ABSTRACT

Objective. Cardiac ischemia/reperfusion (I/R) injury, a necessary consequence of transplantation, is probably related to the formation of reactive oxygen species (ROS). The ROS burst within the first moments of reperfusion is associated with injury, continuously generate O$_2^{-}$ at about 3% to 5% of total O$_2$ consumption owing to electron leak by mitochondrial oxidoreductases, especially complexes I and III. 3-nitro-N-methyl-salicylamide (NNMS) displays inhibitory effects on succinate-cytochrome C reductase, but also reduces effects on creation of O$_2^{-}$/H$_2$O$_2$ radical and H$_2$O$_2$ by isolated rat mitochondria. Presumably NNMS inhibits electron leakage from the mitochondrial respiratory chain. We investigated effect of NNMS on heart protection after hypothermic ischemia.

Methods. A Langendorff-prepared rat heart model was employed after the heart had been preserved for 4 hours under hypothermic conditions of ischemia with subsequent reperfusion/rewarming for 60 minutes.

Results. The group of hearts treated with NNMS showed increased recovery of heart function compared with a group of mEC. The lactate dehydrogenase (LDH) activity in coronary flow (CF) by hearts treated with NNMS was lower than that with mECs, as was the content of malondialdehyde (MDA) and conjugated diene (CD).

Conclusions. NNMS improved heart physiology after reperfusion following 4 hours of hypothermic ischemia.
Complexes I and III of the respiratory chain are responsible for leaking electrons, thereby generating \( \text{O}_2^- \). \( \text{O}_2^- \) and \( \text{H}_2\text{O}_2 \) are the primary sources of ROS. They play prominent roles in diverse pathophysiologic processes, such as aging, neurodegeneration, as well as heart and lung toxicity. It is probable that addition of certain substances to classic cardioprotectant and storage solutions may exert beneficial effects against ROS, thereby preserving the functional capacity of the myocardium. The substances may reduce leakage of electrons from complexes I and III of the respiratory chain.

3-Nitro-N-methyl salicylamide (NNMS), which was synthesized by our laboratory, was previously shown to mitigate I/R injury in the rat brain. In this study, we observed that NNMS improved recovery of heart function during 4 hours of cold storage and reperfusion using an isolated rat heart model.

MATERIALS AND METHODS

We employed hypothermic conditions to reduce metabolism and thereby limit ischemic damage. Theoretically, lowering preservation temperature by 10°C lowers the cells’ metabolic demand by approximately 50%. The generally accepted method of preserving donor heart integrity is arrest with cold (4°C) cardioplegic solution followed by storage in an electrolyte solution immersed in ice. The temperatures attained by this technique are believed to reach 4°C.

Model of Isolated and Perfused Rat Heart

Male Sprague Dawley (SD) rats (weighing 300 to 350 g) were anesthetized using thiopental sodium and injected IV with heparin (500 IU). After the chest was opened using bilateral sternocostal sections, the heart was immediately excised and placed into a cold bath (4°C) containing Krebs-Henseleit buffer (K-HB). It was fixed through the aortic root and left atrium on to perfusion cannulas of a Langendorff apparatus for perfusion at a constant pressure of 60 mm Hg. The K-HB solution had the following composition (mmol/L): NaCl 119.0; NaHCO\(_3\) 25.5; KCl 4.3; KH\(_2\)PO\(_4\) 1.2; MgSO\(_4\) 1.2; CaCl\(_2\) 2.5; and glucose 11.0. K-HB was used as the perfusion medium. Saturated with 95% O\(_2\) and 5% CO\(_2\) at pH 7.4 at stable temperature of 37°C.

A water-filled latex balloon was inserted into the left ventricle. The balloon was adjusted to a left ventricular end-diastolic pressure (LVEDP) of 6 to 9 mm Hg. We continuously monitored the left ventricle developed pressure (LVDP), its first derivatives \( \frac{dp}{dt}_{\text{max}} \), and heart rate (HR). The resultant electrical signals were digitized by MacLab analog to digital converter and recorded by computer. Coronary flow (CF) was measured by timed collection.

After a 20-minute stabilization period, the heart was arrested with 30 mL cardioplegic solution (modified Euro-Collins solution, mECs) at 4°C using a constant pressure of 60 mm Hg. The mEC solution had the following composition (mmol/L): K\(_2\) HPO\(_4\) 3; H\(_2\)O 42.5; NaHCO\(_3\) 10.0; KCl 14.0; KH\(_2\)PO\(_4\) 15.0; MgSO\(_4\) 1.7; H\(_2\)O 5.0; CaCl\(_2\) 0.025; and glucose 139.0. Then the heart was disconnected from the circuit, immersed in storage solution, and stored in an icebox for 4 hours. Thereafter, the organ was reperfused at 37°C in Langendorff mode for 60 minutes during which LVEDP, LVDP, \( \frac{dp}{dt}_{\text{max}} \), and HR were monitored, and CF measured by timed collections. The experiment was divided into 3 groups: group C \((n = 8)\); (2) group mEC \((n = 8)\); and (3) group N+m \((n = 8)\). The group C hearts were not immersed in storage solution. The group mEC hearts were immersed in mEC solution. The group N+m hearts were treated with NNMS (8 \( \mu \)g/mL) added to the mEC solution. To observe whether mEC solution had protective actions, the C group hearts were not immersed in a preservation solution.

Myocardial Water Content

To obtain heart wet weight (WW), the hearts were dried to a constant weight in a 80°C oven for more than 48 hours and reweighed to obtain heart dry weight (DW). We calculated percent myocardial water content (MWC) as follows:

\[
\text{MWC(\%)} = \left( \frac{\text{WW} - \text{DW}}{\text{WW}} \right) \times 100\%
\]

Malonyldialdehyde Levels

The malonyldialdehyde (MDA) assay method has been described by Askawa. MDA was an estimate of the extent of oxidative damage. In brief, heart biopsy samples (0.3 g) homogenized with 0.7 mL of 0.9% NaCl at 4°C were incubated with 2.25 mL of thiobarbituric acid-reactive substances (TBARS). In 100 mL TBARS were thiobarbituric acid (0.67 g) and trichloroacetic acid (TCA 12 g). Successive procedures included heating to 100°C for 30 minutes followed by cooling and centrifugation (3000 rpm) for 10 minutes. The MDA concentration was determined at 532-nm excitation using a spectrophotometer (721 type made in Shanghai).

Results were shown as nanomoles of MDA per mg of protein. The protein content of myocardial tissue was measured by the Bradford method.

Assay of Conjugated Diene

Myocardial conjugated diene (CD) was measured by the method of Corongiu et al. Homogenized heart tissue was extracted; the OD was determined at 233 nm excitation. The results are shown as nanomoles of CD per mg of protein.

Contents of Coronary Flow

The protein content in coronary flow was measured by the Bradford method; myoglobin, (Mb) by the method of Elz et al, lactate dehydrogenase (LDH), by an automated biochemistry instrument.

RESULTS

Effects of NNMS on Functional Parameters

There was no significant difference in the preischemia values of LVEDP. The post-storage values of LVEDP were significantly lower among hearts treated with NNMS than the group C \((P < .05; \text{Fig } 1\text{C})\). Mean values of recovery (% prestorage values) of LVEDP were 23.19%, 16.71%, and 52.51%, respectively, a significant improvement in LVDP, \( \frac{dp}{dt}_{\text{max}} \) and \( -\frac{dp}{dt}_{\text{max}} \) among hearts treated with NNMS compared with both groups C and mEC both \( P < .05; \text{Fig. 1A, B, and D} \). These groups also showed improvements in HR mean value of recovery upon reperfusion compared with groups C and mEC, although the result, did not reach statistical significance. Although NNMS showed 71.28% of the values recorded in the pre-ischemic period; C and mEC groups displayed 65.67% and 59.39%, respectively. There was no significant difference in the mean value of recovery of CF. These results suggested a beneficial role of NNMS in ischemia-reperfusion injury.
Myocardial Water Content Levels Evaluation

There was no significant effect of NNMS treatment on mean values of MWC. The MWC in the NNMS group was 81.006% ± 1.316%, whereas in the C group it was 80.895% ± 2.098%, and in the mEC group, 81.676% ± 1.189%.

Evaluation of Mb, Protein Content, and LDH Activity

There was no significant influence of NNMS on the mean values of Mb content in coronary flow. A significantly lower protein content was in coronary flow of the hearts treated with NNMS compared with group C at 5 and 60 minutes; and significantly reduced compared with both ‘C’ and mEC groups at 30 minutes (Fig 2A). There was significant improvement of LDH content in the CF of hearts treated with NNMS compared with groups C and mEC (P < .05 and P < .001, respectively).

LDH activity in CF in hearts treated with NNMS is shown in Figure 2 B.

Evaluation of MDA and Conjugated Diene

The formation of MDA as an indicator of oxidative injury revealed significant differences in both groups, with the hearts treated with NNMS significantly lower than those stored in only mEC solution: 23.30 ± 7.79 nmol/100 mg Pr in group N+m versus 27.31 ± 6.49 nmol/100 mg Pr in mEC group (P < .05; Fig 3 A). The formation of CD is also an indicator of oxidative injury. The mean values of CD that the hearts were treated by NNMS was significant lower than groups C and mEC (P < .05; Fig 3 B).

DISCUSSION

Cardiac I/R injury, a necessary consequence of transplantation, is associated with a burst of ROS, which contributes to tissue damage. The molecule mechanism of I/R injury has been explained as due to electrons leakage by the respiratory chain mitochondria. The hypoxia of tissue ischemia places the mitochondrial respiratory chain in a high reducing state, which disposes to easy leakage of electrons. Reoxygenation (Reox) of the respiratory chain in a high reducing state may lead to a burst of leaking electrons, generating ROS, which contribute to serious tissue damage. Many studies have demonstrated that mitochondria represent a primary source of ROS in mammalian cells. Two pathways of oxygen consumption are available in mitochondria: One

Fig 2. Protein content and LDH activity of coronary flow: coronary flows were obtained at 5, 10, 30, and 60 minutes after reperfusion. *N+m group compared with ‘C’ group, P < .05; **N+m group compared with mEC group, P < .05. *mEC group compared with ‘C’ group, P < .05. C, ‘C’ group; m, mEC group; N+m, NNMS was added to mEC solution.
is O2 consumption by electrons transferred inside the chain used for ATP synthesis. The other is the O2 consumed by the electrons leaked out of the chain, which are used in the radical metabolism. The generation of O2− radical and H2O2 in the substrate side of respiratory chain results from electron leakage of the chain. Complexes I and III of the respiratory chain are responsible for electron leakage and O2− radical generation (Fig 4). Hence, it may be beneficial for heart transplantation to use drugs that reduce O2− radical generation by electron leakage in the mitochondrial respiratory chain.

NNMS is a methyl salicylate that has undergone nitrification, with addition of salicylamide by methylic acylation (Fig 5). The crystals of NNMS change to yellow raphide. With a melting point of 132°C to 133°C.

We devised a method to measure electron leakage of mitochondria respiratory chain complexes I and III, showing that NNMS reduced generation of O2− radical and H2O2 by isolated rat myocardial mitochondria. In addition, we found that NNMS had an inhibitory effect on succinate-cytochrome C reductase (Fig 6).

Effect of NNMS on Heart Function
Reperfusion of the heart following an ischemic period generates free radicals such as superoxides and hydroxyl radicals. The generated free radicals may induce peroxidation of the intracellular components, such as phospholipids, proteins, and DNA, thus provoking cellular damage. Free radical attack may be one cause of contractile dysfunction of ischemic reperfused hearts.30,31

Our results demonstrated that NNMS added to a cardioplegic and storage solution attenuated contractile dysfunction of heart. Possible mechanisms to explain the effect of NNMS on diastolic dysfunction after reperfusion injury include reduced impairment of calcium homeostasis, specifically calcium sequestration and release by the sarcoplasmic reticulum, a step that is vulnerable to oxygen-derived free radical injury. In addition, free radical disruption of mitochondrial membranes may interfere with adenosine triphosphate production with subsequent ischemic contracture and loss of distensibility.12

Differences in MWC between mEC hearts and those treated with NNMS were not significant, although there was a trend toward greater edema in the mEC hearts. David et al12 suggested the possibility that myocardial edema played a role in diminished distensibility.

Because free radicals generated during I/R periods are considered to peroxidate membrane phospholipids, excessive ROS may cause cell damage by oxidizing DNA, proteins, carbohydrates, and membrane phospholipids. We determined MDA and CD content in the ischemic-reperfused myocardium. There was a reduced myocardial MDA content in hearts treated with NNMS (8 μ g/mL) compared with mEC (23.30% ± 7.79% versus 27.31% ±
mice, preservation and that lonidamine-treated hearts show functional recovery of the heart after long-term hypothermia. MPT opening by lonidamine markedly attenuates the effect of NNMS on heart preservation. Experimental evidence strongly suggests that MPT inhibition by cyclosporine (CsA) enhanced protection against reperfusion injury. These effects suggested a contribution of NNMS to protect the heart against free radical attack during ischemia and reperfusion.

Possible mechanisms of a significant influence of NNMS on heart preservation included: (1) prevention of mitochondrial (m) Ca\(^{2+}\) overload by reduced ROS generation. ROS generators decrease myofilament Ca\(^{2+}\) sensitivity. Although ROS scavengers given after ischemia improve Ca\(^{2+}\) sensitivity,25 (2) Reduced ROS generation preventing opening of mitochondrial permeability transition pores (MPTP). Mitochondrial permeability transition causes mitochondrial to become uncoupled and capable of hydrolyzing ATP rather than synthesizing ATP. Unrestrained, this effect leads to loss of ionic homeostasis and ultimately necrotic cell death. The functional recovery of the Langendorff-perfused heart from ischemia inversely correlated with the extent of pore opening. Inhibition of MPTP provides protection against reperfusion injury.35 Recent studies have demonstrated that generation of intracellular ROS was enhanced prior to the onset of mitochondrial membrane permeability transition (MPT).34 Rajesh et al35 suggested that MPTP inhibition by cyclosporine (CsA) enhanced functional recovery after long term hypothermic heart preservation. Experimental evidence strongly suggests that MPTP opening by lonidamine markedly attenuates the functional recovery of the heart after long-term hypothermic preservation and that lonidamine-treated hearts showed low myocardial ATP levels.6

In conclusion, NNMS seemed to be different from other free radical scavengers, since it partially inhibited electron leakage from complex I and III of the mitochondria respiratory chain. If the ROS burst cannot occur at the onset of reperfusion, tissue damage is significant reduced. The present study suggested a new mechanism to eliminate I/R injury due to mitochondrial generation of ROS.

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