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Protamine-induced Cardiotoxicity Is Prevented by Anti-TNF-α Antibodies and Heparin

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Background: **We investigated the role of tumor necrosis factor α (TNF-α) in protamine-induced cardiotoxicity and the possibility of preventing or decreasing this effect by anti TNF-α antibodies and heparin.**

Methods: **Isolated rat hearts were perfused for 60 min with Krebs-Henseleit solution (KH). The control group was perfused with KH alone, the KH > protamine > KH group was treated from the 20th to the 40th minute with protamine, and the KH + anti-TNF > protamine + anti-TNF > KH + anti-TNF group was treated the same as the KH > protamine > KH group but with anti-TNF-α antibodies added throughout perfusion. The KH + heparin > protamine + heparin > KH + heparin group was treated the same as the KH > protamine > KH group but with heparin added to KH throughout perfusion. The KH > protamine > KH + heparin was perfused the same as the KH> protamine > KH group but with heparin added to KH for the last 20 min. Left ventricular (LV) function and coronary flow were measured every 10 min. TNF-α was measured in the coronary sinus effluent. Left ventricular TNF messenger RNA was determined in the control and KH > protamine > KH groups at baseline and after the 40-min perfusion.**

Results: **Protamine caused a significant decrease of peak systolic pressure and dP/dt (to 25% of baseline). Significant amounts of TNF-α in the effluent in the KH > protamine > KH group (102.3 ± 15.5 pg/min) and TNF messenger RNA expression in left ventricular samples were detected. TNF-α was below detectable concentrations in the control, KH + anti-TNF > protamine + anti-TNF > KH + anti-TNF, and KH + heparin > protamine + heparin > KH + heparin groups. TNF-α concentrations correlated with depression of LV peak systolic pressure ($r = 0.984$; $P = 0.01$) and first derivate of the increase of LV pressure ($r = 0.976$; $P = 0.001$). Heparin improved LV recovery and decreased protamine-induced TNF-α release (KH > protamine > KH + heparin group).**

Conclusions: **Anti-TNF-α antibodies and heparin prevent protamine-induced TNF-α release and depression of LV function. Heparin improves protamine-induced depression of cardiac function.**

PROTAMINE sulfate (protamine) is widely used to reverse heparin anticoagulation in the practice of cardiovascular surgery. However, protamine administration has occasionally been associated with clinically significant side effects, including systemic hypotension, bradycardia left ventricular (LV) dysfunction, and pulmonary edema.[1–5]

Two mechanisms have been suggested to be responsible for protamine-induced vasodilation or hypotension: an immunological reaction and endothelium-dependent relaxation. Several studies have demonstrated an association between the adverse reaction to protamine and fish allergy or treatment with protamine-Hagerone insulin.[2,4,6,7] These patients had elevated plasma concentrations of immunoglobulin E and immunoglobulin G antibodies specific to protamine, as well as complement system activation. Recent investigations reported that protamine, as an arginine-rich protein, causes endothelium-dependent relaxation through the supply of L-arginine, a physiologic precursor of nitric oxide.[4,8,9]

Some authors have reported that protamine decreases contractility of isolated LV myocytes and β-adrenergic responsiveness.[10] Wakefield *et al.*[11] proposed that protamine toxicity is related to its excessive positive charge. Katiricioglu *et al.*[12] found that protamine causes increased plasma concentrations of cardiotoxic cytokine–tumor necrosis factor α (TNF-α). It was recently found that the isolated rat myocardium itself can release TNF-α during ischemia.[13]

Despite these recent investigations, the pathophysiology of the direct cardiotoxic effect of protamine remains unclear. The purpose of the current study was to examine whether protamine induced a direct cardiotoxic effect related to the production of TNF-α in isolated perfused hearts. Anti–tumor necrosis factor antibodies (anti-TNF Ab) and heparin were used to prevent protamine-induced cardiotoxicity.

Materials and Methods

The present study protocol was approved by the Animal Care Committee at Tel Aviv University, Tel Aviv, Israel. The influence of protamine on the heart was investigated with the use of a modified Langendorff perfusion system. Male Wistar rats (weight, 350–410 g) were anesthetized by intraperitoneal injection of phenobarbital sodium (30 mg/kg). Their hearts were excised rapidly, immersed in ice-cold saline, and mounted on the 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 buffer solution (KH) containing 118 mM NaCl, 4.7 mM KCl, 2.0 mM $CaCl_2$, 1.2 mM $MgSO_4 7H_2O$, 1.2 mM KH_2PO_4, 11.1 mM glucose, and

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25 mM $NaHCO_3$. The perfusate was bubbled continuously with O_2, 95%, and CO_2, 5%, maintaining a pH of 7.4–7.5. Heart temperature was measured by a thermistor implanted in the right ventricular wall and carefully maintained at 37°C by water-jacketing 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, Welwin Garden City, UK), ensuring an identical heart rate for all hearts. A water-filled latex balloon was placed in the left ventricular (LV) cavity *via* a small incision in the left atrium and connected to a pressure transducer (Model PI 32284; Mennen Medical, Hamburg, Germany). The balloon was tied and inflated to a volume that produced 0 mmHg diastolic pressure.

Protocol

Thirty-five rats hearts were assigned randomly to five subgroups. After a 15-min stabilization period, all hearts were perfused with KH for 60 min. This perfusion consisted of three 20-min intervals. The control group was given KH solution alone. The KH > protamine > KH group was given a 20-min perfusion with KH solution, after which protamine (12 μg/ml) was added to the KH solution for 20 min, and the hearts were rinsed with KH solution alone during the final 20 min. The KH + anti-TNF > protamine + anti-TNF > KH + anti-TNF group was treated the same as the KH > protamine > KH group, but 0.6 μg/min anti–tumor necrosis factor α antibodies (anti-TNF Ab) was added to all periods of perfusion (60 min). The KH + heparin > protamine + heparin > KH + heparin group was perfused the same as the KH > protamine > KH group, but 5 U/ml heparin, 0.5 ml/min, was added to all periods of perfusion. The KH > protamine > KH + heparin group was given the same treatment as the KH > protamine > KH group, but 5 U/ml heparin, 0.5 ml/min, was added to KH solution during the final 20 min.

Concentration of protamine was chosen at the time of a pilot study. In the pilot experiments that we conducted before embarking on the current investigation, ventricular fibrillation was caused by concentrations of 25–50 μg/ml protamine in the perfusate, equivalent to a clinical dose of 1.5–3.0 mg/kg body weight, assuming equal distribution throughout circulating blood volume.[14] After titration, we used a dose of 12 μg/ml, which caused cardiac depression without ventricular fibrillation. A dose of 5 U/ml heparin, 0.5 ml/min, is approximately equivalent to the clinical dose. Concentration of anti-TNF Ab was chosen according to specific activity: Approximately 1 μg of these antibodies completely neutralized 25 pg rat TNF. In our study, we found that the TNF-α concentration in the coronary sinus effluent was 102 pg/ml at 20 min after the addition of protamine. When we used 0.6 μg/min anti-TNF-α Ab (total dose, 36 μg), before adding protamine, for total neutralization of TNF-α in the heart tissue, no TNF-α was found in the effluent from the coronary sinus of protamine-treated hearts.

Heparin, protamine, and anti-TNF-α Ab were added to the perfusate using separate infusion pumps. The effluent for TNF-α determination was taken in all groups at baseline and after the 40th, 50 th, and 60th min of perfusion. Left ventricular hemodynamic parameters (peak systolic pressure, dP/dt_{max} and dP/dt_{min} [first derivative of increase and decrease, respectively, in LV pressure], and coronary flow) were measured every 10 min.

Additional hearts for the KH > protamine > KH and the control groups were assayed for LV TNF messenger RNA (mRNA) expression at the 20th min after protamine action (40th min after baseline) and at baseline or at the 40th min of perfusion with KH alone for control group, approximately.

TNF-α Analysis

Effluent samples from the coronary sinus for TNF-α measurement were drawn at baseline measurements (the 15th min of stabilization), at the 40th min (after protamine addition), and at the 60th min (protamine washout) and were immediately stored at −70°C until assayed. TNF-α activity was measured using a commercially available ELISA kit (Cytoscreen TM rat kit TNF-α; Biosource, Camarillo, CA). The limit of detection was 4 pg/ml. The coefficients of variation for the dosage of TNF-α in our laboratory were as follows: The interassay coefficient of variation was less than 3.9%, and the intraassay coefficient of variation was less than 2.5%.

Immediately after a stabilization period and 40 min after baseline (control and KH > protamine > KH groups), the myocardium was excised and placed in a cold Hanks' balanced solution. Total RNA was extracted from myocardial samples using the guanidinium thiocyanate method.[15] RNA pellets were maintained at −20°C with 75% ethanol until assay. Dried sediments were dissolved in sterile ribonucleose-free water and quantitated spectrophotometrically at $\lambda = 260$ nm.

Two micrograms of total RNA were subjected to reverse transcription reaction in 20 μl using a transcription system (Promega Corporation, Madison, WI). After completion of the reaction, 5 μl of the reaction mixture was used for TNF complementary deoxyribonucleic acid (cDNA) amplification by polymerase chain reaction (PCR), and 5 μl of 1:10 diluted reaction mixture was used for reduced glyceraldehyde-phosphate dehydrogenase (GAPDH) cDNA PCR amplification. Our PCR negative control contained H_2O instead of cDNA, and the reverse transcription negative control contained H_2O instead of RNA. For TNF cDNA amplification, the following primers were used: sense, CACGCTCTTCTGTCTACTGA, and antisense, GGACTCCGTGATGTCTAAGT, producing a 546 bp fragment.[16] An annealing tempera-

Table 1. Baseline Measurements

Variable	Control	Protamine	AntiTNF Ab–Protamine	Heparin–+ Protamine	Protamine–Heparin
Rat weight (g)	383 ± 17	376 ± 10	380 ± 11	368 ± 15	376 ± 8
Peak systolic pressure (mmHg/S)	118 ± 7	125 ± 6	145 ± 8	120 ± 7	129 ± 10
dP/dt max (mmHg/S)	3,942 ± 361	4,043 ± 209	4,736 ± 238	3,894 ± 182	3,786 ± 380
Coronary flow (ml/min)	18 ± 1	16 ± 1	19 ± 2	18 ± 1	16 ± 1

Data presented are mean ± SD. No significant differences were found between variables in any experimental groups.
dP/dt max = first derivative of the increase of left ventricular pressure; dP/dt min = first derivative of the decrease of left ventricular pressure.

ture of 57°C was chosen for this reaction. For GAPDH cDNA amplification the primers were sense, AATGCATCCTGCACCACCAA, and antisense, GTAGCCATATTCATTGTCATA, producing a 515-bp fragment.[17] The annealing temperature in this case was 60°C. The optimal cycle number for the TNF-α and GAPDH was 30. For PCR amplification and reverse transcription reaction, a mini cycler TM (MJ Research Inc., Watertown, MS) was used.

Quantitative Analysis

Polymerase chain reaction products (10 μl) were separated in 1.8% agarose gel, stained with ethidium-bromide, visualized by ultraviolet irradiation, and photographed with Polaroid film (Kodak Co., New Haven, CT). The film was used to evaluate band densities using Fujifilm Thermal Imaging System (Model FTJ-500; Fuji Photo Film Co., LTD., Osaka, Japan), the computer-based Image Capture Software (Pharmacia Biotech, Jerusalem, Israel), and the TINA program package (Raytest Isotope Messgerate, GmbH, Staubenhardt, Germany). Intensities of the bands were expressed in arbitrary densitometry units. All TNF-α band intensities were normalized by respective GAPDH values. Each PCR reaction was performed at least twice, and four or five hearts were used for each experimental group.

Drugs

Protamine sulfate and heparin sodium were purchased from Kamada (Bei-Kama, Israel). Polyclonal rabbit anti-rat TNF Ab were purchased from R&D Systems (Minneapolis, MN). The specific activity is that approximately 1 μg of these antibodies completely neutralizes 25 pg rat TNF-α.

Statistical Analysis

Results are presented as the mean ± SD. Measurements of TNF-α in the coronary effluent were normalized to 1-min volume of coronary flow. All measurements were subjected to two-way analysis of variance with repeated measures. This design includes one between-subject factor (the experimental group) and one within-subject factor (the time of measurement). Whenever a significant time trend was demonstrated, we used contrast analysis to compare each measurement with its successive one (SIMPLE; SPSS Inc., Chicago, IL). Only one contrast was used to eliminate the problem of multiplicity. We report the results of the comparison of several treatments with the control group. Although the results are represented separately for each treatment group, the significant values are based on a joint statistical analysis followed by a multiple comparison test. The *P* values for the analysis of variance F tests are one-sided, and the *P* values for the correlation coefficient tests are two-sided. A one-way analysis of variance was performed to compare TNF-α concentrations between the experimental groups in each time point separately.

Pearson correlation coefficient analysis was calculated between TNF-α concentrations in the effluent, and the percentage of cardiac function injury was measured at the end of the protamine action. This correlation coefficient was calculated for the KH > protamine > KH group. Significance was established at the level of $P < 0.05$. All the statistical analyses were performed with the SPSS computer program in the Statistics Department at Sourasky Tel-Aviv Medical Center.

Results

Hemodynamic Changes

The baseline values for the different LV hemodynamic parameters are presented in table 1. No significant differences were found between any experimental groups. We also did not find any differences in the LV hemodynamic parameters in any of the experimental groups before protamine was added (*i.e.*, baseline values and at 10 and 20 min). No *P* values attained statistical significance (fig. 1).

Protamine was found to cause a significant depression of LV function and a decrease of coronary flow (fig. 1) for all variables of the KH > protamine > KH group in comparison with the control group ($P < 0.05$). Thus, the addition of protamine caused a decrease of peak systolic pressure (to 25 ± 3%), dP/dt_{max} (to 25 ± 3%), coronary flow (to 63 ± 4%), and an elevation of end-diastolic pressure (to 22 ± 6 mmHg).

TNF-α Release and TNF mRNA Expression

Significant amounts of TNF-α in the KH > protamine > KH group (*i.e.*, 102.3 ± 15.5 pg/min) were detected in

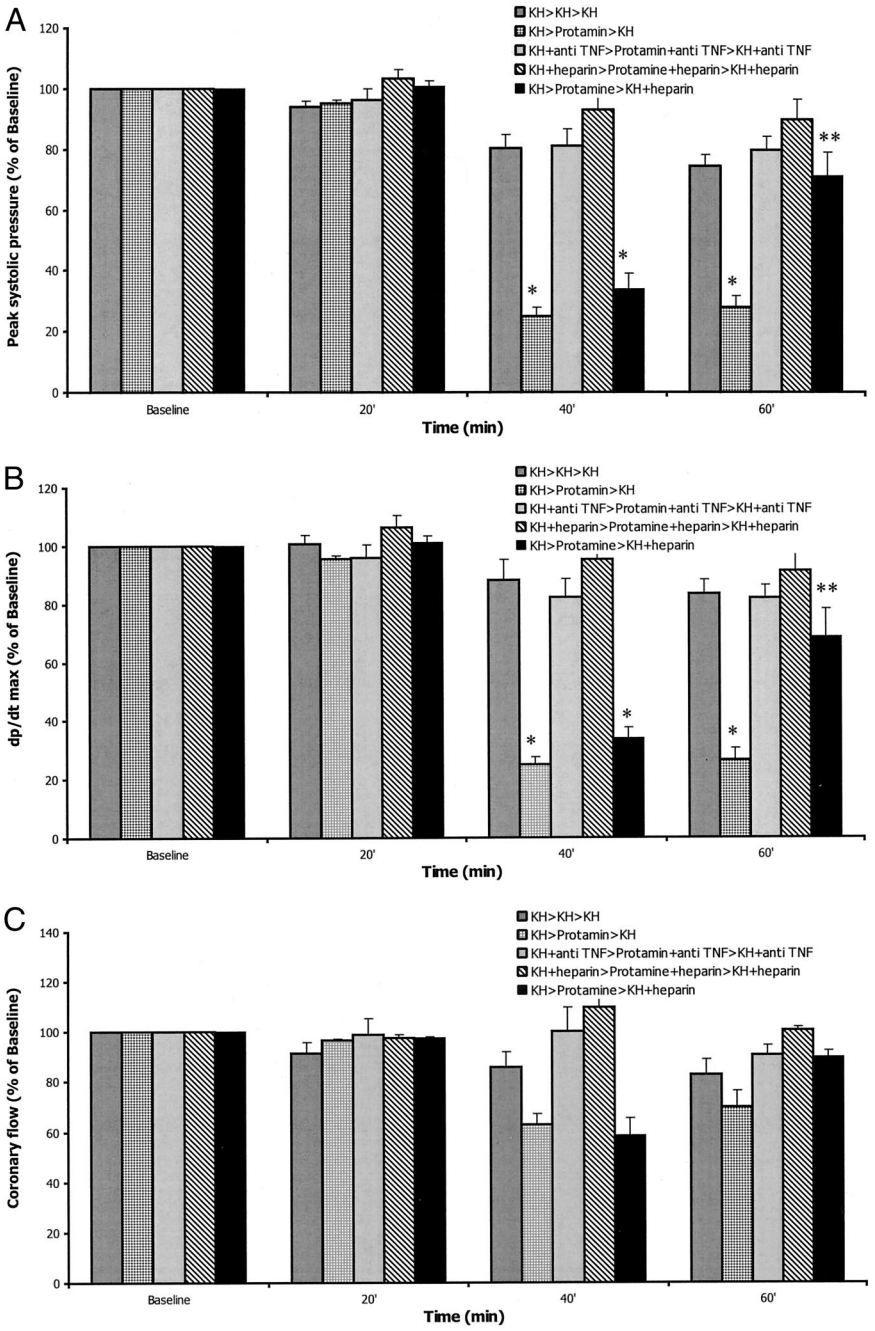

Fig. 1. Hemodynamic performance of isolated rat hearts during 1 h of perfusion: Control group perfused with Krebs-Henseleit solution (KH), KH > protamine > KH group treated with protamine from 20 to 40 min, KH + anti-TNF > protamine + anti-TNF > KH + anti-TNF group treated with anti-TNF antibodies at all perfusion time, KH + heparin > protamine + heparin > KH + heparin group treated with heparin at all perfusion time, KH > protamine > KH + heparin group treated with heparin at time of washout (last 20 min). Results are presented as the percentage of baseline measurements for each heart. No significant differences found between control group and groups treated with anti-TNF antibodies or heparin throughout perfusion. *$P < 0.05$ *versus* control group. **$P < 0.05$ for measures on 60 *versus* 40 min for KH > protamine > KH + heparin group.

the effluent after a 20-min perfusion with protamine. Protamine-induced effluent TNF-α concentrations significantly correlated with the mechanical deterioration of the hearts after protamine action in the KH > protamine > KH group (fig. 2). No significant correlation between the TNF-α concentration and coronary flow was found ($r = -0.206$; $P = 0.658$).

In samples taken from the control group after baseline and at the perfusion times of 40, 50, and 60 min, TNF-α was below detectable concentrations. Basal TNF mRNA expression (control group) was detected in the LV samples after the stabilization period and did not change after 40 min of perfusion (fig. 3A). Intensities of the bands at baseline and after the 40th min of perfusion were 0.41 ± 0.03 and 0.42 ± 0.04, respectively (P = not significant) (fig. 3B). After the 20th min of protamine perfusion, TNF mRNA expression increased to 0.64 ± 0.05 and was significantly higher than the concentrations detected in the control group ($P = 0.005$) (fig 3).

Neutralization of Protamine-induced Cardiotoxicity by Anti–TNC Antibodies

The anti-TNF Ab that had been added before protamine (in the KH + anti-TNF > protamine + anti-TNF > KH + anti-TNF group) completely prevented protamine-induced depression of LV function in comparison with

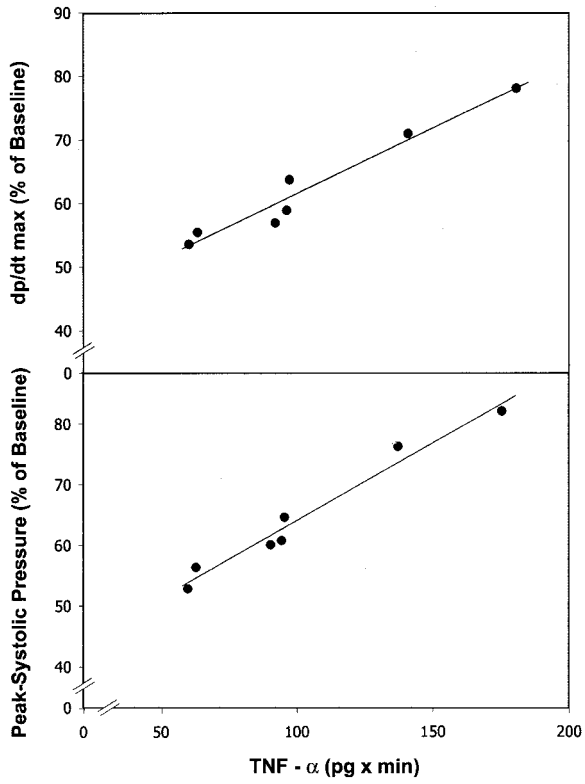

Fig. 2. Correlation of tumor necrosis factor α concentrations in the effluent with the deterioration (relative to baseline measurements) in peak systolic pressure (r = 0.984; *P* = 0.01), dP/dt max (r = 0.976; *P* = 0.001) after exposure to protamine.

the LV function of the KH > protamine > KH group ($p<0.05$) (fig. 1). No significant differences were observed in any of the measured data between the control and the KH + anti-TNF > protamine + anti-TNF > KH + anti-TNF groups. All measurements of effluent TNF-α in these groups were below detectable concentrations.

Influence of Heparin on Protamine-induced Cardiotoxicity

Heparin added to perfusate before protamine (KH + heparin > protamine + heparin > KH + heparin) prevented protamine-induced depression of LV function in comparison with the KH > protamine > KH group ($P < 0.05$) (fig. 1). No significant differences were found in any hemodynamic data between the control group and the KH + heparin > protamine + heparin > KH + heparin group. All concentrations of TNF-α in the effluent of the KH + heparin > protamine + heparin > KH + heparin group were below detectable concentration. The addition of heparin also improved LV function recovery after protamine-induced LV depression. Washout with heparin (the KH > protamine > KH + heparin group) significantly improved the recovery of LV function compared with the KH > protamine > KH group ($P < 0.05$) (fig. 1). Washout with heparin in this group caused a decrease of the TNF-α concentration from 128.0 ± 23.7 to 35.6 ± 7.8 pg/min ($P = 0.018$) at the 50th min of perfusion; at the 60th min of perfusion TNF-α was below detectable concentration.

Discussion

The results of the present study suggest that protamine induced a cardiotoxic effect related to TNF-α production by non–blood-perfused rat hearts. Most investigators suggest that positively charged protamine, like other polycations, causes a direct cardiotoxic effect by destroying cell membranes and causing mitochondrial damage.[11,18,19] Recent studies have shown that negatively charged heparin decreases the protamine-induced cardiotoxic effect, probably *via* formation of a nonactive complex.[11] Other authors[12] have found that protamine causes leukocyte activation and release of cardiotoxic cytokine TNF-α. TNF-α also acts through the nitric oxide pathway.

Tumor necrosis factor is a proinflammatory polypeptide hormone with potent negative inotropic properties.[20,21] These TNF-α concentration- and time-dependent effects are reversible[21] and can be initiated by the activation of TNFR 1 cell surface receptors.[22] We also found that the direct addition of TNF-α to the isolated perfused heart using a dose of 700 pg/min caused a 40–50% depression of LV function (unpublished data, D. Pevni, M.D., I. Frolkis, M.D., Ph.D., Laboratory of Department of Thoracic and Cardiac Surgery, Tel Aviv Sourasky Medical Center, Israel, July 1999). Treatment with anti-

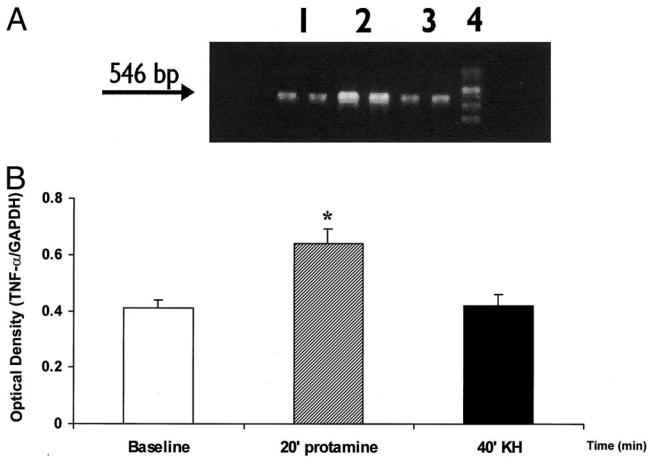

Fig. 3. Effect of protamine on myocardial tumor necrosis factor (TNF) messenger RNA (mRNA) expression: representative polymerase chain reaction (PCR) analysis of RNA samples (in duplicate) (*A*) and relative optical density of TNF PCR signal (*B*). Data were normalized to relative reduced glyceraldehyde-phosphate dehydrogenase PCR signal (*B*). Samples drawn at the beginning of perfusion (1 or baseline), 20 min after protamine addition to 40-min perfusion from baseline (2 or 20' protamine), and after 40-min perfusion with Krebs-Henseleit solution (KH) (3 or 40' KHw) served as control. Four- to 100-base pair DNA ladder. Significantly increased TNF mRNA expression was observed after protamine addition. Band intensities were considerably higher than in baseline group and in group perfused with KH ($P < 0.005$).

TNF Ab has been shown to prevent myocardial dysfunction during experimental burn shock and in several cardiac diseases such as viral myocarditis and acute allograft rejection.[23,24]

In the current study, we deliberately used a blood-free model to exclude the possibility of involvement of systemic blood factors in protamine-induced cardiotoxicity and TNF-α formation**.** In our earlier investigation, we found that the rat myocardium can release significant amounts of TNF-α after prolonged ischemia.[13] At present, we have shown that protamine causes significant elevation of TNF-α, which is detected in the effluent of the coronary sinus, and that it stimulates the expression of TNF mRNA in the LV myocardium. The TNF-α concentration correlated with a depression of LV contractility. Anti-TNF Ab completely prevented the protamine-induced cardiotoxic effect. In the KH + anti-TNF > protamine + anti-TNF > KH + anti-TNF group, in which isolated hearts were given anti-TNF Ab, TNF-α was below a detectable concentration.

The findings of an increase of the TNF mRNA and TNF-α concentrations, correlation of the TNF-α concentration with depression of the LV function, and prevention of protamine-induced cardiotoxic effect by specific anti-TNF Ab are strong lines of evidence that the protamine-induced depression of the LV function depends on TNF-α formation and release. Pearson *et al.*[8] reported that protamine caused coronary artery dilation. The coronary flow decrease that was found in our study was probably associated with LV diastolic dysfunction. We found an increase in LV diastolic pressure and no correlation between the TNF-α concentration and coronary flow. In the heparin-pretreated hearts, protamine did not cause any depression of LV function and TNF-α release**.** Heparin at a dose of 5 U/ml, 0.5 ml/min, which had been given after protamine, completely bound free protamine and markedly improved LV recovery. This was accompanied by a decreased TNF-α concentration. The finding that heparin prevents or decreases protamine cardiotoxicity suggests that probably only free (nonbinding) protamine causes depression of LV function. The findings of local TNF-α synthesis in the rat myocardium and anti-TNF Ab and heparin protective action can shed new light on our understanding of the cardiotoxic effect of protamine.

Possible Mechanisms for Anti–TNF Antibody and Heparin Myocardial Protection

Protamine, like other polycations, is a membrane-disorganizing agent.[18,19] This action on cardiomyocytes can be a trigger for TNF-α production. The negative inotropic effect of TNF-α is associated with decreased concentrations of intracellular calcium during the systolic contraction sequence.[21] TNF-α is involved in the release of free radicals from the myocardium,[25] a self-amplifying process, because free radical production has been shown to cause further increase of TNF-α.[26] Antibodies against TNF-α neutralize TNF-α release by the myocardium and probably prevent binding of TNF-α to TNFR 1 receptors. Thus, a reasonable explanation for the cardioprotective effect of anti-TNF Ab might be that elimination of TNF-α prevents the release of free radicals and decreases the snowball effect. The negatively charged heparin binds to the positively charged protamine resulting in the formation of a nonactive complex.[11] The formation of the nonactive heparin–protamine complex is probably the reason for the decrease in TNF-α production and the observed improvement in LV function. When heparin is added before protamine, protamine binds to heparin and forms a nonactive complex, preventing protamine-induced triggering of TNF-α formation.

Our study has several limitations. First, it was performed *in vitro*. Second, protamine was administrated before heparin, whereas it is usually administrated in the presence of heparin in clinical practice. Nonblood perfusion excluded the possibility for protamine to bind to blood protein. Our non–blood-perfused isolated rat heart model provided high partial oxygen pressure that can induce coronary vasoconstriction and low oxygen transport.[27,28] The present investigation does not exclude factors contributing to protamine-related adverse side effects, such as direct damage to cell membranes and mitochondria and the influence of immunological factors.

In the present study, we found that the protamine-induced cardiotoxicity is related to TNF-α synthesis (expression of TNF mRNA) and release (significant amounts of TNF-α detected in the effluent of coronary sinus) by isolated rat heart. We also found that anti-TNF Ab completely prevented this adverse affect of protamine. The detected TNF-α concentrations were directly correlated with protamine-induced depression in myocardial mechanical performance.

Heparin was found to prevent protamine-induced cardiotoxic effect. It also improved the protamine-induced depression of LV function and decrease in TNF-α release. It would appear that only free protamine (not bound with heparin) caused the depression of LV function in our animal model.

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