Available online at www.sciencedirect.com
ScienceDirect
Bioorganic & Medicinal Chemistry Letters 18 (2008) 1788–1794
Bioorganic & Medicinal Chemistry Letters

Protective effect of nitronyl nitroxide–amino acid conjugates on liver ischemia–reperfusion induced injury in rats

Wei Bi,[a,*] Jianhui Cai,[a] Ping Xue,[a] Yanrong Zhang,[a] Sanguang Liu,[a] Xiang Gao,[a] Meng Li,[a] Zhibo Wang,[a] Michèle Baudy-Floc'h,[c] Sarah A. Green[b] and Lanrong Bi[b,*]

[a]*Department of Surgery, Second Hospital of HeBei Medical University, Shijiazhuang 050000, PR China*
[b]*Department of Chemistry, Michigan Technological University, 1400 Townsend Drive, Houghton, MI 49931, USA*
[c]*UMR 6226 CNRS, ICMV, Sciences Chimiques de Rennes, Université de Rennes I, F-35042 Rennes Cedex, France*

Received 18 October 2007; revised 8 February 2008; accepted 13 February 2008
Available online 16 February 2008

Abstract—Stable nitroxides are potential antioxidant drugs. In this study, we have linked nitroxide to natural amino acids with the aim to improve therapeutic activity. The radical scavenging activities of two nitronyl nitroxide–amino acid conjugates (**NNR** and **NNK**) were evaluated in PC 12 cell survival assays. The NO scavenging activities of these compounds were confirmed in the acetylcholine-induced vasorelaxation assay. In addition, the protective effect of **NNR** was demonstrated in an in vivo rat model of hepatic ischemia–reperfusion (I/R) induced injury and oxidative change. Because **NNR** reduced hepatic I/R injury by minimizing oxidative stress, it might be possible to develop it into a possible therapeutic agent for hepatic I/R injury.

Hepatic ischemia/reperfusion (I/R) is a problem commonly encountered in many clinical conditions including liver transplantation, hepatic failure after shock, and liver surgery for trauma and cancer. To reduce hemorrhagic complications, temporary clamping of the hepatic blood supply is required. When the blood supply is restored, the organ is usually subjected to a further insult, aggravating the injury created within the ischemic period. I/R injury is a complex pathophysiological process involving the reaction of oxygen-derived free radical, cytokines, and neutrophils. This can eventually lead to cell damage, cell death, increased vascular permeability, tissue necrosis, and multi-organ dysfunction.[1–5] Reactive oxygen species (ROS) are one of the most important components of tissue injury after reperfusion of ischemic organs. The major ROS include the superoxide and hydroxyl radicals, and hydrogen peroxide. ROS induced injury targets enzymes and other proteins, lipids, nucleic acids, the cytoskeleton, and cell membranes, resulting in decreased mitochondrial function, and lipid peroxidation.[6,7]

Endogenous antioxidant compounds, such as superoxide dismutase, catalase, glutathione, and β-carotene, may limit the effects of ROS but these systems can become overwhelmed by large quantities of ROS. Recently, several successful therapeutic strategies have been developed to prevent liver tissue damage after I/R. These include alternative clamping techniques as well as pharmacological intervention with antioxidants.[8] Enhancing the liver's antioxidant capacity might be a promising therapeutic strategy to prevent I/R injury.[9–16] More recently, various antioxidants have been developed to counteract I/R injury. For example, α-tocopherol, ascorbic acid, allopurinol, coenzyme Q 10, and superoxide dismutase have been reported to prevent hepatic I/R injury. It has been suggested that the antioxidant therapy is a promising approach to ameliorate liver injury during I/R.[17–20]

Stable nitroxides have found a wide range of applications in biology and medicine.[21] As a unique class of antioxidants, nitroxides have recently been explored as a complementary strategy for modulating oxidative injury. It has been suggested that nitroxides possess potential therapeutic benefits in a variety of diseases including I/R injury.[22–28] They have been shown to attenuate oxidative damage in various experimental models.[26–28] The protective effects of nitroxides can be attributed to their antioxidant capacities. In addition to directly scavenging free radical, nitroxides have also been shown to attenuate the formation of other reactive oxygen and nitrogen species.[29–36]

Keywords: Free radical scavenger; Ischemia–reperfusion injury.
* Corresponding authors. Tel.: +1 906 487 1868; fax: +1 906 487 2061 (L.B.); e-mail: lanrong@mtu.edu

0960-894X/$ - see front matter © 2008 Elsevier Ltd. All rights reserved.
doi:10.1016/j.bmcl.2008.02.030

The cellular and in vivo pharmacology of stable nitroxides has been investigated in previous studies.[31,32] It has been reported that nitroxides can be reduced in vivo to hydroxylamines or oxidized to oxo-ammonium cations via electron transfer reactions. Thus, all these three forms (nitroxide radical, oxo-ammonium cation, hydroxylamine) can be present in the tissue.[31,32] In addition, it was also suggested that the hydroxylamine and oxo-ammonium cation might com-proportionate, yielding two nitroxide molecules. In other words, the non-radical species might also com-proportionate to yield the more stable radical form and thus the nitroxides can replenish itself.[31–35] As a result, unlike antioxidants that act in a sacrificial mode, nitroxides can provide protection in a catalytic way. Through the continuous exchange between the two forms they can act as self-replenishing antioxidants thus bestow catalytic protective activity (Fig. 1). This key feature indicates the potential of this unique class of antioxidants against I/R injury.

Contrary to exogenously added SOD or catalase and several common antioxidants, nitroxides readily cross the blood–brain barrier and permeate the cell membrane. Thus, nitroxides would seem to have unique therapeutic potential for diseases and injuries related to oxidative stress.[36–46] In addition, it has been reported that some amino acids can be useful in preventing oxidative damage during surgery.[47–58] For example, pretreatment with L-arginine has a protective effect on multiple organs I/R injury.[51,52] To develop new therapeutic agents against hepatic ischemia–reperfusion injury, we sought to link an antioxidant moiety (nitronyl nitroxide) to a series of amino acids in the hope that the resulting nitronyl nitroxide–amino acid conjugates would provide a synergistically protective effect for hepatic ischemic-reperfusion injury. It turned out that some of these newly synthesized nitronyl nitroxides–amino acid conjugates displayed significantly protective effects against liver I/R injury. The detailed synthesis and structure–activity relationship studies of those newly synthesized nitronyl nitroxides–amino acid conjugates will be reported elsewhere. In the present study, we report the synthesis and biological studies of two nitronyl nitroxide–amino acid (Arg, Lys) conjugates (**NNR** and **NNK**) (Fig. 2). Moreover, the protective effect of the newly synthesized nitronyl nitroxide–amino acid conjugate (i.e., **NNR**) on hepatic I/R induced tissue damage was evaluated by the measurement of biomedical parameters: serum aspartate aminotransferase (AST) (a nonspecific marker for hepatic injury), alanine aminotransferase (ALT) (a specific marker for hepatic parenchyma injury) and liver malondialdehyde (MDA) levels (an end product of lipid peroxidation).

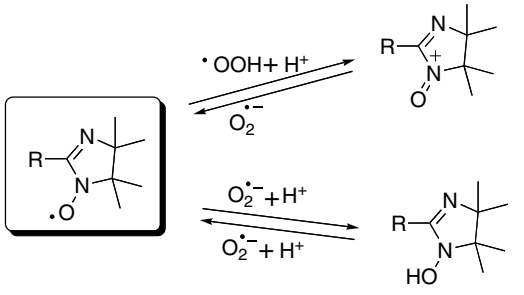


Figure 1. Schematic representation of the redox transformation of nitroxide, hydroxylamine, and the oxo-ammonium cation.

The nitronyl nitroxide derivatives were synthesized according to Ullman's procedure with minor modification as shown in Scheme 1.[53] Synthesis was initiated with dinitro compound **1**, followed by reduction with zinc in an ammonium chloride buffered solution to yield the key intermediate bis(hydroxyamine) compound **2** (63% yield), which was subsequently subjected to condensation with 4-hydroxyl-benzaldehyde to generate the corresponding dihydroxy-imidazolidine **NN-3** (51% yield). Oxidation by PbO_2 gave the nitronyl nitroxide derivative **NN-4** (52% yield). Subsequent treatment of **NN-4** with $BrCH_2COOC_2H_5$ followed by hydrolysis in the presence of NaOH provided **NN-6** (90% yield).

Synthesis of nitronyl nitroxide–amino acid conjugates was straightforward, involving a simple incorporation of the amino acid residue via the phenyloxyacetyl moiety on the 2-position of the imidazolyl ring. After the coupling and deprotection, the target compounds, **NNR** and **NNK**, were obtained in moderate to good yield (73–90%).

ESR spectroscopy is the best tool for the study of free radical, due to the sensitivity and accuracy of the method. As shown in Figure 3, different types of radicals show different patterns in the ESR spectra.

The NO trapping ability of **NNR** and **NNK** were monitored by the direct trapping experiment, as shown in Figure 3. It was observed that the initial spectrum of the nitronyl nitroxide–amino acid conjugates (**NNR** and **NNK)** had five lines (with an intensity ratio of 1/2/3/2/1 and the same a_N coupling constants). When NO gas was bubbled into the solution of **NNR** and **NNK** in deaerated phosphate buffer, the ESR spectrum was

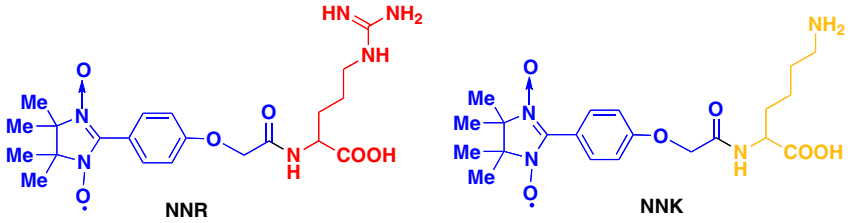
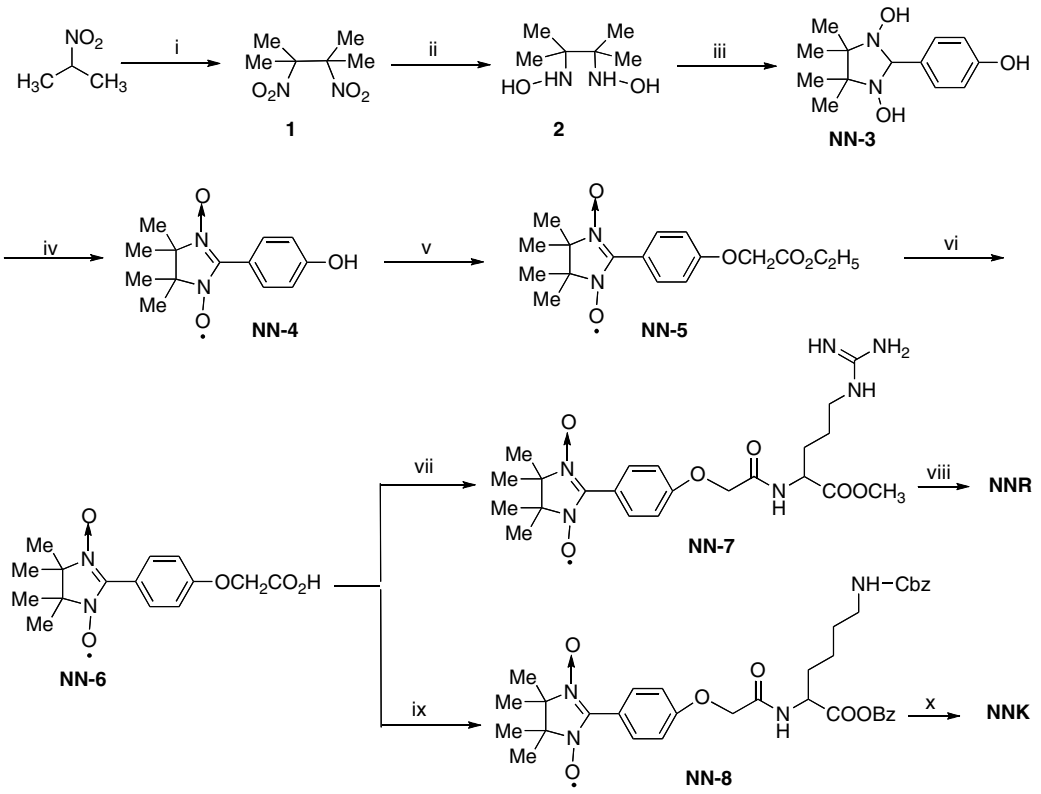


Figure 2. Structure of nitronyl nitroxide–amino acid conjugates (**NNR** and **NNK**).



Scheme 1. Synthesis of nitronyl nitroxide–amino acid conjugates (**NNR** and **NNK**). Reagents: (i) Br_2, NaOH (6 mol/L); (ii) Zn, NH_4Cl; (iii) 4-hydroxyl-benzaldehyde/MeOH; (iv) PbO_2; (v) $BrCH_2COOC_2H_5$, NaOEt, THF; (vi) NaOH (2 mol/L); (vii) HCl·Arg-OMe, DCC, HOBt, and NMM, pH 9; (viii) NaOH (2 mol/L); (ix) HCl·Lys(Z)-OBzl, DCC, HOBt, and NMM, pH 9; (x) trifluoroacetic acid/trifluoro-methylsulfonic acid (4:1).

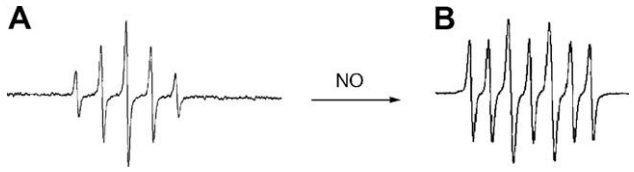
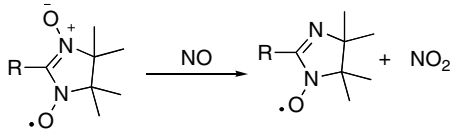


Figure 3. ESR spectra of **NNR** at 10^{-5} mol/L in phosphate buffer (pH 7.4), prior to and after the introduction of NO. (A) ESR spectra of the nitronyl nitroxide derivative, (B) ESR spectra of the imino-nitroxide.



Scheme 2. The reaction of nitronyl nitroxides radical with NO.

changed to a seven-line pattern (with an intensity ratio 1/1/2/1/2/1/1 and different a_N coupling constants). This is due to an electron interacting with two inequivalent nitrogens. Imino nitroxides are produced by the reaction between nitronyl nitroxide derivatives and NO (Scheme 2).

Various free radicals are formed during the ischemia and reperfusion phases. The free radical scavenging properties of **NNR** and **NNK** against NO, H_2O_2· and ·OH were evaluated using PC 12 cell survival assay following the published method with minor modifications.[55] The EC_{50} (μM) values are summarized in Table 1. It was observed that **NNR** and **NNK** exhibited comparable scavenging capacity with their parent compound **NN** (nitronyl nitroxide) toward different active radical species. Compared with **NN**, the differences were not statistically significant.

The NO scavenging activities of **NNR** and **NNK** were further evaluated in the acetylcholine (Ach)-evoked, endothelium-medicated relaxation assay.[53,56] The results expressed as the percentage inhibition of acetylcholine (Ach)-induced vasorelaxation by the test compounds are summarized in Table 2.

The animal experiments were performed in compliance with the 'Guide for the Care and Use of Laboratory Animals' published by the US National Institutes of Health. Male Wistar rats were randomly allocated into three groups: (1) Sham group: rats subjected to the surgical procedures (see Supporting Information), except for liver I/R ($n = 4$); (2) I/R group: rats subjected to

Table 1. Free radical scavenging activities of **NNR** and **NNK**

Compound	EC_{50} (μM) values (±SD)*		
	NO	H_2O_2·	·OH
NN	90.1 ± 2.03	32.0 ± 2.69	57.2 ± 2.41
NNR	92.8 ± 2.95	49.0 ± 2.68	98.9 ± 3.12
NNK	85.5 ± 4.64	26.8 ± 3.66	92.7 ± 3.06

* SD, standard deviation, $n = 6$; NN = nitronyl nitroxide.

Table 2. Inhibition of Ach-induced vasorelaxation

Compound	Inhibition percentage ($\overline{X}$±SD%)*		
	100 μmol	10 μmol	1 μmol
NS		1.63 ± 1.42	
NN	31.0 ± 2.6[a]	8.6 ± 3.2[a]	1.8 ± 1.4
NNR	98.9 ± 4.9[a,b]	74.0 ± 3.7[a,b]	28.7 ± 5.4[a]
NNK	85.0 ± 5.3[a,b]	60.7 ± 5.5[a,b]	24.8 ± 5.6[a]

[a] Compared with NS (normal saline), $P < 0.001$.
[b] Compared with NN (nitronyl nitroxide), $P < 0.001$.
* SD, standard deviation, $n = 6$.

the surgical procedures underwent liver ischemia for 30 min followed by reperfusion for 2 h ($n = 8$); (3) I/R + drug treatment group: rats received **NNR** (30 mg/kg) or L-Arg (30 mg/kg) just 10 min before the reperfusion and again 1 h after initiation of reperfusion ($n = 8$). Animals in groups (1) and (2) were administrated saline solution in place of drug. All animals were maintained under anesthesia (sodium pentobarbital, 80 mg/kg) for the duration of the experiment (i.e., 30 min + 2 h). At the end of the animal experiments, rats were sacrificed by a sodium pentobarbital overdose. Blood and liver samples were collected immediately, and frozen at −70 °C until analysis.

The steady-state level of malondialdehyde (MDA), which is the end product of lipid peroxidation, in the liver mitochondria was determined by measuring the level of thiobarbituric acid reactive substances spectrophotometrically according to the Buege and Aust method.[60] The serum aspartate aminotransferase (AST) and alanine aminotransferase (ALT) levels were measured to assess liver function. The results are presented in Tables 3 and 4.

There was a significant increase in MDA level in the I/R group (4.86 ± 2.43 nmol/mg, $P < 0.05$) compared with the sham-operated control group (1.75 ± 0.26 nmol/mg). Treatment with L-Arg or **NNR** reversed the elevations in the MDA level. The MDA level in the **NNR** + I/R group (2.76 ± 0.74 nmol/mg) was lower than that in the I/R (4.86 ± 2.43 nmol/mg) and I/R + L-Arg treatment groups (3.82 ± 1.79 nmol/mg), but the differences were not statistically significant.

Table 3. MDA levels in the hepatic tissues of sham, ischemia/reperfusion (I/R), I/R + L-Arg and I/R + **NNR** groups

	MDA (nmol/mg protein)
Sham	1.75 ± 0.26
I/R	4.86 ± 2.43[a]
I/R + L-Arg	3.82 ± 1.79[a]
I/R + **NNR**	2.76 ± 0.74[a]

[a] $P < 0.05$ compared with sham group; $n = 8$.

Table 4. Serum levels of ALT and AST in sham, ischemia/reperfusion (I/R), I/R + L-Arg and I/R + **NNR** groups of rats

	AST (U/L)	ALT (U/L)
Sham	182 ± 30	52 ± 4
I/R	1894 ± 875[a]	1530 ± 750[a]
I/R + L-Arg	789 ± 214[b,c]	493 ± 180[b,c]
I/R + **NNR**	510 ± 138[b,c]	173 ± 50[b,c]

[a] $P < 0.001$ compared with sham group.
[b] $P < 0.05$ compared with sham group.
[c] $P < 0.05$ compared with I/R group; $n = 8$.

Serum ALT level was 1530 ± 750 U/L in the I/R group, which is much higher than that in the sham-operated control group (52 ± 4 U/L, $P < 0.001$). The ALT level in the **NNR** + I/R group was 173 ± 50 U/L, which was significantly lower than that of the I/R group ($P < 0.05$) but still higher than that in the sham group. The serum AST level was 1894 ± 875 U/L in the I/R group, which was much higher than that in the sham group (182 ± 30 U/L, $P < 0.001$). Compared with the I/R group, after **NNR** treatment, ASL level of **NNR** + I/R group was dramatically reduced (510 ± 138 U/L, $P < 0.05$), but still much higher than that of the sham-operated control group. Although the ALT and AST levels in the **NNR** treatment group were lower than that in the L-Arg treatment group, the difference was not statistically significant.

Tissue sections (4–5 μm) were made using a cryostat microtome (Leica CM1850 UV clinical cryostat) at −30 °C, and were stained with hematoxylin–eosin and examined under a light microscope (Olympus-BX51).

ESR spectrometry revealed the existence of unpaired electrons, suggesting that **NNR** and **NNK** were of the same free radical characteristics as their parent compound, nitronyl nitroxide (**NN**). In addition, it was shown that they are reactive with NO as demonstrated by the ESR results (Scheme 2 and Fig. 3). Likewise, **NNR** and **NNK** retained the free radical scavenging activity of **NN** against NO, H_2O_2· and ·OH as demonstrated in the PC 12 cells survival assay (Table 1).

Rat pheochromocytoma (PC 12) cells, originated from the adrenal medulla, synthesize and release catecholamines. These cells are very sensitive to oxidative stress. PC 12 cells model system has been established for in vitro ischemia studies.[55] Cell survival as determined by MTT reduction was markedly decreased after PC 12 cells were exposed to free radical. In our present study, pre-incubation of PC 12 cells with **NNR/NNK**, or the incubation with the test compounds after exposure to free radical, both methods could prevent the reduction of viability caused by free radical. However, when the cells were pre-incubated with the test compounds, free radical induced cell toxicity was significantly attenuated. This presumably was due to a synergetic effect between antioxidant activity and membrane permeability. Free radical accumulation leads to cellular oxidative stress. Therefore, the elimination of free radical is critical for reducing oxidative stress. Decreasing PC 12 cells viability was suppressed when cells incubated with **NNR/NNK**. These results indicate that **NNR** and **NNK** have good scavenging capacity toward different active radical species. One possible mechanism underlying the effectiveness of **NNR/NNK** against cellular death induced by free radical involved their nitr-

oxide structures since it is known that nitroxides are potent antioxidants and free radical scavengers. Alternatively, a possible direct scavenging of free radical by **NNR/NNK** during the incubation period cannot be excluded. On the other hand, we speculated **NNR/NNK** might preferentially associate with the plasma membranes at the cellular surface.

The endothelium controls the tone of the underlying vascular smooth muscle through the production of vasodilator mediators. In Ach-induced relaxation of the rat thoracic aorta assay, Ach acts on the endothelium to elicit the release of nitric oxide (NO), a potent vasodilator.[53,56] A decreased relaxation response in the rat aortic strip could be attributed to a reduction in NO synthesized by the endothelium. Therefore, this assay was undertaken to assess the NO scavenging capability of **NNR** and **NNK**. Nitronyl nitroxide (**NN**) was found to be a weak inhibitor of Ach-induced vasorelaxation, whereas Ach-induced relaxation was significantly reversed by **NNR** and **NNK**. At the concentration of 10 μmol, compared to **NN** (8.6 ± 3.2%), **NNR** and **NNK** showed good inhibition ability with 74.0 ± 3.7% and 60.7 ± 5.5%, respectively (Table 2). We speculate that the nitric oxide scavenging activity of **NNR** and **NNK** led to attenuation of the NO concentration in vitro, thereby leading to inhibition of Ach-induced vasorelaxation.

Oxygen radical formation is harmful for biomolecules such as nucleic acids, membrane lipids, enzymes, and receptors. Oxygen radicals tend to attack the membrane-associated polyunsaturated fatty acids and to form lipid peroxides.[57–59] Peroxidation of membrane lipids can disrupt membrane fluidity and cell compartmentation, which can further lead to cell lysis. In fact, lipid peroxidation is implicated in the pathogenesis of various liver injuries and subsequent liver fibrogenesis in experimental animals and humans.[57–59] MDA is a major reactive aldehyde that appears during the peroxidation of biological membrane polyunsaturated fatty acids. Therefore, the hepatic content of MDA can be used as an indicator of liver tissue damage.[60] In this study, hepatic I/R caused a significant increase in MDA (Table 3). This observation is in agreement with previous studies.[61,62] Tissue damage may result in lipid peroxidation and necrosis, and the increased tissue MDA levels can be accepted as a criterion of tissue injury. Interestingly, **NNR** treatment caused a significant inhibition in MDA production (Table 3). This decrease indicated that lipid peroxidation of liver tissue and cellular injury was reduced. This protective effect of **NNR** is most likely due to its ability to scavenge the very reactive hydroxyl and peroxyl radicals thereby limiting hepatic injury.

Serum aminotransferase activities have long been regarded as indicators of hepatic injury.[63] Damage to the hepatocytes alters their transport function and membrane permeability, leading to leakage of enzymes from the cells.[64] Therefore, the marked release of AST and ALT into the circulation indicates severe damage to hepatic tissue membranes during the reperfusion process. Compared with sham-operated rats, the I/R of the liver resulted in significant increases in AST and ALT levels (Table 4), demonstrating development of hepatic cellular injury. In hepatic I/R injury, it has been reported that excessive amounts of free radical are generated in the early phase of reperfusion. To obtain an optimal therapeutic effect, **NNR** was administrated just before the reperfusion and again 1 h after initiation of reperfusion. We found that **NNR** administrated 10 min before and again 1 h after initiation of reperfusion caused a substantial reduction in the I/R induced increase in ALT and AST (Table 4).

From the histological results, the sham group revealed regular morphology of liver parenchyma with intact hepatocytes and sinusoids (Fig. 4A). In the hepatic I/R group, there was severe sinusoidal congestion and hemorrhage, the enlarged central vein, subendothelial edema, and degenerated hepatocytes with perinuclear vacuolization (Fig. 4B). In the I/R + **NNR** treatment group, there was moderate sinusoidal dilatation, and the central vein and hepatocytes appeared normal in most areas (Fig. 4C). The histological results indicated that, although the congestion, necrosis, and hepatocellular changes were still observed in the hepatic I/R + **NNR** treatment groups, the histological improvement was prominent with **NNR** treatment in the hepatic I/R + **NNR** group.

In the present study, it was confirmed that L-Arginine could reduce lipid peroxidation thereby ameliorating hepatic ischemia–reperfusion injury, which was in accor-

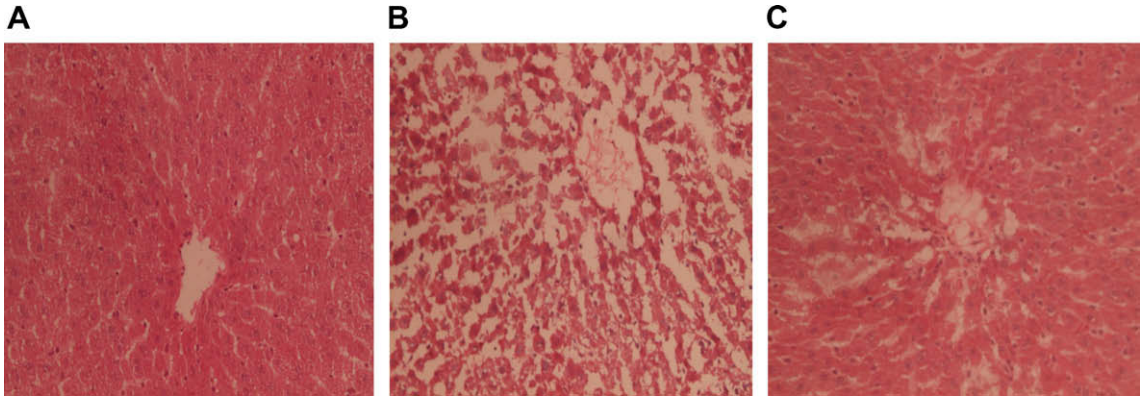


Figure 4. Histological analysis of livers. Representative photographs (original magnification: 200×) were taken from rat livers 2 h after treatment with sham-operation (Sham) (A), hepatic I/R (I/R) (B), and hepatic I/R + treatment with **NNR** (C). $N = 8$.

dance with the previous reports.[65] Our present results indicate that **NNR** administered before the onset of the reperfusion and again 1 h after initiation of reperfusion significantly reduced the liver injury after the I/R. Liver ischemic-reperfusion injury pathogenesis is multifactorial. The effects of other factors maybe involved in the pathogenesis of liver I/R injury, therefore, the precise acting mechanism of these newly synthesized nitronyl nitroxide–amino acid conjugates should be further explored and the interaction between the different pathways also should be considered in our further studies.

Acknowledgments

This work is dedicated to Professor Nicholas J. Turro on the occasion of his 70th birthday with all best wishes for his future endeavors. The authors are grateful to China Natural Science Foundation of HeBei Province (No. 062761266), Administration of Traditional Chinese Medicine of HeBei Province (No. 2007134), and Second Hospital of HeBei Medical University for support of this research. The authors are also grateful to Dr. Michael R. Neuman and Dr. James Russo for many helpful discussions, and the Department of Chemistry at MTU for funding.

Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2008.02.030.

References and notes

1. Urakami, H.; Abe, Y.; Grisham, M. B. *Clin. Exp. Pharmacol. Physiol.* **2007**, *34*, 912.
2. Valko, M.; Leibfritz, D.; Moncol, J.; Cronin, M. T.; Mazur, M.; Telser, J. *Int. J. Biochem. Cell Biol.* **2007**, *39*, 44.
3. Brady, N. R.; Hamacher-Brady, A.; Westerhoff, H. V.; Gottlieb, R. A. *Antioxid. Redox Signal.* **2006**, *8*, 1651.
4. Galaris, D.; Barbouti, A.; Korantzopoulos, P. *Curr. Pharm. Des.* **2006**, *12*, 2875.
5. Zweier, J. L.; Talukder, M. A. *Cardiovasc. Res.* **2006**, *70*, 181.
6. Wiseman, A. *Med. Hypotheses* **2006**, *66*, 329.
7. Jaeschke, H. *Chem. Biol. Interact.* **1991**, *79*, 115.
8. Fan, C.; Zwacka, R. M.; Engelhardt, J. F. *J. Mol. Med.* **1999**, *77*, 577.
9. Zhou, W.; Zhang, Y.; Hosch, M. S.; Lang, A. R.; Zwacka, M.; Engelhardt, J. F. *Hepatology* **2001**, *33*, 902.
10. Muller, M. J.; Vollmar, B.; Friedl, H. P.; Menger, M. D. *Free Radic. Biol. Med.* **1996**, *21*, 189.
11. Yabe, Y.; Nishikawa, M.; Tamada, A.; Takakura, Y.; Hashida, M. *J. Pharmacol. Exp. Ther.* **1999**, *289*, 1176.
12. Yabe, Y.; Kobayashi, N.; Nishihashi, T., et al. *J. Pharmacol. Exp. Ther.* **2001**, *298*, 894.
13. Seal, J. B.; Gewertz, B. L. *Ann. Vasc. Surg.* **2005**, *19*, 572.
14. Siemionow, M.; Arslan, E. *Microsurgery* **2004**, *24*, 468.
15. Hines, I. N.; Harada, H.; Wolf, R.; Grisham, M. B. *Curr. Med. Chem.* **2003**, *10*, 2661.
16. Gross, E. R.; Gross, G. J. *Expert Opin. Emerg. Drugs* **2007**, *12*, 367.
17. Glantzounis, G. K.; Yang, W.; Koti, R. S.; Mikhailidis, D. P.; Seifalian, A. M.; Davidson, B. R. *Curr. Pharm. Des.* **2006**, *12*, 2891.
18. Cesaratto, L.; Vascotto, C.; Calligaris, S.; Tell, G. *Ann. Hepatol.* **2004**, *3*, 86.
19. Hines, I. N.; Harada, H.; Wolf, R.; Grisham, M. B. *Curr. Med. Chem.* **2003**, *10*, 2661.
20. Amersi, Farin; Nelson, Sally K.; Shen, Xiu Da; Kato, Hirohisa; Melinek, Judy; Kupiec-Weglinski, Jerzy W.; Horwitz, Lawrence D.; Busuttil, Ronald W.; Horwitz, Marcus A. *Proc. Natl. Acad. Sci. U.S.A.* **2002**, *99*, 8915.
21. Soule, B. P.; Hyodo, F.; Matsumoto, K.; Simone, N. L.; Cook, J. A.; Krishna, M. C.; Mitchell, J. B. *Free Radic. Biol. Med.* **2007**, *42*, 1632.
22. Fink, M. P.; Macias, C. A.; Xiao, J.; Tyurina, Y. Y.; Delude, R. L.; Greenberger, J. S.; Kagan, V. E.; Wipf, P. *Crit. Care Med.* **2007**, *35*, S461.
23. Soule, B. P.; Hyodo, F.; Matsumoto, K. I.; Simone, N. L.; Cook, J. A.; Krishna, M. C.; Mitchell, J. B. *Antioxid. Redox. Signal.* **2007** (Epub ahead of print).
24. Zeltcer, G.; Berenshtein, E.; Kitrossky, N.; Chevion, M.; Samuni, A. *Free Radic. Biol. Med.* **2002**, *32*, 912.
25. Steinbauer, M.; Guba, M.; Buchner, M.; Farkas, S.; Anthuber, M.; Jauch, K. W. *Shock* **2000**, *14*, 163.
26. Udassin, R.; Haskel, Y.; Samuni, A. *Gut* **1998**, *42*, 623.
27. Martin, J.; Lutter, G.; Sarai, K.; Senn-Grossberger, M.; Takahashi, N.; Bitu-Moreno, J.; Haberstroh, J.; Beyersdorf, F. *Eur. J. Cardiothorac. Surg.* **2001**, *19*, 321.
28. Kato, N.; Yanaka, K.; Hyodo, K.; Homma, K.; Nagase, S.; Nose, T. *Brain Res.* **2003**, *979*, 188.
29. Zhang, R.; Pinson, A.; Samuni, A. *Free Radic. Biol. Med.* **1998**, *24*, 66.
30. Shankar, R. A.; Hideg, K.; Zweier, J. L.; Kuppusamy, P. *J. Pharmacol. Exp. Ther.* **2000**, 838.
31. Krishna, M. C.; Grahame, D. A.; Samuni, A.; Mitchell, J. B.; Russo, A. *Proc. Natl. Acad. Sci. U.S.A.* **1992**, *89*, 5537.
32. Krishna, M. C.; Russo, A.; Mitchell, J. B.; Goldstein, S.; Dafni, H.; Samuni, A. *J. Biol. Chem.* **1996**, *271*, 26026.
33. Cighetti, G.; Allevi, P.; Debiasi, S.; Paroni, R. *Chem. Phys. Lipids* **1997**, *88*, 97.
34. Mitchell, J. B.; DeGraff, W.; Kaufman, D.; Krishna, M. C.; Samuni, A.; Finkelstein, E. *Arch. Biochem. Biophys.* **1991**, *289*, 62.
35. Mohsen, M.; Pinson, A.; Zhang, R.; Samuni, A. *Mol. Cell. Biochem.* **1995**, *145*, 103.
36. Monti, E.; Cova, D.; Guido, E.; Morelli, R.; Oliva, C. *Free Radic. Biol. Med.* **1996**, *21*, 463.
37. Hahn, S. M.; Krishna, C. M.; Mitchell, J. B. *Adv. Exp. Med. Biol.* **1994**, *366*, 241.
38. Samuni, A. M.; DeGraff, W.; Krishna, M. C.; Mitchell, J. B. *Mol. Cell. Biochem.* **2002**, *234-235*, 327–333.
39. Gelvan, D.; Saltman, P.; Powell, S. R. *Proc. Natl. Acad. Sci. U.S.A.* **1991**, *88*, 4680.
40. Oliver, C. N.; Starke-Reed, P. E.; Stadtman, E. R.; Liu, G. J.; Carney, J. M.; Floyd, R. A. *Proc. Natl. Acad. Sci. U.S.A.* **1990**, *87*, 5144.
41. Aronovitch, Y.; Godinger, D.; Israeli, A.; Krishna, M. C.; Samuni, A.; Goldstein, S. *Free Radic. Biol. Med.* **2007**, *42*, 1317.
42. Dragutan, I.; Mehlhorn, R. J. *Free Radic. Res.* **2007**, *41*, 303.
43. Gariboldi, M. B.; Terni, F.; Ravizza, R.; Meschini, S.; Marra, M.; Condello, M.; Arancia, G.; Monti, E. *Free Radic. Biol. Med.* **2006**, *40*, 1409.
44. Schubert, R.; Erker, L.; Barlow, C.; Yakushiji, H.; Larson, D.; Russo, A.; Mitchell, J. B.; Wynshaw-Boris, A. *Hum. Mol. Genet.* **2004**, *13*, 1793.

45. Kinugasa, Y.; Ogino, K.; Furuse, Y.; Shiomi, T.; Tsutsui, H.; Yamamoto, T.; Igawa, O.; Hisatome, I.; Shigemasa, C. *Circ. J.* **2003**, *67*, 781.
46. Lanteri, R.; Acquaviva, R.; Di Giacomo, C.; Caltabiano, R.; Li Destri, G.; Vanella, L.; Santangelo, M.; Lanzafame, S.; Di Cataldo, A. *Microsurgery* **2006**, *26*, 25.
47. Fotiadis, C.; Adamis, S.; Misiakos, E. P.; Genetzakis, M.; Antonakis, P. T.; Tsekouras, D. K.; Gorgoulis, V. G.; Zografos, G. C.; Papalois, A.; Fotinou, M.; Perrea, D. *Acta Chir. Belg.* **2007**, *107*, 192.
48. Spanos, C. P.; Papaconstantinou, P.; Spanos, P.; Karamouzis, M.; Lekkas, G.; Papaconstantinou, C. *J. Gastrointest. Surg.* **2007**, *11*, 247.
49. Schuster, H.; Blanc, M. C.; Neveux, N.; Bonnefont-Rousselot, D.; Le Tourneau, A.; De Bandt, J. P.; Cynober, L. *Scand. J. Gastroenterol.* **2006**, *41*, 1342.
50. Chander, V.; Chopra, K. *J. Surg. Res.* **2005**, *128*, 132.
51. Burra, P.; Ferraresso, M.; Cadrobbi, R., et al. *Transplant. Proc.* **1997**, *29*, 2992.
52. Nilsson, B.; Yoshida, T.; Delbro, D.; Andius, S.; Friman, S. *Transplant. Proc.* **1997**, *29*, 3111.
53. Wu, Y.; Bi, L.; Bi, W.; Li, Z.; Zhao, M.; Wang, C.; Ju, J.; Peng, S. *Bioorg. Med. Chem.* **2006**, *14*, 5711.
54. Shimono, S.; Tamura, R.; Ikuma, N.; Takimoto, T.; Kawame, N.; Tamada, O.; Sakai, N.; Matsuura, H.; Yamauchi, J. *J. Org. Chem.* **2004**, *69*, 475.
55. (a) Desole, M. S.; Sciola, L.; Sircana, S.; Godani, C.; Migheli, R.; Delogu, M. R.; Piras, G.; De, N. G.; Miele, E. *Neurosci. Lett.* **1998**, *247*, 1; (b) Hillion, J.; Takahashi, K.; Maric, D.; Ruetzler, C.; Barker, J.; Hallenbeck, J. *J. Cereb. Blood Flow Metab.* **2005**, *25*, 154; (c) Greene, L. A.; Tischler, A. S. *Proc. Natl. Acad. Sci. U.S.A.* **1976**, *73*, 2424; (d) Boniece, I. R.; Wagner, J. A. *J. Neurosci.* **1993**, *13*, 4220; Boniece, I. R.; Wagner, J. A. *J. Neurosci. Res.* **1995**, *40*, 1; (e) Mosmann, T. *J. Immunol. Methods* **1983**, *65*, 55.
56. Cowart, M.; Kowaluk, E. A.; Daanen, J. F.; Kohlhaas, K. L.; Alexander, K. M.; Wagenaar, F. L.; Kerwin, J. F. *J. Med. Chem.* **1998**, *41*, 2636.
57. Paradis, V.; Kollinger, M.; Fabre, M.; Holstege, A.; Poynard, T.; Beddosa, P. *Hepatology* **1997**, *26*, 135.
58. Liu, J. Y.; Chen, C. C.; Wang, W. H.; Hsu, J. D.; Yang, M. Y.; Wang, C. J. *Food Chem. Toxicol.* **2006**, *44*, 336.
59. Vaca, C. E.; Wilhelm, J.; Harms-Rihsdahl, M. *Mutat. Res. Rev. Genet. Toxicol.* **1988**, *195*, 137; Ohkawa, H.; Ohishi, N.; Yagi, K. *Anal. Biochem.* **1979**, *95*, 351.
60. Buege, J. A.; Aust, S. D. *Methods Enzymol.* **1978**, *52*, 302.
61. Omar, R.; Nomikos, I.; Piccorrelli, G.; Savino, J.; Agarwal, N. *Gut* **1989**, *30*, 510.
62. Sumimoto, K.; Oku, J.; Dohi, K.; Kawasaki, T. *Transplant. Proc.* **1990**, *22*, 2023.
63. Molander, D. W.; Wroblewsk, F.; La Due, J. S. *Clin. Res. Proc.* **1955**, *3*, 20.
64. Zimmerman, H. J.; Seeff, L. B. Enzymes in Hepatic Disease. In *Diagnostic Enzymology*; Goodley, E. L., Ed.; Lea and Febiger: Philadelphia, 1970; p 1.
65. Shiraishi, Y.; Lee, J. R.; Laks, H.; Waters, P. F.; Meneshian, A.; Blitz, A.; Johnson, K.; Lam, L.; Chang, P. *Ann. Thorac. Surg.* **1996**, *62*, 1580; Nakanishi, K.; Vinten-Johansen, J.; Lefer, D. J.; Zhao, Z.; Fowler, W. C., 3rd; McGee, D. S.; Johnston, W. E. *Am. J. Physiol. Heart Circ. Physiol.* **1992**, *263*, H1650; Lass, A.; Suessenbacher, A.; Wolkart, G.; Mayer, B.; Friedrich, B. *Mol. Pharmacol.* **2002**, *61*, 1081.