Heparin Added to Cardioplegic Solution Inhibits Tumor Necrosis Factor-α Production and Attenuates Myocardial Ischemic-Reperfusion Injury*

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Objectives: Tumor necrosis factor (TNF)-α is known to be a proinflammatory cytokine that has a pronounced negative inotropic effect and plays an important role in ischemic-reperfusion injury. Methods: Twenty isolated rat hearts were randomly divided equally into two groups (heparin and nonheparin) and were perfused with a Krebs-Henseleit solution using a modified Langendorff model. The influence of heparin on the synthesis and release of TNF-α by isolated rat hearts after 1 h of global cardioplegic ischemia and on left ventricular (LV) performances during 30 min of postischemic reperfusion was investigated.

Results: Significant mean (± SEM) amounts of TNF-α in myocardial tissue (1,149 ± 33.7 pg/g) and effluent (951.8 ± 27.3 pg/mL) from the coronary sinus were detected after global cardioplegic ischemia. The addition of heparin to the cardioplegic solution significantly improved the recovery of LV function in the postischemic heart (p < 0.0001 for all measurements). TNF-α protein production in the heparin-treated hearts was below detectable levels despite a postischemic increase of TNF-α messenger RNA expression in both heparin-treated hearts and non-treated hearts (0.71 ± 0.06 and 0.8 ± 0.12 relative optical density, respectively).

Conclusion: This study shows, for the first time, that heparin causes the inhibition of TNF-α protein synthesis and release from the isolated ischemic rat heart within the posttranscriptional stage, and that it prevents the depression of LV function caused by ischemic-reperfusion injury.

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Key words: cardioplegia; isolated perfused heart; messenger RNA; tumor necrosis factor-α

Abbreviations: ANOVA = analysis of variance; bp = base pair; CF = coronary flow; CS = coronary sinus; dP/dt max = first derivative of the rise of the left ventricular pressure; ELISA = enzyme-linked immunosorbent assay; GAPDH = glyceraldehyde-phosphate dehydrogenase; KH = Krebs-Henseleit; LV = left ventricle, ventricular; MAP = mitogen-activated protein; PCR = polymerase chain reaction; TNF = tumor necrosis factor

Tumor necrosis factor (TNF)-α plays an important role in the pathogenesis of a number of inflammatory states, among them septic shock, myocarditis, glomerulonephritis, Crohn disease, insulin resistance, and others, and has a clear negative inotropic effect.1–4 Some investigations4 have confirmed that the level of TNF-α was increased in patients with chronic heart failure and have demonstrated a direct relation between circulating levels of TNF-α and clinical features of the disease. TNF-α is an important element in reperfusion injury after myocardial revascularization.5 The hemodynamic effects of TNF-α are characterized by decreased myocardial contraction, reduced ejection fraction and hypotension, and decreased systemic vascular resistance.4

TNF-α is a trimeric 17-kd polypeptide that is produced by monocytes and macrophages as well as by cardiomyocytes.4 Our group reported that global cardioplegic ischemia caused TNF-α gene expression and TNF-α release from isolated non-blood-perfused ischemic hearts. This postischemic TNF-α level significantly correlated with the deterioration of left ventricular (LV) function during the time of reperfusion.6 Moreover, the administration of mono-
clonal antibodies to TNF-α eliminated this cytokine in effluent and attenuated the posts ischemic myocardial injury.6

The ability of heparin to bind antithrombin III and heparin cofactor II in order to inhibit the coagulation cascade is widely exploited in clinical practice. Heparin has been shown to possess antiinflammatory action, including the modulation of some of the pathophysiologic effects of endotoxin and TNF-α, such as neutrophil migration, edema formation, reduction of eosinophil migration, and decrease of vascular permeability.7,8 Salas et al8 have reported an antiinflammatory effect of heparin associated with the attenuation of a CD11b-dependent adherent mechanism induced by TNF. Heinzelmann et al9 on the other hand, have reported that heparin enhances TNF-α production in human monocytes. Most of these studies were performed on tissue cultures of WBCs. The main aims of the present study were to explore whether heparin treatment affects paracrine TNF-α-associated mechanisms of ischemic-reperfusion injury in a blood-free environment in an isolated perfused rat heart model, and whether it improves posts ischemic heart recovery.

Materials and Methods

The present study protocol was approved by the Animal Care Committee of Tel Aviv University, Tel Aviv, Israel. Male Wistar rats (weight, 350 to 400 g) were anesthetized by intraperitoneal injection of pentobarbital sodium (30 mg/kg). The hearts were rapidly excised, immersed in cold saline solution (4°C), and mounted on the stainless steel cannula of a modified Langendorff perfusion apparatus. Retrograde aortic perfusion was initiated at a perfusion pressure of 85 mm Hg with an oxygenated modified Krebs-Henseleit (KH) buffer solution composed of the following: NaCl, 115 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; NaHCO3, 25 mmol/L. The perfusate was bubbled continuously with 95% O2 and 5% CO2, maintaining a pH of 7.4 to 7.5. The PO2 and PCO2 values in the perfusion solution were 450 to 550 mm Hg and 25 to 30 mm Hg, respectively. The temperature of the heart was monitored by a thermistor implanted in the right ventricular wall, and was carefully maintained at 37°C or 31°C (at the time of ischemia) by warming the perfusate reservoir and the isolated heart in a water jacket. The right atrium was removed, and the heart was paced to 300 beats/min at 4 V using an external pacemaker (type E4162; Devices Limited, Implants Division; Garden City, UK), ensuring an identical heart rate for all of the hearts studied. A water-filled latex balloon was placed in the LV cavity through a small incision in the left atrium and was connected to a pressure transducer (model PT 32254; Mennen Medical; Hamburg, Germany). The balloon was tied and inflated to a volume that produced 5 mm Hg diastolic pressure. Zero calibration of the pressure transducer was maintained throughout the experiment.

LV peak systolic pressure and coronary flow (CF) were measured, and the first derivative of the rise of the LV pressure (dP/dt max) and the area under the LV-developed pressure curve (i.e., the pressure-time integral) were calculated. These variables were continuously recorded, and each measurement was taken at 10-min intervals.

Protocol

Twenty rats were randomly divided equally into two groups (heparin and nonheparin groups). Baseline hemodynamic measurements were recorded after a 15-min period of stabilization. The hearts in the heparin and nonheparin groups were perfused for 30 min. Hemodynamic parameters and CF were assessed every 10 min during this perfusion period. Warm cardioplegia (temperature, 37°C) was initiated for 2 min (perfusion pressure, 60 mm Hg; KCl, 16 mEq/L in KH solution) with 5 U/mL heparin (heparin group) or without heparin (nonheparin group), and a 60-min period of global cardioplegic ischemia at 31°C was applied to the arrested heart. The administered dose of heparin 5 U/mL was close to the equivalent clinical dose. The total heparin dose given during 2 min of warm cardioplegia was 150 U. Thereafter, measurements of LV function were made every 10 min throughout the 30-min reperfusion period. The TNF-α protein levels were determined in the effluent from the coronary sinus (CS) by a cytotoxic activity assay method at baseline and at the end of ischemia (ie, the first milliliter of reperfusion effluent) in the heparin and nonheparin groups. The TNF-α protein level also was determined from the effluent of five additional hearts after 90 min of nonischemic perfusion (controls).

Fifty additional hearts were used for the detection of TNF-α messenger RNA expression at baseline (n = 5), after 1 h of global ischemia (n = 5 in each group), and after 90 min of perfusion without ischemia (hearts from the control group). TNF-α protein content in the effluent from the CS was determined by a cytotoxic activity assay method and a commercially available enzyme-linked immunosorbent assay (ELISA) kit (Cytoscreen rat kit TNF-α; Biosource; Camarillo, CA) in the heparin group (n = 10) and the nonheparin group (n = 10) at the end of ischemia (ie, the first milliliter of reperfusion effluent) and after 90 min of nonischemic perfusion (control group, n = 5). TNF-α protein content was determined with the ELISA kit in LV tissue after 1 h of global ischemia in the heparin group (n = 5) and nonheparin group (n = 5), and at the end of 90 min of nonischemic perfusion in the control group.

TNF-α Messenger RNA Analysis

The LV myocardium was excised and placed in a cold Hanks balanced solution. Total RNA was extracted from LV myocardial samples using the guanidine thiocyanate method.10 RNA pellets were maintained at −20°C with 75% ethanol until assayed. Dried sediments were dissolved in sterile ribonuclease-free water and quantitated spectrophotometrically at a λ of 260 nm.

Two micrograms of total RNA was subjected to reverse transcription reaction in 20 μL of reaction mixture using a transcription system (Promega Corporation; Madison, WI). After completion of the reaction, 5 μL of the reaction mixture was used for TNF complementary DNA amplification by polymerase chain reaction (PCR), and 5 μL of a 1:10 diluted reaction mixture was used for glyceraldehyde-phosphate dehydrogenase (GAPDH) complementary DNA PCR amplification. Our PCR negative control contained H2O instead of complementary DNA, and the transcription reaction negative control contained H2O instead of RNA.

For TNF complementary DNA amplification, the following primers were used: sense, CAGCCGTCTCTGTCTACTGA; antisense, GGACTCCGTGATGTCTAAGT. These produced a 546-base pair (bp) fragment.11 An annealing temperature of 57°C was chosen for this reaction.

For GAPDH complementary DNA amplification, the following primers were used: sense, ATGCTACCTCGACGCACCAA; antisense, GTAGCCATATGTCATATA. These produced a 515-bp fragment.12 The annealing temperature in this case was
60°C. The optimal number of cycles for the TNF-α and GAPDH was 30. For PCR amplification and reverse transcription reaction, a minicycler (MJ Research Inc; Watertown, MA) was used.

Quantitative Analysis

PCR products (10 μL) were separated in a 1.8% agarose gel, which was stained with ethidium-bromide, visualized by ultraviolet irradiation, and photographed using film (Polaroid; Cambridge, MA). The film was used to evaluate band densities (Thermal Imaging System FTJ-500; Fujifilm; Osaka, Japan), a computer-based image capturing software (Image Capture Software; Pharmacia Biotech; Jerusalem, Israel), and a software package (TINA; Raytest Isotope Messgerate, GmbH; Staubenhardt, Germany). The 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 five hearts were utilized for each experimental group.

TNF-α Determination

Cytotoxic Activity Assay Method: Effluent samples from the CS for TNF-α protein determination were drawn at baseline (ie, 15 min after stabilization) and immediately after ischemia (ie, the first milliliter) in the heparin and nonheparin groups and after 90 min of perfusion (ie, controls) and were immediately stored at −70°C until assayed. TNF-α levels were determined on the basis of a cytotoxic activity assay on mouse L929 cells according to the methods described by Yuhas et al. Each assay included a standard curve of recombinant human TNF-α (specific activity, 2.5 × 10^7 U/mg protein [1 U = 40 pg of TNF-α]). The limit of detection was 40 pg/mL.

ELISA Method: The effluent samples from the CS and LV myocardial tissue that were taken for TNF-α measurements were immediately stored at −70°C until assayed. TNF-α activity was measured by a commercially available ELISA kit. The limit of detection was 4 pg/mL. The coefficients of variation for the dosage of TNF-α in our laboratory were an interassay coefficient of variation of <3.9% and an intraassay coefficient of variation of <2.5%.

Drugs

Heparin sodium was purchased for use (Kamada; Beit-Kama, Israel).

Statistical Analysis

The results are presented as the mean ± SEM. Measurements of LV function were subjected to two-way analysis of variance (ANOVA) with repeated measures. Significance was established at a level of p < 0.05. The Wilcoxon test was performed to compare the measurements of LV function between separate time points in the heparin and nonheparin groups. The unpaired Student t test was applied to compare normalized TNF-α messenger RNA band intensities between the heparin and nonheparin groups at each time point separately. All of the statistical analyses were performed with a statistical software package (SPSS; SPSS Inc; Chicago, IL) in the statistics department at our medical center.

RESULTS

Hemodynamic Changes

The baseline values for the nonheparin and heparin groups for the different LV hemodynamic parameters are given in Figure 1. No significant differences were found between these two groups before global cardioplegic ischemia.

Global cardioplegic ischemia was found to cause a significant depression of LV function and a decrease of CF in the nonheparin group (Fig 1). Following 60 min of ischemia and 30 min of reperfusion, the mean peak systolic pressure was decreased to 47.26 ± 3% (p < 0.0001 [ANOVA]), the dP/dt max to 51.1 ± 3.9% (p < 0.001 [ANOVA]), and the CF to 62.2 ± 2% (p < 0.001 [ANOVA]) of baseline values.

Heparin was found to have essentially preserved cardiac function in the reperfusion period. After ischemia, the hearts treated with heparin developed higher pressure, greater dP/dt max, a higher pressure-time integral, and a greater CF (p < 0.0001 for all measurements) compared to the hearts of the nonheparin group (Fig 1). Moreover, the addition of heparin to the cardioplegic solution yielded no significant differences between any measured parameters of LV function and CF before ischemia (ie, baseline values and after 10, 20, and 30 min of perfusion) and after reperfusion (ie, after 10, 20, and 30 min of reperfusion) [Fig 1].

TNF-α Messenger RNA Expression and TNF-α Protein Synthesis and Release

Basal TNF-α messenger RNA expression was detected in the LV samples after the stabilization period (Fig 2), and there were no differences in baseline TNF-α messenger RNA expression compared to 90 min of the nonischemic normally perfused hearts (intensities of the bands, 0.28 ± 0.06 and 0.31 ± 0.09, respectively). After 1 h of global cardioplegic ischemia (Fig 2), TNF-α messenger RNA expression significantly increased in both the nonheparin group (0.81 ± 0.12; p < 0.002) and the heparin group (0.71 ± 0.06; p < 0.005). No differences were found between the band intensities of TNF-α messenger RNA in these groups after 1 h of global cardioplegic ischemia.

Significant mean amounts of TNF-α (by cytotoxic assay, 596 ± 178.3 pg/mL; by ELISA, 951.8 ± 27.3 pg/mL) were detected in the effluent from the CSs of the hearts in the nonheparin group immediately following 1 h of global cardioplegic ischemia. The TNF-α in the effluent from the CS was below a detectable level at the same time point in the heparin hearts and after 90 min in the control hearts. The level of TNF-α protein in myocardial tissue was elevated in the nonheparin group after ischemia (1.149 ± 33.7 pg/g), but it was below detectable
levels in the heparin group (at the same time point) and the control group (90 min after nonischemic perfusion).

**Discussion**

The results of the present study demonstrate the direct effect of heparin on the myocardium during global cardiac ischemia. Heparin added to a cardioplegic solution prevents TNF-α protein production and release from cardiac myocytes after global cardiac ischemia and improves myocardial recovery during reperfusion but does not influence global cardiac ischemia-induced TNF-α messenger RNA expression. A blood-free model was intentionally used in this study to exclude the possibility of involvement of systemic blood factors in endogenous TNF-α formation and in exogenous TNF-α action on the heart. A model of cardioplegic ischemia was chosen because of the extensive use of cardioplegic solutions in cardiopulmonary bypass procedures.

TNF-α has a potent negative inotropic effect. Kapadia et al demonstrated that myocardial cells could themselves produce TNF-α in isolated perfused hearts under endotoxin treatment. Our group and others have reported that global cardiac ischemia caused TNF-α gene expression and TNF-α release from isolated ischemic hearts. This postischemic TNF-α level significantly correlated with the deterioration of LV function during the time of reperfusion, and the administration of monoclonal antibodies to the TNF-α eliminated this cytokine in effluent and attenuated the postischemic myocardial injury.

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**Figure 1.** Hemodynamic performance of isolated rat hearts (nontreated hearts, white bars; heparin-treated hearts in cardioplegia, black bars). Before ischemia, both groups demonstrated similar peak systolic pressures (top left, A), dP/dt max (top right, B), pressure-time integral (bottom left, C), and CF (bottom right, D). Following ischemia, hearts in the nonheparin group underwent significant deterioration in myocardial performance, while hearts treated with heparin in cardioplegia exhibited improved recovery compared to those in the nonheparin group (p < 0.0001 for all variables). Rep = reperfusion.
The search for a TNF-α inhibitor for clinical use is one of the main challenges of contemporary research. TNF-α inhibitors could potentially deter or at least attenuate several treatment-resistant diseases. The administration of the TNF-α inhibitors anti-TNF-α antibodies has been shown to prevent myocardial dysfunction during experimental burn shock in animal models and to ameliorate several other pathologic conditions such as viral myocarditis, acute allograft rejection, and glomerulonephritis.

Some studies have shown that heparin could cause the inhibition of some TNF-α-induced pathologic processes. Several uncontrolled studies have suggested that it may be potentially therapeutic in the clinical management of refractory ulcerative colitis and active Crohn disease. Heparin has been found to protect skeletal muscle against ischemia-reperfusion injury in vivo. A number of studies have shown that it has an antiinflammatory action that is associated with the inhibition of TNF-α-induced pathologic effects. Salas et al have reported that heparin attenuates TNF-α-induced inflammatory response via a CD11b-dependent mechanism, and Wan et al have reported that low-molecular-weight heparin inhibits TNF-α-induced leukocyte rolling. Hoffman et al demonstrated that the addition of heparin decreases the TNF level in the plasma of septic patients and in monocyte culture stimulated by endotoxin cells.

Most of these studies were performed on tissue cultures of WBCs. The direct effect of heparin on myocardial TNF-α was not investigated. Our current findings demonstrated that the addition of heparin to

![Figure 2](image.png)

**Figure 2.** Effects of heparin on myocardial TNF-α messenger RNA expression in ischemic hearts, as follows: representative PCR analysis of RNA samples (top, A); relative optical density of the TNF PCR signal (bottom, B). The data were normalized to the respective GAPDH PCR signals. Samples withdrawn at the beginning of perfusion (top, A, 1; bottom, B, Baseline) served as controls. TNF-α messenger RNA expressions increased (top, A, 2; bottom, B, ischemic [Isch]) in hearts that underwent global cardioplegic ischemia (p < 0.005). No differences were found in TNF-α messenger RNA band intensities for heparin-treated (top, A, 3; bottom, B, Heparin) and untreated hearts.
a cardioplegic solution prevents the synthesis and release of TNF-α and preserves LV function at the time of reperfusion. These findings of local TNF-α synthesis and release from the isolated ischemic-reperfused myocardium, and the protective role of heparin on ischemia-reperfusion injury in a blood-free and leukocyte-free environment can shed new light on previous hypotheses. Heparin acts not only to prevent the recruitment of circulating leukocytes to sites of inflammatory lesions, but it probably causes the down-regulation of TNF-α synthesis and release from the myocardium itself. TNF-α possesses a local insult or deleterious paracrine effect (myocardial depression), and heparin achieves its protective action without any essential need for systemic involvement in this process.

Possible Mechanisms of Heparin-Induced Inhibition of TNF-α Synthesis

In the present study, global cardiac ischemia caused the transcriptional activation of the TNF-α gene. The LV myocardium showed a significant (2.5-fold to 3-fold) increase of TNF-α messenger RNA expression (compared to baseline) in the myocardium of hearts in both the heparin group and the control group. In the heparin group, however, TNF-α protein was not detected in the effluent from the CS. The absence of TNF-α protein release after ischemia, despite an elevated expression of TNF-α messenger RNA, may be the result of the down-regulation of the translational stage of TNF-α synthesis or it may be related to posttranslational events. TNF-α is initially synthesized as a membrane-anchored prohormone, which is processed proteolytically to yield active cytokine. The TNF-converting enzyme has been identified as a metalloproteinase-like enzyme. We performed ELISA studies of myocardial tissue in order to determine whether translational or posttranslational mechanisms were involved in the elimination of TNF-α protein in the cardiac postischemic effluent after heparin treatment. Our results showed that the addition of heparin to the cardioplegic solution eliminated TNF-α protein production in the isolated heart after ischemia. This, we think, allows us to propose that the down-regulation of the translational stage of TNF-α synthesis had occurred.

The inhibition of TNF-α synthesis at the translational level is believed to be an important mechanism for protecting the host from the harmful effect of TNF-α. In response to lipopolysaccharide stimulation, macrophages or monocytic cells pretreated with dexamethasone or guanilylhydrazone failed to produce TNF-α protein, but messenger RNA was induced dramatically. A key element for the translational regulation of TNF-α has been identified in the 3’ untranslated region of TNF messenger RNA (ie, the AU-rich sequence). Han et al demonstrated that the 3’ untranslated region of TNF-α messenger RNA caused a 600-fold decrease in its translational efficiency. Several factors (eg, an inducible cytoplasmic factor and an AUUA-specific messenger RNA-binding protein) have been identified as being capable of binding to the AU-rich sequence and may mediate the enforced inhibition of TNF-α translation. We speculate that heparin can activate these intracellular factors in the same way. Another potential target of heparin action is p38 mitogen-activated protein (MAP) kinase. It has been suggested that MAP kinase may be critical in the translational control of TNF-α synthesis. The activation of the MAP kinase cascade enhances the translational efficiency of TNF-α messenger RNA. It is possible that heparin may down-regulate the activity of this kinase.

In the present study, heparin treatment caused an increase of CF. In our previous experiments, we had shown a significant decrease in endothelial nitric oxide synthase messenger RNA expression after global cardiac ischemia. The addition of anti-TNF-α antibodies was shown to increase CF and endothelial nitric oxide synthase messenger RNA expression. This may represent an additional mechanism of heparin to ameliorate posts ischemic recovery via the aborting of TNF-α production.

Our study has several limitations. By having been performed in vitro, the nonblood perfusion excluded the possibility for heparin binding to blood protein. Our non-blood-perfused isolated rat heart model provided high partial oxygen pressure, which can induce coronary vasoconstriction and low oxygen transport.

Conclusion

We have shown that heparin, widely used in clinical practice, improved the recovery of LV function following global cardiac ischemia. The addition of heparin to a cardioplegic solution before ischemia prevented the paracrine myocardial TNF-α protein production and release caused by heparin-related down-regulation of the posttranscriptional stage of TNF-α synthesis. TNF-α is considered to be involved in the pathogenesis of a number of pathologic states, including chronic heart failure and ischemic-reperfusion injury. The inhibitory effect of heparin on TNF-α synthesis may have therapeutic benefit.

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REFERENCES


4 Meldrum DR. Tumor necrosis factor in the heart. Am J Physiol 1998; 274:R577–R595


