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TUMOR NECROSIS FACTOR-α AND TROPONIN I RELEASE IN PORCINE CARDIAC LYMPH AND CORONARY SINUS BLOOD BEFORE AND AFTER CARDIOPULMONARY BYPASS

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ABSTRACT

To assess the concentrations of cardiac troponin I (cTnI) and tumor necrosis factor-α (TNFα) in cardiac lymph compared with coronary sinus (CS) blood and to measure cardiac lymph flow before and after cardiopulmonary bypass (CPB). In 21 pigs, the main cardiac lymph trunk was cannulated before institution of standardized CPB. Lymph flow, cTnI and TNFα in cardiac lymph and CS blood were measured before and after CPB for 6 hours. Before CPB, cTnI concentration was 215 ± 36 ng/ml in cardiac lymph and 0.5 ± 0.1 ng/ml in CS blood, respectively. After aortic declamping a significant elevation of cTnI values was measured in cardiac lymph and CS blood. cTnI concentration in cardiac lymph and CS blood peaked 6 hrs after CPB (10,556 ± 4,735 vs. 22.2 ± 3.7 ng/ml, p<0.01). TNFα concentration at baseline was 23.2 ± 5.6 pg/ml in lymph and 18.7 ± 9.5 pg/ml in CS blood, and there was no significant release of TNFα up to the end of the experiment. Baseline cardiac lymph flow was 3.07 ± 0.35 ml/h and declined after aortic clamping (0.72 ± 0.16 ml/h; p<0.01) and peaked one hour after CPB (5.66 ± 0.97 ml/h; p<0.01).

In conclusion, very high cTnI concentrations in cardiac lymph suggest serious perioperative myocardial damage after CPB with cardioplegia, which is underestimated by cTnI release into the bloodstream. In our study, the myocardium was not a major source of TNFα release.

Cardiac surgery with cardiopulmonary bypass (CPB) is associated with perioperative myocardial cell damage (PMD) due to ischemia-reperfusion injury (1) and induction of a systemic inflammatory response with complement activation (2), leukocytosis (3) and the release of proinflammatory cytokines (4) such as IL-1, IL-6 and tumor necrosis factor α (TNFα). Myocardial damage and the release of proinflammatory cytokines are partially responsible for patient morbidity and mortality due to perioperative myocardial infarction, increased capillary permeability with myocardial interstitial fluid accumulation and subsequent low cardiac output-syndrome, hypotension, cardiac arrhythmias and pulmonary hypertension (5,6).

Cardiac lymph reflects accurately the contents of the myocardial interstitial space (6). Lymph examinations provide sensitive information about intramyocardial changes after cardiac operations with CPB (7) and indicate ischemic episodes of the myocardium with an early release of the classic cardiac markers such as creatinine phosphokinase, and lactate dehydrogenase (8).

TNFα is produced by activated monocytes, lymphocytes and Kupffer cells. Factors

triggering the synthesis include C5a, IL-1β and gram-negative endotoxin (9) and it is known that TNFα can be expressed *de novo* by cardiac myocytes. (10).

To date, reports on plasma levels of TNFα after cardiac operations with CPB have been controversial. In some studies it remained undetectable (11) although in others significant plasma values have been recorded (12). A significant TNFα release has been associated with postoperative hypotension, tachycardia (13), and myocardial depression due to apoptosis and necrosis of the myocardial cell (14). Cytokine levels correlate also positively with the degree of myocardial injury after CPB and with creatinine kinase MB isoenzyme or cTnI levels (15).

Cardiac Troponin I (cTnI) is a highly specific marker for myocardial tissue. Unlike creatinine kinase MB isoenzyme, it is only present in the myocardial cell, not detectable in healthy persons, and is rapidly released after myocardial injury remaining elevated for 7-10 days (16). It is documented to be a specific marker for the diagnosis of peri- or postoperative myocardial damage in patients undergoing coronary bypass surgery with CBP (1).

The purpose of our study was to examine if cardiac lymph was an accurate reflection of the myocardial interstitial space and was a more sensitive indicator of TNFα release during CPB than coronary sinus blood (CS). Furthermore we evaluated the perioperative myocardial cell damage (PMD) expressed by the release of cTnI into cardiac lymph and CS blood.

MATERIALS AND METHODS

The study was conducted according to the guidelines of the German Animal Protection Law ensuring humane care and was approved by the supervising state agency for animal experiments.

In twenty-one healthy female pigs (body weight 39.4 ± 3.6 kg, body surface area or BSA 1.1 ± 0.1 m^2), the main efferent cardiac lymph trunk was cannulated before CPB.

Anesthesia was uniform and consisted of a combination of ketamine (4 mg/kg) i.m. and pentobarbital (5 mg/kg) i.m. and maintained with boluses of pentobarbital as required. After endotracheal intubation, lungs of the pigs were mechanically ventilated with a Servo A ventilator (Siemens 900B, Solna, Sweden) using an air/oxygen mixture (FiO_2: 0.5). Esophageal and myocardial probes were placed for temperature measurements. Catheters were placed preoperatively in the carotid artery and jugular vein for pressure monitoring and intraoperatively for blood sampling in the coronary sinus.

CPB and myocardial protection

After successful cannulation of the main cardiac lymphatic trunk and anticoagulation with bovine lung heparin (400 IU/kg), the aorta (18 F, Argyle THI, Brunswick Company, St. Louis, USA) and both venae cava (20/24 F, Stöckert GmBH, Munich, Germany) were cannulated and CPB instituted. Anticoagulation was controlled by activated clotting time, which was maintained at a value higher than 450 seconds throughout the duration of CPB.

CPB equipment was uniform and consisted of a roller pump inducing a non-pulsatile flow (Stöckert GmbH, Munich, Germany), a disposable pediatric hollow fiber oxygenator (Dideco D4000, Italy), a hard-shell cardiotomy reservoir (Dideco D754, Italy), an arterial blood filter and a bubble trap. The extracorporeal perfusion circuit was primed with a 1000 ml of crystalloid solution (Delta Pharma, Pfullingen, Gemany). Cooling and rewarming were performed with a heat exchanger. Total duration of CPB was set at 120 minutes in each pig with nonpulsatile flow index of 2.4-2.7 L/min/m^2 at a moderate hypothermia of 28°C. Mean systemic arterial pressure was maintained between 40 and 60 mmHg. After a perfusion time of 30 minutes (pre AoX; baseline) , the aorta was cross-

clamped (AoX) for a duration of 60 minutes and cardioplegic cardiac arrest was achieved with a single dose of cold (4°C) crystalloid "Brettschneider" solution (Custodiol; Dr. Köhler Chemie GmbH, Germany) injected into the aortic root. After removal of the aortic clamp, reperfusion was continued for 30 minutes (post AoX). Then the pigs were weaned from CPB and all cannulas were removed. Anticoagulation was then reversed by 1:1 protamine administration, chest tubes were placed and the sternum closed.

Postoperative care

Perioperative monitoring was performed with an ECG monitor (Siracust 404, Siemens, Germany). Monitoring included continuous registration of heart rate and rhythm, mean arterial blood pressure (MAP), central venous pressure (CVP) myocardial and esophageal temperature. Inotropic support consisting of dopamine and fluid administration with a crystalloid solution were adapted to optimize hemodynamics. Routine blood examinations included blood gas analysis, arterial lactate concentrations and serum electrolytes. The experiment in each pig was terminated six hours after CBP.

Cannulation of the cardiac lymphatic trunk and lymphatic flow measurements

After sternotomy, the main efferent cardiac lymph trunk was identified by epicardial injection of Evans-blue (0.1 ml, 0.5% aqueous solution; bioMerieux sa., Marcy l'Etoile, France) and cannulated with a 18G Cavafix-Certo-Basilica® catheter (B.Braun Melsungen AG, Melsungen, Germany) as previously described by our group (17). Lymphatic flow was collected in scaled 2.7ml EDTA tubes (Sarstedt, Germany) before aortic cross-clamping (AoX) (baseline), after aortic declamping and every hour after CPB and was expressed in ml/hour.

Sampling of blood and lymph

Blood samples for determination of cTnI were taken intra- and postoperatively from the coronary sinus. Lymph samples and blood samples were collected in EDTA tubes and separated after centrifugation for 3 min at 3000 rpm. The supernatant was stored at -80°C until cTnI and TNFα assay determination. Samples were taken before AoX (baseline), after aortic declamping and 2, 4 and 6 hours after CPB.

TNFα determination

TNFα concentrations were analyzed by an enzyme-linked immunosorbent assay (ELISA) according to the manufacturer recommendations (Enodogen Inc., Cambridge, USA). This assay is specific for measurement of natural and recombinant pig TNFα. It detects <10 pg/ml of pig TNFα.

cTnI determination

cTnI was measured by a fluorometric enzyme immunoassay according to the manufacturer recommendations (Stratus: Dade International; Miami, USA). This cTnI assay is an automated two-site immunoassay that utilizes two monoclonal antibodies specific for the cardiac isotype of troponin I. The minimum detectable concentration is 0.35 ng/ml.

Statistical analysis

Statistical analysis was performed with the SPSS Software package (SPSS Software GmbH, München, Germany). Results are expressed as mean values ± standard error of the mean (mean ± SEM). The quantitative analysis of the group was done with the Wilcoxon nonparametric test. Correlation analyses were done by Pearson's correlation test. P-values <0.05 were considered significant.

RESULTS

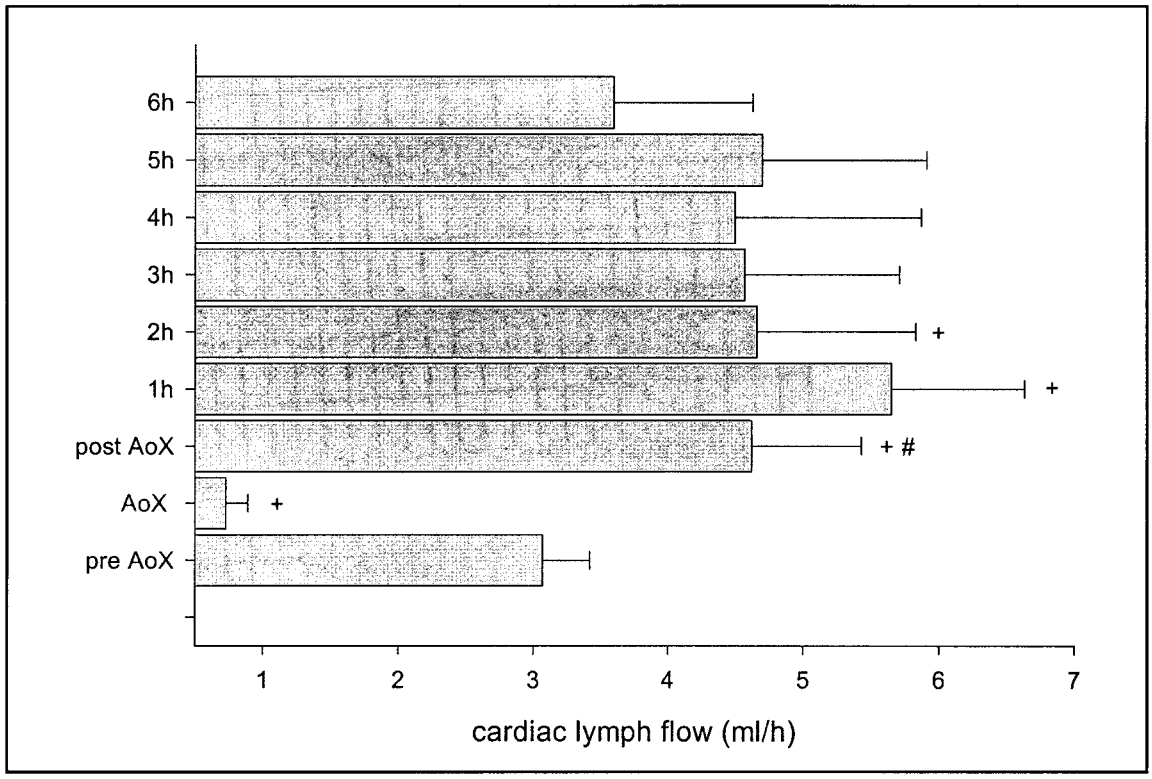

Fig. 1: Cardiac lymph flow (mean ± SEM) before, during and after aortic cross-clamping (AoX) and during (1 hour) and after cardiac pulmonary bypass up to 6 hours (h).+ = levels compared with baseline (P<0.05) ; # = higher than pre AoX (P<0.05).

Lymph flow rate *(Fig. 1)*: Baseline (preAoX) cardiac lymph flow was 3.07 ± 0.35 ml/h. With AoX lymph flow significantly declined to 0.72 ± 0.16 ml/h (p<0.01 vs baseline) and increased after aortic declamping to 4.6 ± 0.81 ml/h (p<0.05 vs baseline). In each pig, lymph flow curve tended at the end of the experiment towards preoperative values. Cumulative lymphatic flow rate during the experiment was 36.1 ± 8.2 ml. Peak values were observed 1h after CPB (5.66 ± 0.97 ml/h, p<0.01 vs baseline). Hemolytic cardiac lymph staining was observed during and after AoX until the fourth to sixth postoperative hour. Cardiac lymph flow did not correlate with BSA, heart rate, MAP or CVP.

TNFα *(Fig. 2)*: At baseline (preAoX) there was no significant difference between the concentration measured in cardiac lymph and coronary sinus blood (23.2 ± 5.6 vs. 18.7 ± 9.5 pg/ml). After aortic declamping (postAoX) and until the end of the experiment, TNFα did not increase significantly either in cardiac lymph or in CS blood and was measured at the sixth postoperative hour at 18.1 ± 4.9 pg/ml in cardiac lymph vs 9.8 ± 4.3 pg/ml in CS blood. TNFα release in coronary sinus blood did not correlate with cardiac lymphatic values.

Baseline cTnI concentration was 215 ± 36 ng/ml in cardiac lymph and 0.47 ± 0.05 ng/ml in coronary sinus blood *(Fig. 3)*. After aortic declamping an elevation of the cTnI values in cardiac lymph (570 ± 293 ng/ml; ns) and a significant increase in coronary sinus blood was noted (3.9 ± 0.62 ng/ml, p<0.01 vs baseline). cTnI concentration in cardiac

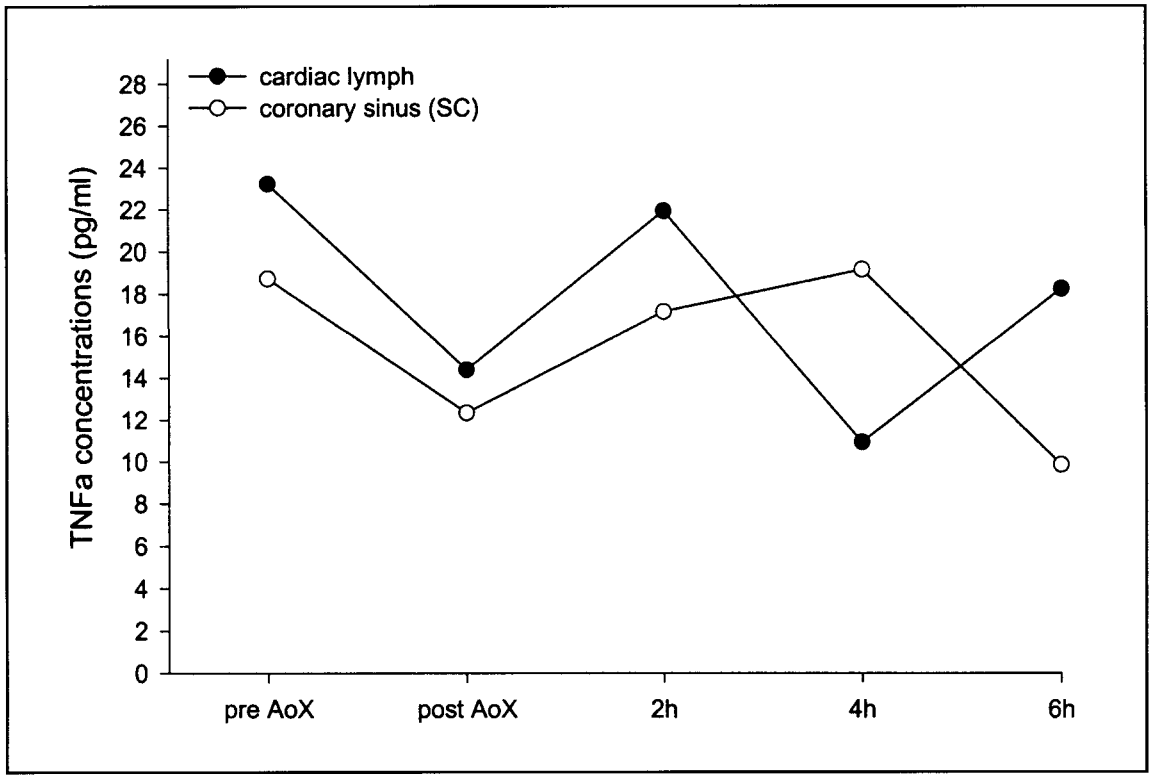

Fig. 2: Levels of TNFα in cardiac lymph and cardiac sinus blood (mean ± SEM) before (baseline) and after aortic cross-clamping (pre AoX , post AoX) and for 2h-6h after cardiopulmonary bypass.

lymph increased significantly during the postoperative hours ($p<0.01$ vs baseline) and peaked 6 hours after AoX (10,556 ± 4,735 ng/ml, $p<0.01$ vs baseline). In CS blood, the release pattern of cTnI was similar to cardiac lymph with a highly significant elevation at the second and fourth postoperative hours ($p<0.01$ vs baseline) and peak values at the end of the experiment (22.16 ± 3.7 ng/ml, $p<0.01$ vs baseline). A significant positive correlation was found between the lymphatic and coronary sinus cTnI values at each postoperative hour ($p<0.01$). There was no correlation of cTnI to TNFα levels in CS blood or cardiac lymph.

DISCUSSION

Cardiac lymph flow

Perioperative myocardial cell damage and interstitial myocardial edema due to ischemia-reperfusion injury and the induction of a systemic inflammatory response are known to cause postoperative myocardial depression after CPB (6).

The observed decrease of cardiac lymph flow during AoX and cardioplegic arrest with total loss of ventricular contractility and lymphatic driving pressure hastens the formation of myocardial edema. Edema in the myocardial interstitial space during AoX derives from an imbalance of increased capillary permeability and fluid filtration into the interstitial space and decreased fluid drainage via the cardiac lymphatics. (2,6,18). The marked decline in lymph flow during AoX (23% of the baseline value) followed by an elevation (150% of baseline value) after

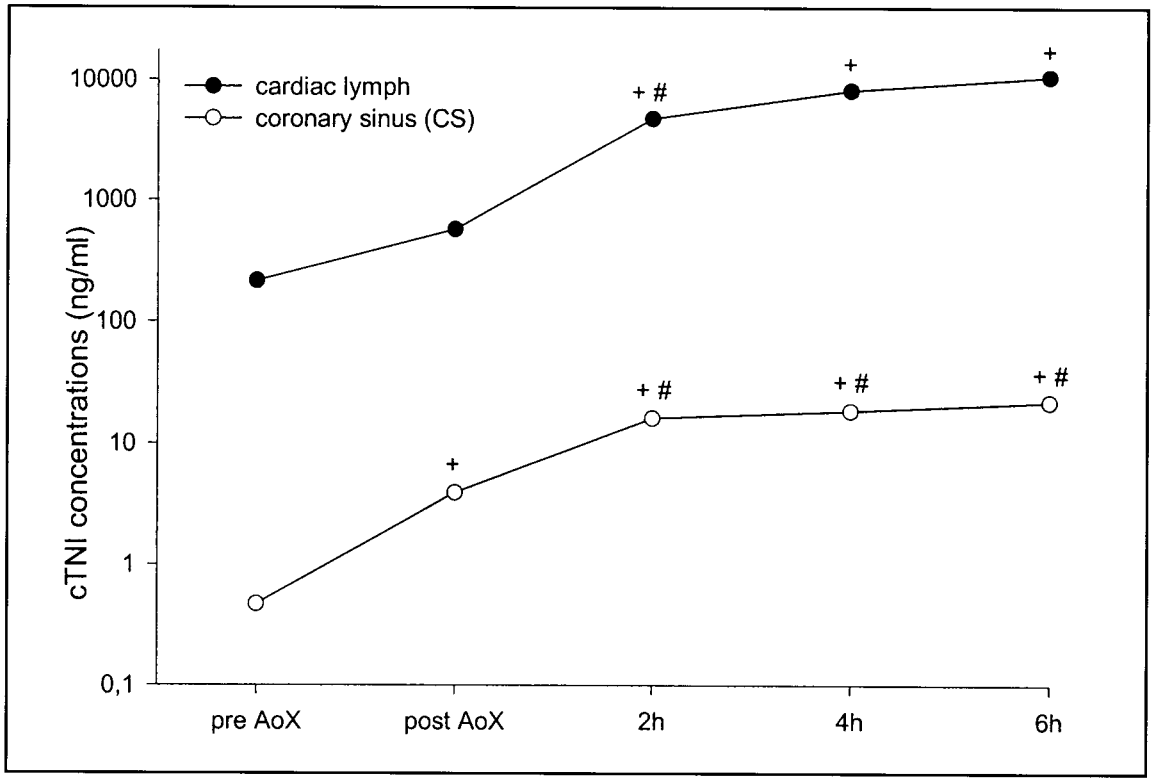

Fig. 3: Changes in logarithmic levels of cTnI in coronary sinus blood and cardiac lymph (mean ± SEM) before and after aortic cross-clamping (pre AoX, post AoX) and 2h-6h after cardiopulmonary bypass. + = significantly higher levels compared with base (P<0.05); # = higher levels compared with pre AoX values (P<0.05).

aortic declamping in each pig underlines the ongoing edema formation during CPB. Cumulative lymph drainage after CPB until the end of the experiment was 32.3 ± 7.7 ml showing the accumulation of interstitial fluid during cardioplegic cardiac arrest. Furthermore, there was prolonged red blood cell staining of the cardiac lymph after aortic declamping until the end of the experiment, indicating damage to the blood capillary endothelium. These findings are consistent with previous observations in dogs (7).

TNFα

The effect of TNFα on the heart relates to increased capillary permeability, myocardial depression and even myocardial cell damage with apoptosis and necrosis (14,19). Elevated TNFα concentrations seem to desensitize cardiac myofilaments to [Ca^{2+}], an effect that may be due to phosphorylation of troponin I producing myocardial depression (19).

TNFα can directly induce cellular death by necrosis and apoptosis in the myocardium contributing to cardiac myocyte loss and ischemia-reperfusion injury (15,19). Additionally TNFα increases synthesis of myocardial cell damaging nitric oxide and triggers the release of other pro- and antiinflammatory cytokines such as IL-8 and IL-10 (20). Soluble TNF-receptors (sTNF-Rp55; sTNF-Rp75) are known to act as a physiological TNF antagonist, and elevated concentrations have been measured after CPB (21). Furthermore, preoperative dexamethasone applications can prevent

significant perioperative TNFα and cytokine release (22).

To date reports on plasma levels of TNFα after cardiac operations with CPB have been discordant. Serial measurements of cytokines in CS, pulmonary artery and arterial blood in patients undergoing cardiac operation with CPB have suggested that the myocardium is a major source of TNFα and IL-6 (20). TNFα release and concentration in cardiac lymph, however, has not been previously assessed.

In contrast to Wan et al (15,20), our results do not show a significant release of TNFα into CS blood during hypothermic CPB. These findings imply that the myocardium is not a source of synthesis of this proinflammatory cytokine. There was no correlation between TNFα release and cTnI concentration or cardiac lymph flow underlining that TNFα is not a cause of myocardial cell damage in our pig model. These results seem surprising compared with work in humans which show a profound release of TNFα during CPB (4,22). The discrepancies might be explained by several possibilities. First, due to the short half-life of TNFα, a rise in our 6 hour study may have been missed (23). Second, hypothermic conditions, as used in our study, are known to be associated with lower cytokine release than normothermic CPB (13). Third, non-comparable methods of TNFα determination in pigs and humans (ELISA vs Bioassay) may lead to false negative or positive levels. We used a pig-specific TNFα-ELISA-kit which detects TNFα concentration less than 10 pg/ml. A dose dependent decrease of measured TNFα concentrations in the presence of sTNF-Rp55 has been noticed by the manufacturer, which may lead to false negative values. Fourth, the absence of detectable circulating TNFα does not exclude local production by activated cells (11).

cTnI

Cardiac Troponin I is a highly specific and established marker for myocardial damage. In addition, cTnI has been shown to be a specific marker for the diagnosis of peri- or postoperative myocardial damage in patients undergoing coronary bypass surgery with CBP (1) and to correlate with myocardial infarction size (24).Because of its origin in the myocardial interstitial space, cardiac lymph has been often used in a canine model for examination of metabolic and functional changes in the myocardium (7,8).

The higher cTnI concentrations in cardiac lymph and CS blood combined with the increased lymph flow rates after aortic declamping in our study suggest perioperative myocardial damage with high fluid extravasation due to increased permeability of the capillary and myocardial cell membrane. The ischemia-reperfusion during CPB leads to a release of oxygen free radicals and cytokines which damage the cell membrane leading to a leak of cTnI and cTnT from the intracellular space into the interstitial space and from here into the cardiac lymphatic system (25). The progressive loss of cTnI may result in depressed myocyte contractility with impaired relaxation and increased myocardial stiffness (26,27).

CTnI levels in cardiac lymph were much higher than in CS blood throughout the experiment, underlining the superiority of cardiac lymph analysis compared to CS in reflecting functional and metabolic changes in the myocardial interstitial space and the severity of contractile protein loss.

In conclusion, in our study the myocardium was not a major source of TNFα release during or after hypothermic CPB. As this finding is in contrast to previous work, it needs to be further evaluated. Myocardial cell damage with pronounced release of cTnI in cardiac lymph and altered cardiac lymphatic flow with AoX and cardiopulmonary bypass occurred throughout the experiment. The extremely high concentrations of cTnI in cardiac lymph reflect the seriousness of myocardial damage, which is easily underestimated by the lower concentrations of cTnI

in CS blood and its lack of correlation with TNFα levels in either blood or lymph.

These findings further support the relevance of monitoring cardiac lymph constituents and dynamics for insight into changes in the myocardial interstitium.

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