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Plasma Prohepcidin as a Negative Acute Phase Reactant after Large Cardiac Surgery with a Deep Hypothermic Circulatory Arrest

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Summary

Hepcidin is a key regulator of iron metabolism and a mediator of anemia in inflammation. Recent *in vitro* studies recognized prohepcidin as a type II acute phase protein regulating *via* interleukin-6. The aim of the present study was to investigate the time course of plasma prohepcidin after a large cardiac surgery in relation to IL-6 and other inflammatory parameters. Patients with chronic thromboembolic hypertension (n=22, males/females 14/8, age 51.9±10.2 years) underwent pulmonary endarterectomy using cardiopulmonary bypass and deep hypothermic circulatory arrest were included into study. Arterial concentrations of prohepcidin, IL-1β, IL-6, IL-8, tumor necrosis factor-α, and C-reactive protein were measured before/after sternotomy, after circulatory arrest, after separation from bypass, and then 12, 18, 24, 36, 48 h and 72 h after the separation from bypass. Hemodynamic parameters, hematocrit and markers of iron metabolism were followed up. Pulmonary endarterectomy induced a 48 % fall in plasma prohepcidin; minimal concentrations were detected after separation from cardiopulmonary bypass. Prohepcidin decline correlated with an extracorporeal circulation time ($p<0.01$), while elevated IL-6 levels were inversely associated with duration of prohepcidin decline. Postoperative prohepcidin did not correlate with markers of iron metabolism or hemoglobin concentrations within a 72-h period after separation from CPB. Prohepcidin showed itself as a negative acute phase reactant during systemic inflammatory response syndrome associated with a cardiac surgery. Results indicate that the evolution of prohepcidin in postoperative period implies the antagonism of stimulatory effect of IL-6 and contraregulatory factors inhibiting prohepcidin synthesis or increasing prohepcidin clearance.

Key words

Hepcidin • Interleukin-6 • Acute phase proteins • Cytokines • Surgery

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Introduction

Hepcidin is a cytokine-induced antibacterial protein which is produced in the liver, released into plasma and excreted in urine. Hepcidin is a homeostatic regulator of iron absorption in the intestinal mucosa, iron recycling by macrophages, and iron mobilization from hepatic stores (Ganz 2006). It appears to have a significant role in the pathogenesis of hemochromatosis and related disorders (Papanikolaou *et. al.* 2005) and to be a major factor in the systemic iron abnormalities seen in anemia of chronic diseases (Rivera *et al.* 2005).

Hepcidin originates from extrahepatic enzymatic cleavage of its prohormone prohepcidin. The regulation of prohepcidin seems to be a complex signaling network. The various protein factors activate and inhibit the translation of prohepcidin, thus achieving fine-tuned regulation of this peptide. Prohepcidin and its active form hepcidin can be upregulated by multiple stimuli including iron, interleukin-6 (IL-6), IL-1α, and IL-1β, and bone

morphogenetic proteins (BMP), particularly BMP-2, BMP-4 and BMP-9 (Truksa *et al.* 2007). Due to dominant regulation by IL-6, hepcidin was classified as a type II acute phase protein (APP). During infections and inflammation, hepcidin-mediated decrease in extracellular iron concentrations probably limits iron availability to invading microorganisms.

Knowledge of hepcidin regulation is foremost gained by *in vitro* studies. Only few clinical studies reported prohepcidin or hepcidin disturbances in acute or chronic inflammatory status in humans. While quantitative methods have been used for the determination of urinary hepcidin and serum prohepcidin, no definitive methods have been published for the determination of hepcidin in plasma (Murphy *et al.* 2007).

In this study, the large cardiac surgery – pulmonary artery endarterectomy (PEA) – was used as *in vivo* model of cytokine network hyperstimulation. PEA is an effective therapeutic approach for patients with chronic thromboembolic pulmonary hypertension (CTEPH), whose prognosis would otherwise be very poor. After successful endarterectomy, pulmonary artery pressure and pulmonary vascular resistance fall and the cardiac output increases (Roscoe and Klein 2008). Cardiac surgery leads to a more pronounced activation of cytokines than some other surgical procedures (Langer *et al.* 2004, Chachkhiani *et al.* 2005). This cytokine "burst" mediates a systemic response by the body's inflammatory system, well known as the systemic inflammatory response syndrome (SIRS).

The aim of the present study was to investigate the time course of plasma prohepcidin in the perioperative and early postoperative periods in patients undergoing PEA using cardiopulmonary bypass (CPB). IL-6 and other inflammatory parameters – IL-1β, IL-8, tumor necrosis factor-α (TNFα), and C reactive protein (CRP) – were measured to evaluate potential relations between plasma prohepcidin and cytokine network status. The selection of measured inflammatory parameters came from experimental studies supporting the role of IL-6, IL-1β and TNFα in prohepcidin expression.

Material and Methods

The prospective study was realized on the Second Department of Surgery – Department of Cardiovascular Surgery of the First Faculty of Medicine in Prague from January 2007 to June 2008. The ethical committee of the institution approved a study protocol and a written informed consent was obtained from the subjects.

Patients

Patients with CTEPH (consecutive series of patients) were followed with PEA with uncomplicated postoperative course. Exclusion criteria were the combination of PEA with other surgical procedure, postoperative bleeding, thromboembolic complication, local and systemic infection. Definitions of infections were based on the guidelines published from the Center for Disease Control and Prevention (Horan and Gaynes 2004). Twenty-two patients were enrolled during 18 months of the trial – 14 males and 8 females, mean age being 51.9±10.2 years, preoperative New York Heart Association (NYHA) class 3.5±0.4 (mean ± S.D.), and mean pressure in the main pulmonary artery (mPAP) was 54 mm Hg.

Surgical procedures

Following median sternotomy, cardiopulmonary bypass (CPB) was established with cannulation of the ascending aorta and the inferior and superior vena cava. Cooling began immediately using CPB cooling blankets, cooled to a bladder temperature of 18-20 °C. Cardiac arrest was induced after aortic cross-clamping by infusion of cardioplegic solution (mostly St. Thomas). Approach to the pulmonary artery had to be bilateral; both pulmonary arteries had to be substantially involved. Pulmonary artery was open; a correct dissection plane was made and pursued to the segmental branches of pulmonary artery. For precision visualization during peripheral dissection, repeated periods of deep hypothermic circulatory arrest (DHCA) with reestablishment of CPB between them were necessary. If other cardiac procedures were required, these were performed during the systemic rewarming. After rewarming period patient was weaned from CPB by the stepwise reduction of pump flow.

Monitoring

Radial and femoral artery cannulae, triple lumen central venous cannula, Swan-Ganz catheter, and single lumen jugular bulb catheter were inserted for continuous monitoring of hemodynamic parameters and jugular bulb blood saturation. Left atrial catheter was surgically placed for both measurement and norepinephrine administration.

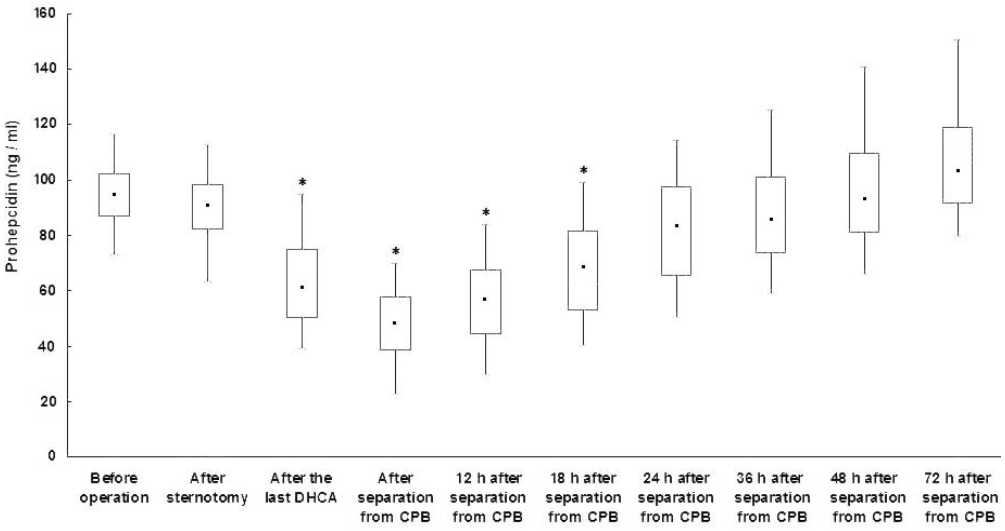

Fig. 1. Prohepcidin plasma levels in perioperative period. The same setting used for Fig. 1 – 3: Box and whisker plot depicting the mean values, interquartile range and full range. * significant differences to preoperative values, $p<0.05$.

Prohepcidin, cytokine and CRP analysis

Arterial blood samples were drawn from femoral artery catheter before operation, after sternotomy, after DHCA, after separation from CPB, then 12, 18, 24, 36, 48 and 72 h after separation from CPB. For all measurements, 5 ml of arterial blood was drawn into a vacutainer heparin tube and immediately centrifuged at 5000 rpm for 15 min Plasma was stored at –80 °C until analysis. Plasma prohepcidin concentration was measured by enzyme-linked immunoassay using a commercially available kit (DRG Diagnostics, Marburg, Germany) in duplicates. Plasma concentrations of TNFα, IL-6, IL-8 (ELISA, Immunotech, Paris, France), and CRP (Kryptor - TRACE technology, ultrasensitive analysis, BRAHMS AG, Hennigsdorf, Germany) were measured in duplicates. The intra- and inter-assay coefficients of variation were below 5 %. Plasma concentrations of prohepcidin, cytokines and CRP (Figs 1-3), were cleared from the effect of perioperative hemodilution and corrected to hematocrit.

Hemodynamic parameters were recorded according the standard surgical protocol. Laboratory markers of iron metabolism – plasma iron (colorimetric analysis, Pliva-Lachema a.s., Brno, Czech Republic), ferritin, and transferrin (immunoturbidimetry, Dialab Gmbh, Wr. Neudorf, Austria) – were examined preoperatively and repeatedly within 72 h after the end of surgery.

Statistical analysis

was carried out using SPSS software (version 12.0) for Windows (SPSS, Chicago, USA). Analysis of covariance (ANCOVA) was used. The normal distribution of all data was examined using the Kolmogorov-Smirnov normality test to determine subsequent use of tests for statistical comparison. As variables were not normally distributed, the data were reported as median and interquartile range. Correlation between the monitored indicators was evaluated by the Pearson's correlation coefficient and the Spearman's rank correlation. For all the tests, $p<0.05$ was defined as statistically significant.

Results

All patients underwent satisfactory clearance of intra-arterial obstruction, and there were no intra-operative deaths. No patients required allogeneic blood transfusion. Mean duration of CPB was 332.0± 46.9 min; mean duration of cross-clamping time was 122.4±20.0 min and circulatory arrest time 40.6±7.5 min Extracorporeal circulation (ECC) time was 332.1±59.7 min; duration of mechanical ventilation was 52.6±32.7 h. There was considerable improvement in hemodynamic variables. PEA significantly decreased mPAP (from 54.2±8.72 to 24.6±7.11 mm Hg, $p<0.001$) as well as pulmonary vascular resistance (from 1106.8±314.6 to 206.4±96.0 dynes.s.cm^{-5}, $p<0.001$). Cardiac index increased within first 24 h after surgery (from 1.93±0.36 to 3.02±0.46 l.min^{-1}m^{-2}, $p<0.001$).

The time course of prohepcidin in perioperative period is shown in Figure 1. Cardiac surgery with CPB induced a 48 % fall in plasma prohepcidin. Prohepcidin decreased from preoperative level 96.2 ng/ml (87.0-103.6) to minimum 49.9 ng/ml (37.8-58.0). The initial decline was revealed after DHCA, and minimal concentrations were detected after separation from CPB ($p<0.001$ in relation to preoperative levels) after which

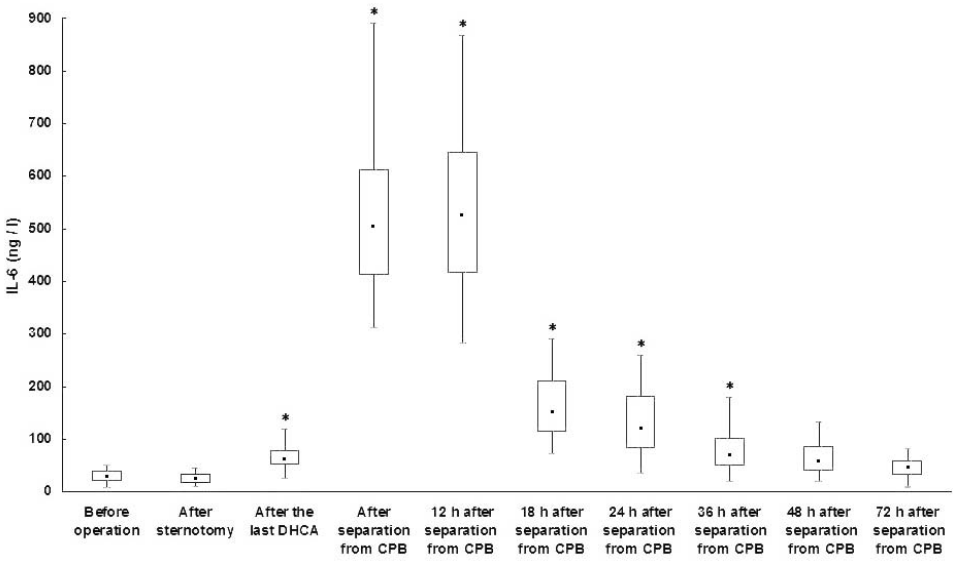

Fig. 2. IL-6 plasma concentrations in perioperative period.

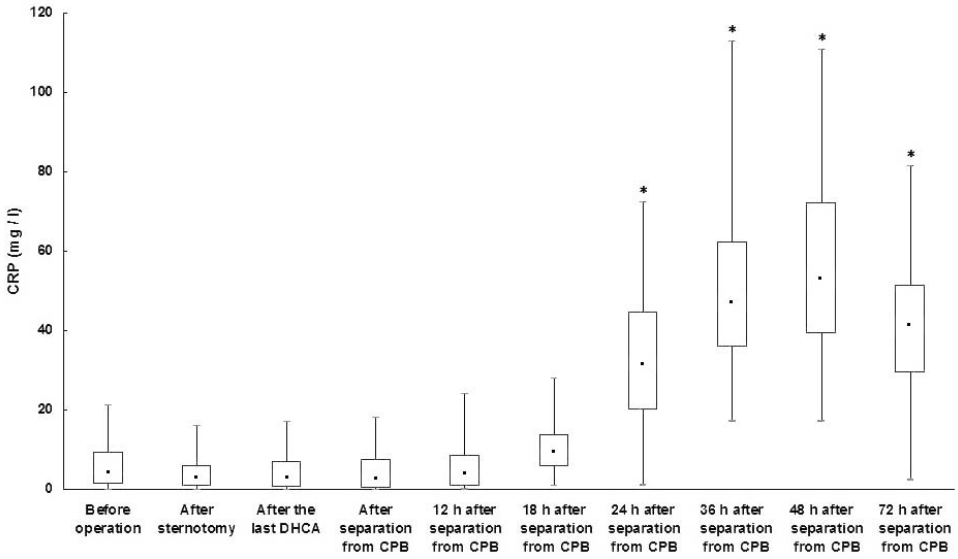

Fig. 3. CRP plasma concentrations in perioperative period.

the levels started to rise. Concentrations returned to initial levels within 24-48 h after the separation from CPB. The following increase was found in samples at 72 h, but without a statistical significance to preoperative levels on $p<0.05$.

The time course of prohepcidin differed from all tested cytokines. As expected, all inflammatory cytokines (with the exception of IL-1β) increased after surgery. IL-6 rise was maximal 12 h after separation from CPB from 28.1 ng/l (20.0-39.4) to 526.0 ng/l (191.5-748.9) with a following decline (Fig. 2). IL-6 transient initial decline with minimum levels after DHCA was related to hemodilution and cleared after correction to hematocrit. TNFα rose from 16.4 ng/l (18.9-46.4) to 222.7 ng/l (140.1-418.2) with a maximum at 12 h after separation from CPB. IL-8 with preoperative levels 45.5 ng/l (18.4-88.1) culminated later, at 18 h after separation from CPB (438.6 ng/l, 284.0-648.9) with a following decline. IL-1β elevation with maximum at 6 h after separation from CPB was not significant. CRP showed prolonged elevation with a peak level at 48 h after separation from CPB (78.2 mg/l, 44.7-118.2) (Fig. 3).

Plasma ferritin levels (46.2 μg/l, 17.2-76.4 preoperatively) increased only slightly at 18 h after separation from CPB (84.7 μg/l, 52.2-146.7) and stayed elevated without significant dynamics until the end of tested period. Plasma ferritin did not significantly correlate with prohepcidin or any other tested inflammatory parameter. There was no significant correlation of plasma prohepcidin with plasma iron concentrations, plasma transferrin concentrations or hemoglobin concentration within a 72-h time frame after

separation from CPB.

Minimal perioperative prohepcidin concentrations correlated inversely with ECC time ($r = –0.82$, $p<0.01$) (Fig. 4). Postoperative peak values of IL-6 and IL-8 correlated closely ($r = 0.80$, $p<0.01$), as well as peak values of IL-6 and CRP ($r = 0.72$, $p<0.01$). However no significant correlation was revealed between prohepcidin preoperative levels and IL-6 concentrations as well as between postoperative minimum concentrations of prohepcidin and IL-6 levels.

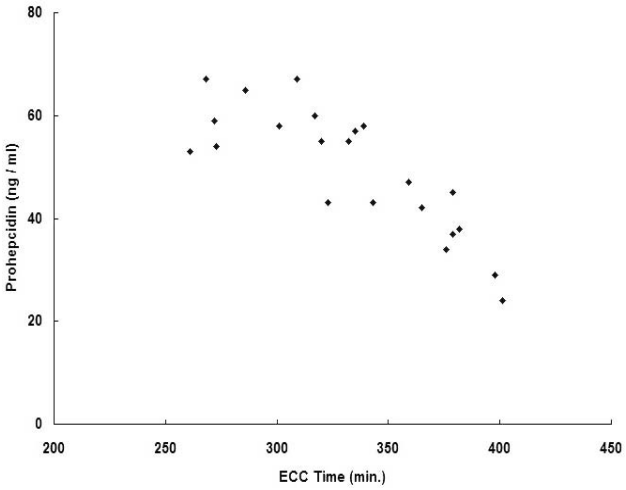

Fig. 4. Correlation between ECC time and minimal perioperative prohepcidin concentrations, $r = –0.82$, $p<0.01$.

When evaluating patients with a quick normalization of prohepcidin into preoperative range within 24 h after separation from CPB (Subgroup 1, n=11), higher peak plasma levels of IL-6 (622.1 ng/l, 474.7-794.6) were revealed in this subgroup compared to patients with delayed prohepcidin normalization (Subgroup 2, n=11), their IL-6 peak levels were 429.9 ng/l (359.6-496.0) ($p<0.05$ between subgroups). Similarly, IL-8 peak postoperative concentrations tended to be higher in Subgroup 1 in relation to Subgroup 2, but the differences were not significant on $p<0.05$.

Discussion

This study has demonstrated that the large cardiac surgery with DHCA and CPB is an inductor of a deep transient decrease of prohepcidin plasma concentrations. Minimal postoperative prohepcidin levels were related to extracorporal circulation time and did not correlate with IL-6 or other tested inflammatory parameters. Nevertheless higher IL-6 concentrations advanced prohepcidin normalization after its initial decline. Postoperative changes of plasma iron or transferrin concentrations and hemoglobin concentration did not correlate with plasma prohepcidin levels.

Hepcidin, a small cysteine-rich cationic peptide, originates from extrahepatic enzymatic cleavage of its prohormone prohepcidin (Kulaksiz *et al.* 2004). Prohepcidin gene is expressed in different tissues, but the liver is a main source of a peptide detected in plasma and urine. Hepcidin is the key regulator of iron metabolism and the mediator of anemia of chronic diseases. Iron overload and inflammation are main inductors of hepcidin (Flanagan *et al.* 2007), where the latter has been linked to hepcidin *via* increased IL-6 (Hoppe *et al.* 2008). In humans, increased urinary hepcidin levels were detected in patients with chronic infections or severe inflammatory diseases. In chronic inflammatory conditions, increased hepcidin levels correlate with increased ferritin levels (Nemeth and Ganz 2006).

To the best of our knowledge, the present study evaluates for the first time the effect of cardiac surgery on plasma prohepcidin concentrations on a larger group of patients. Cardiac surgery with CPB induced a cytokine response characteristic for inflammation. Due to the combination of local trauma, ECC, and pulmonary and myocardial reperfusion, PEA leads to substantial changes in the immune system resulting to cytokine network activation with consequent endocrine, metabolic and cardiovascular signs of SIRS.

Recently, Hoppe *et al.* (2008) reported serum hepcidin and serum prohepcidin concentrations in five male patients before and after heart surgery. There were significant alterations in both serum hepcidin and serum prohepcidin. Serum prohepcidin decreased after 48 h compared with preoperative values, whereas serum hepcidin increased within a 144-h time frame. In our patients, minimal prohepcidin concentrations were revealed already after separation from CPB. The following return to initial levels was observed within 24-48 h after the separation from CPB.

Our results showed prohepcidin as a negative acute phase reactant with a strong initial decrease after PEA. Currently, relations between IL-6 levels and duration of prohepcidin disturbance were revealed in our study. It remains to be determined whether the initial decrease of prohepcidin documented in our study is due to proteolytic trimming of serum prohepcidin as recently hypothesized Hoppe *et al.* (2008) or there are other factors restraining prohepcidin elevation or inhibiting its production.

Contradictions between *in vitro* studies, illustrating direct stimulation of prohepcidin gene *via* inflammatory signals, and *in vivo* findings were reported recently. Kemna *et al.* (2005) pointed out the lacking correlation between the urinary hepcidin elevation and serum prohepcidin on human endotoxemia model. Serum prohepcidin concentration did not reflect inflammation or iron metabolism changes. Authors discussed technical limitations of serum assays for an explanation. Due to methodological problems, no definitive method has been used for hepcidin assessment in plasma.

Minimal perioperative prohepcidin concentrations correlated inversely with ECC duration in our patients. We consider hypoxia as a potential down-regulating factor of prohepcidin synthesis explaining its decrease after the cardiac surgery with circulatory arrest. Our hypothesis is supported the recent experimental findings of Benedict *et al.* (2007), who demonstrated that transient hypoxia causes a reduction in circulating prohepcidin concentrations in men. In their study, circulating prohepcidin but not hepcidin concentrations were distinctly lower 150 min after the end of hypoxia.

The importance of the IL-6-hepcidin axis *in vivo* is not fully understood and the knowledge of IL-6 role in prohepcidin regulation is based mainly on the *in vitro* experiments. IL-6 stimulates directly prohepcidin expression *in vitro*. In human hepatocyte cultures, prohepcidin expression was induced after direct exposure to lipopolysaccharide and this response could be partially ablated by the addition of anti-IL-6 antibodies (Kemna *et al.* 2005).

Prohepcidin mRNA was dramatically induced by IL-6 *in vitro*, but not by IL-1 or TNFα, demonstrating that human hepcidin is a type II APP (Nemeth *et al.* 2003). Nevertheless, Lee *et al.* (2005) described that other cytokines such as IL-1α and IL-1β strongly stimulate hepcidin transcription in incubating murine hepatocytes. Wrighting and Andrews (2006) recently reported signal transducer and activator of transcription 3 (STAT-3) as the crucial transcription factor in the upregulation of human hepcidin by IL-6. Involvement of STAT-3 is a common mechanism for induction of all APP belonging to type II proteins. Our results showed that the time course of plasma prohepcidin after aseptic surgery is distinct from CRP, another type II APP tested in our patients. CRP elevation after one-time inflammatory stimulus was prolonged and delayed to all other inflammatory parameters.

Prohepcidin failed to correlate with parameters of iron metabolism postoperatively. Our findings are in agreement with the results of Kemna *et al.* (2008). Similarly Taes *et al.* (2004), Hadley *et al.* (2006), and Benedict *et al.* (2007) concluded that hepcidin, but not prohepcidin, is significantly related to the iron status.

In summary, strong decrease of plasma prohepcidin concentrations was induced by PEA with DHCA. Amplitude of prohepcidin decline was related to the time of CPB, while elevated IL-6 levels were inversely associated with duration of prohepcidin decline. Postoperative prohepcidin did not correlate with markers of iron metabolism or hemoglobin concentrations within a 72-h period after separation from CPB.

Conflict of Interest

There is no conflict of interest.

Acknowledgements

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Abbreviations

APP – acute phase proteins, BMP – bone morphogenetic protein, CPB – cardio-pulmonary bypass, CRP – C-reactive protein, CTEPH – chronic thromboembolic pulmonary hypertension, DHCA – deep hypothermic circulatory arrest, ECC – extracorporeal circulation, IL – Interleukin, mPAP – mean pulmonary artery pressure, NYHA – New York Heart Association, PEA – pulmonary endarterectomy, SIRS – systemic inflammatory response syndrome, TNFα – tumor necrosis factor-α.

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