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Original Article

Combination of ischemic post-conditioning with a synthetic glycine derivative improves functional outcome after ischemic-reperfusion injury in rats

Xiang Gao[1,2], Wei Bi[2], Shuxia Liu[1], Qingjuan Liu[1], Yanrong Zhang[3], Huijun Duan[1]

[1]Department of Pathology, Hebei Medical University, Key Laboratory of Kidney Diseases of Hebei Province, Shijiazhuang, Hebei Province, China; [2]Department of Surgery, The Second Hospital of Hebei Medical University, Shijiazhuang, Hebei Province, China; [3]Department of Surgery, The Third Hospital of Hebei Medical University, Shijiazhuang, Hebei Province, China

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Abstract: To investigate the effects of combined treatment with ischemic post-conditioning and infusion of nitroxide derivative glycine-nitronyl nitroxide conjugate (GNN) in hind limb ischemic-reperfusion animal model, male Wistar rats were randomly allocated into 5 groups: 1) sham-operated group; 2) Ischemia/reperfusion (I/R) group; 3) I/R injury + GNN alone; 4) Ischemic post-conditioning at the onset of reperfusion combined with saline; 5) Ischemic post-conditioning combined with i.v. administration of GNN (ischemic post-conditioning + GNN). At 4 h post-insult, the apoptotic cell distribution in liver, kidney and muscle tissues, the levels of malondialdehyde (MDA), myeloperoxidase (MPO) and serum tumor necrosis factor-alpha (TNF-α) levels, as well as the degree of muscle damage were evaluated. Compared to controls, animals treated with GNN could significantly attenuate cellular apoptosis in kidney and muscle tissues. Meanwhile, muscular inflammation, edema and damage levels were also decreased in the combined treatment group. On the other hand, the upregulation of MDA, MPO and TNF-α induced by ischemia were also significantly suppressed by combined treatment. These results demonstrate that combination of ischemic post-conditioning and the pharmaceutical agent (GNN) may provide an effective therapeutic strategy in acute and transient ischemic damage.

Keywords: Ischemic post-conditioning, glycine-nitronyl nitroxide conjugate, limb ischemia/reperfusion injury, apoptosis

Introduction

As a life threatening condition, acute lower limb ischemia is frequently encountered in many disease states and surgical procedures, including native thrombosis, cardiovascular embolism, occlusion of a bypass graft or angioplasty site, thrombosis of a popliteal aneurysm, other traumatic or iatrogenic injuries [1]. Although the primary effect of acute ischemia is local tissue hypoxia, multiple cardiovascular pathways will be impacted after a complex vicious circle [2]. Prolonged ischemia reperfusion (I/R) injury leads to severe and irreversible tissue damage/necrosis and results in multiple organ dysfunctions. For instance, I/R of a lower limb leads to noncardiogenic pulmonary edema, which may be a major cause of morbidity and mobility [3, 4]. Although restoration of blood flow can save the extremity, reperfusion itself may induce additional cellular injury via introducing a vicious cascade including inflammatory cytokines release, reactive oxygen species (ROS) over-production. As a type of by-product of cellular metabolism, ROS play an important role in the pathogenesis of I/R injury [5]. Reactive oxygen species are important intermediates of the normal metabolism of oxygen involved in many normal physiological reactions and processes, including ATP generation in mitochondria, protein and lipid degradation, and inflammatory responses [6]. I/R induces the generation of excessive ROS, thus initiating a cascade of cellular injury, necrosis, apoptosis and subsequent inflammation [7]. Antioxidant therapy or suppression of post-ischemic neutrophil infiltration provides a usefully therapeutic method to attenuate the damage caused by ROS in vari-

ous organs during ischemia/reperfusion [8]. Previous studies have demonstrated antioxidant therapy is an effective pharmacological approach in alleviating ischemia/reperfusion injuries in multiple organs and tissues [9-12]. Stable nitroxides deliver potent antioxidant action and attenuate ROS in multiple models of oxidative stress induced by superoxides, H_2O_2 and organic hydroperoxides. Nitroxides have been validated to reduce oxidative damage in various experimental models including I/R injury. Nitroxides directly removing free radicals (carbon, oxygen, nitrogen, sulphur, and protein radicals) [13, 14]. The terminal products produced by the reaction of nitroxides with free radicals are less damaging to cells and tissues than the free radicals themselves [15]. On the other hand, Nitroxides also attenuate the formation of other ROS and reactive nitrogen species (RNS). Unlike other antioxidants that act in a sacrificial mode, nitroxides act as a catalyst. Briefly, nitroxides go through one-electron redox reactions to yield the corresponding hydroxylamines and oxo-ammonium cations via one electron transfer reactions. The hydroxylamine and oxo-ammonium cations can comproportionate and yield two nitroxide molecules. The non-radical species might also comproportionate and yield stable radical form, and thus replenish themselves. As a result, nitroxide radical, hydroxylamine cation and oxo-ammonium would be accumulated in this tissue. Through continuous exchange, these three forms can act as self-replenishing antioxidants, thus conferring catalytic protective activity. Unlike exogenously administrated SOD and several other common antioxidants, nitroxides readily pass through the blood-brain barrier and permeate the cell membrane. Therefore, nitroxides could provide a novel and effective antioxidant therapy for diseases and injuries related to oxidative damage [11, 12, 16].

It has been reported that Ischemic pre-conditioning and post-conditioning are one of the most powerful innate mechanism to protect against ischemia-reperfusion injury [17-19]. Briefly, these processes involve a brief period of sub-lethal local tissue ischemia which confers protection against a lethal ischemia. That is to say short cycles of I/R prior to a lethal episode of ischemia or a burst of short shots of I/R at the onset of restoration of the flow (post-conditioning) render the organ tolerant to I/R injury. Mechanisms involved in ischemic pre-conditioning are very complex, which involve the reduction of neutrophil accumulation and the intracellular Ca^{2+} overload, as well as the delayed restoration of neutral pH with a reduction of oxidative damage [20-23]. As the onset of ischemia is difficult to be predicted, the clinical application of preconditioning is much limited. Since then, post-conditioning is a more practical intervention, which could be applied immediately after a long ischemic period. However, sometimes the effect is not ideal enough clinically. On the other hand, pharmacological agents are also much limited to achieve protection for I/R injury as they need to be administered before the onset of ischemia. Considering the clinical relevance, a combination of ischemic post-conditioning and pharmacological agent given by intravenous injection during ischemia may provide an effective protection against I/R injury. In this study, we evaluated the effects of the combination of pharmacological agent (GNN, glycine-nitronyl nitroxide) conjugate [12] and post-conditioning in an acute limb I/R injury animal model.

Materials and methods

Cell culture

The human umbilical vein endothelial cells were purchased from ATCC and maintained following the standard protocol. Briefly, HUVECs cells were cultured in Endothelial medium (DMEM, Life Technologies, Shanghai, China) containing 10% fetal bovine serum (FBS, Life Technologies), 1% streptomycin (100 g/mL) and 1% penicillin (100 U/mL) at pH 7.4 in a 5% CO_2 incubator at 37°C.

Oxygen-glucose deprivation and cell culture treatment

After adhering overnight, The Culture medium was replaced by glucose-free Earle's balanced salt solution (EBSS, pH 7.4) bubbled with 95% N_2 and 5% CO_2. The cells were then placed in an anaerobic chamber containing a mixture of 95% N_2 and 5% CO_2 humidified at 37°C for 8 h. Oxygen-glucose deprivation was terminated by replacing the anoxic medium with fresh ECM, and returning to normoxia for an additional 4 h.

Hind limb ischemia-reperfusion injury

Male Wistar rats (average weight, 275 ± 25 g) were housed in a standard animal room with

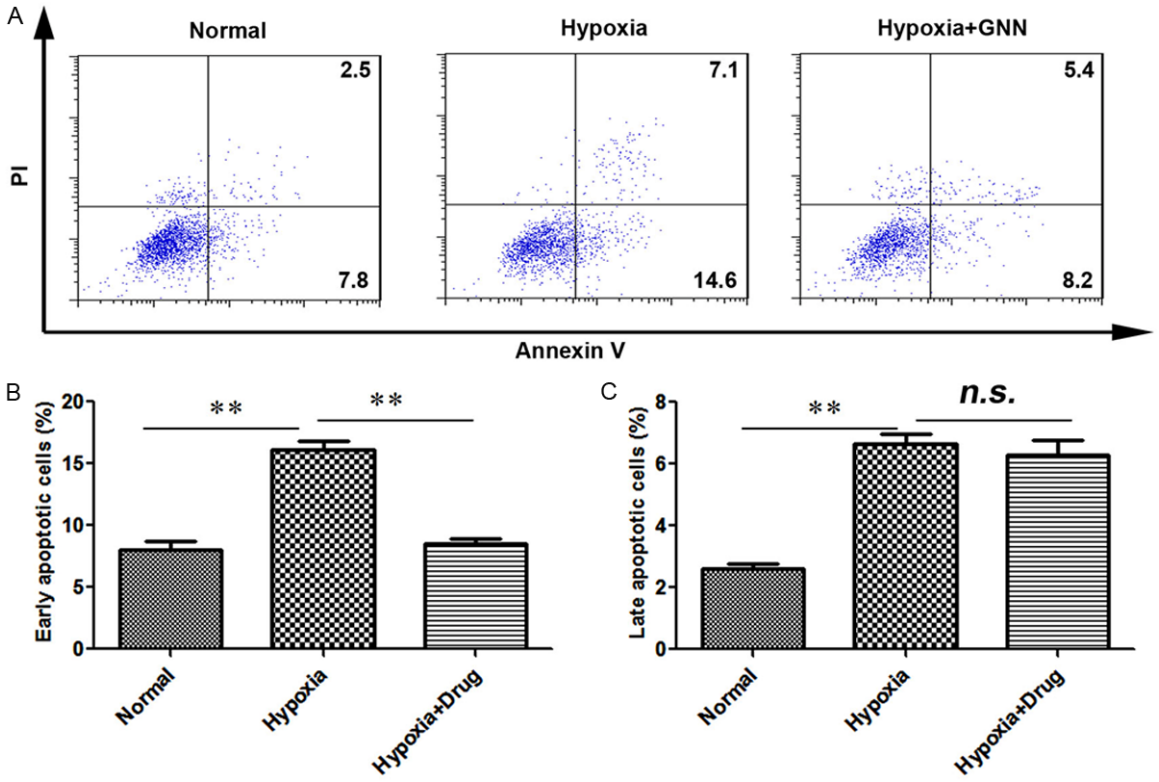

Figure 1. GNN effectively suppresses cellular apoptosis induced by hypoxia *in vitro*. (A) The apoptotic cells were determined by Annexin V and PI staining in HUVECs. (B) and (C) Early apoptotic cells (B) and late apoptotic cells (C) in different treatment group were analyzed. **indicates $P<0.01$.

food and water ad libitum under controlled conditions of humidity and temperature and were randomly allocated into 5 groups: 1) sham-operated group-rats subjected to the procedures described below (without I/R [n=6]); 2) I/R group-rats subjected to I/R (described below) were exposed to limb ischemia for 3 h followed by reperfusion for 4 hours (n=8); 3) I/R injury (as in group 2) + GNN alone - rats received GNN given as a bolus 30 mg/kg followed by i.v. infusion of 10 mg/kg/min for 15 min (i.v. GNN; n=8); 4) Ischemic post-conditioning (four cycles of 60 s reperfusion and 60 s of re-occlusion) at the onset of reperfusion combined with i.v. saline (n=8); and 5) Ischemic post-conditioning (four cycles of 60 s reperfusion and 60 s of re-occlusion) combined with i.v. administration of GNN (ischemic post-conditioning + GNN). Saline or GNN administration was started 5 minute before the onset of reperfusion.

For induction of I/R injury, anesthesia was achieved via intraperitoneal injection of sodium pentobarbital (80 mg/kg), and body temperature was maintained at 37°C with a heating pad. A unilateral rubber band was applied above the greater trochanter to disrupt arterial blood flow to the hind limbs. Following 3 h of hind limb ischemia, the rubber band was removed, inducing hind limb reperfusion. Upon conclusion of experiments, rats were euthanized via sodium pentobarbital overdose. Tissue samples were immediately isolated, and blood was rapidly obtained from the ascending aorta. Tissue samples were divided into two sections, one for determination of lipid peroxidation, and the other for tissue sections employed for histological examination (Leica CM1850 UV clinical cryostat) at -30°C. This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The animal use protocol has been reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) of the First Affiliated Hospital of Zhengzhou University.

Flow cytometric analysis of apoptosis

Apoptosis assay was performed using the FITC-Annexin V Apoptosis detection kits (eBiosciences, San Diego, CA, USA), following the standard protocol.

Biochemical assay

Serum levels of alanine aminotransferase (ALT), aspartate aminotransferase (AST), blood urea nitrogen (BUN) and creatinine (Cr) were measured with an auto-biochemical analyzer (Toshiba, Japan). The serum levels of TNF-α were measured with a TNF-α ELISA kit according to the manufacturer's instruction.

The extent of tissue edema was determined by measuring tissue wet to dry weight (W/D) ratio. Immediately after harvest, tissue samples were blotted, weighed, and placed in a drying oven at 55°C until a constant weight was obtained. Tissue edema is suggested by an increase in the W/D ratio. MDA levels were determined following Beuge's method (Beuge and Aust 1978).

Statistical analysis

A two-way ANOVA followed by Scheffé's test was first carried out using the Origin Program to test for differences between groups. If differences were established, the values were compared using Student's *t*-test for paired data. The values were expressed as means ± SE. The results were considered significant if P was <0.05.

Results

GNN suppresses cell apoptosis induced by hypoxia in vitro

To evaluate the function of GNN in ischemia, we established an in vitro model by using human umbilical vein endothelial cells (HUVECs). We found that apoptosis could be induced in HUVECs while these cells were cultured in low-oxygen cell culture conditions. The nitroxide derivative could effectively suppress cellular apoptosis induced by hypoxia (**Figure 1**). It's interesting that GNN attenuated the apoptotic process in HUVECs induced by hypoxia only at the early phase, not at the late phase (**Figure 1B** and **1C**).

GNN could attenuate cellular apoptosis in kidney and muscle tissues induced by ischemia in in vivo model

Next, we want to determine the function of combined treatment of post-conditioning and GNN in in vivo model. After detecting the apoptotic cell distribution in different organs, we found that ischemia could induce apoptosis in multiple organs (liver, kidney and muscle) (**Figure 2**). Meanwhile, GNN treatment suppressed cellular apoptosis induced by ischemia, especially in kidney and muscle tissues (**Figure 2A**, **2C** and **2D**).

Evaluation of tissue oxidative damage-malon dialdehyde (MDA) levels

As an end product of membrane lipid peroxidation, MDA presents a surrogate biomarker of lipid peroxidation. To assess plasma and tissue lipid peroxidation, MDA levels were quantified in plasma and tissue samples. **Table 1** shows that MDA levels in both plasma (3.45 ± 0.44 nmol/ml) and lung (84.5 ± 3.7 nmol/g) tissue were significantly increased in the I/R group of rats compared to the animals of the sham-operated group (plasma: 1.67 ± 0.13 nmol/ml; lung: 41.8 ± 3.2 nmol/g). However, the plasma and lung MDA levels in the treatment with post-conditioning alone group of rats were not significantly lower than that of the animals of I/R group. Treatment with the GNN alone (plasma: 2.28 ± 0.38 nmol/ml; lung: 60.3 ± 3.5 nmol/g) or combination of GNN + ischemic post-conditioning caused a remarkable inhibition of MDA production (plasma: 1.89 ± 0.20 nmol/ml; lung: 52.7 ± 4.9 nmol/g), which suggested an attenuation of lipid peroxidation and cellular injury.

Evaluation of tissue oxidative damage--myeloperoxidase (MPO) activity

MPO assay is routinely measured as an index of neutrophil infiltration and a marker for acute inflammation when polymorphonuclear cell infiltration occurred. **Table 1** showed that the lung MPO activity in the I/R group of rats (MPO: 19.3 ± 1.9 U/g) was significantly increased compared to the sham-operated group (MPO: 8.0 ± 0.6 U/g). The rats treated with GNN alone (MPO: 10.4 ± 1.2 U/g) or the combined treatment (MPO: 9.3 ± 1.3 U/g) with GNN + ischemic post-conditioning both led to a significant reduction of lung MPO activity when compared with that of the animals of I/R group.

Measurement of serum tumor necrosis factor-alpha (TNF-α)

TNF-α is an important mediator for local and remote organ injury after hind limb I/R. As shown in **Table 1**, the TNF-α levels were dra-

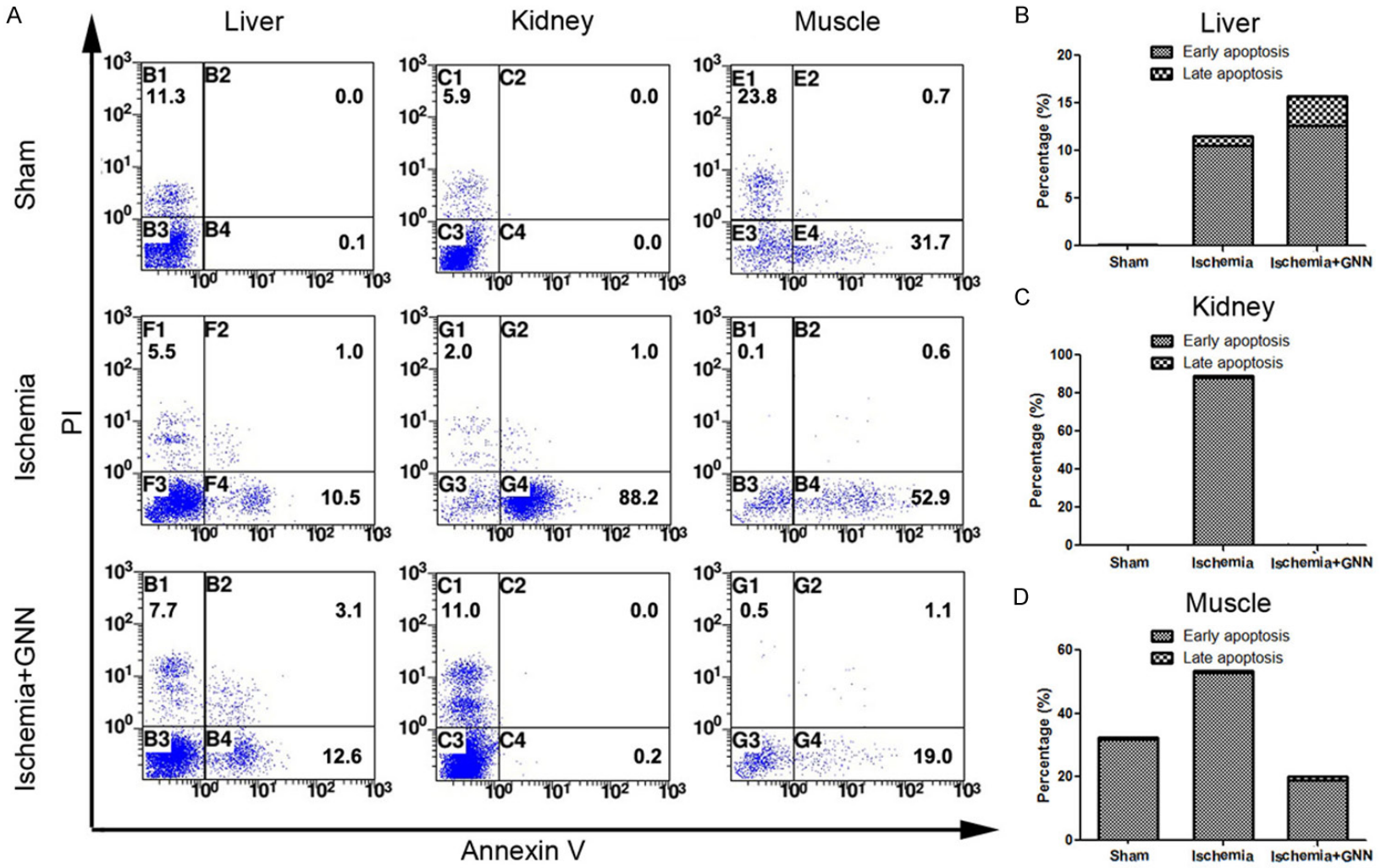

Figure 2. GNN significantly attenuates cellular apoptosis in kidney and muscle tissues induced by ischemia. (A) Flow cytometry to show the percentages of early apoptotic and late apoptotic cells of liver, kidney and muscle tissues treated with Ischemia and GNN. (B-D) Stacked bars to present the percentage distributions of early apoptotic and late apoptotic cells of liver (B), kidney (C) and muscle (D) tissues treated with Ischemia and GNN.

Table 1. Serum tumor necrosis factor-α (TNF-α) levels, Plasma MDA levels, lung MDA levels and lung myeloperoxidase (MPO) activities in corresponding five groups

Groups	TNF-α (pg/ml)	Plasma MDA (nmol/ml)	Lung MDA (nmol/g tissue)	MPO (U/g)
Sham	51.2±6.9	1.67±0.13	41.8±3.2	8.0±0.6
I/R	130.6±5.7[a]	3.45±0.44[a]	84.5±3.7[a]	19.3±1.9[c]
I/R + GNN	85.8±9.1[b]	2.28±0.38[b]	60.3±3.5[b]	10.4±1.2[d]
I/R + PC	90.4±7.6[b]	2.96±0.57	70.4±4.2	16.5±1.7[d]
I/R + PC + GNN	72.2±9.5[b]	1.89±0.20[b]	52.7±4.9[b]	9.3±1.3[d]

[a]Compared with sham-operated group, P<0.01; [b]Compared with I/R group, P<0.01; [c]Compared with sham-operated group, P<0.05; [d]Compared with I/R group, P<0.05; n=8.

Table 2. Determination of edema in lung and muscle tissues in corresponding five groups

Groups	Edema in lung tissue (W/D)	Edema in muscle tissue (W/D)
Sham	3.66±0.32	4.02±0.26
I/R	5.73±0.38[a]	6.07±0.37[a]
I/R + GNN	4.50±0.27[b]	4.86±0.38[b]
I/R + PC	5.01±0.39[b]	5.42±0.41[b]
I/R + PC + GNN	4.02±0.34[b]	4.45±0.39[b]

[a]Compared with sham-operated group, P<0.05; [b]Compared with I/R group, P<0.05; n=8.

matically increased in the rats of I/R group (TNF-α: 130.6 ± 5.7 pg/ml) at the end of reperfusion, compared with that of the sham-operated group (TNF-α: 51.2 ± 6.9 pg/ml). The findings indicated that the inflammatory damage induced by hind limb I/R seemed to be systemic. In the GNN alone (TNF-α: 85.8 ± 9.1 pg/ml) or the combined treatment (GNN + post-conditioning) group (TNF-α: 72.2 ± 9.5 pg/ml), there was a significant fall in the serum levels of pro-inflammatory cytokines at the end of reperfusion compared to the animals of I/R group. This implied that TNF-α increased in systemic circulation after I/R, which could be inhibited by the treatment of GNN alone or by the combined treatment (GNN + post-conditioning). Clearly, the combined treatment is a more effective strategy than the GNN treatment alone. However, there were no statistically significant differences between these groups.

Determination of tissue edema

The extent of tissue edema was determined by measuring tissue wet to dry weight (W/D) ratio. Immediately after autopsy, tissue samples were blotted, weighed, and placed in a drying oven at 55°C until a constant weight was obtained. Tissue edema is suggested by an increase in the W/D ratio. The results are shown in **Table 2**.

Evaluation of liver and renal function

The serum aspartate aminotransferase (AST) and alanine aminotransferase (ALT) levels were measured to assess liver function. The impairment of renal function was assessed by blood urea nitrogen and creatinine levels. Serum levels of ALT, AST, BUN and Cr were measured with an auto-biochemical analyzer (Toshiba, Japan). Compared with the sham-operated rats, the acute limb I/R resulted in a significant increase in AST and ALT levels demonstrating development of severe hepatic cellular injury. GNN administration alone or the combination treatment (GNN + post-conditioning) led to a substantial reduction in the I/R-induced increase in ALT and AST (**Table 3**).

Moreover, compared to sham-operated rats, animals of ischemia/reperfusion group exhibited a significant increase in the serum urea nitrogen levels, suggesting a severe degree of glomerular dysfunction caused by acute limb ischemia/reperfusion injury. After the treatment with GNN alone or the combined GNN + post-conditioning treatment, serum levels of urea nitrogen were significantly reduced compared to animals of the ischemia/reperfusion group. Furthermore, acute limb I/R injury did result in higher creatinine (Cr) concentrations in the animals with saline + I/R groups compared to the sham-operated group. However, treatment with GNN alone or the combined treatment did lead to a significant drop in Cr concentrations (**Table 3**).

Discussion

Besides the regional injury, limb I/R injury may also yield a systemic inflammatory response resulting in multiple organ failure. Limb I/R injury drives the generation and secretion of numerous pro-inflammatory cytokines (TNFα, IL-1β, IL-6 etc.), chemokines (MCP, IL-8 etc.)

Table 3. Serum alanine aminotransferase (ALT), aspartate aminotransferase (AST), blood urea nitrogen (BUN) and creatinine (Cr) in all the five groups

Groups	ALT (μM)	AST (μM)	BUN (mM)	Cr (mM)
Sham	110.4±43.9	63.6±13.4	7.3±1.9	34.9±2.5
I/R	302.3±54.3[a]	147.3±21.3[a]	14.2±2.1[a]	62.3±2.4[a]
I/R + GNN	203.9±43.9[b]	92.2±23.6[b]	9.9±2.5[b]	45.3±2.1[b]
I/R + PC	240.4±43.5	113.7±32.5	11.0±1.6	50.5±2.6
I/R + PC + GNN	177.3±33.6[b]	78.7±16.8[b]	8.7±1.6[b]	40.9±1.8[b]

[a]Compared with sham-operated group, P<0.01; [b]Compared with saline + I/R group, P<0.01.

and ROS into the systemic circulation, which further promotes local and systemic inflammation [24-27]. Remote organ injury, induced by hind limb I/R injury, is characterized by accumulation of inflammatory infiltrates and increased microvascular permeability [28, 29]. Since then, An effective procedure method to attentuate the outcome of I/R injury is clinically meaningful. In this study, a potential effective therapeutic method for I/R injury was developed, which improves functional outcome after ischemic-reperfusion injury in rats and a successful example was provided for combination of surgical operation with a pharmaceutical agent.

As mentioned above, previous studies have shown that ischemic pre-conditioning and post-conditioning can protect ischemic and reperfused organ from I/R injury. Although ischemic pre-conditioning provides a useful tool against I/R injury, its utilization in clinical setting is really limited mainly due to the onset of ischemia is not predictable. Although the effectiveness of post-conditioning against cardiac I/R injury is convincing clinically, there have been only few attempts to adapt this ischemic post-conditioning in other reperfused organs. Recently we have demonstrated the protective effect of nitroxides in an ischemia/reperfusion injury animal model [11, 12, 16]. In the present study, we demonstrated that when ischemic post-conditioning combined with administration of GNN i.v. significantly minimized the oxidative damage induced by ischemia reperfusion injury. This finding suggests that the combination of the two treatments could significantly enhance the beneficial effect of the nitroxide derivative. This might happen because the cyclic reperfusion and re-occlusion of the blood vessel during the ischemic post-conditioning procedure enabled rapid distribution of the circulating GNN into the ischemic organ before the completed reperfusion is established. During the early stage at the onset of reperfusion, GNN might be trapped in the ischemic reperfused organ. Ischemic post-conditioning may further increase the availability of the pharmaceutical agent, GNN. We hypothesized that the combination of the pharmaceutical agent, GNN with ischemic post-conditioning could inhibit the generation of reactive oxygen species by neutralizing NO and thereby the formation of the peroxynitrite from NO and superoxide might be minimized.

Free radical formation and inflammation play an important role in I/R injury [28]. Therefore, we investigated the putative beneficial effects of the combination of GNN (i.v.) and post-conditioning in limb I/R injury by monitoring the changes in serum tumor necrosis factor-α (TNF-α), malondialdehyde (MDA) and myeloperoxidase (MPO) in tissues. Hind limb I/R promotes the inflammatory response by stimulating the production of inflammatory cytokines and chemokines. MDA is an important intermediate of lipid peroxidation and therefore is generally taken as an indicator of lipid peroxidation [30]. In plasma and tissue samples from the test animals, we found that MDA levels significantly increased following I/R damage as compared to sham-operated animals, and these increases were significantly attenuated with GNN alone or the combined treatment (I/R + GNN + post-conditioning). During hind limb ischemia/reperfusion, leukocytes may re-enter the systemic circulation. The activated neutrophils may cause local and remote organ damage, especially in the lungs. After adhering to the pulmonary microvascular endothelium, neutrophils exert their toxic effects through the release of proteolytic enzymes and free radicals (Lakhan et al. 2009). The decrease in lung MPO activity and attenuation of lung histopathology indicated that the combination of GNN and ischemic post-conditioning could protect the remote organ (lung) against limb I/R. Clearly, the combination treatment (GNN + post-conditioning) could significantly inhibit the I/R-induced increase in lung MPO activity and MDA levels, suggesting an inhibition of neutrophil infiltration and lipid peroxidation. This inhi-

bition was also associated with tissue edema as evaluated by W/D ratio (wet to dry tissue weight ratio) (**Table 2**). The inhibition of neutrophil infiltration and lipid peroxidation by the GNN alone or the combined treatment (GNN + post-conditioning) led to attenuate lung damage associated with limb I/R injury.

Urea is the product of protein metabolism made in the liver and excreted via the kidneys. Blood urea nitrogen (BUN) level is used as a marker of renal function. The serum levels of urea nitrogen were significantly reduced after the treatment with GNN alone or the combined GNN + post-conditioning treatment, when compared with the animals of ischemia/reperfusion group. In addition, compared to I/R group of rats, Cr concentrations dropped to a significantly after the treatment with GNN alone or the combined treatment.

In conclusion, ischemic post-conditioning alone with four cycles of 60 s did not result in significant protection. However, when ischemic post-conditioning was employed in combination with nitroxide derivative, GNN (i.v.), the protective effects of the combination of these two treatments were found to be significant. This observation is of clinical interest because it demonstrates that the combination of ischemic post-conditioning and GNN may provide a synergistic protection against reperfusion damage.

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Disclosure of conflict of interest

None.

Address correspondence to: Huijun Duan, Department of Pathology, Hebei Medical University, Key Laboratory of Kidney Diseases of Hebei Province, Shijiazhuang 050017, Hebei Province, China. E-mail: duanhj246@hotmail.com

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