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The effect and pharmacokinetics of nafamostat mesilate adjunct to cold nondepolarizing cardioplegia in a canine model of cardiac preservation

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Abstract We examined the effects of nafamostat mesilate (NM) on myocardial, biochemical, and functional changes in canine hearts. An isolated heart was preserved for 6 h at 5 °C and then reperfused for 2 h at 37 °C. NM was added to the cardioplegic solution. At concentrations of both 10^{-7} M ($n = 8$) and 10^{-6} M ($n = 6$), NM was able to maintain myocardial cyclic adenosine monophosphate (cAMP) at a normal level and to reduce guanosine monophosphate (cGMP) concentrations at the end of both preservation and reperfusion. The serum N-acetyl-b-D-glucosaminidase (NAG) concentration during reperfusion was lower in hearts treated with NM 10^{-6} or 10^{-7} M than in those without NM ($P < 0.05$). Although NM failed to preserve myocardial concentrations of adenine nucleotide compounds, NM 10^{-7} M maintained the ± dp/dt of the left ventricle after reperfusion at the same level as in the nonischemic control group and better than NM 10^{-6} M or no NM ($P < 0.05$). Myocardial uptake of NM 10^{-5} M (higher concentration) was 55 % ± 8 % (6-h preservation) and 29 % ± 15 % (2-h reperfusion). We conclude that NM 10^{-7} M adjunct to nondepolarizng solution does not preserve myocardial adenine nucleotide concentrations but does facilitate the recovery of left ventricular function. NM 10^{-5} M (higher concentration) seems to have a high affinity for the myocardium and may depress the recovery of left ventricular function.

Key words Cardioplegia, nafamostat mesilate · Preservation, heart, nafamostat mesilate · Heart preservation, nafamostat mesilate · Nafamostat mesilate, heart preservation

Introduction

Improved techniques for cardiac preservation are essential to increase the availability of donor hearts [6]. Although several types of preservation solutions are presently in clinical use, all are depolarizng solutions, and when combined with hypothermia, as they frequently are, they have disadvantages related to membrane instability. To overcome these problems, both membrane stabilization and inhibition of protease activation are needed to preserve myocardial viability [16]. We have also developed a new, nondepolarizing solution that has been shown to enhance myocardial viability in a 12-h preservation model using the canine heart [16, 17].

Nafamostat mesilate (NM), a synthetic protease inhibitor, suppresses the activity of several inflammatory processes involved with the plasmin system [12, 13, 19], the kinin system [1, 7, 8], and the complement system [4]. Activation of proteases is affected by cyclic nucleotides [5]. It has been found that lysosomal enzymes suppress phosphorylation of mitochondria [9, 11], and that myocardial ischemia leads to an accumulation of lysosomal enzymes after 45 min of normothermic is-

chemia in the myocardial infarct model [3, 23] and after 4–6 h of hypothermic global ischemia [17, 18]. Moreover, lysosomal enzymes are activated based on their own pH levels and affect biochemical and morphological changes in the myocardium [23]; N-acetyl-D-glucosaminidase (NAG) is released even after a short period of myocardial ischemia in significant correlation with the elevation of the MB fraction of creatine phosphokinase (MB-CPK) [22]. Although protease inhibition with aprotinin through suppression of the cyclic guanosine monophosphate (cGMP) may enhance myocardial viability following hypothermic cardioplegia induced with a depolarizing solution followed by reperfusion [15], the effect of NM adjunct to nondepolarizing cardioplegia on the myocardium has yet to be established. Furthermore, no information exists on the pharmacokinetics of NM in the myocardium during cardioplegic arrest. Our hypothesis was that NM would inhibit proteases by suppressing the release of cGMP and prevent depletion of myocardial adenine nucleotide compounds.

This study examined the myocardial pharmacokinetics of NM and tested whether the addition of NM to our nondepolarizing solution would enhance myocardial viability in a 6-h hypothermic preservation, 2-h reperfusion canine model of cardiac preservation.

Materials and methods

Twenty-eight mongrel dogs weighing 9.2–17.0 kg (mean 11.8 kg) were anesthetized with intravenous pentobarbital (30 mg/kg) and maintained by mechanical ventilation. Animals received care according to the "Principles of Laboratory Animal Care" formulated by the National Society for Medical Research and the "Guide for the Care and Use of Laboratory Animals" prepared by the National Academy of Sciences.

Procurement of the heart

A median sternotomy was performed, and the superior and inferior vena cavae were isolated with 2–0 silk ligatures, both proximally and distally. The azygous vein was ligated and divided. Both common carotid arteries, the left subclavian artery, and the descending aorta were isolated proximally and distally with 2–0 silk ligatures, and the hila of the lungs were encircled bilaterally with 2–0 silk ligatures. A 10 Fr arterial cannula was inserted in the proximal right subclavian artery, and a 24 Fr venous cannula was placed in the right ventricle through the right atrial appendage. Approximately 500 ml of blood was withdrawn from the venous cannula, heparinized, and saved for transfusion during reperfusion. The previously isolated arteries were ligated, as were the pulmonary hila after ventilation was terminated. Immediately following aortic occlusion, cardioplegia was induced by infusing cold (5 °C) cardioplegic solution via the arterial cannula. The volume of the initial infusion was 15 ml/kg in all dogs. The superior and inferior vena cavae were ligated and divided, and the heart was removed.

Preservation of the heart

Each heart was immersed for 6 h in cold (5 °C) saline, and cardioplegic solution (3 ml/kg) was infused every 60 min. The composition of this solution was NaCl 60 mM, Mg-l-aspartate 8 mM, $CaCl_2$ 1 mM, mannitol 50 mM, lidocaine hydrochloride 2 mM, glucose 245 mM, and betamethasone 250 mg/l. The osmolarity was 450 mosmol, and a pH of 7.50 was maintained by the addition of sodium bicarbonate 10 mM.

A latex balloon was placed in the left ventricle and secured with a holding apparatus sutured in the mitral position. The balloon was connected to a transducer (Statham P23DB, Statham Instruments, Los Angeles, Calif., USA) and a polygraph (Nihon Kohden, Tokyo) was used to measure the developed left ventricular pressure during reperfusion. Special care was taken to avoid mechanically induced aortic regurgitation.

Reperfusion

A second dog was anesthetized with pentobarbital (intravenously, 30 mg/kg as an initial dose, and increased sufficiently to suppress corneal reflex), mechanically ventilated, heparinized, and maintained hemodynamically by the infusion of Ringer's lactate solution. Both carotid arteries were cannulated (10 Fr) and connected to the arterial cannula placed in the preserved heart. A second pressure transducer and a magnetic flowmeter (Nihon Kohden) were connected to the circuit to measure the perfusion pressure and flow. Coronary sinus blood flow was also measured with a magnetic flowmeter to estimate coronary blood flow. Blood from the cannulae in the right and left ventricles was collected in a reservoir and infused back into the supporting dog with a pump; a heat exchanger maintained normothermia. Myocardial temperature was measured with a thermistor placed in the right ventricle. Rewarming myocardial temperature to 37 °C took from 10 to 15 min. Reperfusion was continued for 2 h. Defibrillation was performed when ventricular fibrillation developed during the early phase of reperfusion. After 5 min of reperfusion, all dogs were paced at 130 beats per minute. No cardiotonic drugs were administered to any of the dogs.

At the end of cardiac arrest and during reperfusion, while the heart was beating, biopsy specimens of left ventricular subendocardium were obtained using frozen Wollenberger clamps and then kept in liquid nitrogen until processing for biochemical analysis took place. Concentrations of adenosine triphosphate (ATP), adenosine diphosphate (ADP), adenosine monophosphate (AMP), creatine phosphate (CP), cyclic adenosine monophosphate (cAMP), and cyclic guanosine monophosphate (cGMP) were measured in the left ventricular tissue specimen [2].

Samples of coronary sinus blood were collected following 5, 60, and 120 min of reperfusion and were analyzed for the serum concentrations of N-acetyl-b-D-glucoaminidase (NAG) [20]. The left ventricular (LV) pressure was determined by inflating the intraventricular balloon with saline volumes of 0–20 ml. The LV end-systolic (diastolic) pressure-volume relationship, and the LV + dp/dt and –p/dt were calculated to evaluate LV function.

Pharmacokinetics of nafamostat mesilate

Myocardial uptake of NM was studied using canine hearts ($n = 6$) in the same experimental protocol as that described earlier with NM 10^{-5} M in the cardioplegic solution. Myocardial uptake of NM was calculated during the 6-h preservation (initially and hourly) and during the 2-h reperfusion (following 5, 60, and 120 min) by subtracting the difference in NM concentration between the infusate (the blood

Table 1 Canine myocardial concentrations of metabolic parameters following preservation and reperfusion as a function of the concentration of nafamostat mesilate to nondepolarizing cardioplegia. Values represent mean ± SEM (*B*, baseline, *P*, at end of preservation, *R*, at end of reperfusion)

		Group 1 No NM ($n = 8$)	Group 2 NM 10^{-7} M ($n = 8$)	Group 3 NM 10^{-6} M ($n = 6$)
Adenosine triphosphate (μmol/g dry)	B	22.35 ± 0.87	22.36 ± 0.87	22.36 ± 0.87
	P	14.25 ± 2.27	8.15 ± 1.77*	8.47 ± 5.69*
	R	19.80 ± 2.03	22.08 ± 2.04**	17.56 ± 2.43**
Adenosine diphosphate (μmol/g dry)	B	6.47 ± 0.52	6.47 ± 0.52	6.47 ± 0.52
	P	5.44 ± 0.57	4.32 ± 0.35	3.64 ± 0.71
	R	5.34 ± 0.52	6.79 ± 0.83	5.04 ± 0.54
Adenosine monophosphate (μmol/g dry)	B	1.22 ± 0.25	1.22 ± 0.25	1.22 ± 0.25
	P	1.57 ± 0.25	1.08 ± 0.16	1.09 ± 0.13
	R	0.86 ± 0.21	1.45 ± 0.34	1.08 ± 0.18
Total adenine nucleotide (μmol/g dry)	B	30.05 ± 1.2	30.05 ± 1.2	30.05 ± 1.2
	P	21.27 ± 2.36	13.55 ± 1.86*	13.20 ± 6.31*
	R	25.99 ± 1.72	30.32 ± 2.68	23.68 ± 2.63
Creatine phosphate (μmol/g dry)	B	34.32 ± 3.12	34.32 ± 3.12	34.32 ± 3.12
	P	6.15 ± 1.79*	6.53 ± 3.66*	5.66 ± 5.04*
	R	37.78 ± 5.60	48.93 ± 6.79	43.26 ± 10.33
Cyclic adenosine monophosphate (pmol/g wet tissue)	B	586 ± 87	586 ± 87	586 ± 87
	P	2617 ± 260*	1306 ± 135*, ***	1372 ± 283*, ***
	R	1030 ± 117*, **	484 ± 65**, ***	427 ± 52**, ***
Cyclic guanosine monophosphate (pmol/g wet tissue)	B	10.9 ± 3.3	10.9 ± 3.3	10.9 ± 3.3
	P	13.3 ± 1.7	4.1 ± 1.0*, ***	3.8 ± 0.9*, ***
	R	10.8 ± 1.1	6.0 ± 2.8	3.6 ± 1.9*, ***

* $P < 0.05$ to baseline;
** $P < 0.05$ to end of preservation; *** $P < 0.05$ vs group 1

withdrawn from the aorta or cardioplegic solution) and coronary effluent. The concentration of NM was determined with the high-performance liquid chromatographic technique. Uptake (%) was calculated as follows: (A–C)/A × 100, where A is the infusate concentration of NM and C the coronary sinus concentration of NM.

Experimental groups

Experimental groups were established based on the concentration of NM in the cardioplegic solution: group 1, no NM ($n = 8$); group 2, NM 10^{-7} M ($n = 8$), and group 3, NM 10^{-6} M ($n = 6$). A control group ($n = 12$) was added to obtain baseline hemodynamic and biochemical data. Animals in the control group underwent the same procedure as those in the experimental groups until the induction of cardioplegia. Therefore, these hearts did not experience ischemia.

Statistics

Data are reported as the mean ± SEM. Differences between groups were determined via an analysis of variance (ANOVA) with the Bonferroni-Dunn test, and differences within groups were analyzed with Student's *t*-test. *P* values below 0.05 were considered statistically significant.

Results

Coronary flow, hematocrit, temperature during reperfusion, and the weight of the LV did not differ between the groups. All animals in all groups survived reperfusion.

The myocardial ATP, ADP, and total adenine nucleotide (TAN) concentrations in groups 2 and 3 at the end of preservation were lower than at baseline ($P < 0.05$). The myocardial cAMP concentration was lower at the end of both preservation and reperfusion in groups 2 and 3 than it was in group 1 ($P < 0.05$). The myocardial cGMP concentration was lower in groups 2 and 3 than in group 1 at the end of preservation and at the end of reperfusion in group 3 than in group 1 ($P < 0.05$; Table 1).

The serum concentration of NAG increased following reperfusion in group 1 but not in groups 2 and 3. The serum concentration of NAG at 60 and 120 min of reperfusion was lower in groups 2 and 3 than in group 1 ($P < 0.05$; Table 2).

Myocardial uptake of NM 10^{-5} M was 54.7 % ± 8.4 % during the 6 h of preservation and 29.1 % ± 15.2 % during the 2 h of reperfusion.

Hearts treated with NM 10^{-7} M (group 2) showed the best recovery of LV + dp/dt at 10–20 ml of LV volume after 120 min of reperfusion (Table 3). The LV –dp/dt after 120 min of reperfusion at 15 ml of LV volume was higher in group 2 than in groups 1 and 3 ($P < 0.05$; Table 3). LV end-diastolic pressure was higher in group 3 than in the control group and groups 1 and 2 at 20 ml of balloon inflation at the end of reperfusion ($P < 0.05$; Table 3). The LV systolic pressure-volume relationship after 120 min of reperfusion showed that the slope of each group was similar, but the intercept in group 2 was lower

Table 2 Canine coronary sinus concentrations of metabolic parameters during reperfusion as a function of concentration of nafamostat mesilate in nondepolarizing cardioplegia. Values represent mean ± SEM (*B*, baseline)

	Reperfusion (min)	Group 1 No NM ($n = 8$)	Group 2 NM 10^{-7} M ($n = 8$)	Group 3 NM 10^{-6} M ($n = 6$)
N-acetyl-β-D-glucosaminidase (IU/dl/per 100 g)	B	6.5 ± 1.0	6.5 ± 1.0	6.5 ± 1.0
	5	4.9 ± 1.0	2.4 ± 1.4	0.6 ± 0.2*
	60	12.9 ± 2.0	6.2 ± 1.4*	1.5 ± 0.3*
	120	31.6 ± 8.8	9.1 ± 2.8*	3.5 ± 0.6*

* $P < 0.05$ versus group 1

Table 3 Canine left ventricular function following 2 h of reperfusion as a function of the concentration of nafamostat mesilate in nondepolarizing cardioplegia. Values represent mean ± SEM (*LV*, left ventricular balloon volume; *ESP*, end-systolic pressure; *EDP*, end-diastolic pressure)

*[1] $P < 0.05$ vs group 1;
*[2] $P < 0.05$ vs group 2;
*[3] $P < 0.05$ vs group 3;
*[4] $P < 0.05$ vs control group

	LV (ml)	Group 1 No NM ($n = 8$)	Group 2 NM 10^{-7} M ($n = 8$)	Group 3 NM 10^{-6} M ($n = 6$)	Control No ischemia ($n = 12$)
ESP (mm Hg)	10	139 ± 9	97 ± 16	129 ± 13	121 ± 3
	15	158 ± 9	116 ± 17	153 ± 12	150 ± 3
	20	182 ± 6	138 ± 19*[4]	182 ± 18	184 ± 3
EDP (mm Hg)	10	7.8 ± 4.3	4.5 ± 2.1	17.5 ± 4.2	7.1 ± 0.7
	15	7.1 ± 1.8	9.6 ± 2.7	31.7 ± 8.4	11.7 ± 0.7
	20	10.7 ± 3.2	13.6 ± 4.0	56.7 ± 13.3*[1, *2, *4]	21.7 ± 0.7
LV +dp/dt (mm Hg/sec)	10	667 ± 32*[4]	875 ± 78*[1], *[3]	575 ± 59*[2, *4]	950 ± 21
	15	705 ± 29*[4]	1043 ± 87*[1], *[3]	608 ± 83*[2, *4]	1176 ± 25
	20	711 ± 30*[4]	1081 ± 79*[1], *[3]	658 ± 138*[2, *4]	1243 ± 32
LV-dp/dt (mm Hg/sec)	10	633 ± 79	656 ± 72	446 ± 85	688 ± 18
	15	612 ± 44*[4]	919 ± 107*[1, *3]	454 ± 80*[2, *4]	913 ± 24
	20	728 ± 21*[4]	919 ± 104*[3]	441 ± 94*[1, *2, *4]	1088 ± 18

than in the control group and groups 1 and 3 (Fig. 1). The LV diastolic pressure-volume relationship after 120 min of reperfusion showed better curves in in the control group and groups 1 and 2 (Fig. 1).

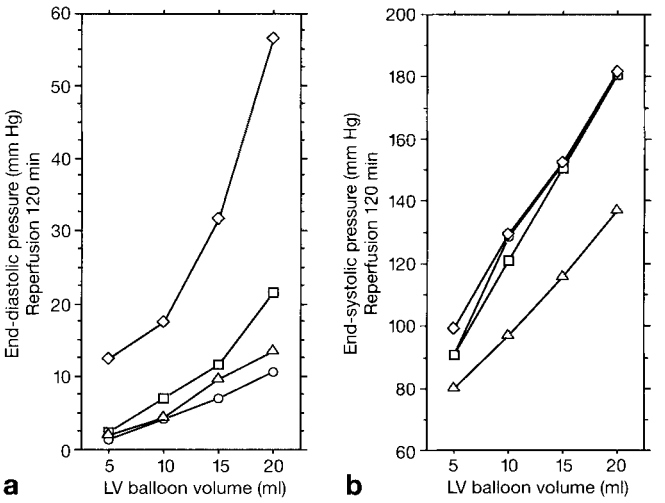

Fig. 1a End-diastolic pressure-volume relationship and **b** end-systolic pressure-volume relationship of left ventricle after 120 min of reperfusion. A latex balloon placed in the left ventricular cavity was inflated with saline solution from 0 to 20 ml and the corresponding pressure was measured (□ control group; ○ group 1, no NM; △ group 2, NM 10^{-7} M; ◇ group 3, NM 10^{-6} M)

Discussion

Although its precise mechanisms of action are not yet well understood, NM, a synthetic protease inhibitor, acts on a number of biochemical pathways. NM suppresses the action of trypsin, endotoxin, and bradykinin [1, 7, 10, 11], and inhibits complement activation during cardiopulmonary bypass. NM 10^{-6} M has been studied as a myoprotectant in experimental myocardial ischemia [4]. Finally, NM 10^{-7} M has been used as an anticoagulant during phoresis [19] and the implementation of left ventricular assist devices [13].

We performed a preliminary study to define the relationship between the dose of NM in cardioplegic solution and the functional response of the left ventricle. It showed that the optimal concentration of NM in cardioplegic solution to obtain the best systolic and diastolic function of the left ventricle was 10^{-7} M. Therefore, the present study compared the biochemical and functional effects of NM 10^{-7}or 10^{-6} M in cardioplegic solution with that of no NM in the cardioplegic solution. The addition of NM to the cardioplegic solution suppressed an increase in the myocardial cAMP concentration at the end of both preservation and reperfusion, and reduced the myocardial cGMP concentration at the end of preservation. Since cyclic nucleotides affect myocardial biochemical pathways such as calcium, energy metabolism, inflammation, and membrane func-

tion, cAMP stimulates cardiac contractility in balance to cGMP. It is possible that NM 10^{-7} M in cardioplegic solution affected myocardial cyclic nucleotides and led to improved cardiac function.

The source of lysosomal enzymes is the Golgi apparatus, one of the subcellular organelles in tissues. The release of lysosomal enzymes is activated by physicochemical changes such as acidosis, ischemia-reperfusion [25], and proteolysis triggered by blood. It is well known that lysosomal enzymes increase with cellular and subcellular damage. The plasma NAG concentration following reperfusion was reduced with NM 10^{-7} and or 10^{-6} M. This result is consistent with that of our previous report [15], in which aprotinin, another protease inhibitor, suppressed cGMP and subsequently reduced the release of lysosomal enzymes. These findings are supported by a report that cAMP stabilizes and cGMP destabilizes lysosomal membranes [5]; therefore, a reduction in cGMP inhibits the release of lysosomal enzymes. Since the hearts in all three of our groups received a multidose injection of cardioplegic solution in hypothermia, subsequently depleting all hearts of leukocytes the effect of proteolysis by leukocytes on the release of NAG would appear to be negligible. Therefore, our results suggest that NM 10^{-6} or 10^{-7}M may play a role as a protease inhibitor, even under hypothermia.

In general, NM did not preserve the myocardial concentrations of adenine nucleotides. Lysosomal enzymes deplete myocardial ATP by suppressing phosphorylation in the mitochondria [9]. In this study, however, the decrease in NAG produced by NM treatment did not improve the myocardial ATP concentration. Nevertheless, NM 10^{-7} M enhanced the recovery of left ventricular diastolic function during reperfusion, even though left ventricular systolic function was not affected. This finding is consistent with that of another report that the myocardial ATP concentration does not necessarily correlate with the recovery of left ventricular function [18, 21]. Our result also suggests that NM improved the left ventricular function in mechanisms other than adenine nucleotides. Since diastolic dysfunction is a frequent complication of long-term cardiac preservation [14], our observation that diastolic function is enhanced when NM 10^{-7} M is added to the nondepolarizing cardioplegic solution is potentially of great clinical value.

One factor not considered is the pharmacological interaction between NM and the other substances in the cardioplegic solution. Although we did not encounter adverse reactions in this study, this topic requires further investigation. It is important that during the administration of NM, even repeatedly, one avoid any anaphylactic reaction since NM is a synthetic substance.

The myocardial uptake of NM was 54.7 % ± 8.4 % during preservation and 29.1 % ± 15.2 % during reperfusion when NM 10^{-5} M was added to the cardioplegic solution. NM was detected in the coronary sinus blood of the donor heart even following reperfusion, although NM was only used during cardioplegia. This pattern suggests the existence of a high-affinity binding site for NM in the myocardium. NM 10^{-5} M was not the optimal concentration to preserve cardiac function and suppressed left ventricular diastolic function (preliminary study, unpublished data). For anticoagulation during extracorporeal circulation [24], NM is used at a concentration of 10^{-5}–10^{-4} M, administered by continuous infusion or multidose injection [13]. As ischemia-reperfusion is common during cardiac surgery, NM is likely to accumulate in the myocardium. Our findings warn against the use of NM at higher concentrations, as NM may accumulate within the myocardium and depress ventricular function.

We conclude that NM 10^{-7} M, adjunct to nondepolarizing solution, enhances the recovery of left ventricular function. Furthermore, NM 10^{-6} and 10^{-7} M seem to inhibit NAG by suppressing myocardial cGMP.

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