Role of Neopterin, C-Reactive Protein and Myeloperoxidase in Patients Undergoing Cardiopulmonary Bypass

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Key Words

Neopterin · C-reactive protein · Cardiopulmonary bypass · Myeloperoxidase · Cell-mediated inflammation

Abstract

Objective: The aim of the present study was to investigate the role of neopterin (NP), C-reactive protein (CRP) and myeloperoxidase (MPO) in patients undergoing coronary artery bypass grafting with or without cardiopulmonary bypass (CPB). **Patients and Methods:** Forty patients submitted for elective coronary artery bypass grafting were included in this prospective study. Patients were divided into two groups of 20 individuals, those who did not undergo CPB (group 1), aged 54.1 ± 13.5 years, and those who did (group 2), aged 60.2 ± 11.7 years. In group 1, there were 17 males and 3 females, while in group 2, there were 16 males and 4 females. Serum CRP, serum and urine NP and leukocyte MPO activity were measured preoperatively, at the end of surgery, and 4, 24 and 72 h after surgery using high-performance liquid chromatography, immunoturbidimetry and the reduction in o-dianizidine, respectively. **Results:** The level of serum NP was higher preoperatively and at the end of surgery (0 h), 4, 24, and 72 h after the operation in those who underwent CPB compared to those who did not. However, there was no significant difference in NP concentrations between the two groups at any time except 24 h after surgery ($p = 0.002$). Urine

NP concentrations showed similar values preoperatively but increased postoperatively in both groups of patients. The only significant difference in urine NP concentration between the two groups occurred at 0 and 24 h after surgery ($p = 0.001$, $p = 0.000$). Serum CRP concentrations showed similar values preoperatively, at the end of surgery and 72 h after the operation and increased at 4 and 24 h postoperatively in both groups. The only significant difference in CRP concentration between the two groups occurred 4 and 24 h after surgery ($p = 0.024$ and $p = 0.000$, respectively). MPO levels were found to be increased in the CPB patients when compared to those patients who did not undergo CPB. However, the difference between the groups was not statistically significant. **Conclusion:** Our data show that CPB induced a rise in NP and CRP levels.

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Introduction

Cardiac surgery with cardiopulmonary bypass (CPB) induces an acute phase reaction that has been implicated in the pathogenesis of several postoperative complications [1]. A recent report [2] indicated that a complex sequence of events leads to activation of leukocytes and endothelial cells, which are responsible for cell dysfunction in different organs. Contact of blood components with

the artificial surface of the CPB equipment leads to activation of inflammatory cells, ischemia-reperfusion injury, endotoxemia and operative trauma, which induces an inflammatory response [1]. Exposure of blood to extracorporeal artificial surfaces during CPB induces alterations in the cell-mediated immune response [3, 4] and a systemic inflammatory reaction, which is characterized by complement activation [4] and cytokine release such as interferon- γ (IFN- γ) and tumor necrosis factor- α [5, 6].

Neopterin (NP) is produced by activated monocytes/macrophages from guanosine triphosphate via guanosine triphosphate cyclohydrolase-1 [7]. The activity of this enzyme is greatly enhanced by IFN- γ . IFN- γ released by activated helper T lymphocytes type 1 is the most potent inducer of NP production, and the concentration of NP indicates the presence of IFN- γ in body fluids [8]. Because IFN- γ is released by active T cells, NP may be a sensitive marker of cell-mediated immunity [9, 10]. NP concentration rises in the early stage of different diseases and increased levels usually correlate with the severity of the disease [11]. It has therefore been suggested that measuring NP concentration is helpful in the follow-up of pathological states associated with the activation of cell-mediated immunity [12].

In this study, we have investigated the role of NP and C-reactive protein (CRP) concentration and leukocyte myeloperoxidase (MPO) activity in CPB in patients undergoing coronary artery bypass grafting (CABG) with or without CPB.

Subjects and Methods

Study Protocol

Forty patients with stable angina pectoris who had triple vessel disease were divided into two equal groups: group 1, 20 patients (16 males and 4 females, mean age 60.15 ± 11 years) who underwent CABG without CPB; group 2, 20 patients (17 males and 3 females, mean age 54.1 ± 3.5 years) who underwent CABG with CPB. Exclusion criteria were patients with poor ventricular function (ejection fraction $<30\%$), diabetes mellitus, renal failure and those requiring emergency surgery; exhibiting remarkably abnormal pulmonary, endocrine, metabolic, or neurological pathology that could constitute significant comorbidities; all cardiac medications were continued until the day before the surgery. After the completion of CABG, patients were transferred to the intensive care unit (ICU). Postoperative care was standardized for all patients, and extubation was done as early as possible. Informed consent was obtained from all subjects, and the study was approved by the Ethics Committee of Mersin University, Mersin, Turkey.

Surgical Procedure

A standardized anesthetic protocol was used throughout the study. All patients received premedication with midazolam, 0.05

mg/kg intravenously, in the operating room. The intraoperative anesthetic technique was the same for both groups. Anesthesia was induced with etomidate 0.3 mg/kg i.v. and remifentanyl 1–2 $\mu\text{g/kg}$ bolus followed by 0.2–0.5 $\mu\text{g/kg/min}$ infusion, and vecuronium 0.1 mg/kg i.v. was used to facilitate intubation. Anesthesia was maintained with remifentanyl infusion 0.2–0.5 $\mu\text{g/kg}$ and sevoflurane 0.5–1%, both titrated according to the patient's hemodynamic response. In group 2, before the initiation of extracorporeal circulation and rewarming, 2–4 mg midazolam was given. After completion of surgery, the remifentanyl infusion was stopped and a loading dose of 0.05 mg/kg morphine was given in the operating room. Propofol infusion, 1–2 mg/kg/h, was started in both groups once surgery was completed. CPB was established in a standardized manner, including the use of membrane oxygenator, roller pump and cardiectomy suction. The heart was exposed through a median sternotomy incision. The Octopus stabilizer (Medtronic) was used for the on-pump group. During on-pump surgery, patients were cooled to 32°C , whereas during off-pump surgery, patients were actively warmed to maintain a core temperature not lower than 35°C . Cold blood cardioplegia was accomplished with antegrade delivery through the aortic root and retrograde delivery through the coronary sinus. A heparinization protocol of 300 U/kg for on-pump surgery and half of the given dose of heparin for off-pump surgery was followed. Protamine was used to reverse the effects of heparinization only in the on-pump group. All anastomoses were sutured by hand. In the off-pump group, intracoronary shunts were not used routinely; indications for use included poor visibility, ST segment changes, and hemodynamic instability. A standardized protocol for immediate postoperative care was followed in the adult ICU, including antiplatelet therapy (300 mg of aspirin 6 h after surgery, followed by a daily dose of 150 mg).

Blood and Urine Sampling

Venous blood was withdrawn into both plain tubes and also tubes containing EDTA anticoagulant. The blood and urine samples were collected preoperatively (t_0), at the end of surgery (t_1), and at 4 h (t_2), 24 h (t_3), and 72 h (t_4) postoperatively. Serum samples were protected from light during the clotting period. They were then centrifuged and the separated serum stored at -80°C until analysis of NP. Following collection, urine samples were stored frozen at -80°C until analysis. EDTA-treated peripheral blood samples were freshly collected from patients for leukocyte MPO activity.

Biochemical Assay

Measurement of NP. Serum and urine NP concentrations were determined using high-performance liquid chromatography (an HP Agilent 1100 series system, München, Germany). The analytical column was a 5 μm pore size Spherisorb ODS-2 C_{18} reverse-phase column (4.6 $\times$ 250 mm; HICHRON, Waters Spherisorb, UK). The pre-column was a C_{18} cartridge (HICHRON). Urinary NP was determined by reverse-phase chromatography as described by Werner et al. [13]. The mobile phase was potassium phosphate buffer (15 mM, pH 6.4) at a flow rate of 0.8 ml/min. A sample of 10 μl of untreated urine was diluted with 990 μl of mobile phase containing disodium EDTA, and 20 μl of this mixture was injected onto the column. Fluorometric detection was performed at an excitation wavelength of 353 nm and emission at 438 nm. For the quantification of NP in serum, the same method

was modified as described: 500 μ l serum and 500 μ l of 2 M trichloroacetic acid were vortex mixed and centrifuged at 10,000 g. The injection volume was 20 μ l. NP peaks were determined according to its retention time and the peaks were confirmed by spiking with added exogenous NP (250 nM). Concentrations of NP were calculated from a prepared NP standard curve and expressed as nM. Urinary NP concentrations are related to creatinine and expressed as μ mol NP/mol creatinine. Urinary creatinine concentrations were determined using standard routine laboratory methods on an automatic analyzer (Cobas Integra 800, Roche Diagnostic, Germany).

Measurement of CRP. Serum CRP concentrations were determined using immunoturbidimetry on a Cobas Integra 800.

Measurement of Leukocyte MPO Activity. 2 ml of EDTA-anticoagulated whole blood was mixed with four volumes of 0.84% cold ammonium chloride. The mixture was then incubated at 4°C for 5 min. To eliminate all erythrocytes, the procedure was repeated 3–4 times. Following centrifugation at 1,500 g for 10 min, the white cell pellet was washed and re-suspended in phosphate buffered saline (PBS) and tubes were then centrifuged at 800 g for 23 min. The white cell fractions were collected and resuspended in PBS pH 7.2. Leukocyte MPO activity was determined by measuring the reduction of o-dianizidine at 410 nm using a spectrophotometer [14]. Whole blood count analysis was with a Sysmex XT 2000I. Results were calculated as U/ μ l cell.

Statistical Analysis

Statistical analysis was performed with SPSS software package, version 11.5 for Windows (SPSS Inc., Chicago, Ill., USA). Categorical data were analyzed using the χ^2 test or Fisher's exact test, where appropriate. Biochemical results were controlled for normal distribution by Shapiro-Wilks test, and according to test result all data were normally distributed. For all biochemical data, statistical analysis was performed using repeated measurements of ANOVA followed by post-hoc analysis with the Bonferroni test to detect differences between the with and without CPB groups. Results are expressed as mean $\pm$ standard deviation (SD). A p value <0.05 was considered significant.

Results

The demographic features, clinical characteristics, and intraoperative data are shown in table 1. None of the patients required re-operation or prolonged intensive care. All patients were transferred from the ICU to a hospital ward. Both length of ICU stay and total length of hospital stay are also given in table 1.

Serum NP concentrations were increased preoperatively and at the end of surgery, 4, 24, and 72 h after the operation in those who underwent CPB, compared to those who did not. There was no significant difference in NP concentrations between the two groups at any time except 24 h after surgery (p = 0.002; fig. 1). Urine NP concentrations also increased postoperatively in both groups

Table 1. Demographic, clinical and surgical data of the study groups

	Group 1 (n = 20)	Group 2 (n = 20)	p value
Age, years	54.1 $\pm$ 13.54	60.15 $\pm$ 11.7	0.053
Males/females	17/3	16/4	0.120
BMI			
$\leq$ 25	13 (65%)	14 (70%)	
$\geq$ 25	7 (35%)	6 (30%)	
Preoperative treatment			
Digoxin	4	2	
Diuretic	3	2	
Nitrate	11	9	
Calcium channel blockers	5	4	
Beta-blockers	6	5	
CPB duration, min	–	112 $\pm$ 16	
Duration of aortic cross-clamping, min	–	35 $\pm$ 4	
Lowest temperature, °C	28.0 $\pm$ 0	28.0 $\pm$ 0	0.912
Hematocrit before induction	0.42 $\pm$ 0.3	0.43 $\pm$ 0.4	0.714
Min. hematocrit during CPB	–	0.27 $\pm$ 0.3	
ICU LOS, median (range), days	2 (1–6)	2 (1–7)	0.651
Total hospital stay, median (range), days	5 (2–10)	5 (2–12)	0.474
Surgical data			
Saphenous vein grafts	2.4 $\pm$ 0.4	2.5 $\pm$ 0.3	0.816
Internal mammary artery	16	17	
Inotropic support after CPB	4	3	

BMI = Body mass index; LOS = length of stay; NS = nonsignificant.

of patients, with the highest concentrations occurring between 0 and 24 h after surgery, then declined to values approaching those observed before surgery and at 72 h. The only significant difference in urine NP concentrations between the patients in the two groups occurred at 0 and 24 h after surgery (p = 0.001, p = 0.000, respectively; fig. 2). MPO activities reached their maximum levels at the end of surgery in both groups and declined thereafter to preoperative values 72 h after surgery. There was no significant difference between MPO activities in the patients in the two groups at any time (fig. 3). Serum CRP concentrations started to increase at 4 h postoperatively, and reached maximum concentration 24 h postoperatively in both groups. CRP concentrations were significantly higher in patients in group 2 than in those in group 1 at 4 and 24 h after operation; significantly higher CRP levels were observed postoperatively at 4 and 24 h in the CPB patient group (p = 0.024 and p = 0.001, respectively; fig. 4).

Fig. 1. Serum NP values during the operative and postoperative periods. t₁ = At the end of surgery; t₂ = 4 h after surgery; t₃ = 24 h after surgery; t₄ = 72 h after surgery. Values are given as mean $\pm$ SD. Group 1: without CPB; group 2: with CBP. * p = 0.002.

Fig. 2. Urine NP values during the operative and postoperative periods. Values are given as mean $\pm$ SD. * p = 0.001; ** p = 0.000.

Discussion

It has been known for several years that CPB is associated with a generalized inflammatory response [3]. The exposure of blood cells and plasma to artificial membranes and the activation of several cell types in the setting of ischemia and reperfusion are believed to play an important role in the development of this generalized inflammatory reaction [15]. Endotoxin and various mediators have been reported to be involved in CPB-induced reactions, which can lead to postoperative organ dysfunction [16]. It is generally accepted that many pro- and anti-inflammatory mediators may become predominant and lead to a deficiency of immunological function during and after CPB [16, 17]. Soluble adhesion molecules released into the circulation are believed to be markers of cellular activation and reflect the extent of inflammation and endothelial damage [18]. Some studies have measured cell-mediated inflammation during the surgery in order to determine its association with systematic inflammatory response syndrome (SIRS). Some have measured adhesion molecules such as CD11a/CD18, CD11b/CD18, integrin and L-selectin. Using flow cytometry, they have shown that either CD11a/CD18 or CD11b/CD18 surface antigen concentrations are increased during and/or after CPB surgery. It has also been proposed that filtering the circulatory macro-

phages may reduce the oxidative burst during the CPB [19–21].

Our results showed that serum NP concentrations increased in both groups of patients postoperatively, reached a plateau and then decreased by 72 h after surgery. Concentrations were only significantly different between the two groups at 24 h after surgery, similar to those reported by Johansson-Synnergren et al. [22] and Brkic et al. [23] but not to Hensel et al. [24], who reported that there was no significant difference in serum NP concentrations between the groups which were without SIRS, SIRS with acute lung injury and SIRS with acute lung injury in CPB patients, 4 h after operation. In this study, the insignificance of results can be explained by the small number of patients. We believe this is the first report measuring urine NP concentrations in patients undergoing CABG with or without CPB. We found that urine NP concentrations were higher in those patients that had undergone CPB than in those who had not, but the difference was only significant at the end of surgery and 24 h after surgery.

NP is produced by activated monocytes/macrophages from guanosine triphosphate via guanosine triphosphate cyclohydrolase-1 [7]. The activity of this enzyme is greatly enhanced by IFN- γ . IFN- γ released by activated T lymphocytes type 1 is the most potent inducer of NP production, and the concentration of NP indicates the presence

Fig. 3. MPO values during the operative and postoperative periods. Values are given as mean $\pm$ SD.

Fig. 4. CRP values during the operative and postoperative periods. Values are given as mean $\pm$ SD. * $p = 0.024$; ** $p = 0.000$.

of IFN- γ in body fluids [8]. Because IFN- γ is released by active T cells, NP may be a sensitive marker of cell-mediated immunity [8, 9]. NP concentrations rise in the early stage of different diseases and increased levels usually correlate with the severity of the disease [11]. Therefore, measuring NP levels is helpful for follow-up of pathological states associated with the activation of cell-mediated immunity [12]. The increase in NP concentrations observed postoperatively in the on-pump group could represent a cumulative consequence of blood being in contact with the tubing associated with the CPB equipment and subsequent activation of monocytes/macrophages [16, 25].

Measurements of NP in serum by HPLC is flawed by the fact that the reduced NP derivative, 7,8-dihydro-neopterin, undergoes oxidation to NP in acidic solution and the presence of air oxygen [13, 26]. This fact may explain why the urine NP concentrations are in accord with data in the literature [27] (in this case no acidification is necessary/performed), whereas the serum concentrations are approximately twice as high compared with literature data on the basis of immunoassays.

In the present study, CRP concentrations did not change during surgery in either group of patients. However, concentrations started to rise 4 h postoperatively, reaching a peak at 24 h, before decreasing at 72 h. Concentrations were significantly higher in group 2 patients at 4 and 24 h after surgery, similar to a previous report [28], in

which CRP concentrations were observed 4 h after operation in both groups. However, 24 h after surgery the increase in CRP in the CPB group was significantly greater than in the non-CPB group. In contrast, in another study, only non-significant increases in CRP concentration were observed after surgery [25]. MPO is a specific marker of primary neutrophil granules that are released in response to strong stimuli, and high activities of MPO at the end of CPB indicate a profound activation of neutrophils.

In the present study, MPO activities peaked at 0 and 4 h after surgery before decreasing to presurgery values at 72 h. MPO activities were higher in the patients who had undergone CPB, but there was no difference in MPO levels among the groups at any time points in this study.

Kutay et al. [29] showed that heparin-coated CPB circuits exhibit a favorable effect on MPO reduction. Studies have shown that heparin possesses anti-inflammation and anti-oxidation properties [30, 31]. In our study, all patients, including the control group, received heparin as an anticoagulant during the surgery. No difference in MPO levels between groups in this study can be explained by the anti-inflammatory and anti-oxidative properties of heparin that is used during surgery.

In this study, those patients who underwent surgery with CPB demonstrated an increased inflammatory response compared to those who did not. This was reflected by the observed increases seen in both CRP and NP.

Conclusion

Because urine NP levels were increased postoperative-ly at 0 h, urine NP may be a marker for early response in CPB; accordingly, we suggest that NP concentration in serum and urine could be used as a marker of inflammation in patients undergoing CPB.

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