Research paper

The heart-protective mechanism of nitronyl nitroxide radicals on murine viral myocarditis induced by CVB3

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A B S T R A C T

Our previous researches showed that nitronyl nitroxyl derivatives, NNP and NNVP were good anti-oxidants and provided radioprotective effects in C6 cells. The objective of the present study is to investigate the possible antiviral effects and underlying pharmacological of the two nitronyl nitroxide radicals against CVB3 in vitro and in vivo. The results showed that NNP and NNVP were some of the most potent compounds in terms of their antiviral effects by protecting myocardial cells against oxidative damage of free radicals. Treatment with NNP or NNVP could decrease the intracellular ROS level in vitro. They could lead to a significant decrease in activities of biochemical markers AST, CK and LDH in infected murine serum and could increase SOD and CAT activities and decreased MDA activities compared with infected control in vivo. NNP and NNVP could reduce NO production in infected mice by reacting with NO to produce the imino nitroxides which was confirmed by ESR spectrometry. In addition, NNP and NNVP could both decrease the mRNA expression of proinflammatory cytokines, TNF-α, IL-2 and IL-6. In conclusion, nitronyl nitroxide radicals NNP and NNVP were shown to have antiviral activities against CVB3 and they may represent potential therapeutic agents for viral myocarditis.

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1. Introduction

Coxsackieviruses are small, positive strand-RNA viruses that belong to the Picornaviridae family and to the Enterovirus genus [1]. Coxsackie viral protease cleaves dystrophin and hence affects the structural integrity of myocytes [2]. It is reported that oxidative stress has been implicated as a possible mechanism leading to selection of more virulent viral genotypes. A myocarditic stain of CVB3 passed through selenium or vitamin E selection of more virulent viral genotypes. A myocarditic stain of CVB3 and they may represent potential therapeutic agents for viral myocarditis.

Nitronyl nitroxides, stable organic radicals, synthesized more than 30 years ago, have received considerable attention recently because of their capability of scavenging free radical such as OH, H2O2, and O2, protecting endothelial cells from the attack of free radicals [5–9]. They have been shown to attenuate oxidative damage in various experimental models [10–12]. They can shuttle between the nitroxide radical, the reduced hydroxylamine and the oxidized oxoammonium cation form with 1 and 2 electron transfer reactions (Fig. 1). Similar to endogenous SOD, the nitroxide acts as a catalyst and is not consumed in the process of dismutation of O2*− to H2O2 and oxygen. In this process, all these three forms (nitroxide radical, oxoammonium cation, hydroxylamine) can be present in the tissue [13,14]. Contrary to exogenously added SOD or catalase and several common anti-oxidants, nitroxides can provide protection in a catalytic way and can act as self-replenishing anti-oxidants. Thus, this key feature indicates that nitroxides would seem to have unique therapeutic potential for diseases and injuries related to oxidative stress [15–23]. However, little information is available in the literature about nitronyl nitroxide’s effects against CVB3.

We previously synthesized a series of chiral nitronyl nitroxyl derivatives, and found NNP and NNVP were good anti-oxidants and provided radioprotective effects in C6 cells [24,25]. Herein, we plan to investigate the protective effect of NNP and NNVP (Fig. 2) on...
myocardial cells against the harmful effects of CVB3 by scavenging the very reactive hydroxyl and peroxyl radicals. The activities of enzymes such as Aspartate transaminases (AST), creatine kinase (CK) and Lactic dehydrogenase (LDH) in infected murine serum were studied. Histopathological changes induced by CVB3 and serum malondialdehyde (MDA), superoxide dismutase (SOD), and catalase (CAT) activities were determined. The effect of NNP and NNVP on TNF-α, IL-2 and IL-6 mRNA expression was detected in hearts of infected BALB/c mice by RT-PCR. NNP and NNVP can decrease the mRNA expression of the three proinflammatory cytokines. In conclusion, nitronyl nitroxides are shown to have antiviral activities against CVB3 and they may represent potential therapeutic agents for viral myocarditis.

2. Material and methods

2.1. Reagents and chemicals

3-[4,5-Dimethyl-2-thiazolyl]-2,5-diphenyl-2-tetrazolium bromide (MTT) were purchased from Sigma. Guanidine hydrochloride (Gu HCl) was provided by the China Medicine (Group) Shanghai Chemical Reagent Corporation, China. Ribavirin (Injection, 100 mg/ml) was purchased from the Shanghai Fenghe Pharmaceutical Group Corporation (Lot. No. 010902). Twice distilled de-ionized water was used throughout the experiments.

2.2. Antivirus experiment

2.2.1. Animals

BALB/c mice (4 weeks old, 14–16 g, male) and Sprague-Dawley (SD) rats (180–220 g, male, for pharmacokinetic and serum pharmacological experiments) were purchased and maintained at Experimental Animal Center, the Fourth Military Medical University (cleaning grade, certificate No. 2002-005). They were housed under constant conditions at a temperature of 23 ± 1 °C, a humidity of 40 ± 5%, and on a 12 h light/12 h dark cycle. They had free access to pellet feed and tap water. The animal experiments were performed according to the European Community guidelines for care and use of animals, and approved by the Ethic Committee for Animal Use of Shanghai Institute of Materia Medica.

2.2.2. CVB3 virus

Coxackievirus B3 Nacy strain which was generously provided by the Department of Microbiology, the Fourth Military Medical University, was propagated in Hela cell monolayers and stored at −70 °C. Viral titers were determined by plaque assay, and the concentration of the CVB3 titer used for the infections was 1 × 10^6 p.f.u./ml (plaque forming units/ml).

2.2.3. Cell culture

Hep-2 cells were kindly provided by the Institute of Traditional Chinese Medicine, China Academy of Traditional Chinese Medicine. They were grown at 37 °C in a humidified atmosphere with 5% CO₂ in Eagle’s minimum essential medium (MEM) supplemented with 10% fetal bovine serum, and 2 μM glutamine, 10,000 units/ml penicillin, 50 μg/ml streptomycin, and 2.5 μg/ml amphotericin B. Primarily cultured myocardial cells from the hearts of neonatal day 1–3 SD rats were cultured in MEM supplemented with 20% fetal bovine serum, and 2 μM glutamine, 10,000 units/ml penicillin, 50 μg/ml streptomycin, and 2.5 mg/ml amphotericin B.

2.2.4. In vitro experiments [26]

Primarily cultured myocardial cells were prepared from the hearts of neonatal day 1–3 SD rats, following disaggregating into individual cells with sequential digests with trypsin. Myocytes (1 × 10^5 cells/well) were cultured in each well of a 24-well plate with a final volume of 2 ml including 20% fetal bovine serum. For cytotoxicity assays, myocytes were seeded into 24-well plates at a density of 1 × 10^5 cells per well and incubated at 37 °C in a humidified atmosphere with 5% CO₂ for 24 h. The medium was removed and fresh DMEM containing the appropriate dilution of NNP or NNVP was added into subconfluent myocytes. These compounds were dissolved in water and added to the medium at a final concentration of 50, 100, 150, 300, 600, 1200 mg/l respectively. After 3 days of incubation, the myocytes showed obvious Cytopathic Effect (CPE) characterized by crimple and refractive brightness and quantified by the high proportion of cells with CPE. The growth medium was removed and viability of the drug was determined in a standard neutral red dye assay [27]. The neutral red dye uptake was determined by measuring the optical density (OD) of the eluted neutral red at 540 nm in a spectrophotometer. The cytotoxic concentration of the drug which reduced viable cell number by 50% (TC50) was determined from dose–response curves [28].

Cells were exposed to the virus (0.5 × TCID50, 0.5 ml/well) for 1 h and washed with PBS three times and then incubated for 72 h with 5 μg/ml NNP, NNVP or Tempol for the infected and treated cells or without any treatment for the infected controls. Myocytes cultured without viral infection served as the normal control. As required by The State Food and Drug Administration, infected cells treated with 1 mg/ml Gu HCl, which suppresses virus propagation in vitro but is banned for in vivo use because of toxicity, were used as negative controls. The infected myocytes showed obvious CPE characterized by crimple and refractive brightness and quantified by the high proportion of cells with CPE. Maximal viral CPE occurred at 72 h post-infection. Virus titers were determined with Hep-2 cells by the plaque assay for whole cell lysates. Briefly, whole cell lysates were 10-fold serially diluted and mixed with hep-2 cells. At a certain dilution, countable plaques are formed, whereas either no plaques or uncountable plaques are formed at other dilutions.

![Fig. 1. The redox transformation of the various oxidation states of nitroxyls.](image)

![Fig. 2. Structures of nitronyl nitroxide radicals NNP and NNVP.](image)
The negative logarithm of the dilution at which countable plaques are formed (–lg TCID₅₀) was defined as an indicator for virus titers of the whole cell lysate samples.

With the fluorescent probe DCDFH-DA to measure ROS changes. DCDFH-DA (2,7-dichlorofluorescein diacetate) is a non-fluorescent material which can be through cell and oxidized to highly fluorescent DCF (2,7-dichlorofluorescin) by ROS. Briefly, cells were loaded with 10 μM DCDHFDA in serum free phenol-red-free medium, then incubation for 30 min at 37 °C. Afterward, cells were washed and lysed with 0.1 N NaOH. Fluorescence was measured using a microplate fluorometer (Spectra MAX, Gemini EM, Molecular Devices) using wavelengths of 480 and 530 nm for excitation and emission, respectively [29].

2.2.5. In vivo experiments

BALB/c male mice at the age of 4 weeks were inoculated intraperitoneally with CVB3 (10 × TCID₅₀). The inoculated mice were given NNP or NNVP orally at a dose of 20 mg/kg (n = 60), or injected intraperitoneally with Ribavirin at a dose of 10 mg/kg (n = 60) daily for 7 days and observed carefully. Sixty inoculated mice were treated with 0.9% NaCl solution daily and used as infected controls. Mice without inoculation (n = 12) were given orally 0.9% NaCl solution daily and used as normal controls. Mice were killed on days 7 and 14 (n = 30, in each group), mortalities and the body weight (BW) and heart weight (HW) of each animal were measured to calculate the heart to body weight ratio (HW/BW). Since Gu HCl cannot be used in in vivo experiments, Ribavirin (RBV), which is able to alleviate symptoms of viral myocarditis and widely accepted in clinical practice in China, was used for the negative controls. The right basal part was fixed in 10% buffered formalin. The remainder of heart was homogenized in 1 ml of MEM. After centrifugation, the supernatant was 10-fold sequential diluted with MEM to determine virus titers by the plaque assay with Hep-2 cells, as described above. The heart tissue fixed in formalin was sectioned (4 μm thick) and stained with hematoxylin and eosin. Photographs for the histological changes were taken by SPOT software.

Blood was collected from the eye sockets and separated into serum to measure the activities of lactic dehydrogenase (LDH), aspartate transaminases (AST), creatine kinase (CK) by using commercially available kits. The left basal part of the heart of mice in each group was frozen in liquid nitrogen for RNA extraction, and the right basal part was divided into two parts. One part was homogenized in 0.9% NaCl solution and centrifuged to determine the activities of malondialdehyde (MDA) (end product of lipid peroxidation), superoxide dismutase (SOD), nitric oxide (NO), and catalase (CAT). The MDA content was determined spectrophotometrically by measuring the presence of thiobarbituric acid reactive substances (TBARS) [30]. SOD enzyme activity determination was based on the production of H₂O₂, from xanthine by xanthine oxidase and reduction of nitroblue tetrazolium as previously described [31]. The product was evaluated spectrophotometrically. CAT activity was determined according to Aebi’s method [32].

Since serum nitrite (NO₂⁻) and nitrate (NO₃⁻) levels can be used to estimate NO production, we measured the concentration of NO₂⁻ and NO₃⁻. Quantitation of NO₂⁻ and NO₃⁻ was based on the Griess reaction, in which a chromophore with a strong absorbance at 545 nm is formed by reaction of NO₂⁻ with a mixture of naphthylethylene diamine and sulfanilamide [33]. Results are expressed as μmol/l.

2.2.6. Detection of cytokine expression by reverse transcriptase polymerase chain reaction

Total cytoplasmic RNA was prepared from the heart tissues of mice from each group by Trizol reagents (Sango Shanghai, China) and reversely transcripted into cDNA. Real-time PCR was performed to evaluate the relative expression of TNF-α, IL-2, IL-6, TNF-α (171 bp); Forward 5′-GGCACGGTTGCTCTGGAAAG-3′, Reverse 5′-GATCCCGCTGAGCTATGAC-3′; IL-2 (243 bp) : Forward 5′-GCCACGTCCTTCCTTTGCA-3′, Reverse 5′-CAAACTCTGAGGAGTGG-3′; β-actin (331 bp): Forward 5′-TGAGCGCCCAGCAGGAA-3′, Reverse 5′-ATGGCAACGTGGGGTGAC-3′. PCR products were electrophoresed on a 1.5% ethidium bromide-stained agarose gel. Band intensity was measured under ultraviolet light, and the intensity of cytokine PCR products was compared in a semiquantitative fashion as a ratio to that of β-actin product derived from the same cDNA sample.

2.2.7. Statistical analysis

Data was analyzed by using a commercially available statistics software package (SPSS for Windows, version 14.0, Chicago, USA). One-way analysis of variance (ANOVA) test performed and Post Hoc multiple comparisons were done with least significant difference (LSD). Results were presented as means ± standard deviation (S.D.); p values < 0.05 was regarded as statistically significant.

3. Results

3.1. In vitro experiments

As shown in Table 1, the TC₅₀ of the NNP and NNVP was 493.5 ± 6.1 mg/l and 478.2 ± 5.9 mg/l respectively. Gu HCl was demonstrated to have stronger toxicity than nitronyl nitroxides. Virus titers in the infected group were significantly increased compared with the normal control. While the virus titers were declined evidently by the nitronyl nitroxide derivatives in vitro (p < 0.01, compare with the infected group, Table 2). The results showed that the two derivatives both had potent antiviral activity in reducing the virus titers in primarily cultured myocardial cells. The antiviral activity is higher with nitronyl moieties on alicyclic ring than that of on the benzene ring, for example, virus titers of NNVP is 4.05 ± 0.27, which is lower than that of NNVP (4.71 ± 0.53) (p < 0.05). The results also showed that the antiviral activity of NNP and NNVP is higher than that of Tempol (p < 0.05).

In addition, we also detected the effect of the derivatives on the intracellular ROS content. As shown in Fig. 3, the CVB3 infection can lead to significantly increase intracellular ROS content (p < 0.01). While treatment with the nitronyl nitroxides could decreased the intracellular ROS level (p < 0.01, compare with the infected group). Moreover, the activity of NNP and NNVP to scavenge ROS was more effective than that of Tempol (p < 0.05).

3.2. In vivo experiments

3.2.1. Effect on mortality and HW/BW ratios

We used the Nacy variant of CVB3 to induce mild myocarditis in a BALB/c mice strain model. The HW/BW ratios in the infected control group on days 7 and 14 were significantly increased compared with those in the normal control group. However, the ratios were significantly decreased in mice treated with NNP or Tempol. The HW/BW ratios in the infected group were significantly increased compared with the normal control (p < 0.01). While treatment with the nitronyl nitroxides could decreased the intracellular ROS level (p < 0.01, compare with the infected group). Moreover, the activity of NNP and NNVP to scavenge ROS was more effective than that of Tempol (p < 0.05).

<table>
<thead>
<tr>
<th>Group</th>
<th>TC₅₀ (mg/l, means ± S.D.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gu HCl</td>
<td>380.5 ± 5.8</td>
</tr>
<tr>
<td>Tempol</td>
<td>410.2 ± 4.7</td>
</tr>
<tr>
<td>NNP</td>
<td>493.5 ± 6.1</td>
</tr>
<tr>
<td>NNVP</td>
<td>478.2 ± 5.9</td>
</tr>
</tbody>
</table>
Effects of nitronyl nitroxide radicals NNP and NNVP on mortality, HW/BW and virus titers of infected BALB/c mice on the 7th and 14th day post-infection.

### Table 3

<table>
<thead>
<tr>
<th>Group</th>
<th>Mortality (%)</th>
<th>HW/BW (means ± S.D.)</th>
<th>Virus titers (−lg TCID50, means ± S.D.)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Normal control</td>
<td>0</td>
<td>0.42 ± 0.42</td>
<td>0</td>
</tr>
<tr>
<td>Infected control</td>
<td>6.39 ± 0.41</td>
<td>4.45 ± 0.42</td>
<td>4.45 ± 0.42</td>
</tr>
<tr>
<td>Gu HCl</td>
<td>3.28 ± 0.35</td>
<td>6.95 ± 0.33</td>
<td>6.27 ± 0.82</td>
</tr>
<tr>
<td>NNP</td>
<td>4.05 ± 0.27</td>
<td>5.42 ± 0.41</td>
<td>5.03 ± 0.71</td>
</tr>
<tr>
<td>NNVP</td>
<td>4.71 ± 0.53</td>
<td>4.43 ± 0.37</td>
<td>4.61 ± 0.69</td>
</tr>
<tr>
<td>Tempol</td>
<td></td>
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</table>

*p < 0.01 compared to the infected control.

### 3.3. Results of mRNA expression of proinflammatory cytokines

The mRNA expression of proinflammatory cytokines, TNF-α, IL-2 and IL-6 mRNA both was decreased in mice treated with NNP or NNVP compared with those of the infected mice (Fig. 6A–D).

### 3.4. Pathological findings

The Pathological findings show that the cell nucleolus of normal myocardium was obvious and clear and the cytoplasm was enriched. The cell membrane held integrity and there were no infiltrating cells among cardiac fibrin (Fig. 7A). In infected murine hearts, mononuclear cell infiltration and necrosis could be observed obviously (Fig. 7B). Compared with the infected control group, the damage of myocardium was relieved and scores of necrosis and infiltration were decreased significantly with treatment of NNP or NNVP (Fig. 7C–F).

### 4. Discussion

The majority cases of viral myocarditis (VMC) are due to enterovirus infection and active replication of CVB viruses appeared in a significant proportion of patients with idiopathic DCM and heart failure as well [34–37]. Increasing evidence supports the role of cardiomyocytes apoptosis in the development of VMC and myocardium remodeling [39] and free radicals relate to the pathogenesis [39–41]. Previous work has shown that reactive oxygen species (ROS) plays a crucial role in the development of CVB3-induced pathogenesis in VMC [42–44]. ROS are intermediates formed during one-electron reduction of molecular oxygen (dioxigen, O2). Some important ROS in biological systems include superoxide radical anion (O2•−), hydrogen peroxide (H2O2), and hydroxyl radical (•OH). Most free radicals are highly reactive and extract electrons from neighboring molecules to complete their own orbital, which results in oxidation of the biological molecules [45–48]. Since ROS are continuously being produced in cells, oxidative stress occurs not as a result of the production of ROS, but rather when the biosynthesis of ROS exceeds the capacity of various intrinsic anti-oxidant defense systems to detoxify these reactive species. Although many enzymatic processes can lead to the formation of ROS, the mitochondrial electron transport chain is thought to be the principal source of partially reduced forms of oxygen. Therefore, there has been considerable interest among scientists and clinical researchers in the development of effective ROS (or electron) scavengers as therapeutic agents to resist ROS damage in VMC. However, many of the most widely studied compounds have their limits. For example, when used as therapeutic agents, proteins like superoxide dismutase (SOD) and catalase fail to penetrate cell membranes and are therefore ineffective compared with infected control (p < 0.01). Meanwhile, they decreased MDA activities and NO production to protected myocardial cells against the harmful effects of CVB3.

### Table 2

In vitro effects of nitronyl nitroxide radicals NNP and NNVP on virus titers in myocardial cells 72 h after infection.

<table>
<thead>
<tr>
<th>Group</th>
<th>Virus titers (−lg TCID50, means ± S.D.)</th>
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<tr>
<td></td>
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<td>Normal control</td>
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<td>Tempol</td>
<td></td>
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</table>

*a p < 0.01, compared to the infected controls.
b p < 0.05, compared to the Tempol group.
c p < 0.05, compared to the NNP group.

### Fig. 3

Effects of NNP and NNVP on the intracellular ROS changes. *p < 0.05 vs. normal control group, **p < 0.05 vs. infected control group, ***p < 0.01 vs. infected control group.

### 3.2.2. Effect on LDH, AST, and CK in infected murine serum

Several biochemical markers, such as AST, CK and LDH were used to identify myocardial injury. Our research confirmed that CVB3 has a significant effect on the various membrane-bound enzymes in terms of increasing activities of plasma AST, CK and LDH. The results showed that treatment with NNP or NNVP (20 mg/kg) could lead to a significant decrease in activities of AST, CK and LDH in infected murine serum compared with infected control groups (Fig. 4A–C).

### 3.2.3. Effects on level of SOD, MDA, CAT and NO in infected murine serum

As shown in Fig. 5A–D, SOD and CAT activities significantly decreased while MDA activities and NO production were obviously increased due to CVB3 infection. Treatment with NNP or NNVP (20 mg/kg) could increase SOD and CAT activities significantly compared with infected control (p < 0.01). Meanwhile, they decreased MDA activities and NO production to protected myocardial cells against the harmful effects of CVB3.

### Table 3

Effects of nitronyl nitroxide radicals NNP and NNVP on mortality, HW/BW and virus titers of infected BALB/c mice on the 7th and 14th day post-infection.

<table>
<thead>
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<th>Group</th>
<th>Mortality (%)</th>
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*a p < 0.01, compared to the infected control.
b p < 0.01, compared to the normal control.
against ROS that are produced intracellularly. Vitamin E and Coenzyme Q are very lipophilic. Therefore, these compounds tend to be retained within the cytosolic membrane, and may fail to achieve pharmacologically significant intracellular concentrations [49].

In the present study, we aimed at investigating the novel ROS scavengers, nitronyl nitroxides, as a potential therapeutic agent for VMC. Stable nitroxyls include two types: TEMPO (2,2,6,6-tetramethylpiperidine-N-oxyl) and NIT (nitronyl nitroxyl) [50]. At present, NIT radicals for against CVB3 effects have not been well characterized. Therein, we carried out experiments to elucidate the antiviral effect of chiral NIT nitroxyl compounds NNP and NNVP both in vitro and in vivo.

In vitro experiment, when the infected primarily cultured myocardial cells were treated with NNP and NNVP, virus titers decreased significantly (Table 2). This observation indicated that nitronyl nitroxide radicals had excellent antiviral activity in vitro and had a protective effect on myocardial cells against CVB3 infection. It is suggested that NNP and NNVP can shuttle between the nitroxide radical, the reduced hydroxylamine and the oxidized oxoammonium cation form with 1 and 2 electron transfer reactions (Fig. 1). They exert SOD-mimetic activity [51], which contributes to

Fig. 4. Effects of NNP and NNVP on the activities of AST (A), LDH (B), and CK (C) in infected murine serum on the 7th day and 14th day post-infection. *p < 0.05 vs. normal control group, **p < 0.01 vs. infected control group.

Fig. 5. (A) The effects of NNP and NNVP on serum SOD activity in infected murine hearts on the 7th and 14th day post-infection. (B) The effects of NNP and NNVP on serum MDA levels in infected murine hearts on the 7th and 14th day post-infection. (C) The effects of NNP and NNVP administration on serum catalase (CAT) in infected murine hearts on the 7th and 14th day post-infection. (D) The effects of NNP and NNVP administration on serum nitric oxide (NO) levels in infected murine hearts on the 7th and 14th day post-infection. ##p < 0.01 vs. normal control group, **p < 0.01 vs. infected control group.
the prevention of the reaction of \( \text{O}_2^\cdot \) with nitric oxide, thereby inhibiting formation of the highly toxic species. The one-electron oxidation of the nitroxide radical (\( \text{RR}'\text{NO}^\cdot \)) by the protonated form of superoxide (\( \text{O}_2^\cdot - \)) converts the nitroxide to the corresponding oxoammonium cation (\( \text{RR}'\text{NO}^+ \)) [52]. The oxoammonium cation can then be reduced back to the nitroxide radical:

\[
\text{RR}'\text{NO}^+ + \text{O}_2^- + 2\text{H}^+ \rightarrow \text{RR}'\text{NO}^\cdot + \text{H}_2\text{O}_2
\]

The oxoammonium cation (\( \text{RR}'\text{NO}^+ \)) form can convert to the corresponding hydroxylamine (\( \text{RR}'\text{NOH} \)) if it is reduced continuously through obtaining 2 electrons. Hydroxylamines act as effective ROS scavengers and in the process are converted back into nitroxides. In other words, these compounds undergo redox recycling [53].

Therefore, nitroxides combine several important anti-oxidant and electron-scavenging properties in a single functional moiety. A recent study reported that the effectiveness of Tempol in catalyzing the metabolism of cellular \( \text{O}_2^\cdot - \) was similar to native SOD and only slightly less than the cell permeabilized pegylated form of SOD (PEG-SOD). Tempol was significantly more effective than other frequently used “anti-oxidants” and was far more effective than vitamins. Among a group of 6-member ring nitroxides, the in vitro SOD-mimetic activity in metabolizing \( \text{O}_2^\cdot - \) closely paralleled their in vitro actions [54], consistent with the concept that the biological efficacy of Tempol depended on its facility to promote the metabolism of \( \text{O}_2^\cdot - \).

Serum aminotransferase activities have long been regarded as indicators of tissue injury. Injury of myocardium alters the structure and function of myocardium in rats, leading to leakage of enzymes from the cells. Therefore, the marked release of biochemical markers into the circulation indicates severe damage to heart tissue membranes during the reperfusion process. Therefore, several biochemical markers, such as AST, CK and LDH were used to identify myocardial injury in our research in vivo experiment. It is confirmed that CVB3 has a significant effect on the various membrane-bound enzymes in terms of increasing activities of plasma AST, CK and LDH. The presence of NNP or NNVP protected myocardial cells against the harmful effects of CVB3 by decreasing the leakage of the enzymes from the myocardial cells into the blood stream and maintaining the levels of these enzymes at normal values.

We also studied the effects of NNP or NNVP on the level of MDA, SOD and CAT in infected murine serum. The results show that NNP or NNVP can protect heart muscles against harmful effects of free radicals by modulating SOD, CAT and MDA activities (Fig. 4). To protect cells against oxidative damage by free radicals, an anti-oxidant system, including SOD and CAT enzymes [55–57] has evolved in aerobic organisms (reviewed in Ref. [58]). These enzymes work in tandem to scavenge ROS. The SOD catalyzes the dismutation of superoxide anion (\( \text{O}_2^\cdot - \)) to \( \text{H}_2\text{O}_2 \). Subsequently, \( \text{H}_2\text{O}_2 \) is reduced to \( \text{H}_2\text{O} \) and \( \text{O}_2 \) by peroxidases or CAT. Under conditions of increased ROS production or when the anti-oxidant system is compromised, cells are unable to efficiently scavenge the free radicals, leading to ROS accumulation [45]. MDA is a major reactive aldehyde that appears during the peroxidation of biological membrane polyunsaturated fatty acids. Therefore, the content

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**Fig. 6.** (A) The representative expression of TNF-\( \alpha \), IL-2 and IL-6 in the hearts of infected BABL/c mice with or without NNP and NNVP treatment as detected by RT-PCR on the 14th day post-infection. The mRNA expression of TNF-\( \alpha \) (B), IL-2 (C) and IL-6 (D) in the hearts of mice, \( n = 15, ^{\#}p < 0.05 \) vs. normal control group, **\( ^{\#\#}p < 0.01 \) vs. infected control group.
of MDA can be used as an indicator of heart tissue damage. In this study, CVB3 infection caused a significant decrease in SOD and CAT and increase in MDA (Fig. 5). Tissue damage may result in lipid peroxidation and necrosis, and the increased tissue MDA levels can be accepted as a criterion of tissue injury. Our experiment proved that treatment with NNP or NNVP (20 mg/kg) could increase SOD and CAT activities and decreased MDA activities and NO production significantly compared with infected control (p < 0.01). This protective effect of nitronyl nitroxides is still considered due to their ability to scavenge the very reactive hydroxyl and peroxyl radicals thereby limiting injury. The nitroxide moiety can be reduced to hydroxylamines or oxidized to oxoammonium cations via electron transfer reactions in vivo. Through the continuous exchange between the two forms they can act as self-replenishing anti-oxidants thus bestow catalytic protective activity. The results are in accord with the reference data. For instance, Tempol given to rats after coronary ligation and reperfusion decreased the infarct size of the myocardium by up to 60% when given during the reperfusion period [59,60], protected the heart against oxidative damage due to ischemia–reperfusion injury [61–66].

It has been reported that nitronyl nitroxide radicals can be useful in scavenging NO in the acetylcholine-induced vaso-relaxation assay. In the present study, ESR spectrometry revealed the existence of unpaired electrons in NNP or NNVP (Fig. 8). As shown in Fig. 8, different types of radicals show different patterns in the ESR spectra. Treatment with NNP or NNVP at a dosage of 20 mg/kg can reduce NO production significantly (p < 0.01, compared with infected control). This is due to an electron interacting with two inequivalent nitrogens. NNP or NNVP may react with NO to produce the imino nitroxides (Scheme 1). In order to confirm this mechanism, we conduct the direct trapping experiment to evaluate the NO trapping ability of NNP and NNVP using PC 12 cell survival assay following the published method with minor modifications [67–72]. It was observed that the initial spectrum of the NNP or NNVP had five lines with an intensity ratio of 1:2:3:2:1. When NO gas was bubbled into the solution of NNP or NNVP in deaerated phosphate buffer, the ESR spectrum was changed to a 7-line pattern with an intensity ratio of 1:1:2:1:2:1:1.

Though CVB3 can directly disrupt dystrophin and sarcolemmal integrity in infected myocytes [2]. However, this is a nonspecific viral response, as the dystrophin cleavage does not take place in adenovirus-infected myocytes. Another potential mechanism is an elevation of pro-inflammatory cytokines, such as tumor necrosis factor-α (TNF-α), IL-2 and IL-6 that influence ventricular remodeling in myocarditis which can lead to heart failure [73–75]. In our study, we found that TNF-α, IL-2 and IL-6 mRNA expression increased significantly in the infected control group on days 7 and days 14 compared with those in the normal control group (Fig. 6). Oxidative stress has been proposed as one of these mechanisms. Beck et al. [76] found that the originally nonvirulent
CVB3 isolated from GPx-1−/− mice after infection showed mutations in the most oxidation sensitive bases, resulting in virulence of the virus. An explanation for this process is that viral RNA may be oxidatively damaged when GPx-1 is deleted, supporting a protective effect of GPx-1. Therefore cytokines play a critical role in maintaining this balance as they are known to modulate lymphocyte and myocardial cell functions [77,78]. Mariappan reported that TNF-α released during sepsisemia enhanced ROS in cardiomyocytes, reduced cardiac contractility, impaired the efficiency with which the cells used oxygen, reduced cardiomyocyte ATP levels and caused cardiac mitochondrial dysfunction by opening the mitochondrial membrane pore [79,80]. The results of the effect of nitronyl nitroxides NNP and NNVP on the mRNA expression of cytokines in our experiment indicate that these derivatives can lead to a significant decrease of TNF-α, IL-2 and IL-6 mRNA expression, suggesting that nitronyl nitroxides may prevent inflammatory responses and suppress myocardial apoptosis and myocardial dysfunction by inhibiting TNF-α and IL-2, IL-6 mRNA expression.

In summary, two chiral novel nitronyl nitroxide derivatives NNP and NNVP were evaluated as a potential therapeutic agent for viral myocarditis. The derivatives displayed higher effective inhibitory potencies against CVB3 than Guanidine hydrochloride in vitro. Further investigations of these compound by antiviral experiment in vivo confirmed that NNP and NNVP can both significantly increase the survival rate and reduce mortality induced by CVB3 infection. Furthermore, NNP displayed better effect on inhibiting the activities of AST, CK and LDH. These observations indicated that nitronyl nitroxide derivatives may represent potential therapeutic agents for viral myocarditis.

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References

Scheme 1. The reaction of nitronyl nitroxide radicals with NO.

![Scheme 1](image-url)