitro-L-arginine methyl ester on intestinal permeability following intestinal ischemia reperfusion injury in a rat model. *Biol Neonate.* 2001;80:60-63.
- Chen JC, Chen HM, Shyr MH, Fan LL, Chi TY, Chi CP, et al. Selective inhibition of inducible nitric oxide in ischemia-reperfusion of rat small intestine. *J Formos Med Assoc.* 2000;99:213-218.