

Original Article

Nitric oxide donor, NOC7, reveals dose dependently and cGMP pathway independently biphasic effects on contractile force of isolated rat heart after global ischemia

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Abstract: Purpose: Our purpose was to investigate whether the 3-(2-hydroxy-1-methyl-2-nitroso-hydrazino)-N-methyl-1-propanamine (NOC7), an ideal NO donor was dose dependently and cGMP-independent in restored cardiac function after global ischemia in an isolated rat heart model. Methods: Langendorff preparations of an isolated rat heart model were established. Isolated rat hearts (n = 40) were randomly divided into 5 groups (ischemic control group, NOC7 groups and NOC7+NS2028 groups). All groups were subjected to 35 min global ischemia, followed by 30 min reperfusion with Krebs-Henseleit bicarbonate buffer (KHB), and NOC7, NOC7+NS2028 at 2 and 200 μ M, respectively. Left ventricular developed pressure (LVDP), the maximum and the minimal rate of rise in LVP (\pm dP/dt), and coronary flows were measured continuously. Cyclic adenosine monophosphate (cAMP) and cyclic guanosine monophosphate (cGMP) levels were measured in myocardium homogenate, using enzyme immunoassay (EIA). Results: 30 min of global ischemia increased LVDP to $121.9 \pm 11.5\%$ at 35 min of reperfusion of 2 μ M NOC7 group and 2 μ M NOC7 associated with NS2028 group from the ischemic control group ($P < 0.05$). While in 200 μ M NOC7 group and 200 μ M NOC7 associated with NS2028 group, the LVDP value only slightly reduced, resulting in a value of only $45.3 \pm 10.4\%$ and $35.3 \pm 6.0\%$ of baseline ($P > 0.05$). Conclusion: NOC7 has biphasic effect on isolated rat heart after ischemia and reperfusion myocardial contractility. This biphasic effect shows neither concentration-dependent nor the cGMP-dependent characteristics.

Keywords: Myocardial contractility, ischemia/reperfusion, post-resuscitation period, soluble guanosine cyclase inhibition, cyclic nucleotides

Introduction

Post-cardiac arrest care is now recognized as a critical link in the chain of survival since it has the potential of cardiac dysfunction. Although numerous clinical pre-arrest can efficiently reduce the cardiac dysfunction caused by cardiac ischemia, however, does not suitable for the cardiac arrest out of the hospital and unpredictable cardiac arrest [1]. Hemodynamic abnormalities after cardiopulmonary resuscitation treatment usually treat with the rehydration therapy, vasoactive (noradrenaline), inotropes (dobutamine), inotropic vasodilators (phosphodiesterase 3 inhibitor agent) based on individual clinical circumstances. Unfortunately, according to the report from < 2010 American Heart Association Guidelines for Cardiopul-

monary Resuscitation and Emergency Cardiovascular Care >, these treatments have not significantly improve this situation [2].

Nitric oxide (NO) is exerts a variety of biological actions under both physiological and pathological conditions. NO is a powerful cardio-protective mediator by regulate the myocardial contractility and the heart rate, which also has the protective effect of the injury after myocardial ischemia-reperfusion and can inhibit the myocardial infarction after ventricular remodeling. NO has the positive inotropic effect which mechanism by the NO donors regulated the concentration of cGMP and cAMP in cardiomyocytes [4]. Our previous study illustrated that the NO donor -NOC7 (3-(2-hydroxy-1-methyl-2-nitroso-hydrazino)-N-methyl-1-propanamine)

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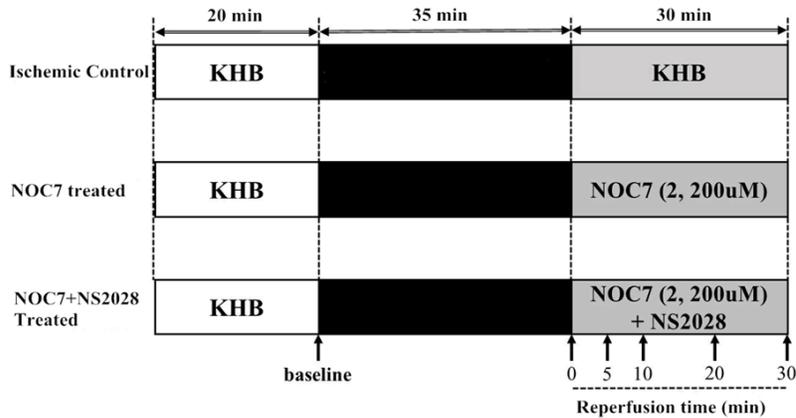


Figure 1. Experiment protocol. After an equilibration period of 20 min, in all groups, the hearts were subjected to 35 min of global ischemia, followed by 30 min of reperfusion. The ischemic control group only perfused with modified Krebs-Henseleit bicarbonate buffer (KHB) alone during the reperfusion period. In the 3-(2-hydroxy-1-methyl-2-nitroso-hydrazino)-N-methyl-1-propanamine (NOC7) groups and the NOC7+NS2028 groups, the hearts were perfused with KHB solution containing different concentrations of NOC7 or NOC7+NS2028 (at 2 μ M and 200 μ M). sGC inhibitor, NS2028 which pump velocity under the 2% coronary blood flow (approximately 10 μ M). Myocardial function was assessed at 5, 10, 20 and 30 min of the reperfusion period; $n = 8$ in each group.

appeared to exert a biphasic effect on the contractile force of the isolated rat heart. NOC7 at 2 μ M rescued myocardial performance while NOC7 at 200 μ M turned to perform negative inotropic effect [5]. However, the mechanism of exogenous NO donors affect myocardial contractility still remains controversial. Except the exogenous NO donors have biphasic effect on the controversial through the cGMP pathway, another explanation is confirmed in recently reports that the NO has the positive inotropic effect by activated a non-cGMP pathway-adenosine cyclase (AC) to increase the concentration of cGMP [6].

The study aims to investigate the role and mechanism of NO donor NOC7 affect the myocardial contractility in isolated rat during the reperfusion period after hearts ischemia, and by giving the use of sGC inhibitor NS2028 to blocking the cGMP pathway, which can demonstrate the inotropic effect whether depends on cGMP pathway.

Materials and methods

Isolated perfused rat heart and perfusion solution

Langendorff preparations of an isolated heart model and the perfusion solution according to the protocol. 40 male Sprague-Dawley rats, weighing 250 to 280 g, were used. All rats were

anesthetized with pento-barbital (50 mg kg^{-1} , administered heparin (500 IU kg^{-1}) intraperitoneally 30 min before surgery [7].

The perfusion solution was a modified Krebs-Henseleit bicarbonate buffer (KHB) gassed continuously with oxygen at 37°C that was composed as follows (mmol l^{-1}) NaCl, 118; NaHCO_3 , 25; KCl, 4.7; CaCl_2 , 1.25; MgSO_4 , 1.2; KH_2PO_4 , 1.2; and glucose, 11. The constant pressure was maintained at 80 cm H_2O and the pH approximately at 7.4. The LB-2 latex balloon (Nihon Koden, Tokyo, Japan) was inserted into the left ventricular cavity via a left atrial incision for

the measurement of pressure variables. Heart rate (HR), Pressure parameters (left ventricular systolic pressure [LVSP], left ventricular end-diastolic pressure [LVEDP], maximum rate of rise in LVP [dP/dt_{max}], and the minimal rate of rise in LVP [dP/dt_{min}]) were recorded continually. LV developed pressure (LVDP) was calculated as follows: $\text{LVDP} = \text{LVSP} - \text{LVEDP}$ (mmHg).

Experimental protocol

All heart materials were subjected to an equilibration period of 20 min before baseline measurements. Those hearts which LV systolic pressure was higher than or reach 45 mmHg were selected as the subject for the study. Subsequently, these hearts were divided into 5 groups ($n = 8$): 1. Ischemic control group, the hearts were subjected to 35 min of global ischemia by stopping perfusion with the oxygenated KHB solution and then reperused for 30 min. 2, 3. NOC7 groups, after 35 min of global ischemia, in which the hearts were perfused with KHB solution containing NOC7, at final concentrations of 2, 200 μ M for 30 min. 4, 5. NOC7 associated with sGC inhibitor NS2028 groups, the same protocol as group 3, 4 while associated with sGC inhibitor NS2028 which pump velocity under the 2% coronary blood flow (approximately 10 μ M). Myocardial function was assessed at 5, 10, 20 and 30 min of the reperfusion period (**Figure 1**). The heart apex

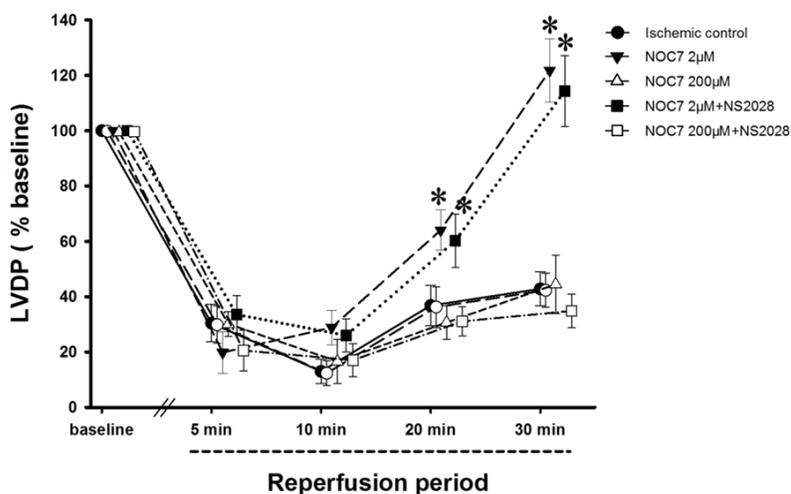


Figure 2. Recovery of left ventricular developed pressure (LVDP) measured at 5, 10, 20, 30 min of reperfusion after 35 min of global ischemia. Data values are presented as mean percentage changes \pm SD; $n = 8$ in each group. * $P < 0.05$ compared with ischemic control.

was collected after surgery, frozen immediately in liquid nitrogen then stored at -80°C until cyclic nucleotide analysis.

Cyclic nucleotide determination

Myocardial samples were homogenized and suspended in modified Krebs-hydroxyethylpiperazine ethanesulfonic acid (HEPES) buffer (pH 7.4; 30°C). After the reactions were terminated with the addition of ice-cold trichloroacetic acid (TCA; final concentration, 6%), the samples were centrifuged at 2000 g for 15 min at 4°C . The supernatants were collected, frozen, and stored at -80°C until cAMP and cGMP were determined, using an acetylation protocol for cAMP and cGMP (Biotrak enzyme immunoassay system [RPN225 and RPN226]; Amersham Biosciences, Piscataway, NJ, USA) according to the manufacturer's instruction. Calculated the cyclic nucleotides level of samples as pmol cyclic nucleotide-mg $^{-1}$ protein. The protein were used BCA Assay Kit (Pierce, Rockford, IL, USA) to measure.

Statistical analysis

The values for results for each group were expressed as means \pm SD. Differences within and between groups were determined by one-way analysis of variance and multiple comparisons, using the Tukey test with SPSS statistical analysis software 11.5 (SPSS, Chicago, IL, USA). A P value < 0.05 was regarded as statistically significant.

Results

Hemodynamic function

30 min of global ischemia increased LVDP to $121.9 \pm 11.5\%$ at 35 min of reperfusion of 2 μM NOC7 group and 2 μM NOC7 associated with NS2028 group from the ischemic control group ($P < 0.05$). While in 200 μM NOC7 group and 200 μM NOC7 associated with NS2028 group, the LVDP value only slightly reduced, resulting in a value of only $45.3 \pm 10.4\%$ and $35.3 \pm 6.0\%$ of baseline ($P > 0.05$; **Figure 2**).

In all groups, the recovery of dP/dt max and dP/dt min as a paralleled the recovery of LVDP, LVEDP increased in all groups, indicating the development of myocardial contracture. NOC7 group and NOC7 associated with NS2028 group recovered LVEDP, decreased to 42.0 ± 4.2 and 45.9 ± 4.4 mmHg, respectively, in comparison to the ischemic control group of 67.6 ± 9.1 mmHg at 30 min of reperfusion ($P < 0.05$; **Table 1**).

Cyclic nucleotide concentrations

The heart apex myocardial cGMP level of NOC7 group significantly increased to 3.89 ± 0.32 pmol mg $^{-1}$ protein at 200 μM , whereas that in the ischemic control group was 1.03 ± 0.36 pmol mg $^{-1}$ protein ($P < 0.05$; **Figure 3**).

The average cAMP level, in the ischemic control group was 0.63 ± 0.24 pmol mg $^{-1}$ protein, and this was significantly increased after 35 min ischemia, especially in NOC7 group at 2 μM , to 2.63 ± 0.24 pmol mg $^{-1}$ protein, respectively ($P < 0.05$; **Figure 4**).

Discussion

We used the Langendorff-perfused isolated rat heart model in this study which excludes the most humoral effects and the sympathetic or parasympathetic control to the central nervous systems, which can be affected by NO donors. This method we chosen for the reason regard

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Table 1. Hemodynamic variables during experiments

	Reperfusion period				
	Baseline	5 min	10 min	20 min	30 min
LVEDP (mmHg)					
Ischemic control	7.7 (0.5)	78.6 (12.5)†	88.2 (8.4)†	72.6 (10.4)†	67.6 (9.1)†
NOC7 2 μ M	8.1 (0.5)	74.3 (7.1)†	78.6 (6.2)†	62.3 (4.6)†	42.0 (4.2)†,*
NOC7 200 μ M	7.9 (0.4)	75.4 (5.4)†	81.4 (5.8)†	78.4 (8.6)†	72.6 (8.7)†
NOC7 2 μ M+NS2808	8.0 (0.6)	69.7 (4.0)†	77.8 (4.7)†	65.3 (7.9)†	45.9 (4.4)†,*
NOC7 200 μ M+NS2808	8.0 (0.8)	79.7 (8.9)†	84.8 (6.1)†	79.7 (5.1)†	69.4 (4.2)†
dP/dt_{max} (mmHg s⁻¹)					
Ischemic control	3335 (459)	713 (180)†	420 (142)†	844 (151)†	1311 (232)†
NOC7 2 μ M	3191 (138)	530 (221)†	748 (193)†	1988 (221)†,*	3485 (263)*
NOC7 200 μ M	3428 (518)	519 (169)†	567 (274)†	958 (415)†	1245 (491)†
NOC7 2 μ M+NS2808	3295 (147)	546 (258)†	726 (185)†	1782 (204)†,*	3185 (386)*
NOC7 200 μ M+NS2808	3367 (580)	769 (218)†	543 (103)†	768 (191)†	1234 (255)†
dP/dt_{min} (mmHg s⁻¹)					
Ischemic control	-2349 (276)	-356 (245)†	-198 (174)†	-636 (132)†	-653 (428)†
NOC7 2 μ M	-2395 (272)	-513 (135)†	-748 (265)†	-1376 (306)†,*	-2775 (458)*
NOC7 200 μ M	-2353 (124)	-490 (197)†	-419 (145)†	-767 (197)†	-790 (297)†
NOC7 2 μ M+NS2028	-2441 (418)	-776 (181)†	-659 (128)†	-1265 (117)†,*	-2474 (334)*
NOC7 200 μ M+NS2028	-2478 (399)	-727 (241)†	-461 (173)†	-681 (182)†	-719 (227)†
Coronary flow (ml min⁻¹)					
Ischemic control	14.3(0.9)	13.5 (1.3)	12.7 (1.2)	11.6 (1.1)	11.5 (1.3)
NOC7 2 μ M	13.4 (0.8)	12.2 (1.1)	12.1 (1.3)	12.5 (1.1)	11.6 (0.9)
NOC7 200 μ M	12.9 (1.3)	12.6 (1.6)	12.3 (1.5)	11.8 (1.2)	11.4 (1.1)
NOC7 2 μ M+NS2028	12.7 (1.5)	12.5 (0.7)	13.2 (0.8)	12.4 (0.9)	11.4 (1.2)
NOC7 200 μ M+NS2028	13.0 (1.5)	11.4 (1.2)	11.9 (1.8)	12.2 (1.9)	10.8 (1.6)

*: $P < 0.05$ vs. ischemic control group; †: $P < 0.05$ vs. baseline. Results were reported as mean values \pm (SD); $n = 8$ in each group. Differences within and between groups were determined by one-way analysis of variance and multiple comparisons using the Tukey test.

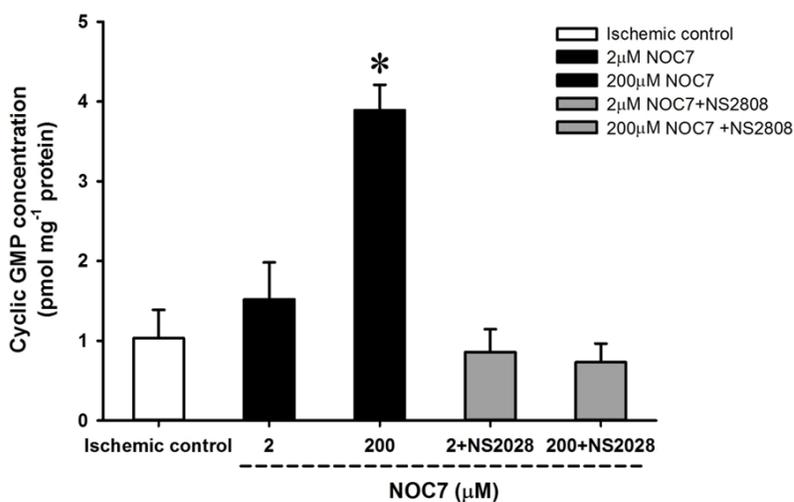


Figure 3. Effects of NOC7 and NOC7+NS2808 on intracellular cyclic guanosine monophosphate (cGMP). * $P < 0.05$ compared with ischemic control.

as the best choice among of heart-model to examine the direct myocardial effects of NOC7 with/without NS2808. The results demonstrated that NOC7 with/without NS2808 at 2 μ M concentration can restore cardiac function after ischemia which increased the myocardial cAMP levels (Figure 4). The LVEDP, dP/dt_{max}, and dP/dt_{min} all increased (Figure 2; Table 1).

Numerous researches shown that the NO donor releases NO which can activate the guanosine cyclase

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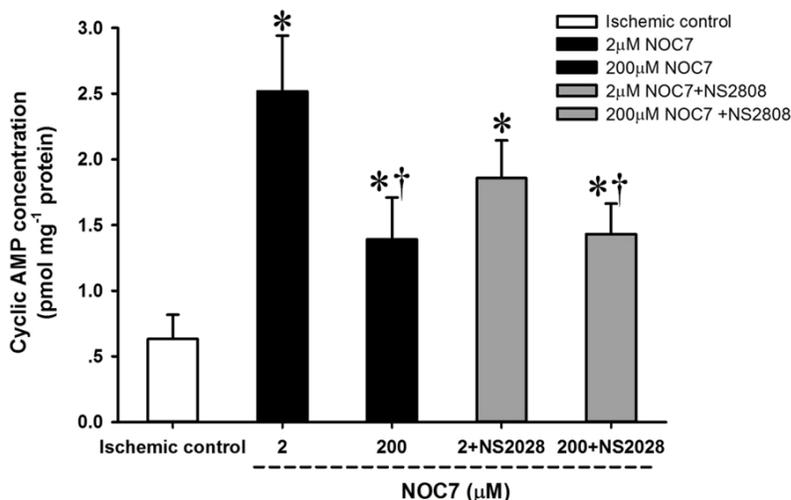


Figure 4. Effects of NOC7 and NOC7+NS2808 on intracellular cyclic adenosine monophosphate (cAMP). * $P < 0.05$ compared with ischemic control.

to derive cGMP. Our previous study have confirmed that the positive inotropic effect of a sGC/cGMP pathway embodied by the moderated increase of cGMP can inhibit myocardial PDE3 while the cAMP degradation is suppressed results in an elevated intracellular cAMP level in the myocardium [7]. This study use sGC inhibitor NS2028 to cutoff sGC/cGMP pathway, however, the NOC7 at 2 μM concentration still showed positive inotropic effect and improvement of cardiac function. The cGMP level of NOC7+NS2028 group was significantly decreased than NOC7 group, while the cAMP level of NOC7 was increased than ischemia control group (Figures 3, 4).

NS2028 is a specific blocker of guanosine cyclase while it has no effect on adenosine cyclase (AC). The positive inotropic effect of NO under this circumstance may indicate that the NO can directly activate AC to increase the cAMP level in Myocardial which in response to an activation of protein-kinase A (PKA) [8, 9].

Despite the positive inotropic effect after cardiac ischemia contributed by some pathways by cGMP, when we blocked the cGMP pathway, there still showing a significant positive inotropic effect which can be considered by NO directly activated AC on the properties of myocardial contractility.

On the other hand, higher concentration of NOC7 (at 200 μM), resulted in a significant increase in cGMP (Figure 3). In spite the level of

cAMP increased than ischemic control group, however, it showed the poor cardiac function after reperfusion and no improvement compared with ischemic control group. It is reported that the exogenous NO donor increased the intracellular cGMP level under such condition, meanwhile activated PKG phosphorylated troponin I [10], which caused myofilament relaxation then dominant our negative effect on myocardial function in our study. The cGMP level of NOC7 + NS2028 group at high concentration (200 μM)

after reperfusion was significantly decreased than the group without the sGC inhibitor NS2028, and similar with ischemic control group (Figure 3). NS2028 blocked the negative inotropic effect mediated by cGMP-PKG pathway. Despite the cAMP was increased than the ischemic control group (Figure 4), however, it is showed that the heart function still not been effectively improved and there were no significant difference with NOC7 group at 200 μM concentration in hemodynamic, which can indicated that the high level NO have the negative inotropic effect through a cGMP-independent pathway.

NO can generate numerous of free radicals which cause nucleic acid and lipid damage and inhibit the enzymes related to mitochondrial electron transport, resulted in inhibition of mitochondrial respiration. The specific mechanism of action may involve such several respects: 1. NO and its derivative N_2O_3 or S-nitrosation can inactivate complex I which located in the mitochondrial respiratory chain thus inhibit the production of ATP and susceptibility to cell death [11, 12]; 2. Large amount of NO can inactivate those enzyme which were related with anti-oxidation and energy metabolism by cGMP-independent pathway; 3. A high concentration NO can binding to the iron ions in heme group thus activate guanosine cyclase, or combined with and inactivated the enzyme located in intracellular iron-sulfide protein which have a direct impact on the cell breathing

thus block the energy generation [8]; 4. Elevated NO may lead to the concomitant production of ONOO⁻ which lead nitration of acid of superoxide dismutase (SOD) thus facilitate cell degeneration after ischemia [13, 14]; 5. NO can directly damage DNA by bases deamination and also can directly cleave DNA; 6. NO through a Cgmp-PKG-independent pathway by occupy the oxygen-binding site of cytochrome oxidase in competition with oxygen in the presence of hypoxia which can reversibly reduce myocardial contractility, and also an important mechanism of cardiac dysfunction caused by high concentration of NO.

Reports shown that NO donors i.e., L-arginine can improves low coronary reflow and postischemic mechanical function [15-17]. In our present study, however, the coronary flows were maintained at constant levels regardless of NOC7 and the sGC inhibitor NS2028 concentration (**Table 1**). Therefore, our result shows no relationship between recovery of cardiac function and cardiac contractility with the theory that the increased coronary flows associated with the enhancement of oxygen supply.

Organic NO donors (sodium nitroprusside, nitroglycerine) are being used as vasodilator for intensive care. Following our present results, the NO donor have biphasic effect on the contractile force of the isolated rat heart and mainly embodied as concentration-dependent: The tiny concentration of NO donor can produce a positive inotropic effect and improve the cardiac function after myocardial ischemia; while the high concentration of NO donor have negative inotropic effect. Although part of the role is to be achieved through cGMP pathway, but this biphasic effect does not seem to depend on the cGMP pathway. The mechanism of NO affected on myocardial contractility needs further research experiment. But we also suggest the appropriate concentration of NO donor (< 2 μ M) or NO itself are applied to the treatment of late-stage clinical cardiopulmonary resuscitation cardiac dysfunction and all good choice for hemodynamic improvement of cardiopulmonary resuscitation.

In summary, the NO donor-NOC7, has biphasic effect on the contractile force of the isolated rat heart after global ischemia. And this mechanism of NOC7 seemed to be involved with the balance between intracellular cAMP and cGMP

levels. The usage of a specific concentration of NO donors may be ideal choice for cardiac function restoration.

Disclosure of conflict of interest

None.

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