

## Original Article

# Evidence of Increased Matrix Metalloproteinase-9 Concentration in Patients Following Cardiopulmonary Bypass

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**ABSTRACT:** Cardiopulmonary bypass (CPB) is associated with a systemic inflammatory response, which can result in acute lung injury known as “postperfusion syndrome.” Neutrophil activation with concomitant serine protease release has been implicated in the pathogenesis of “postperfusion syndrome.” Increased plasma levels of neutrophil elastase (NE) have been demonstrated in patients undergoing CPB, and it is well documented that both NE and matrix metalloproteinase-9 (MMP-9) have a synergistic role in pulmonary injury. We, therefore, hypothesized that plasma levels of MMP-9 would be elevated in patients after CPB. Human plasma was obtained after informed consent from eight patients undergoing CPB. Plasma was collected at the start of CPB, 5 minutes after the initiation of CPB, and at the termination of CPB (156 ± 17 min). All samples were analyzed by both standard enzyme-linked immunosorbent assay

(ELISA) and gelatin zymography for MMP-9 (free and total enzyme) concentration. Data were expressed as means ±SE and assessed by analysis of variance (ANOVA). Plasma MMP-9 concentration was significantly increased at the end of CPB (191±30.4 ng/mL;  $p < .05$ ) as compared to both the start of CPB (28.3±13.2 ng/mL) and 5 minutes after the initiation of CPB (44.3±15.4 ng/mL). Patients undergoing CPB show an increase in serum MMP-9 levels. Prior studies utilizing an animal model of “postperfusion syndrome” have shown that inhibition of MMP-9 and NE prevented pulmonary injury following CPB. The results of the current study suggest that such an approach may also have merit in the clinical setting of cardiopulmonary bypass. **Key-words:** cardiopulmonary bypass, matrix metalloproteinase-9, lung injury. *JECT*. 2001;33:218–222

Development of the acute respiratory distress syndrome (ARDS) following cardiopulmonary bypass (CPB) is referred to as postperfusion syndrome (PPS) (1). The pulmonary pathophysiology of each is similar and includes the initiation of a systemic inflammatory response involving the activation of plasma proteins (complement, coagulation factors) and such cellular defense systems as endothelial cells, macrophages, monocytes, lymphocytes, and neutrophils (2, 3). The resulting alteration in lung function is characterized by endothelial damage and increased microvascular permeability, thus, leading to reduced pulmonary compliance, increased intrapulmonary shunting, and refractory hypoxemia.

Recent reports have emphasized the role of the neutro-

phil as the primary cell involved in the mechanism of lung injury seen after CPB (4–10). In the classic “two-hit” theory of PPS as demonstrated by Picone et al. (11), the “first hit” (CPB) primes the neutrophil. Neutrophil priming consists of up-regulation of both nicotinamide adenosine dinucleotide phosphate (NADPH) oxidase, which catalyzes the formation of superoxide anion, and the adhesion receptors CD11/CD18. It is theorized that neutrophil priming occurs secondary to contact of blood with the foreign surface of the pump–oxygenator system. Priming leads to sequestration of neutrophils in the lung and has been shown to occur following CPB by several investigators (12, 13). The “second hit,” which could be caused by hypoxia, bleeding, ischemia, re-operation, or infection activates the primed neutrophil to release oxygen free radicals and proteases, causing destruction to the alveolar–capillary basement membrane. This tissue damage allows protein-rich plasma to flood the alveolus, causing pulmonary edema typical of ARDS (14).

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Increasing evidence exists that the key component in the disruption of the alveolar–capillary basement membrane during the development of ARDS is the release of proteolytic enzymes by the neutrophil (15, 16). Specifically, neutrophil elastase (NE) is elevated in both plasma and bronchoalveolar lavage (BAL) fluid of patients with ARDS (17, 18). Furthermore, the level of NE correlated with the subsequent degree of lung injury. In addition, an increase in plasma NE has been demonstrated in patients undergoing CPB with evidence of lung dysfunction (19–21).

Matrix metalloproteinases (MMPs) are a family of proteolytic enzymes known to degrade type IV collagen of the alveolar basement membrane and possess the ability to act synergistically with NE (22). Moreover, elevation of MMPs have been demonstrated in both plasma and BAL fluid of patients with ARDS (23–25). Thus, the purpose of our study was to determine if MMP would be elevated in patients following CPB and if this correlates with subsequent lung injury.

## METHODS

The study was approved by the Institutional Review Board, and informed consent was obtained from each patient.

### Patients

Patients, ranging from 28–87 years of age, scheduled for elective cardiac surgery, with preserved left ventricular function, and without pre-existing disease were considered eligible for the study. Seven patients enrolled underwent coronary artery bypass grafting (CABG), and one patient had an atrial septal defect (ASD) repaired at the SUNY Upstate Medical University Hospital.

### Operative Technique

After median sternotomy and heparinization (300 IU/kg of body weight) to maintain an activated clotting time of greater than 400 seconds, standard cannulation techniques of the ascending aorta and right atrium were used to complete the CPB circuit. Following this, a retrograde catheter was placed in the coronary sinus. The aorta was then cross-clamped, and CPB was performed with standard extracorporeal circulation utilizing a conventional membrane oxygenator (Optima, Cobe Cardiovascular, Arvada, CO) and centrifugal pump (Bio-Pump, Medtronic, Parker, CO). The circuit was primed with lactated Ringers, 5000 IU of heparin, 5 g Amicar, 50 Meq sodium bicarbonate, and 12.5 g mannitol. Patients were kept at moderate hypothermia with systemic temperatures maintained between 30–32 EC. After initiation of CPB, non-pulsatile CPB pump flow was adjusted to maintain SvO<sub>2</sub> greater than 70%, and mean arterial pressure was maintained between 50–90 mm Hg. Myocardial protection was

afforded exclusively with a modified Buckberg cardioplegia in the following fashion. Induction with 1 L of warm solution (500 cc antegrade, 500 cc retrograde) and 1 L of cold solution (500 cc antegrade, 500 cc retrograde), followed by intermittent cold retrograde cardioplegia (300 cc/20 min). After cardiac arrest, distal anastomoses were performed first. With completion of the final distal anastomosis, the proximal and left anterior descending (LAD) anastomoses were then performed. In the patient who underwent ASD repair rather than CABG, a pericardial patch was utilized at this stage. The patients were rewarmed, a hot shot was infused retrograde consisting of substrate-rich warm blood cardioplegia with potassium and the aortic cross clamp was removed. The patients were then weaned from CPB. After the operation, patients were transported to the ICU and ventilated in a volume-controlled mode until extubation.

### Sample Collection

The first blood sample (baseline/pre-CPB) was obtained from the patient immediately before the initiation of CPB and before the administration of heparin intraoperatively. The second blood sample was collected 5 minutes after the initiation of CPB, and the final sample was obtained at the termination of CPB and before protamine administration. Fifteen milliliters of arterial blood was collected at each time interval in a heparinized test tube. Blood was immediately centrifuged at 3100 rpm for 5 minutes, and plasma samples were stored at –70 EC until assayed.

### MMP-9 Concentration (ELISA Assay)

We chose to assay MMP-9 in particular, because neutrophils and alveolar macrophages (i.e., inflammatory cells) primarily release MMP-9. Plasma concentrations of both free and total (tissue inhibitors of metalloproteinases [TIMP] bound) MMP-9 were determined by enzyme-linked immunosorbent assays (ELISA). Briefly, MMP-9 concentrations (ng/mL) were measured using a human MMP-9 ELISA kit (Oncogene Research Products CA #QIA56), which is a sandwich enzyme immunoassay employing a mouse monoclonal antibody and a sheep polyclonal antibody. MMP-9 present in the serum first binds to the capture and detecting antibodies. Unbound material was washed away and horseradish peroxidase (HRP)-conjugated streptavidin was added to bind to detector antibody. HRP then catalyzes the conversion of chromogenic substrate tetra-methyl benzidine (TMB) to form a colored solution, which can then be detected by spectrophotometer.

### MMP-9 Concentration (Gelatin Zymography)

The methods for purification of collagenase and gelatin zymography have been fully described elsewhere (26). Briefly, zymography was performed on 2.5 µL of serum samples as directed for precast NOVEX Zymogram

**Table 1.** Clinical characteristics of CPB patients ( $N = 8$ ).

Mean age (yrs)	69.1 ± 6.2
Males	7/8
CPB time (min)	156 ± 17
Cross clamp time (min)	127.6 ± 0.4
# of bypasses	2.8 ± 0.4
Length of stay (days)	7.6 ± 1.4

(Novex Experimental Technology, San Diego, CA) gels to detect and characterize MMP's. These precast gels consist of a 10% SDS-polyacrylamide gel with 0.1% of gelatin incorporated as a substrate. The gels were run under non-reducing conditions with 1X Tris-glycine SDS running buffer at 125 V. The gels were then renatured with 2.5% Triton X-100 for 30 minutes, rinsed briefly, and developed with 50 mm Tris/HCL buffer, pH 7.6, containing 10 mm  $\text{CaCl}_2$  at 37 E overnight. Proteinases were easily identified as clear bands against a dark Coomassie blue stained background. Because of the effect of SDS, this zymography allows the determination of the molecular species of latent, activated, and complexed forms of gelatinases (MMP-9) and is an additional way to quantify concentration of serum MMP-9.

### Pulmonary Function

The alveolar-arterial  $\text{PO}_2$  gradient ( $\text{AaDO}_2$ ) and the respiratory index (alveolar-arterial  $\text{PO}_2$  gradient/ $\text{PaO}_2$ ) were used as a measure of pulmonary dysfunction and were determined immediately postoperatively and at time of extubation.  $\text{AaDO}_2$  was calculated using the equation  $([\text{FiO}_2 \times 713] - [5/4 \times \text{PaCO}_2]) - \text{PaO}_2$ . In addition, time to extubation was recorded.

### Statistics

Data are reported as the mean ± standard error of the mean (SEM). Analysis of variance (ANOVA) was performed with Newman-Keuls post hoc analysis for statistical interpretation of MMP-9 concentration versus CPB time. A  $p$  value of  $< .05$  was considered statistically significant. In addition, simple regression analysis was utilized to define the correlations of variables measured ( $\text{AaDO}_2$ , respiratory index, and time to extubation) as related to MMP-9 concentration at the end of CPB.

**Table 2.** Pulmonary parameters.

	A-a Gradient (mmHg) (Immediately Post-op)	Respiratory Index (Immediately Post-op)	A-a Gradient (mmHg) (Extubation)	Respiratory Index (Extubation)	Time to Extubation (Hours)	MMP-9 Concentration End of CPB (ng/mL)
Patient 1	263	2.2	107	0.78	8	146
Patient 2	230	1.5	129	1.1	15	138
Patient 3	52	0.28	20	0.09	8	104
Patient 4	154	0.69	110	0.82	17	214
Patient 5	182	0.88	105	0.81	12	223
Patient 6	230	1.6	94	0.71	6	140
Patient 7	274	2.6	139	1.4	12	379
Patient 8	241	1.8	147	1.8	19	190

## RESULTS

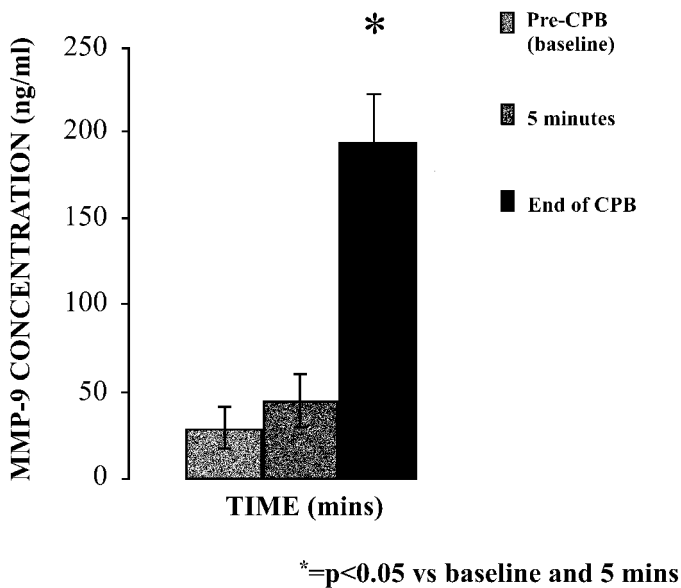
The demographics, CPB time, aortic cross clamp time, number of coronary artery bypasses performed, and length of stay for the patients are shown in Table 1. The alveolar-arterial  $\text{PO}_2$  gradient, respiratory index, and time to extubation of all patients are reported in Table 2. Simple regression revealed no significant influence of these pulmonary parameters to levels of MMP-9 at the end of CPB. Furthermore, all patients were extubated within a normal time period and developed no apparent pulmonary complications. All patients, except one, had an uneventful hospital course and required no further hospitalizations related to their surgery. One patient expired on postoperative day 15 before of sepsis from either a urinary tract infection or cellulitis of the graft site lower extremity. Arterial blood gases and chest X-rays of this patient revealed no pulmonary complications at the time of death.

The increase in plasma concentration of MMP-9 after 5 minutes of CPB was not significantly different from the baseline value (Figure 1). However, at the termination of CPB, a statistically significant increase in MMP-9 concentration was observed when compared to baseline and 5 minutes of CPB values (Figure 1). This significant increase in plasma concentration of MMP-9 seen at the end of CPB was also demonstrated by gelatin zymography (Figure 2).

## DISCUSSION

The significance of this study is that we are the first group, to our knowledge, to investigate a potential relationship between increased plasma levels of MMP-9 in patients undergoing CPB and early signs of pulmonary dysfunction. Recently, Joffs et al. [27] reported increases in a variety of MMPs following CPB (including MMP-9); however, did not attempt to correlate these findings with abnormalities in pulmonary function. In our study, although we clearly demonstrated a significant rise in serum MMP-9 at the termination of CPB, we were unable to demonstrate any pulmonary dysfunction in these eight patients with respect to alveolar-arterial gradient, respiratory index, length of intubation, or pulmonary complica-

MMP-9 PLASMA CONCENTRATION BY ELISA



**Figure 1.** MMP-9 concentration as measured by ELISA at baseline (pre-CPB), 5 minutes after start of CPB, and at termination of CPB. Data are mean ± SE. CPB: Cardiopulmonary bypass; ELISA: Enzyme-linked immunosorbent assay; MMP-9: Matrix metalloproteinase-9.

tions. The lack of correlation between lung injury and an increase in plasma proteases is in concordance with Butler et al., who demonstrated no association between lung dysfunction and increased plasma NE following CPB (28). However, in contrast, other investigators have reported lung dysfunction associated with increases in plasma NE after CPB (19–21).

One explanation for the lack of correlation offered by Butler et al. is that protease concentration in his study was measured intravascularly and, thus, may not reflect intrapulmonary levels (28). Thus, the possibility exists that, although plasma MMP-9 was elevated in this study, the concentration of MMP-9 in pulmonary tissue may not be elevated sufficiently to cause injury. In addition, this highlights the fact that the local action of proteolytic enzymes

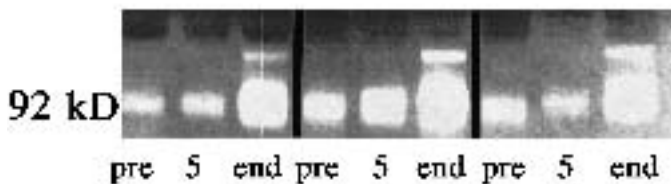
makes it difficult to consistently relate systemic protease concentrations to end organ damage (29). Furthermore, the results of a study by Faymonville et al. (30) demonstrates that pulmonary neutrophil sequestration following CPB is a transient phenomenon, thus potentially explaining why the pulmonary concentration of proteases may not be sufficient to cause lung damage.

Another possible explanation for the lack of correlation between serum protease concentration and lung injury is found in a study by Yamazaki et al. (31). In this study, Yamazaki demonstrated that increased plasma NE correlated with histologic lung injury in dogs subjected to partial CPB. However, when these dogs were given a NE inhibitor, lung injury was ameliorated. Moreover, no significant differences in lung function, expressed as alveolar–arterial gradient, was found between the control and NE inhibitor-treated groups. This suggests that changes in alveolar–arterial gradient may not be sensitive enough to detect early pulmonary damage.

A recent study suggests the incidence of PPS is 1%, although the associated mortality remains unacceptably high (40–60%), continuing to make PPS a clinically relevant problem (4). To reduce the incidence of PPS, the pathogenesis of the syndrome must be thoroughly understood. A major question concerning ARDS pathogenesis is why do the majority of CPB patients who show evidence of low-grade lung injury with increased plasma levels of proteases never develop PPS? We speculate the answer to the above question is related to the “two-hit” theory of ARDS (32). Specifically, we postulate that the initial stimulus (CPB-“first hit”) must be of sufficient strength to lead subsequently to neutrophil priming and sequestration in the pulmonary vasculature. Although these primed neutrophils accumulate in the pulmonary vessels, there is no lung injury until these neutrophils are activated by a second stimulus (hit). In addition, the “second hit” must be of adequate strength to activate the neutrophil causing release of a significant amount of proteases to cause lung damage.

In conclusion, we have demonstrated increased plasma levels of MMP-9 in patients undergoing CPB. Although we did not demonstrate specific tissue injury in this study, evidence exists that proteases (specifically MMP’s) can cause alveolar–capillary basement membrane damage with subsequent lung injury (23–25, 33, 34). Furthermore, amelioration of this lung injury has been demonstrated in animal (35) and human models (36, 37) utilizing protease inhibitors. We suspect an adequate priming stimulus (CPB) followed by sufficient activation of neutrophils will cause a significant release of MMP-9, resulting in lung injury. Although, in our study, an apparent subclinical level of MMP-9 was observed, we feel patients who demonstrate an increased plasma level of MMP-9 are vulnerable to lung injury and the development of PPS if exposed

GEL ZYMOGRAM



**Figure 2.** Representative gelatin zymograms (see MMP-9 concentration section in text for detailed explanation) from three patients demonstrating increased concentration of MMP-9 at termination of CPB as compared to baseline and 5 minutes after CPB. CPB: Cardiopulmonary bypass; MMP-9: Matrix metalloproteinase-9.

to a second inflammatory stimulus. Moreover, because of the small sample size and the fact that the incidence of PPS is low, a study involving a larger patient population (investigating both plasma and lung tissue levels of MMP-9) would be necessary to obtain a correlation between elevated MMP-9 levels and PPS. If such an association could be documented, prophylactic treatment with protease inhibitors for CPB patients at high risk for the development of PPS might reduce the incidence of PPS.

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