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The Effects of Leukocyte Reduction on Matrix Metalloproteinase Release in Cardiopulmonary Bypass

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Abstract: Matrix metalloproteinases (MMPs) are a family of enzymes responsible for degrading the extracellular matrix, a process that likely contributes to the development of altered vascular permeability. Past studies in patients undergoing cardiopulmonary bypass (CPB) have documented increased levels of MMPs with CPB. The purpose of this study was to evaluate the effect of leukocyte reduction on MMP release during CPB. Patients ($n = 17$) undergoing elective coronary revascularization requiring CPB were randomly assigned to either a leukocyte-reducing filter (LRF) group ($n = 9$) or the standard CPB circuit with no LRF ($n = 8$). White blood cell (WBC) counts, MMP-2, and MMP-9 levels were serially measured at baseline and up to 12 hours post CPB. MMP levels were measured by enzyme-linked immunoassay. ProMMP-2 levels increased in both the non-LRF and LRF groups but to a higher degree in the LRF group. ProMMP-9 levels increased by 40% in the non-LRF group. In contrast, proMMP-9 decreased by 30% in the LRF group. The addition of leukocyte-reducing filters in the CPB circuit attenuated the release of MMP-9 but increased release of MMP-2 post-CPB. Because MMPs can degrade the extracellular matrix, leading to increased vascular permeability, attenuation of MMPs may have decreased the local tissue injury known to occur as a result of these enzymes. However, future prospective studies to test this hypothesis directly are warranted. **Keywords:** cardiopulmonary bypass (CPB), matrix metalloproteinases (MMP), inflammatory response, leukocyte reduction, leukocyte reducing filters (LRF), white blood counts (WBC), complete blood counts (CBC), extracorporeal circuit (ECC). *JECT. 2004;36:185–190*

Matrix metalloproteinases (MMPs) are a family of enzymes responsible for degrading the extracellular matrix, including the basement membrane (1), a process that likely contributes to the development of altered vascular permeability and subsequent tissue edema postcardiopulmonary bypass (CPB) (2–4). MMP synthesis and activity are tightly regulated under normal conditions, degrading extracellular matrix in coordination with matrix synthesis (1). However, in disease states, production of this enzyme has been shown to be upregulated (5). Elevated levels of various subtypes of this enzyme family have been observed in cardiovascular disease states associated with tissue structural changes (6). In the post-CPB setting, increased degradative activity directed at the extracellular matrix, especially the basement membrane, can alter endothelial geometrical relationships, compromising the endothelial barrier, ultimately leading to tissue edema and organ dysfunction (2,7). Preliminary data have been generated indicating elevation of select MMPs following CPB, including MMP-9 (7,8). Leukocytes are capable of MMP production and release associated with the inflammatory response (9,10). Previous studies have shown that leukocyte-reducing filters selectively reduce white blood cells while leaving platelets almost unaffected (11,12). This study prospectively evaluates the temporal relationship between MMP release with the use of leukocyte reduction filters.

MATERIALS AND METHODS

Patient Selection and Description

Patients ($n = 17$; 15 men, 2 women) undergoing elective coronary artery revascularization with cardiopulmonary bypass (CPB) were entered into this study after obtaining informed consent. This protocol was reviewed and approved by the Institutional Review Board of the Medical University of South Carolina. The inclusion criteria were elective coronary artery bypass surgery patients, preserved left ventricular function (ejection fraction >50%), and willingness and ability to provide informed consent.

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The exclusion criteria were abnormal ventricular function, severe left ventricular hypertrophy or other cardiac disease, active malignancy, chronic inflammatory disease, required use of medications known to inhibit MMPs (e.g., chronic tetracycline use), redo operation, myocardial infarction within 6 months, recent (within 6 months) catheter-based coronary revascularization, serum creatinine > 1.4, and severe chronic obstructive pulmonary disease (FEV1 < 60% predicted).

Patients were randomly assigned to either having two leukocyte-reducing filters (LeukoGuard-6 and LeukoGuard-BC, Pall Corporation, East Hills, NY) introduced to the CPB circuit or simply receiving conventional therapy without the leukocyte-reducing filters. The patients were assigned by random draw from an envelope by the team's perfusionist. All members of the cardiac team, except for perfusion, were blinded before analysis. For those patients randomized to the LRF group, one filter (Pall LeukoGuard-6) was placed in the arterial line distal to the bifurcation of the blood cardioplegia line and proximal to the standard arterial line filter. A second filter (Pall LeukoGuard-BC) blood cardioplegia filter was placed in the cardioplegia line distal to the roller pump and proximal to the cardioplegia heat exchanger. Both filters were primed according to the manufacturer's recommendations. Blood flow and line pressure were maintained according to hospital protocol for all patients. Patient profiles are presented in Table 1. These profiles were similar between both the non-LRF and LRF groups, with no statistical difference between groups.

Standard induction and maintenance of anesthesia was accomplished with a combination of sufentanil, midazolam, and isoflurane. Temperature management was passive; that is, patient temperatures were allowed to drift during the cross-clamp period, with active rewarming during the last 10 to 15 minutes of the cross-clamp period and as appropriate into the resuscitation period while still on CPB. Priming solution for the pump consisted of approximately 1700 mL of Plasmalyte-A (Baxter Healthcare, Deerfield, IL). Hematocrits were managed with a goal of 25–30 during the pump run. Patients were transfused for hematocrits below 21–23 and active diuresis with mannitol and furosemide for values above this. Systemic heparinization was achieved with a heparin dose of 400 units/kg.

Table 1. Patient profiles.

Height (cm)	174 ± 10
Weight (kg)	95 ± 16
Body surface area (m2)	2.1 ± 0.2
Age (years)	59 ± 9
Graft number	3 ± 1
Duration of CPB (min)	91 ± 21
Duration of cross clamp (min)	70 ± 19

n = 17 patients.

Cardiopulmonary bypass was maintained at a cardiac index of 2.0–2.4 L/min/m^2. Initial cardioplegic arrest was accomplished with antegrade administration of a 500 mL solution of D_5 0.2% NaCl containing 29 mL THAM buffer, 34 mL adenosine citrate phosphate dextrose, and 120 mEq/L K^+. This was followed immediately by retrograde administration of 300 mL of the cardioplegic solution. Approximately every 20 minutes, cardioplegic arrest was maintained with 400–700 mL retrograde administration of the cardioplegic solution with a lower potassium concentration, 60 mEq/L of K^+. At the termination of CPB, heparin was neutralized with protamine at a 1:1 ratio. The terminal concentration of potassium was 60 mEq/L. The ratio between the crystalloid solution and blood was 4:1.

Sample Collection

Blood samples were obtained for matrix metalloproteinase (MMP) levels at baseline (before CPB), 30 minutes into rewarming of the patient, 30 minutes post CPB, 6 hours post CPB, and 12 hours post CPB. All samples were placed in EDTA tubes, centrifuged, and plasma was stored at −70°C until assay. Complete blood counts (CBC) with differentials were run for baseline and 12 hours post-CPB samples. From the CBC with a differential, neutrophil and band (immature neutrophil) levels were measured. CBCs without differentials were run for samples obtained at 30 minute into rewarming, 30 minutes post-CPB, and 6 hours post-CPB.

MMP Plasma Assays

This study focused upon one known class of MMPs, gelatinases, which include MMP-2 and MMP-9. Plasma samples were allowed to thaw on ice. Quantification of respective MMP species was done utilizing an enzyme-linked immunosorbant assay (ELISA) kit (Amersham Pharmacia Biotech, Buckinghamshire, England). The antisera used for MMP-2 reacts against the proform of MMP-2 (proMMP-2) and does not react against the active form. For MMP-9, the antisera detects the proform of the enzyme (proMMP-9). The ELISA procedure was similar for each MMP, using a two-site assay. Plasma was added to precoated wells containing antibody to the MMP of interest and incubated at 20–30°C for 1 hour. The ELISA plate was washed three times and incubated in the primary MMP antisera conjugated to horseradish peroxidase (25°C, 1 h). After three washes, tetramethylbenzadine (TMB)/hydrogen peroxidase was added to the mixture, and the reaction was allowed to proceed for 30 minutes. The ELISA plate was immediately read at a wavelength of 450 nm (Labsystems Multiskan MCC/340, Helsinki, Finland). The concentration of plasma MMP species was determined using known MMP concentrations to generate a standard curve with each set of samples.

Data Analysis

Two approaches were taken to examine leukocyte and MMP release during CPB with or without the use of the LRFs. First, temporal changes in leukocyte and MMP concentrations between the two groups were profiled with respect to the baseline values (before CPB). For the second approach, the leukocyte and MMP plasma concentrations were pooled together at the 30 minutes rewarming time. The percentage change from the 30-min time point was derived by dividing the post-bypass levels for both the non-LRF and LRF groups by the previously calculated mean of the pooled 30-min rewarming time point. The rationale for this time point normalization is that this is the last sampling period before weaning the patient from CPB. This time point is the last plasma withdrawal before the patient is taken off the bypass pump. Therefore, the 30-min rewarming time point serves as a control to profile the changes in leukocyte and MMP levels before and after the final time of blood exposure to the filter. Before statistical analysis, the concentrations for MMPs were corrected for hemodilution by dividing the baseline hematorcrit number by multiplying the MMP concentration for a specific time point by the quotient of the baseline hematocrit number over the relative hematocrit number for the specific time point. The resultant MMP concentrations were evaluated using analysis of variance (ANOVA) for repeated measures, followed by a Bonferroni corrected *t*-test, where appropriate. A matched *t*-test was performed on the neutrophil band data collected post CPB. All statistical procedures were performed using the BMDP statistical software package (BMDP Statistical Software, Inc., Los Angeles, CA). Results are presented as mean ± SEM. Values of $p < .05$ were considered statistically significant.

RESULTS

White blood cells (WBCs) increased up to 6 hours post CPB ($p < .05$) when compared to baseline before decreasing at 12 hours post CPB (Figure 1A). WBCs, measured as a percentage change from 30 minutes into rewarming the patient, showed a trend for WBCs to be higher in the non-LRF group as compared to the LRF group, but did not reach statistical significance (Figure 1B). Systemic arterial plasma levels for proMMP-2 trended upward in the non-LRF group, but fell to within normal levels by 6 hours post CPB (Figure 2A). In contrast, proMMP-2 increased from baseline to 6 and 12 hours post CPB in the LRF group. A similar trend was noted when proMMP-2 was computed as a percentage change from the 30-minute rewarming time point (Figure 2B). Systemic arterial plasma levels for proMMP-9 increased significantly ($p < .05$) from baseline at the 30-minute rewarming time point in the LRF group (Figure 3A). ProMMP-9 levels were computed as a percentage change from the 30-minute rewarming time point to account for patient variability in each group and to assess the potential impact of the leukocyte reduction filters. When proMMP-9 values were computed as a change from the 30-minute rewarming time point, a significant 30% reduction in relative MMP-9 levels was observed when compared to the filter group (Figure 3B). ProMMP-9 levels increased 40% from 30 minutes into rewarming at 6 hours post CPB in the non-LRF group. The percentage of neutrophils of the total leukocyte count was measured from a CBC differential at baseline and 12 hours post CPB. Although there was no significant difference in the percentage of neutrophils at baseline, there was a significant increase ($p < .05$) in both groups 12 hours post CPB as compared to baseline (Figure 4A). The percentage of bands, immature neutrophils, of the total leu-

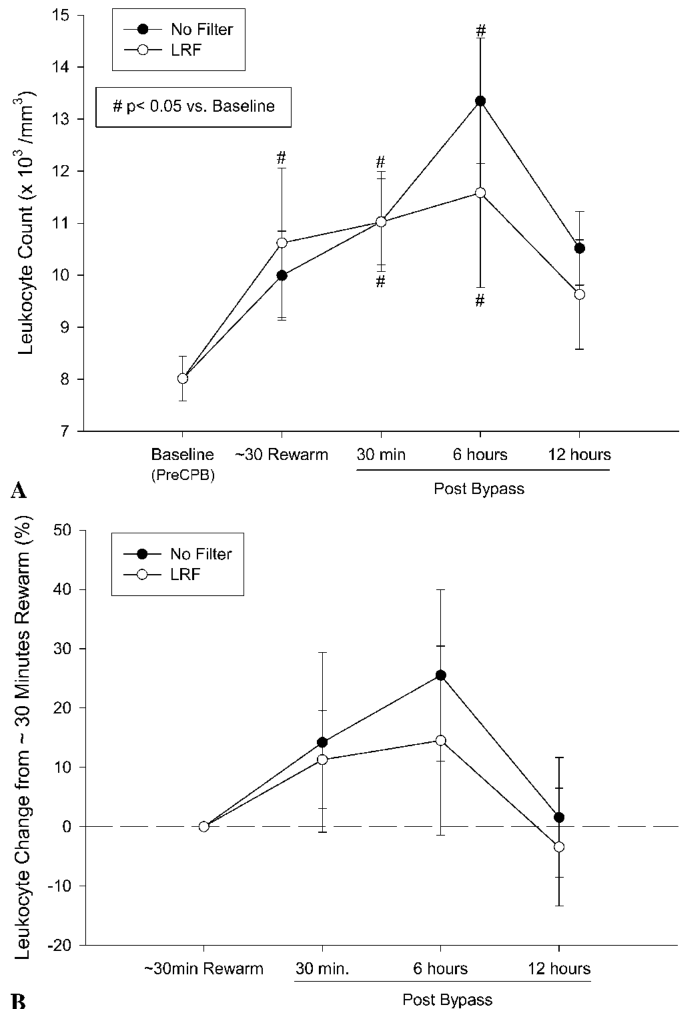
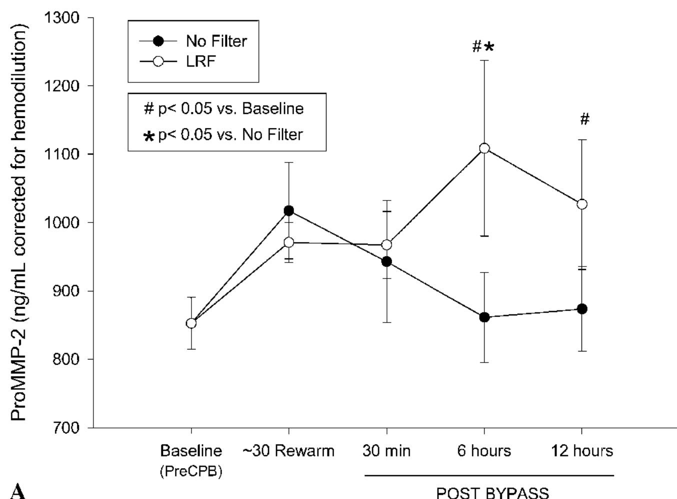


Figure 1. (A) WBCs were measured by complete blood counts, which showed WBCs increased in both the non-LRF and LRF groups. WBCs increased up to 6 hours post CPB ($p < .05$) when compared to baseline before decreasing at 12 hours post CPB. **(B)** WBCs measured as a percentage change from 30 minutes into rewarming the patient. There is a trend for WBCs to be higher in the non-LRF group as compared to the LRF group, though no significant difference was shown.



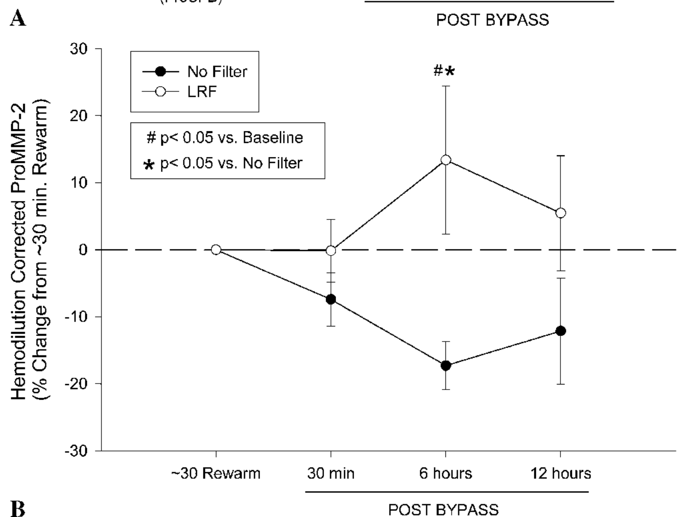
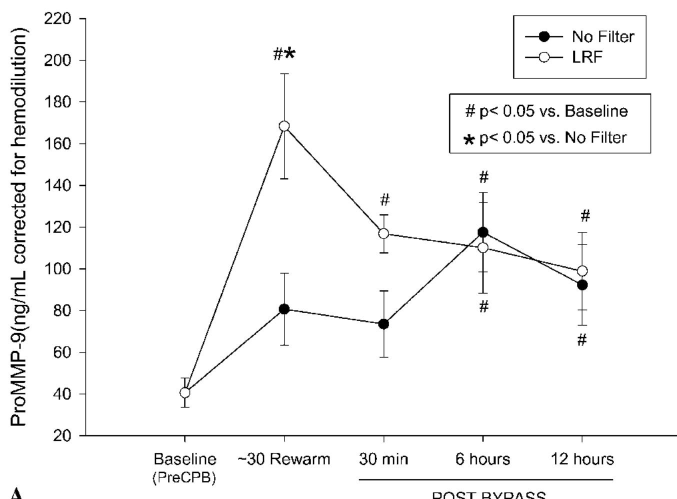


Figure 2. (A) Systemic arterial plasma levels for ProMMP-2 increased from baseline to 30 minutes into rewarming in the non-LRF group before returning to within normal levels by 6 hours post CPB. ProMMP-2 increased from baseline to 6 hours post CPB before decreasing at 12 hours post CPB in the LRF group. ProMMP-2 was significantly higher ($p < .05$) in the LRF group than the non-LRF group at 6 hours post CPB and significantly higher ($p < .05$) in the LRF group when compared to baseline at 12 hours post CPB. **(B)** ProMMP-2 measured as a percentage change from 30 minutes into rewarming the patient. ProMMP-2 increased in both the non-LRF and LRF groups, but to a higher degree in the LRF group. ProMMP-2 is significantly higher ($p < .05$) in the LRF group at 6 and 12 hours post CPB when compared to 30 minutes into rewarming.



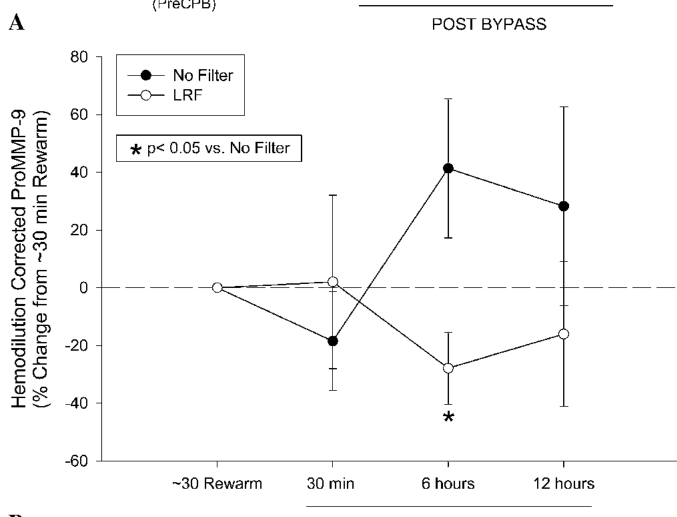


Figure 3. (A) Systemic arterial plasma levels for proMMP-9 increased significantly ($p < .05$) from baseline to 30 minutes into rewarming the patient when compared to the non-LRF group before decreasing continually to 12 hours post CPB. **(B)** ProMMP-9 levels measured as a percentage change from baseline. ProMMP-9 levels decreased 30% from 30 minutes into rewarming the patient at 6 hours post CPB in the LRF group.

kocyte count was measured from a CBC differential at baseline and 12 hours post CPB. There was no significant difference in the percentage of bands at baseline, but there was a significant increase ($p < .05$) in the LRF group at 12 hours post CPB when compared to baseline (Figure 4B).

DISCUSSION

CPB continues to be an integral part of many cardiac surgical procedures. However, the systemic inflammatory response seen in cardiac patients exposed to CPB can lead to intra-operative and post-operative complications. Activation of platelets, neutrophils, monocytes, endothelial cells, and lymphocytes has been shown to mediate the principal complications of CPB: bleeding, thromboembolism, fluid retention, and temporary organ dysfunction (13). Shear stress, apulsatile flow, and other physical factors related to CPB have led to contact activation and the disruption of endothelial cell and neutrophil properties. Free radicals, proteases, and other inflammatory mediators are released by the body as a result.

Matrix metalloproteinases (MMPs) are a family of proteolytic enzymes that are responsible for degrading the extracellular matrix, including the basement membrane (1), a process likely responsible for the development of altered vascular permeability and subsequent tissue edema post-cardiopulmonary bypass (2–4). The effects of the inflammatory response lead to a downstream disrup-

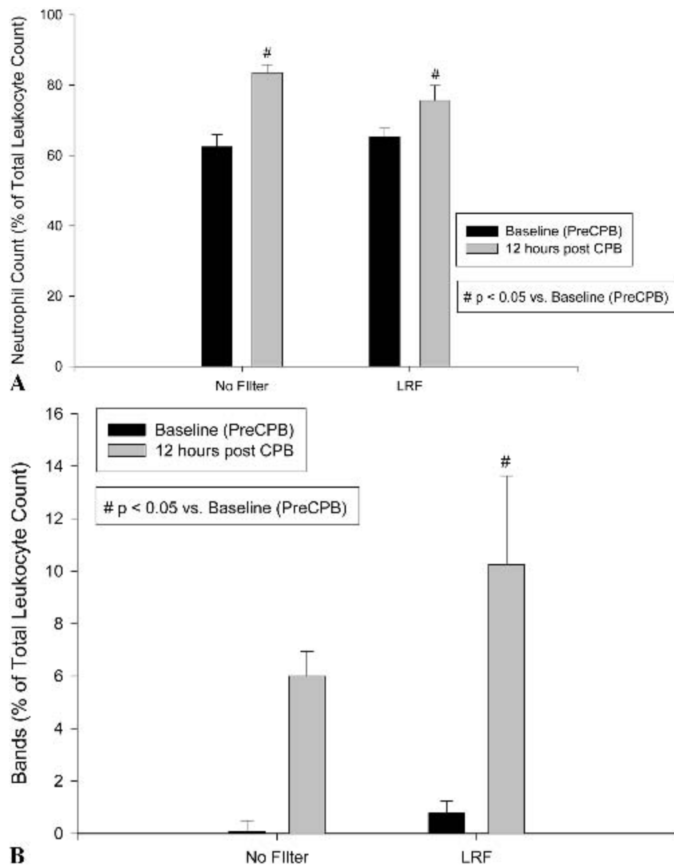


Figure 4. (A) The percentage of neutrophils of the total leukocyte count was measured from a CBC differential at baseline and 12 hours post CPB. Although there was no significant difference in the percentage of neutrophils at baseline, there was a significant increase ($p < .05$) in both groups 12 hours post CPB as compared to baseline. There was no significant difference when comparing the non-LRF and LRF groups to one another at 12 hours post CPB. **(B)** The percentage of bands, immature neutrophils, of the total leukocyte count was measured from a CBC differential at baseline and 12 hours post CPB. There was no significant difference in the percentage of bands at baseline, but there was a significant increase ($p < .05$) in the LRF group at 12 hours post CPB when compared to baseline. This suggests that neutrophil levels had decreased and were attempting to replenish themselves, resulting in an increased number of immature neutrophils, or bands.

tion in the balance of MMPs that exists under normal physiologic conditions. Increased activation and release of MMPs has been demonstrated to occur in several inflammatory processes and result in abnormalities in tissue structure and function (1,3,5,6,14–16). Increased release of several subclasses of MMPs has been shown to occur in the post-CPB setting (7,8). MMP-2 and -9, are from the subclass gelatinases, which have been shown to cause cardiovascular remodeling and were measured for purposes of this study (5). Once synthesized, MMPs are released into the extracellular space in a pro-enzyme, or latent, form. The proMMPs bind to various proteins and remain quiescent until activated. There are two central findings from this study. ProMMP-2 was shown to increase to a higher degree in the LRF group, although it did increase in both the LRF and non-LRF groups. ProMMP-9 levels increased in both the LRF and non-LRF groups as well. However, to examine MMP release following final blood exposure to the filters, proMMP-9 activity was computed as a mean percentage change from the 30-minute rewarming time point to compare trends in the LRF and non-LRF groups. ProMMP-9 levels increased 40% in the non-LRF group. In contrast, a 30% reduction in proMMP-9 levels was observed 6 hours after CPB in the LRF group.

Leukocyte-reducing filters (LRF) have been shown to remove activated neutrophils selectively in patients undergoing cardiopulmonary bypass (11,17) without causing further activation with filtration (18). LRF can be integrated into the arterial line of the ECC as well as into the blood cardioplegia line. A blood cardioplegia LRF has been shown to lower WBCs effectively while leaving platelets almost unaffected (19). Various biochemical markers for myocardial injury, such as Troponin-T and creatinine phosphokinase myocardial band (CPK-MB) have been shown to be lower in patients with an LRF than in control patients (19,20). The goal of this study was twofold. First, interruption or sequestration of neutrophils was attempted with utilization of leukocyte- reducing filters in both the arterial line and blood cardioplegia lines. Second, this study set out to define the effects on MMP production and release as a result of the filters.

Many different variables were measured to determine the effects of leukocyte reduction on patients exposed to cardiopulmonary bypass. White blood cell (WBC) counts increased in both the LRF and non-LRF groups, consistent with the increased inflammatory response previously documented as a result of CPB. MMP-2 and -9 are both of the gelatinase subclass; however, each is synthesized in a unique manner. MMP-2 synthesis and release can be accomplished by many different cell types, including smooth muscle cells, endothelial cells, myocytes, and platelets. It is important to note that although many cell types are capable of the MMP-2 synthesis, production and release of this MMP is not associated with neutrophils. Introduction of two leukocyte-reducing filters to the extracorporeal circuit (ECC) for use during CPB was aimed at decreasing systemic leukocytes, more specifically neutrophils. Because MMP-2 production and release is not associated with neutrophils, the filter additions to the ECC did not effectively attenuate proMMP-2 levels in the LRF patients.

Two important sources of MMP-9 are neutrophils and macrophages. Facilitation of proMMP-9 production may be the result of neutrophil stimulation secondary to increases in the inflammatory response seen with cardiopulmonary bypass. For this reason, addition of the two leukocyte reducing filters, aimed at filtering leukocytes, and thus, neutrophils, decreased the level of proMMP-9 by 30% in the LRF group. However, the early increase in

MMP-9 in the LRF group at the 30-minute rewarming time point may have been caused by the sequestering and degranulation of neutrophils within the filter. Based upon the finding of this study, future studies in this context are warranted.

To investigate the effects of the leukocyte-reducing filters further, neutrophils and bands, immature neutrophils, were measured as a percentage of the total WBC count at baseline and 12 hours post CPB. When compared to baseline, the percentage of neutrophils increased significantly in both groups at 12 hours post CPB. However, the change in neutrophils was not significant when comparing the LRF and non-LRF groups. This suggests that patients in the LRF group had sufficient time to synthesize neutrophils filtered from the ECC by 12 hours post CPB. Furthermore, the percentage of bands, immature neutrophils, was not significantly different between the LRF and non-LRF groups at baseline, but was significantly increased in the LRF group at 12 hours post CPB. The increased percentage of immature neutrophils at 12 hours post CPB suggests that, although neutrophils had sufficient time to replenish themselves, as seen with the neutrophil data, these neutrophils were, in fact, still in an immature state. The purpose of the presentation of these results was to document that the LRF affected neutrophil density. Sequestration and removal of neutrophils would be expected to provoke synthesis and maturation of neutrophils; that is, the presence of neutrophil "bands." Indeed, the LRF induced a relatively higher neutrophil band count at 12 h post CPB.

In summary, introduction of two leukocyte-reducing filters had opposite effects on MMP-2 and MMP-9 levels in patients undergoing elective coronary revascularization utilizing cardiopulmonary bypass. Although MMP-2 and -9 are from the same subclass, they are synthesized in very different manners. The opposite effects seen in this study may be the result of a disruption in the delicate physiological balance that exists between these two enzymes. The results of this study as well as further investigation into the correlation between MMP levels and early clinical outcomes seen following CPB may provide rationale for future intervention aimed at blocking the action of these enzymes in an effort to reduce the amount of tissue edema and organ dysfunction developed during and following CPB.

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