Protamine cardiotoxicity and nitric oxide

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Abstract

Objectives: The purpose of this study is to assess the role of the nitric oxide (NO) pathway in protamine-induced cardiotoxicity and to formulate a possible explanation for this adverse effect.

Methods: Isolated rat hearts were perfused by Krebs–Henseleit (KH) solution using a modified Langendorff model. They were randomized into three groups: A, 40 min perfusion with KH solution; B, 20 min perfusion with KH solution and 20 min with protamine; C, as B but L-N-monomethyl-L-arginine (L-NMMA), a non-selective inhibitor of the NO pathway, was added during 40 min of the perfusion period. Left ventricular (LV) function was measured every 10 min. NO and tumor necrosis factor-α (TNF) were detected in the effluent from the coronary sinus (CS) and in the supernatant of the cardiac myocytes culture. Nitric oxide synthases (NOS) mRNA levels were determined in groups A and B from LV samples at baseline and after 40 min of perfusion.

Results: We found that protamine at a dose of 12 µg/ml causes significant depression of LV function (decreased peak systolic pressure to 22.5 ± 3.2% and dP/dt max to 22.9 ± 3.1%). L-NMMA did not prevent protamine cardiotoxicity. NOS mRNA was not detected from LV samples in any group. The NO in the effluent from the CS and from the supernatant of the cardiomyocytes culture was below detectable levels. However, a significant amount of TNF was measured in the effluent from the CS (108 ± 17 pg/min for group B and 117 ± 13 pg/min for group C) and in the supernatant of the cardiomyocytes culture (65 ± 21 pg/ml).

Conclusions: This study suggests that direct protamine-induced cardiotoxicity does not depend on the NO pathway. Our finding that protamine induced TNF release by cardiomyocytes can shed new light on the understanding of protamine cardiotoxicity. © 2001 Elsevier Science B.V. All rights reserved.

Keywords: Protamine; Nitric oxide; Tumor necrosis factor-α; Isolated perfused heart

1. Introduction

The administration of protamine sulfate (protamine) for heparin reversal may cause several circulatory side effects such as systemic hypotension, pulmonary hypertension and left ventricular (LV) dysfunction [1–3]. Despite its extensive clinical application, the mechanisms of protamine-induced hemodynamic changes and direct cardiotoxic effect have not been fully determined.

One of the mechanisms that has been suggested for protamine-induced systemic vasodilatation is an increased production of nitric oxide (NO). Protamine is an arginine-rich protein, and L-arginine is the physiological substrate of NO production [4,5]. Cardiac myocytes express two types of nitric oxide synthases (NOS), endothelial (eNOS) and inducible (iNOS). eNOS activity is regulated by the contractile state of the heart, while iNOS expression is induced by cytokines [6,7]. Excessive production of NO by cardiomyocytes causes contractile dysfunction and depression of cardiac function [7,8]. Some laboratory studies found that protamine has a direct cardiotoxic effect [9]. Some of the investigators proposed that protamine cardiotoxicity was related to excessive positive charging of protamine [3,10]. On the other hand, it was shown that the protamine causes activation of leukocytes and increases in the blood level of the cardiotoxic cytokine, tumor necrosis factor-α (TNF) [11]. TNF may result itself in an increased NO production [12].

The present study was undertaken to determinate the direct effect of protamine on the NO system in the isolated perfused rat heart. NO release and specific myocardial eNOS and iNOS mRNA were determined, as was the influence of NO release after protamine induced its effect on rat cardiomyocytes culture. TNF was detected in the heart perfusate effluent and in the supernatant of cardiac myocytes culture.

To the best of our knowledge, this is the first investigation of a possible participation of the NO pathway in direct...
protamine-induced heart dysfunction. In addition, we have now shown that protamine causes TNF production in an isolated non-blood perfused rat heart and in cardiac myocytes culture.

2. Materials and methods

The investigation of the mechanism by which protamine induced cardiotoxicity was carried out by using a modified Langendorff perfusion system and employing a rat cardiac myocyte culture.

2.1. Langendorff perfusion system

Adult male Wistar rats (350–400 g) were anesthetized by an intraperitoneal injection of pentobarbital sodium (30 mg/kg). Their hearts were rapidly excised, immersed in ice-cold saline with heparin, and mounted on a stainless steel cannula of a modified Langendorff perfusion system. Retrograde aortic perfusion was initiated at a perfusion pressure of 85 mmHg with an oxygenated modified Krebs–Henseleit (KH) buffer solution: NaCl 118 mmol/l; KCl 4.7 mmol/l; CaCl₂ 2.0 mmol/l; MgSO₄ 7H₂O 1.2 mmol/l; KH₂PO₄ 1.2 mmol/l; glucose 11.1 mmol/l, and NaHCO₃ 25 mmol/l. The perfusate was bubbled continuously with 95% O₂ and 5% CO₂, maintaining a pH of 7.4–7.5. Our Langendorff system contained two separate reservoirs for retrograde aortic perfusion that provided the possibility of changing perfusion solutions during the experiment. The reservoirs were filled with a separate pump.

Cardiac temperature was measured by a thermistor implanted in the right ventricular wall and carefully maintained at 37°C by wrapping a water jacket around the perfusate reservoir and the isolated heart. The right atrium was partially removed, and the heart was paced to 300 beats/min at 4 V using an external pacemaker (type E4162; Devices Limited, Implants Division), ensuring an identical heart rate for all hearts. A water-filled latex balloon was inserted into the LV cavity via a small left atrium incision and connected to a Mennen Medical PI 32284 pressure transducer. The balloon was tied and inflated to a volume that produced 0 mmHg diastolic pressure.

2.2. Protocol

Twenty-four rats were randomized into three subgroups (A, B, and C) of eight animals each. After a 15 min period of stabilization (baseline point) LV pressure, contractility (dP/dt max, dP/dt min, pressure–time integral) and coronary flow were measured every 10 min. All the hearts were perfused for 40 min. Determination of NO and TNF in the effluent from the coronary sinus for all three groups was carried out at baseline and after 40 min of perfusion.

2.3. Experimental groups

Group A (control) hearts were perfused with KH solution alone. In group B, after 20 min of perfusion with KH solution, protamine (12 µg/ml) was added for 20 min. Group C hearts shared the same protocol as group B, but Ng-monomethyl-l-arginine (l-NMMA, 10 µmol/l), a competitive inhibitor of eNOS and iNOS [13], was added to the KH solution after the stabilization period during all stages of perfusion. This dose of l-NMMA was found to cause inhibition of NOS with no influence on the isolated perfused heart performance [14].

Ventricular fibrillation was caused by a concentration of 25–50 µg/ml of protamine in perfusate, equivalent to a clinical dose of 1.5–3.0 mg/kg body weight [15], assuming equal distribution throughout in the circulating volume used in our pilot experiments. After titration, we used a dose of 12 µg/ml, which caused cardiac depression without ventricular fibrillation.

2.4. TNF determination

TNF levels were detected in both the heart effluent and cell culture supernatant. Effluent samples from the coronary sinus for TNF measurement were drawn at the baseline point and after 40 min of perfusion and were immediately stored at −70°C until assayed. TNF activity was measured using the commercially available ELISA kit Cytoscreen™ rat kit TNF, Immunoassay kit (Biosource, Camarillo, CA). The limit of detection was 4 pg/ml.

2.5. NO measurement

Effluent nitrite (NO₂) and nitrate (NO₃), stable metabolites of NO representing NO production, were detected in the heart perfusate samples and cardiac myocytes culture supernatant, as previously described [16]. NO₃ was reduced to NO₂ by nitrate reductase prepared in our laboratory from *Escherichia coli* incubated in anaerobic conditions with KNO₃ [17]. After incubation of samples with *E. coli* prepare, the concentration of total NO₂ (formed from NO₃ and preexisted) was determined using Griess reagent (1% sulfanilamide and 0.1% N-(1-naphthyl)-ethylenediamine dihydrochloride in 2.5% phosphoric acid). Reagent was added to the sample in the proportion of 2:1, absorbance was measured at 546 nm and the NO₂ concentration was calculated from the calibration curve obtained by spectrophotometry of similarly treated standard solutions (2–50 µmol/l Na NO₂). The limit of detection was 2 µmol/l.

2.6. Myocardial tissue eNOS and iNOS determination

Immediately after a stabilization period (baseline, four additional hearts) and after perfusion with protamine (*n* = 5), the myocardium of the LV was excised and placed in cold Hank’s balanced solution. The LV of hearts perfused for 40 min with KH alone served as a control for hearts perfused with protamine (*n* = 5). Total RNA was extracted from myocardial samples using the guanidinium thiocyanate method [18]. RNA pellets were kept at −20°C with
75% ethanol until assay. Dried sediments were dissolved in sterile RNAse-free water and quantified spectrophotometrically at A = 260 nm.

Total RNA (2 µg) was subjected to reverse transcription reaction in 20 µl of reaction mixture using a transcription system (Promega, USA). After completion of the reaction, 5 µl of this reaction mixture was used for eNOS and iNOS cDNA polymerase chain reaction (PCR) amplification, and 5 µl of 1:10 diluted reaction mixture was used for GAPDH cDNA amplification. Our PCR negative control contained H2O instead of cDNA, and the cDNA negative control contained H2O instead of RNA. Primer sequences, annealing temperature, number of cycles and PCR product size [19] are reported in Table 1.

2.7. Quantitative analysis

PCR products (10 µl) were separated in 1.8% agarose gel, stained with ethidium bromide, visualized by UV irradiation and photographed with Polaroid film. The images were taken to evaluate band densities using FujiFilm Thermal Imaging System FTJ-500 computer-based image capture software (Pharmacia Biotech) and the TINA program package (Raytest Isotope Messgerate, GmbH). Intensities of the bands were expressed in arbitrary densitometry units. All eNOS and iNOS band intensities were normalized by respective GAPDH values. Each PCR reaction was performed at least twice.

2.8. Rat cardiac myocyte cultures

Myocyte cultures were derived from the excised hearts of neonatal Wistar rats, which were finely minced. The pieces were immersed in dissociation solution (calcium- and magnesium-free Hank’s balanced salt solution, Gibco, Bassel, Switzerland) containing a 1:200 dilution of RDB enzyme (Institute of Biology, Nes-Ziona, Israel). One million cells were placed in 0.1% gelatin pre-coated 35 mm tissue culture dishes. The medium was replaced on the following day. Cardiac myocyte cultures were treated with cytosine arabinoside (3 mmol/l) 1 day after seeding for 2 days to abort the multiplication of fibroblasts and other dividing cells, but there was no effect on cardiac myocytes since they were essentially post-mitotic. Thus, experiments were done on a highly rich cardiac myocytes population [20]. Using the periodic acid Schiff procedure [21], the amount of fibroblasts in the cardiac myocytes culture never exceeded 10%.

Cardiac myocytes were treated with protamine on the fourth day after seeding, when the cultures were confluent. Fresh medium was introduced and protamine (150 µg/ml) was added to the cardiac myocyte cultures. Measurement of NO was performed after 24 h of storage with protamine, and TNF assessment was performed after 1 h. For the purpose of comparison, NO and TNF were measured in the myocytes culture without protamine after 24 h and after 1 h of incubation, respectively.

2.9. Drugs

Protamine sulfate and heparin sodium were purchased from Kamada (Israel). L-NMMA was purchased from Sigma.

2.10. Ethics

Animal care complied with the ‘Principles of Laboratory Animal Care’ and the ‘Guide for the Care and Use of Laboratory Animals’ (NIH No. 85-23, Revised 1985).

2.11. Statistical analysis

The results are presented as the mean ± SE. All measurements were subjected to one-way analysis of variance (ANOVA) with repeated measures. NO measurements in the coronary effluent were normalized to a 1 min volume of coronary flow. Significance was established at a level of P < 0.05. All statistical analyses were performed by the Statistical Department of our Medical Center using an SPSS computer program.

3. Results

3.1. Hemodynamic changes

The baseline values for different LV hemodynamic parameters are given in Table 2. No significant differences were found between the experimental groups. Protamine was found to cause significant depression of LV function and to decrease coronary flow (for all variables of group B after
protamine addition in comparison to group A: \( P < 0.05 \), Fig. 1). Thus, protamine caused a decrease of peak systolic pressure (to 22.5 \( \pm \) 3.2\%, \( P < 0.005 \)), \( dP/dt \) max (to 22.9 \( \pm \) 3.1\%, \( P < 0.005 \)), pressure–time integral (to 25.1 \( \pm \) 3.7\%, \( P < 0.005 \)), and coronary flow (to 59 \( \pm \) 4\%, \( P < 0.05 \)), and an elevation of end-diastolic pressure (to 22 \( \pm \) 6 mmHg, \( P < 0.05 \)). The \( \text{l-NMMA} \) competitive inhibitor of eNOS and iNOS (group C) did not prevent protamine cardiotoxicity. No significant differences were found between groups B and C (Fig. 1).

### 3.2. Effect of protamine on NO production

The NO\(_2\) and NO\(_3\) levels in the effluent from isolated perfused hearts in all groups were below detectable levels.

After 24 h of cardiomyocytes culture incubation, the control NO supernatant levels were 3.85 \( \pm \) 2.6 \( \mu \)mol/l. There was no significant (\( P = 0.34 \)) increase of NO supernatant levels after 24 h of storage with protamine (4.19 \( \pm \) 2.3 \( \mu \)mol/l).

### 3.3. Effect of protamine on eNOS and iNOS mRNA expression

Basal eNOS and iNOS mRNA expression (group A) was detected in LV samples after the stabilization period and did not change following 40 min of perfusion (Figs. 2A and 3A). The intensities of the bands at baseline and at the end of 40 min of perfusion were 0.41 \( \pm \) 0.03 and 0.39 \( \pm \) 0.03 (\( P = 0.157 \), Fig. 2B), respectively, for eNOS and 0.60 \( \pm \) 0.02 and 0.67 \( \pm \) 0.02 (\( P = 0.136 \), Fig. 3B), respectively, for iNOS.

No significant changes were found after perfusion with protamine (group B) in eNOS mRNA (0.44 \( \pm \) 0.03) or iNOS mRNA (0.51 \( \pm \) 0.12) expression in comparison with these levels at baseline and after 40 min of KH perfusion (\( P = \text{NS} \), Figs. 2 and 3).

### 3.4. Effect of protamine on TNF release

Significant amounts of TNF, i.e. 108.3 \( \pm \) 16.7 pg/min (group B) and 117 \( \pm \) 12.8 pg/min (group C), were detected in the effluent after a 20 min perfusion (i.e. 40 min of perfusion from baseline) with protamine and with \( \text{l-NMMA} \) + protamine, respectively. The TNF was below detectable levels in samples taken before the addition of protamine and in the control group after the 40 min perfusion.

Significant amounts of TNF (65.4 \( \pm \) 21.3 pg/ml) were detected in the supernatant of the cardiomyocytes culture after 1 h of incubation with protamine. The TNF in the supernatant was below detectable levels in the non-protamine-treated cardiomyocytes culture.

![Fig. 1. Hemodynamic performance of isolated rat hearts during 1 h of perfusion: control group (white bars), hearts treated with protamine (dashed bars), and hearts treated with \( \text{l-NMMA} \) before protamine action (black bars). No significant differences were found between the group treated with protamine and the group treated with \( \text{l-NMMA} \) + protamine. Results are presented as each heart’s percentage of baseline measurements.](https://academic.oup.com/ejcts/article-abstract/20/1/147/482794)
4. Discussion

In an excellent review of the heparin protamine interaction, Carr and Silverman [3] summarize the multiple effects of protamine neutralization of heparin. It was suggested that the numerous cardiovascular effects of protamine are mediated by complement activation, histamine, thromboxane antibody formation and by increased NO production [3,22].

The possible effects of protamine on the vascular smooth muscle relaxation by NO have already been suggested [22]. Protamine causes stimulation of NO production by endothelial cells in tissue culture [23]. Removal of the endothelium or the administration of l-NMMA prevented protamine-induced vasodilatation in mammalian arteries. In our laboratory protamine induces endothelial NO-dependent relaxation of the human internal thoracic artery and this was prevented by NOS inhibitors. Recent studies showed that an excessive production of NO by myocytes causes contractile dysfunction and depression of cardiac function [6–8]. Cardiac myocytes express two types of NO synthase: eNOS and iNOS [6,8]. iNOS is not a constitutive form, and needs to be activated. However, in some cells there is low basal-specific iNOS activity. iNOS can be activated by cytokines leading to NO overproduction and causes inappropriate vasodilatation and negative inotropic effects [8,9]. The NO produced endogenously in cardiac myocytes is reversed by NOS inhibition [8]. All these favor a possible protamine–NO system mechanism in the protamine cardiotoxicity. We hypothesized that prota-
radicals or directly binds to the cell membrane and causes activation of the intracellular signaling cascade. Our findings of protamine-induced local TNF synthesis by the rat’s myocytes can shed new light on the understanding of the cardiotoxic effect of protamine.

4.1. Conclusions

In contrast to the findings of many investigators who showed that protamine causes its adverse effects via excessive NO production in isolated vessels, our results demonstrated that its direct cardiotoxic effect is not associated with the NO pathway. We found that protamine induces the release of the cardiotoxic cytokine TNF from cardiomyocytes. This TNF release may be one possible mechanism of protamine-induced cardiotoxicity.

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References