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Plasma cytokines do not reflect expression of pro- and anti-inflammatory cytokine mRNA at organ level after cardiopulmonary bypass in neonatal pigs

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Background: Plasma concentrations of inflammatory markers are increased in response to the trauma of cardiac surgery and cardiopulmonary bypass (CPB). It is, however, unknown whether the plasma cytokine levels and cytokine mRNA expression at organ level reflect each other.

Methods: Twenty-six piglets (17–19 days) were allocated to the sham-group (sternotomy only, n=13) or to the CPB-group (sternotomy, 120 min CPB procedure with 60-min aortic cross-clamp, n=13). The pigs were observed for 0.5 h or 4 h post-CPB. Plasma levels of IL-1 β , IL-6, IL-8 and IL-10 and mRNA expression of TNF- α , IL-1 β , IL-6, IL-8, IL-10 and iNOS in organs were registered with concomitant changes in oxygenation index (OI) and expiratory nitric oxide (NO).

Results: In pigs killed 0.5 h post-CPB there was a significant increase in IL-10 mRNA in the lungs and kidneys compared with the sham-group. IL-1 β mRNA was detectable in the kidneys and lungs of the CPB-pigs, while IL-6 mRNA was up regulated only in lungs. In pigs killed 4 h post-CPB a significantly higher IL-6 mRNA was found in heart tissue and a lower

IL-10 mRNA was found in lungs of CPB pigs compared with the sham-group. There was a concomitant significant increase in OI and increased plasma IL-8 and IL-10 concentrations in the CPB-pigs compared with the sham-pigs.

Conclusion: The cytokine mRNA expression pattern was very different for the pigs killed already 0.5 h after the CPB procedure compared with the pigs killed 4 h post-CPB. The plasma cytokine levels poorly reflected mRNA expression of the pro- and anti-inflammatory cytokines.

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Key words: CPB; cytokines; lung function; mRNA; pigs; RT-PCR.

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CARDIOVASCULAR, pulmonary and renal dysfunction are common complications after cardiac surgery performed with cardiopulmonary bypass (CPB) and are especially pronounced in the paediatric patient (1–3). This postoperative temporary organ dysfunction is thought to be a consequence of the systemic inflammatory response elicited by cardiac surgery and CPB (4).

Systemic inflammation is characterized by activation of the immune system with the release of a number of inflammatory mediators accompanied by simultaneous changes in the cell-mediated immunity.

The systemic inflammatory response has been defined as a Systemic Inflammatory Response Syndrome (SIRS). Some of the initial mediators of SIRS are pro- and anti-inflammatory cytokines, which are mainly synthesized by activated monocytes and macrophages in response to external stimuli such as surgical trauma and CPB.

Cytokine production may remain a local response to a minor tissue injury. In the case of an extensive stimulus, however, cytokines can be detected in peripheral blood. In patients with established SIRS or organ dysfunction both pro- and anti-inflammatory cytokines are detectable in markedly increased amounts in peripheral blood. We have previously demonstrated that the CPB procedure itself induces a well-defined pro- and anti-inflammatory cytokine

The work was carried out at the Institute of Experimental Research, Aarhus University Hospital, Denmark.

response in neonatal pigs (5) analogous to the human response to cardiac surgery performed during CPB (6, 7). However, plasma levels of cytokines do not necessarily reflect tissue concentrations or their effect at organ level and may therefore be a poor reflection of the processes occurring at organ level.

This study was designed to investigate production of cytokines at organ level during systemic inflammation. The mRNA expression of pro- and anti-inflammatory cytokines at organ level was investigated in neonatal pigs exposed to a standardized CPB procedure or sham operation (sternotomy without CPB). Concomitant changes in plasma cytokine levels and pulmonary function estimated as oxygenation index ($= \text{mean airway pressure} \times \text{FiO}_2 \times 100 / \text{PaO}_2$) were monitored during the study period. For pigs killed 0.5 h post-CPB, expiratory nitric oxide (NO) was also monitored.

Material and methods

Animals

Twenty-six piglets aged 17–19 days (3.5–5.5 kg) were divided into a CPB-group ($n = 13$, where seven pigs were killed 0.5 h post-CPB and six pigs were killed 4 h post-CPB) or a sham-group ($n = 13$, where seven pigs were killed time-matched to the 0.5-h post-CPB-group and six pigs were killed time-matched to the 4-h post-CPB-group). Fifteen donor pigs were included in the study.

Anaesthesia

The Danish Inspectorate for Animal Experimentation approved the study, and the investigation conforms to the 'Principles of Laboratory Animal Care' published by the US National Institute of Health (NIH publication no. 86–23, revised 1985).

The anaesthetic procedure was performed as previously described (5), with ketamine (10 mg kg^{-1} i.m.) and midazolam (0.5 mg kg^{-1} i.m.) used as pre-medication. Further ketamine (10 mg kg^{-1}) was given i.v. before intubation and mechanical ventilation. Anaesthesia was maintained with intravenous fentanyl ($50 \mu\text{g kg}^{-1} \text{ h}^{-1}$) and pancuronium (2 mg h^{-1}) for neuromuscular relaxation.

Blood collection

Catheters (5-F, St Jude Medical Company, St Paul, Minnesota, USA) were inserted into the internal carotid artery and the external jugular vein for blood sampling and blood donation, respectively.

Blood samples for cytokine measurements were collected into EDTA-Vacutainers (Becton-Dickinson, Meylan-Cedex, France) at the following times: immediately after induction of anaesthesia (baseline), 5 min after weaning off CPB (end-CPB), and either at 0.5 h after CPB for pigs killed at this time (0.5 h post-CPB) or at 2 h post-CPB procedure and 4 h post-CPB procedure for pigs killed 4 h after the CPB.

Surgery

The surgical procedure and CPB were performed as previously described (5, 8). Briefly, a median sternotomy was performed, heparin was administered and the CPB-pigs were connected to a paediatric cardiopulmonary bypass set-up. Furosemide and sodium bicarbonate was added to the prime volume. Cold cardioplegia arrested the heart. Mannitol and a second cardioplegic dose were administered 30 min after aortic cross-clamping. The aortic cross-clamping time was 60 min (at 25°C) and total bypass time approximately 120 min. The animals were rewarmed to 37°C , mechanical ventilation was resumed, CPB was stopped, and the heart was defibrillated if needed. Protamine sulphate was administered. Donor blood was given intravenously to maintain a mean arterial pressure above 50 mmHg.

In the sham-group, anaesthesia, sternotomy and mechanical ventilation were identical to the CPB-group. All animals in the sham-group were heparinized and protamine sulphate was administered time-matched to the CPB-group. Moreover, they received a donor blood volume equivalent to the CPB-group. The sham group did not receive mannitol and furosemide. The observation period was identical to the CPB-group.

Reverse transcriptase-polymerase chain reaction (RT-PCR)

At the end of the experiment all animals were quickly eviscerated and multiple biopsies from the different organs without macroscopic visible blood were transferred to storage tubes. From the pigs killed 0.5 h post-CPB, biopsies were harvested from the kidneys, lungs, liver and heart, while the pigs killed 4 h post-CPB had biopsies taken from the heart, lung and liver. The biopsies were immediately snap-frozen in liquid nitrogen and stored at -80°C until RNA extraction. RNA was extracted using trizol according to the manufacturer's instructions and reversed transcribed to complementary DNA (cDNA) using a 'First Strand cDNA Synthesis Kit' (Amersham-Pharmacia Biotech AB, Amersham Biosciences, Buckinghamshire, UK).

with random hexamers according to the manufacturer's instructions. Briefly, 3 µg of mRNA in 8 µl of water was heated to 65°C for 10 min, after which it was chilled on ice for 2 min and added to 5 µl of Bulk First Strand cDNA Mix, 1 µl of DTT Solution and 1 µl of pd (N)6 primers (random hexadeoxynucleotides) in a 0.02-µg ml⁻¹ solution. The solution was mixed thoroughly and heated to 37°C for 1 h. Of the resulting solution 1 µl was mixed with 14.6 µl of water and added to 1 µl of forward primer and 1 µl of reverse primer, both in a solution of 0.02 µg µl⁻¹ and 2 µl of 10×PCR buffer (Amersham Pharmacia Biotech AB) and 0.4 µl of *Taq* DNA polymerase (Amersham Pharmacia Biotech AB). The thermal cycling conditions were set to 95°C for 2 min, followed by 95°C for 30 s, 55°C for 45 s and 72°C for 45 s, repeated 30 times and followed by 72°C for 10 min. The specific porcine primers were as follows:

Hypoxanthine phosphoribosyl transferase (HRPT) forward primer: 5'-TGA ACG TCT TGC TCG AGA TG-3', HRPT reverse primer 5'-TCA AAT CCA ACA AAG TCT GGC-3'. IL-10 forward primer: 5'-GCG ACT TGT TGC TGA CCG G-3', IL-10 reverse primer: 5'-GAA CCT TGG AGC AGA TTT TG-3'. IL-1β forward primer: 5'-ATG GCC CCA AAG AGA TGA AG-3', IL-1β reverse primer: 5'-GAA CAC CAC TTC TCT CTT CA-3'. IL-8 forward primer: 5'-ACT TCC AAA CTG GT GTT GC-3', IL-8 reverse primer: 5'-CTG TTG TTG TTG CTT CTC AG-3'.

TNF-α forward primer: 5'-AAC CTC AGA TAA GCC CGT CG-3'. TNF-α reverse primer: 3'-ATG GCA GAG AGG AGG TTG AC-5', IL-6 forward primer: 5'-TGC TCT TCA CCT CTC CGG AC-3', IL-6 reverse primer: 3'-CTT TCT GGA GGT AGT CCA GG-5'. iNOS forward primer: 5'-GTC CAA CCT GCA GGT CTT CG-3', iNOS reverse primer: 5'-CCA TGA TGA TGG TCA CAT TCT GC-3'.

HRPT primers were used for internal standards and the cytokine/HRPT ratio was obtained using IMAGE-QUANT software (Molecular Dynamics, Amersham Biosciences, Buckinghamshire, UK).

Cytokine measurement

Fresh-frozen plasma samples (–80°C) obtained from EDTA-stabilized blood at baseline, end-CPB, 0.5 h post-CPB or 4 h post-CPB were used for cytokine measurements with enzyme-linked immunosorbent assays (ELISA). Commercially available kits specific for porcine IL-1β, IL-6 (R & D systems, Minneapolis, MN, USA) and IL-8 and IL-10 (Biosource International, Camarillo, CA) were used according to the manufacturers' instructions. The detection levels for

the assays were: IL-1β, 10 pg ml⁻¹; IL-6, 10 pg ml⁻¹; IL-8, 10 pg ml⁻¹; and IL-10, 3 pg ml⁻¹.

Clinical parameters

Oxygenation index (OI) was calculated as mean airway pressure × FiO₂ × 100/PaO₂ (mmHg) in all piglets every hour of the experiment. All piglets killed 0.5 h post-CPB had their expiratory NO continuously measured on-line using a chemiluminescent NO analyser (Advanced Pollution Instrumentation, Inc., San Diego, CA). Recommendations for standardized procedures for the online measurement of exhaled lower respiratory NO was followed (9). Mixed tracheal air was continuously sampled (275 ml min⁻¹) through a PVC suction catheter (CH 08, 33 cm; UNO, Copenhagen, Denmark) placed on the outside of the cuffed endotracheal tube. Data was recorded as the average of 5-min continuous samplings after initial signal stabilization. Range was 0–100 p.p.b. Data update occurred every 7 s. Stability (defined as standard deviation of 10 min continuous measurements) at zero and span (90 p.p.b.) was 0017 p.p.b. and 0048 p.p.b., respectively. Requiring a signal/noise ratio of >2, the lower detectable limit was found to be 0.1 p.p.b. at our sample rate. Rise and fall time to a 95% square signal change was <2 min.

Statistics

RT-PCR

Data for mRNA expression were tested for normal distribution using the Kolmogorov-Smirnov test. Using unpaired Student's *t*-test or the Mann-Whitney rank sum test compared the two groups. Data are given as the mean of the ratios of the band intensities of cytokine mRNA over the corresponding HRTP mRNA and standard deviation (SD).

Clinical parameters

Changes in delta values (baseline: end of observation period) in OI and expiratory NO for the two groups were compared with Student's unpaired *t*-test. The OI for the 4-h post-CPB group and the corresponding sham-group have already been published (4).

Cytokines

The groups were logarithmically transformed in order to obtain normality. Repeated measurement analysis was performed and hence changes in cytokine concentrations over time were compared between the two groups. The IL-8 and IL-10 plasma levels for the

4-h post-CPB group and the corresponding sham-group have already been published (5).

P-values <0.05 were considered significant.

Results

Piglets killed 0.5 h after termination of the CPB procedure

RT-PCR

There was a significant up-regulation of mRNA IL-10 in the lungs of the CPB-group compared with the sham group (1.2 ± 0.7 vs. 0.5 ± 0.4 , $P = 0.003$). Furthermore, IL-10 was only detectable in the kidneys from the CPB-pigs (1.2 ± 0.9 , $P = 0.01$). Only the lungs from the CPB-group showed IL-6 mRNA expression (1.5 ± 0.2 , $P < 0.001$). TNF- α was higher in the lungs of the sham-pigs (1.5 ± 0.7 in CPB-pigs vs. 4.0 ± 2.6 in sham-pigs, $P = 0.04$). In the other organs there was no difference between the groups (IL-8 expression was only detectable in the lungs but not in other organs in either group; 1.1 ± 0.5 vs. 1.5 ± 1.5 for CPB-pigs and sham-pigs, respectively, $P = 0.5$). Expression of iNOS mRNA did not differ between the groups in any organ (lungs: 1.3 ± 0.6 vs. 0.7 ± 0.6 , $P = 0.1$; kidneys: 1.9 ± 1.3 vs. 3.5 , $P = 0.1$, heart: 0.8 ± 0.2 vs. 1.2 ± 0.6 , $P = 0.1$, liver: 1.2 ± 0.3 vs. 1.2 ± 0.2 , $P = 0.8$ for CPB-pigs and sham-pigs, respectively). IL-1 β was only detectable in the kidneys (1.33 ± 1.1 , $P = 0.02$) and the lungs (1.4 ± 0.6 , $P = 0.001$) of the CPB-pigs and was not detectable at all in the sham-pigs nor in the liver and the heart of the CPB-pigs. The cytokine mRNA profile for the different organs are shown in Fig. 1.

Plasma cytokines

The CPB procedure elicited a significant increase in plasma IL-10 at the end of the CPB procedure compared with baseline and 0.5 h post-CPB ($P < 0.001$) (Fig. 2). IL-8 peaked immediately after termination of the CPB procedure in the CPB-group (Fig. 3). There was a significant difference between the two groups in plasma IL-8 levels ($P = 0.04$). Only three of seven pigs in the CPB-group and two of seven pigs in the sham-group showed detectable IL-6 levels. IL-1 α was not detectable in any of the groups.

Clinical parameters

The pulmonary function was impaired in the CPB-group estimated as an increased OI between baseline and 0.5 h post-CPB (3.28 ± 2.74 vs. -0.01 ± 1.19 at 0.5 h post-CPB, $P = 0.002$). The expiratory NO concen-

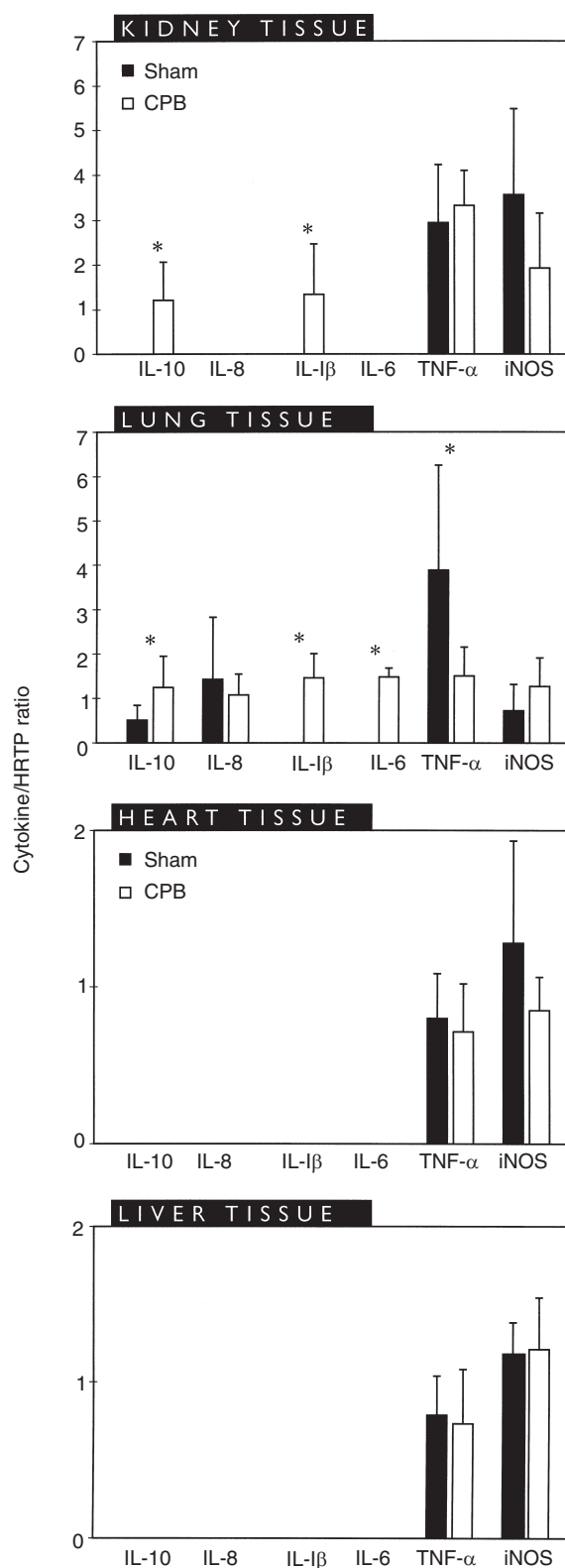


Fig. 1. Cytokine mRNA profile at organ level at 0.5 h after termination of the cardiopulmonary bypass (CPB) procedure. Results are represented as the mean of the ratios of the band intensities of cytokine mRNA over the corresponding HRTP mRNA with standard deviation. * $P < 0.05$ for differences between groups.

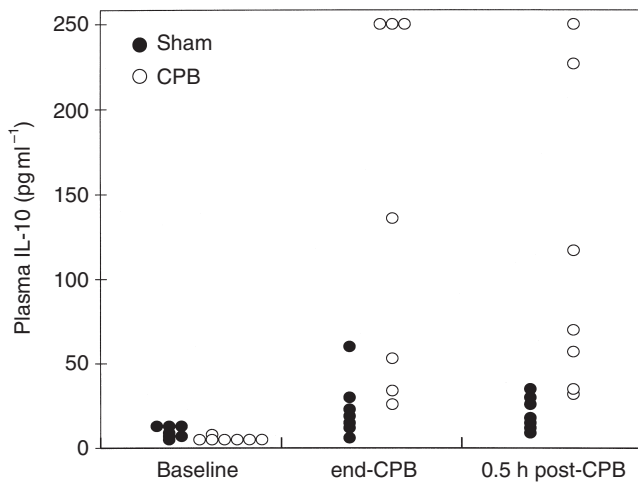


Fig. 2. The individual concentrations of plasma IL-10 in the sham-group (●) and in the cardiopulmonary bypass (CPB)-group (○). Each symbol represents one pig. * $P < 0.05$ for differences between groups.

tration decreased in both groups compared with baseline (delta value of $1.19 \text{ p.p.b.} \pm 0.7 \text{ p.p.b.}$ in the CPB-group vs. $0.49 \text{ p.p.b.} \pm 0.45$ in the sham-group, $P = 0.09$ or NS).

Piglets killed after 4 h

RT-PCR (Fig. 4)

IL-6 mRNA was significantly higher in the heart tissue of pigs subjected to CPB compared with the sham-group (0.56 ± 0.35 vs. 0.18 ± 0.18 , $P = 0.04$), whereas there was no difference between the CPB-group and the sham-group for IL-6 mRNA in the other organs (lung: 0.35 ± 0.36 vs. 0.30 ± 0.30 ; liver: 0.68 ± 0.9 vs. 0.21 ± 0.3 , respectively). In the lungs of the CPB-pigs, IL-10 mRNA was significantly down-regulated compared the lungs of the sham-pigs (1.18 ± 0.6 vs. 1.33 ± 0.7 , $P = 0.04$), whereas there was no difference in IL-10 expression between the CPB-group and the sham-group in the other organs (heart: 0.8 ± 0.5 vs. 0.118 ± 0.6 ; liver: 0.6 ± 0.6 vs. 1.2 ± 0.8 , respectively). mRNA expression of IL-8 was also without statistical significant difference between the CPB-group and the sham-group (lung: 1.0 ± 0.4 vs. 1.1 ± 0.8 ; heart: 1.2 ± 0.6 vs. 1.56 ± 0.8 ; liver: 0.9 ± 0.5 vs. 1.5 ± 1.4 , respectively). IL-1 β expression did not differ between the CPB-group and the sham-group (lungs: 0.5 ± 0.3 vs. 0.5 ± 0.2 ; heart: 0.6 ± 0.3 vs. 0.6 ± 0.4 ; liver: 0.7 ± 0.3 vs. 0.7 ± 0.6 , respectively). TNF- α mRNA expression was without difference between CPB- and sham-pigs (lungs: 2.26 ± 1.37 vs. 1.11 ± 0.94 ; heart: 1.09 ± 0.65 vs. 0.82 ± 0.47 ; liver: 3.74 ± 3.74 vs. 4.16 ± 3.14). Expression of iNOS was not different between the CPB-group and the sham-

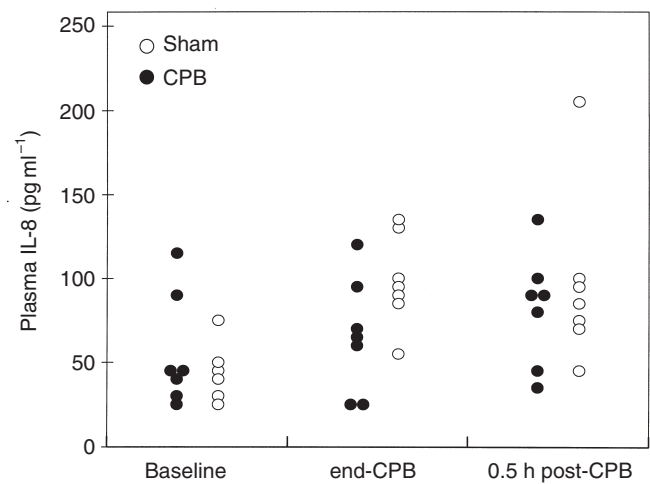


Fig. 3. The individual concentrations of plasma IL-8 in the sham-group (○) and in the cardiopulmonary bypass (CPB)-group (●). Each symbol represents one pig. * $P < 0.05$ for differences between groups.

group (lungs: 0.9 ± 0.6 vs. 1.1 ± 0.4 ; heart: 1.1 ± 0.3 vs. 1.3 ± 1.1 ; liver: 0.8 ± 0.2 vs. 1.0 ± 0.2 , respectively).

Plasma cytokines

Increased plasma cytokine levels of IL-8 were detectable in both groups but to a greater extent in the CPB-pigs compared with the sham-pigs, whereas IL-10 was detectable in the CPB-group only (5).

Clinical parameters

The oxygenation index increased significantly more between baseline and 4 h post-CPB in the CPB group compared with the sham-group (4).

Discussion

The basic finding in this study was a very different cytokine expression at organ level in the pigs killed 0.5 h and 4 h after the CPB procedure. Moreover, the picture of cytokine mRNA at organ level poorly reflects the circulating level of the associated cytokine protein.

It is interesting that IL-6 mRNA was only detectable in the lung tissue of the CPB-pigs killed 0.5 h post-CPB, while IL-6 mRNA 3.5 h later could be demonstrated in the tissue of the lung, liver and heart in both the sham- and CPB-pigs. The heart has previously been shown to be a source of IL-6 synthesis in dogs exposed to CPB (10) or after ischemia and reperfusion (11). Interestingly, plasma IL-6 was hardly found in any of the pigs in the present study.

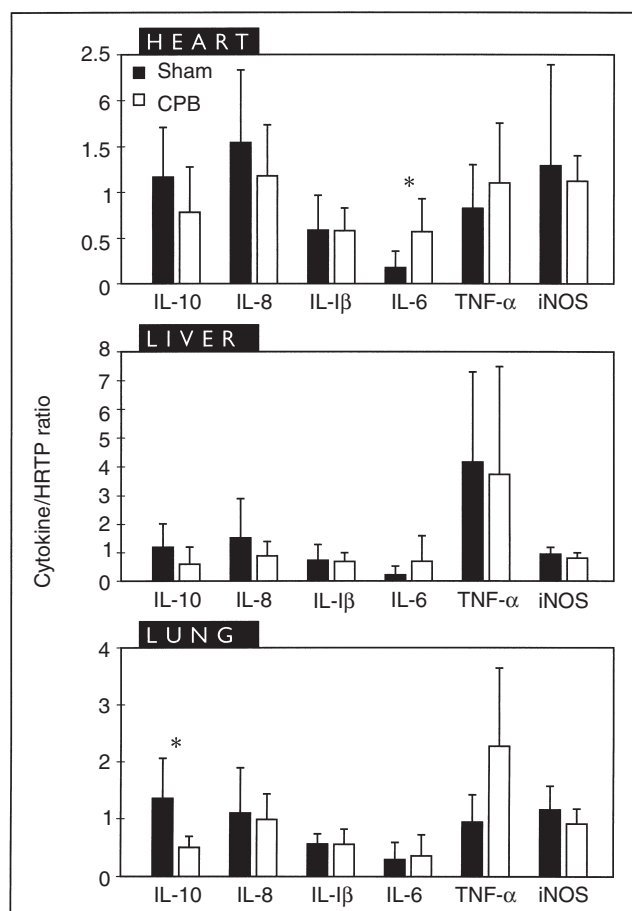


Fig. 4. Cytokine mRNA profile at organ level at 4h after termination of the cardiopulmonary bypass (CPB) procedure. Results are represented as the mean of the ratios of the band intensities of cytokine mRNA over the corresponding HRTP mRNA with standard deviation. * $P < 0.05$ for differences between groups.

Atrial and skeletal muscle biopsies have been harvested from children undergoing heart surgery during CPB. Production of IL-8 mRNA occurred in most children in both myocardium and skeletal muscle (12). In the present study IL-8 mRNA was only observed in the lungs 0.5h post-CPB, while it was found in both the heart, liver and lung tissue 4h later in the CPB- as well as the sham-group. Interestingly, the higher plasma IL-8 concentrations found in the pigs subjected to CPB compared with the sham-pigs were not accompanied by differences at organ level in the two groups.

The finding that mRNA of certain pro- and anti-inflammatory cytokines are detectable only in the lungs and the kidneys of pigs 0.5h after exposure to CPB raises questions as to their physiological and pathophysiological significance. It is well known that the lungs and kidneys are organs at high risk for postoperative dysfunction (1, 4, 13). The exact role of

local cytokine production induced by the CPB procedure remains to be clarified, but the impaired oxygenation found in all CPB-pigs might be a consequence of the inflammatory state manifested as mRNA-cytokine production.

The stimulus for increased mRNA expression is unknown, but ischemia-reperfusion injury is a possibility for the lungs in relation to CPB but is less possible for the kidneys. Other factors such as hypotension, hypoxia, acidosis and reperfusion, which induce cell activation and production of inflammatory mediators, may contribute to renal dysfunction (14).

The fact that iNOS was found in all tissues from the pigs without a difference between the CPB- and sham-group at any time could indicate a constitutive expression without any up-regulation in response to external stimuli (15). Moreover, there was also an equal decrease in expiratory NO in the two groups that terminated 0.5h post-CPB. This decrease in exhaled NO after CPB is in accordance with human studies (16).

Systemic inflammation seems to be a dynamic process that may lead to a wide variety of clinical manifestations from uncomplicated recovery to severe multiorgan failure. However, the contribution of the different organs to cytokine production seems to differ with respect to the type of cytokine. The systemic inflammatory response induced by the CPB procedure reflects a paradox of simultaneous activation and depression of the immune system. In human and animal studies activation is manifested by increased plasma levels of pro-inflammatory cytokines (TNF- α , IL-1 β , IL-6 and IL-8) accompanied by an anti-inflammatory state characterized by increased plasma levels of anti-inflammatory cytokines such as IL-10 (5–7, 14, 17). Plasma IL-1 α was, however, not detectable in plasma in contrast to expression of mRNA of IL-1 α at organ level. TNF- α and IL-1 α are, however, both cytokines that show a very inconsistent release pattern in plasma in patients undergoing cardiac surgery with CPB (6).

It should be kept in mind that plasma cytokines might be regarded as a spillover from organs and, as such, just 'the tip of the iceberg' (18). It should also be kept in mind that increased levels of mRNA do not necessarily result in similarly increased levels of proteins, neither at organ nor at plasma level. However, in a recent CPB study in pigs it was demonstrated that increased expression of porcine mRNA IL-10 and TNF- α correlated with an increased production of IL-10 and TNF- α in the myocardium (19).

In the present study the plasma IL-8 response was found at higher levels in the CPB-group compared with the sham-group. Interestingly, plasma IL-10 was only detectable in the CPB-pigs regardless of the

length of observation. It was demonstrated in a previous study that peak levels occurred immediately after termination of the CPB procedure with a return to baseline levels 4 h after CPB (5). This finding suggests that the IL-10 response to the CPB procedure may be very temporary. As part of the systemic inflammatory response, IL-10 mediates an anti-inflammatory response that balances the pro-inflammatory response. However, down-regulating mediators other than IL-10 are also capable of modifying the SIRS. Such a down-regulating compound may be endogenous morphine, which is detectable in plasma in increasing amounts after CPB in humans and in pigs (20, 21).

In conclusion, the cytokine mRNA expression pattern was very different for the pigs killed already 0.5 h after the CPB procedure compared with the pigs killed 4 h post-CPB. This underlines the relevance of evaluating the time profile of SIRS in organs after CPB. Interestingly, cytokine mRNA up-regulation occurs in kidney, lung and heart tissue after sternotomy and CPB. Plasma cytokine levels, however, poorly reflect mRNA expression of pro- and anti-inflammatory cytokines at organ level.

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