Anesthesiology
1999; 91:355–61

Nitecapone Reduces Cardiac Neutrophil Accumulation in Clinical Open Heart Surgery

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Background: **To study the effect of nitecapone, a novel antioxidant, on cardiac neutrophil activation during cardiopulmonary bypass in patients.**

Methods: **In a double-blind, placebo controlled trial, 30 male patients undergoing coronary artery bypass grafting were randomly assigned to control (crystalloid cardioplegia, n = 15) and nitecapone groups (cardioplegia supplemented with nitecapone, n = 15). Leukocyte differential counts, neutrophil and monocyte CD11b and L-selectin expressions and neutrophil hydrogen peroxide production were measured in blood samples parallelly obtained from the coronary sinus and aorta before cardiopulmonary bypass and at 1, 5, and 10 min after aortic declamping. Myocardial myeloperoxidase activity was analyzed in biopsies taken at 1, 5, and 10 min after declamping.**

Results: **Transcoronary neutrophil difference (*i.e.*, aorta − sinus coronarius) at 1 min after aortic declamping was significantly lower in nitecapone-treated patients (0.41 [−0.42–0.98] × 10^9 cells/l) than in controls (0.68 [−0.28–2.47] × 10^9 cells/l; P = 0.032). At 5 min after aortic declamping, significant transcoronary reduction of neutrophil hydrogen peroxide production and CD11b expression were observed in controls but not in nitecapone patients. At 24 h postoperatively, left ventricular stroke volume was better in nitecapone-treated patients (94 [51–118] ml) than controls (66 [40–104] ml; P= 0.018). Data are median [range].**

Conclusion: **Nitecapone added to cardioplegia solution reduces cardiac neutrophil accumulation and transcoronary neutrophil activation during clinical cardiopulmonary bypass. Reflected by better left ventricular stroke volume, nitecapone treatment may be an additional way of reducing the deleterious effects of neutrophil activation during cardiopulmonary bypass. (Key words: Antioxidant; free oxygen radical; neutrophil; reperfusion.)**

ACTIVATED neutrophils play a significant role in cardiac reperfusion injury. Neutrophil depletion improves cardiac[1] and coronary endothelial cell function,[1–3] and reduces myocardial necrosis[4,5] after reperfusion during experimental cardiopulmonary bypass (CPB). Similarly, inhibition of neutrophil adherence to endothelium with blocking antibodies against CD18[6–8] and intercellular adhesion molecule 1 (ICAM-1)[9] enhances myocardial function during reperfusion. Coronary sequestration of neutrophils occurs also in clinical open heart surgery.[10,11] In emergency coronary artery bypass grafting[12] and in heart transplantation,[13] leukocyte depletion reduces peak creatine kinase MB concentrations and the need for inotropic support.

Nitecapone is a catechol derivative with antioxidative properties. It is an effective scavenger of superoxide, nitric oxide, hydrogen peroxide and hydroxyl radicals.[14,15] In addition, it enhances recycling of vitamin E *via* ascorbate,[14] and reduces oxidation of glutathione (GSH to GSSG).[16] In a reperfusion model of the Langendorff-perfused heart,[17,18] in experimental heart transplantation in the rat,[19] and in experimental CPB in the pig,[20] nitecapone enhances cardiac function and reduces oxidative stress.

The aim of the present study was to evaluate the effect of nitecapone on coronary neutrophil activation during clinical coronary artery bypass surgery. The study hypothesis was that nitecapone as an effective antioxidant might, by reducing oxidative stress, also reduce cardiac neutrophil activation.

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Received from the Division of Infectious Diseases, Departments of Bacteriology and Immunology, Thoracic Surgery, Internal Medicine, and Clinical Chemistry, Hospital for Children and Adolescents, University of Helsinki, Finland. Submitted for publication August 28, 1998. Accepted for publication January 27, 1999. Supported by the Clinical Research Institute of Helsinki University Central Hospital and the Foundation for Pediatric Research, Helsinki, Finland.

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Patients and Methods

Patients

The study was approved by the local ethical committee and the Ministry of Health of Finland. All patients gave their informed consent. Thirty male patients undergoing coronary artery bypass grafting were prospectively entered into this study. The patients were randomly assigned to receive nitecapone (n = 15) or to serve as controls (n = 15). The surgical procedures consisted of coronary artery bypass grafting with venous or arterial grafts.

Anesthesia

Patients were premedicated with lorazepam. Patients received their normal morning dose of long-acting nitrates, β-blockers, and calcium channel blockers. Anesthesia was induced with 30 μg/kg fentanyl citrate and 0.1 mg/kg midazolam and maintained with 0.3 $\mu g \cdot kg^{-1} \cdot min^{-1}$ fentanyl citrate and 0.8 $\mu g \cdot kg^{-1} \cdot min^{-1}$ midazolam infusions. Enflurane or isoflurane was added when needed. Muscle relaxation was achieved with 0.1 mg/kg pancuronium and maintained with 2-mg boluses according to the response to neurostimulation of the ulnar nerve.

Operation

Patients were ventilated with a Siemens Servo Volume Ventilator (Siemens-Elema AB, Solna, Sweden). The aorta and right atrium were cannulated for CPB. The extracorporeal circuit was primed with 2,000 ml of a crystalloid solution containing 5,000 U of heparin. Before cannulation, patients received heparin at a dose of 3 mg/kg body weight. Bypass was conducted at a flow rate of 2.0–2.4 l/m^2 and mean arterial pressure was maintained at 40–80 mmHg with nonpulsatile perfusion in mild hypothermia (30–32°C as a nasopharyngeal temperature). The rectal temperature was rewarmed to a minimum of 34°C and the nasopharyngeal temperature to 36–37°C before weaning from CPB. $F_{I_{O_2}}$ was 100% during the perfusion, and $S_{V_{O_2}}$ was maintained over 75%. Lung ventilation was discontinued after the beginning of perfusion and was subsequently resumed after declamping. Heparin was neutralized after discontinuation of CPB using protamine sulfate. The duration of the reperfusion period before weaning from CPB was 30% of the aortic cross-clamping time.

In the controls, cardioplegia was induced with 15 ml/kg body weight of Plegisol™ (Orion-Pharma, Espoo, Finland; composition: sodium 110 mEq, chloride 160 mEq, potassium 16 mEq, calcium 2.4 mEq, magnesium 32 mEq; temperature 5°C) *via* the aortic root cannula after application of aortic clamp. An additional 2 ml/kg of the cardioplegic solution was infused every 15 min and if ventricular fibrillation was present. In the nitecapone group, 50 mM nitecapone (Orion-Pharma) was added to the cardioplegic solution. Nitecapone was a gift from Orion-Pharma and was provided in the form of sterile powder dissolved in $NaHCO_3$ and then added to Plegisol solution.

Myocardial Biopsies and Analysis of Cardiac Myeloperoxidase Activity

At 1, 5, and 10 min after aortic declamping, myocardial biopsies were taken from the apex of the left ventricle, together with corresponding blood samples (see below). The samples were immediately frozen in liquid nitrogen and stored at −70°C until assayed for myeloperoxidase activity using a modification of the spectrophotometric method described by Suzuki *et al.*[21]

Blood Samples

Parallel blood samples from the coronary sinus and the ascending aorta were taken as follows: immediately before the onset of CPB, and at 1, 5, and 10 min after aortic declamping. In addition, a blood sample was drawn from the radial arterial cannula immediately before aortic declamping. Each blood sample was divided into three 1-ml aliquots as follows. For leukocyte differential counts (Technicon H2 analyzer), 1-ml aliquot of a sample was transferred into a tube containing EDTA. For analysis of adhesion molecule expressions, another 1-ml aliquot of the sample was immediately transferred into a polystyrene tube (prechilled on ice at 0°C to minimize increases in CD11b expression due to sample handling; see references 22 and 23) (Falcon No. 2058, Becton Dickinson Labware, Lincoln Park, NJ) supplemented with 250 μl pyrogen-free citrate (Baxter Health Care, Norfolk, England) and 150 μl dextran (MW 70.000; Sigma Chemicals, St. Louis, MO). After sedimentation on ice at 0°C for a maximum of 60 min, the leukocyte-rich plasma was separated into another ice-cold polystyrene tube and carefully kept at 0°C during cell staining and until analysis with flow cytometry (see below). Finally, for analysis of intracellular hydrogen peroxide production, 1 ml of the blood sample was immediately transferred into a polystyrene tube (prewarmed to 37°C in the dark) supplemented with 250 μl of pyrogen-free citrate, 150 μl of dextran, and 2 μl of 2,7-dichlorofluorescein-diacetate (DCFH-DA, final concentration 100 μM, Eastman Kodak,

Rochester, NY). After 15 min incubation at 37°C in the dark, the leukocyte-rich plasma was separated and kept in an ice-water bath at 0°C in the dark.

Preparation of Leukocytes

Determination of adhesion molecule expression on neutrophils and monocytes was carried out by three-color flow cytometry as previously described, with minor modifications.[22,23] Firstly, 25 μl aliquots of leukocyte-rich plasma were double-labeled (30 min at 0°C) using saturating concentrations of the PE conjugates of the mouse anti-CD11b antibody (IgG2a), the mouse anti-CD62L antibody (anti-Leu8, IgG2a), or as an isotype control mouse anti-keyhole limpet hemocyanin IgG2a-PE, and of the FITC conjugate of the mouse anti-CD14 (IgG2b) antibody. All monoclonal antibodies were purchased from Becton Dickinson (San Jose, CA). After labeling, contaminating erythrocytes were lysed with diluted ice-cold FACS lysing solution (Becton Dickinson). Leukocytes were then washed with ice-cold PBS, fixed with 1% formaldehyde in PBS and, finally, stained with LDS-751 solution (final concentration 100 ng/ml).

Analysis of intracellular hydrogen peroxide production of neutrophils was performed as previously described.[24] Neutrophils were not labeled with monoclonal antibodies. Contaminating erythrocytes were lysed with ice-cold ammonium chloride solution, washed twice with ice-cold PBS, and kept as a cell suspension in PBS without fixation. The samples were analyzed within 6 h after preparation.

Flow Cytometry

A FACScan flow cytometer (Becton Dickinson) and LYSYS II software were used for acquisition and analysis of the data. To analyze adhesion molecule expression, 5×10^3 LDS-751-positive events were collected in live mode. Secondly, a data set of 10^3 CD14-positive events were collected. To study adhesion molecule expression specifically on neutrophils, among LDS-751-positive live gated cells, and specifically on monocytes, among CD14-positive live gated cells, the irrelevant cell populations were electronically eliminated, as described previously.[21,22] Adhesion molecule expression is reported in relative fluorescence units (RFU), *i.e.*, as the mean of the positively fluorescing cell population. To analyze intracellular hydrogen peroxide production of neutrophils, DCF fluorescence intensity was measured after gating in an analog manner with the determination of neutrophil adhesion molecule expression described above.

Table 1. Patient and Operative Data

	Controls	Nitecapone
Age (yr)	62 (44–73)	61 (40–77)
Preoperative EF (%)	52 (35–80)	59 (43–72)
Previous AMI	6	10
Number of bypasses	3 (1–4)	3 (3–5)
Crossclamping time (min)	59 (22–80)	59 (37–106)
Perfusion time (min)	96 (47–120)	95 (63–146)

EF = ejection fraction; AMI = acute myocardial infarction.

Statistical Analysis

Patient data and results are expressed as median and range or as individual values. The Mann-Whitney test was used for comparison between the study groups. The Wilcoxon test was used when comparing values of the sinus coronarius samples with values of simultaneously obtained aortic samples within a study group. Analysis of variance and the Wilcoxon test with Bonferroni correction were used for multiple comparison of repeated measurements, *i.e.*, when comparing changes in the arterial samples within a study group. A P value less than 0.05 was considered statistically significant.

Results

Patient Characteristics, Operative Data, and Clinical Outcome

The nitecapone and control groups did not differ with respect to age, preoperative ejection fraction, previous incidence of acute myocardial infarction, extracorporeal perfusion time, aortic cross-clamping time, or the number of coronary artery bypass grafts (table 1). Neither were there any differences in the use of inotropic agents, mean arterial pressure, cardiac output, or cardiac index during 24 h postoperatively. Heart rate was significantly higher in the control group at 3 h and 6 h (data not shown), but not at 24 h (table 2) postoperatively. After induction of anesthesia, left ventricular stroke volume was comparable in the nitecapone (70 [57–101] ml) and the control groups (64 [38–107] ml). At 24 h postoperatively, left ventricular stroke volume was significantly higher in the nitecapone (94 [51–118] ml) than the control group (66 [40–104] ml; table 2; $P = 0.018$).

Leukocyte Counts

There were no differences in arterial (*i.e.*, aorta or radial artery) neutrophil (table 3), monocyte (table 3), or lymphocyte counts (data not shown) between the study groups. At 1 min after aortic declamping, in the control

Table 2. Hemodynamic Data

		Induction of Anesthesia	24 h Postoperatively
Heart rate (beats/min)	C	61 (51–81)	88 (73–109)
	N	55 (47–71)	81 (55–93)
Cardiac output (l)	C	4.3 (2.9–6.1)	6.2 (3.6–9.7)
	N	4.2 (3.3–5.3)	6.7 (4.5–10.4)
Cardiac index (l/m^2)	C	2.2 (1.7–2.9)	3.0 (2.0–4.9)
	N	2.2 (1.7–2.9)	3.5 (2.2–5.3)
Stroke volume (ml)	C	64 (38–107)	66 (40–104)
	N	70 (57–101)	94 (51–118)*

C = controls; N = nitecapone treatment.

* $P < 0.05$, N *versus* C.

group and to a lesser extent in the nitecapone group, neutrophil counts were significantly lower in the sinus coronarius than in the aorta (control: 4.43 [2.10-6.52] $\times$ 10^9 cells/l *vs.* 5.35 [2.41-8.99] $\times$ 10^9 cells/l, P = 0.001; nitecapone: 4.24 [0.66-9.92] $\times$ 10^9 cells/l *vs.* 4.02 [1.49-9.72] $\times$ 10^9 cells/l, P = 0.021). Consequently, at 1 min after aortic declamping, the transcoronary difference (*i.e.*, aorta − sinus coronarius) of neutrophil counts, *i.e.*, neutrophil sequestration, was significantly lower in the nitecapone (0.41 [−0.42-0.98] x 10^9 cells/l) than in the control group (0.68 [−0.28-2.47] x 10^9 cells/l) (P = 0.032; fig. 1). There were no differences in monocyte or lymphocyte counts across the coronary circulation at any time point in either group (data not shown).

Myocardial Myeloperoxidase Activity

Myocardial myeloperoxidase activity did not differ significantly between the respective nitecapone and control groups at 1 min (26 [3-467] μU/g *vs.* 39 [10-297] μU/g), at 5 min (26 [6-343] μU/g *vs.* 54 [4-400] μU/g), or at 10 min (23 [4-151] μU/g *vs.* 31 [6-520] μU/g) after aortic declamping. Interestingly, 6 of the 15 control patients had both high myocardial myeloperoxidase activity at 10 min and high coronary neutrophil sequestration at 1 min after declamping. In the remaining controls and in each patient of the nitecapone group, myeloperoxidase activity and neutrophil sequestration both were comparatively low (fig. 2).

Intracellular Hydrogen Peroxide Production of Neutrophils

In arterial samples, intracellular H_2O_2 production of neutrophils increased during CPB in both study groups, but there were no differences between the groups (table 3). In the nitecapone group, there was no transcoronary difference (*i.e.*, sinus coronarius − aorta) of H_2O_2 production at any time point. In the control group, neutrophil H_2O_2 production was lower in the sinus coronarius at 5 min (sinus coronarius − aorta: −31 [−244-25] RFU; P = 0.008) and 10 min (sinus coronarius − aorta: −11 (−257-27) RFU; P = 0.045) after aortic declamping but not before CPB. Further analysis of the data indicated that at 5 min after aortic declamping, the transcoronary difference of H_2O_2 production was significantly lower in the six control patients with high coronary neutrophil sequestration and high cardiac myeloperoxide activity as compared with the remaining control patients (−61 (−244 to −28) RFU *vs.* −8 (−77-25) RFU; P = 0.014).

Neutrophil and Monocyte CD11b and L-Selectin Expression

CD11b and L-selectin fluorescence of neutrophils and monocytes in arterial (*i.e.*, aorta or radial artery) samples are presented in table 3. There were no differences in CD11b or L-selectin fluorescence between the study groups (table 3). Neither was there any transcoronary difference in neutrophil CD11b fluorescence in either group prior to CPB (data not shown). In the control group at 5 and 10 min after declamping, CD11b fluorescence intensity was marginally but statistically significantly lower in the sinus coronarius than in the aorta (sinus coronarius − aorta: −4 [−14-5] RFU at 5 min, P = 0.026; and −6 [−11-2] RFU at 10 min, P = 0.008). No transcoronary differences were observed in the nitecapone group (data not shown). Neither were there any transcoronary differences in monocyte CD11b, monocyte L-selectin, or neutrophil L-selectin fluorescence of either group (data not shown).

Discussion

In accordance with previous reports,[10,11] coronary neutrophil sequestration in our patients occurred rapidly during reperfusion after aortic declamping. Nitecapone supplementation to the cardioplegia solution reduced significantly this neutrophil sequestration at 1 min after aortic declamping. Interestingly, in the controls, two subpopulations could be distinguished: in six patients, both coronary neutrophil sequestration and myocardial myeloperoxide content, an index of myocardial neutrophil accumulation, were relatively strong, as compared with the remaining controls, in whom cardiac neutrophil accumulation was as low as in each nitecapone-treated patient.

Table 3. Neutrophil and Monocyte Activation in Arterial Samples

		Before CPB	Before Reperfusion	1 min after Declamping	5 min after Declamping	10 min after Declamping
Neutrophil count						
($\times 10^9$/l)	C	4.00 (2.11–8.67)	5.19 (2.26–7.40)	5.35 (2.41–8.99)	5.77 (2.26–8.12)	6.04 (3.46–9.20)*
	N	3.07 (1.27–6.09)	3.75 (1.49–10.50)*	4.02 (1.49–9.72)*	4.23 (1.79–10.22)*	4.99 (2.05–9.00)*
Monocyte count						
($\times 10^9$/l)	C	0.34 (0.23–0.62)	0.29 (0.07–0.56)	0.31 (0–0.61)	0.35 (0.05–0.48)	0.31 (0.20–0.62)
	N	0.41 (0.24–0.80)	0.23 (0–0.53)*	0.25 (0–0.65)	0.33 (0.07–0.62)	0.31 (0–0.64)
Neutrophil CD11b						
(RFU)	C	51 (27–151)	107 (62–231)*	118 (72–245)*	115 (70–252)*	98 (64–219)*
	N	50 (33–83)	118 (73–242)*	117 (60–248)*	124 (56–229)*	116 (80–196)*
Monocyte CD11b						
(RFU)	C	34 (26–91)	52 (28–87)	65 (30–148)	67 (36–106)	59 (35–133
	N	38 (25–58)	52 (39–154)*	50 (45–129)*	54 (35–116)*	55 (43–132)*
Neutrophil L-selectin						
(RFU)	C	263 (161–387)	236 (177–348)	240 (169–361)	221 (135–336)	228 (167–353)
	N	236 (149–513)	230 (161–297)	238 (172–369)	235 (159–395)	255 (172–398)
Monocyte L-selectin						
(RFU)	C	269 (153–360)	326 (269–491)*	305 (269–500)*	310 (221–475)*	333 (230–496)*
	N	231 (149–359)	271 (187–443)*	290 (203–407)*	301 (221–475)*	344 (218–457)*
Neutrophil H_2O_2						
(RFU)	C	77 (27–263)	147 (60–344)*	244 (74–664)*	193 (83–579)*	158 (61–585)*
	N	95 (24–178)	166 (55–444)*	163 (59–479)*	191 (75–662)*	173 (50–477)*

C = controls; N = nitecapone treatment; RFU = relative fluorescence unit.

*$P < 0.0125$ (Bonferroni correction) *versus* before CPB.

Activated neutrophils are a potent source of proteolytic enzymes and oxygen free radicals. The deleterious effects of neutrophil activation has been well documented in a variety of organs, including the heart.[25,26] The ability to reduce cardiac neutrophil accumulation in our patients is thus therapeutically important and was reflected as significantly better left ventricular stroke volume in the nitecapone-treated patients than the controls at 24 h postoperatively. In accordance with our results, nitecapone enhances cardiac function also in the reperfusion model of Langendorff-perfused heart,[17,18] in experimental heart transplantation in the rat,[19] and in experimental CPB in the pig.[20] Similarly, there are several experimental studies documenting cardioprotective effect of neutrophil inhibition during reperfusion.[1–9] To best of our knowledge, only two clinical intervention

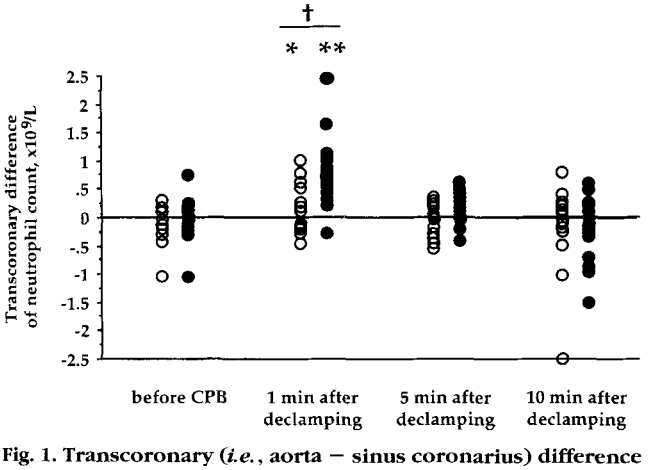

Fig. 1. Transcoronary (*i.e.*, aorta − sinus coronarius) difference of neutrophil count in nitecapone patients (white dots) and controls (black dots). *$P < 0.05$ and **$P < 0.01$, sinus coronarius *versus* aorta; †$P < 0.05$, nitecapone patients *versus* controls.

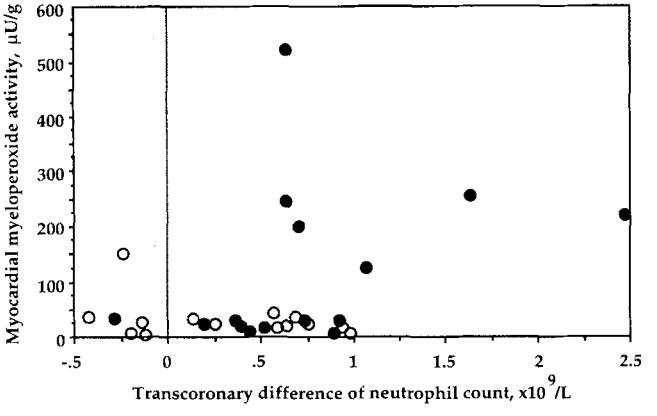

Fig. 2. A dot blot between transcoronary (*i.e.*, aorta − sinus coronarius) neutrophil sequestration at 1 min and myocardial myeloperoxidase activity at 10 min after aortic declamping in nitecapone-treated patients (white dots) and controls (black dots).

studies aimed at reducing neutrophil-mediated cardiac reperfusion injury have been published to date.[12,13] In emergency coronary artery bypass grafting due to developing acute myocardial infarction[12] and in heart transplantation,[13] reperfusion with leukocyte-depleted blood reduces both cardiac release of creatine kinase MB and the need for inotropic support.

Although coronary neutrophil sequestration was significantly lower in the nitecapone-treated patients than in the controls, there were no significant differences between the groups in CD11b or L-selectin expression or in hydrogen peroxide production of peripheral (*i.e.*, circulating) neutrophils. This probably reflects the fact that neutrophil activation required for endothelial adhesion occurs locally within the reperfused coronary vessels. This local activation is supported by the observation that in the controls during reperfusion, hydrogen peroxide production of neutrophils was lower in the coronary sinus samples than in the simultaneously taken aortic samples. This transcoronary reduction of neutrophil hydrogen peroxide production was significantly stronger in those six control patients with the highest coronary neutrophil sequestration and the highest myocardial myeloperoxidase activity, as compared with the remaining controls. Furthermore, in the control patients, there was also a marginal but statistically highly significant decrease in neutrophil CD11b expression across the coronary circulation during reperfusion. Notably, nitecapone supplementation to the cardioplegia solution abolished transcoronary reduction of both hydrogen peroxide production and CD11b expression. Taken together, the results possibly reflect reperfusion-induced coronary sequestration of those neutrophils with the highest hydrogen peroxide production and highest CD11b expression. As the transcoronary reduction of neutrophil hydrogen peroxide production and CD11b expression were observed only in the controls, our results suggest that targeting of nitecapone treatment to the coronary circulation is sufficient and effective in reducing cardiac neutrophil activation.

During reperfusion, free oxygen radicals are produced by activated endothelium and by activated neutrophils. These oxygen free radicals modulate neutrophil adhesion to endothelium: in *ex vivo* models, hydrogen peroxide and superoxide radicals increase adhesion molecule expression both on neutrophils[27] and on endothelial cells,[28] resulting in enhanced leukocyte adherence. Adherent neutrophils, on the other hand, mediate endothelial cell damage by further releasing oxygen free radicals and amplifying the inflammatory process.[29] Thus, during reperfusion, free radicals have an effect on several steps of neutrophil-endothelial cell interaction. In this process, both neutrophils and endothelial cells are simultaneously the source and the target of free radicals. Nitecapone is an effective scavenger of superoxide, nitric oxide, hydrogen peroxide, and hydroxyl radicals.[15,16] In addition, it enhances recycling of vitamin E *via* ascorbate,[15] and reduces oxidation of GSH to GSSG.[17] Importantly, as a hydrophobic molecule, nitecapone is especially effective as a membrane antioxidant, favoring its protective effects on endothelial cells. Thus, although we cannot exclude direct inhibitory effect of nitecapone on neutrophils, various antioxidative properties of this novel molecule seem to be a plausible explanation for the reduction of coronary neutrophil accumulation during reperfusion in our patients. In accordance with our results, pretreatment of endothelial cells with hydroxyl radical scavengers inhibits hydrogen peroxide-induced neutrophil adherence in an *ex vivo* model.[30]

In conclusion, in the present study we demonstrate reduced cardiac neutrophil accumulation and better left ventricular stroke volume after nitecapone treatment in open heart surgery. Inhibition of neutrophil sequestration may be one mechanism by which this antioxidant molecule protects against cardiac reperfusion injury. Nitecapone treatment may be an additional way to reduce the deleterious side effects of neutrophil activation during CPB.

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