THE IMMUNOLOGICAL AND OXIDATIVE EFFECTS OF APPLYING AN EXTRACORPOREAL CIRCUIT IN A NEONATAL SEPSIS MODEL

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ABSTRACT

We aimed to study the changes in cytokines and oxidative mediators in a neonatal sepsis model when applying an extracorporeal circuit (ECC). Of 28 anesthetized and mechanically ventilated 3-day-old piglets, 14 underwent cecal ligation and perforation (CLP), of which 7 underwent ECC for 3

Abbreviations:
ECC: Extracorporeal circuit
CLP: Cecal ligation and perforation
IL-6: Interleukin-6
IL-10: Interleukin-10
TNF: Tumor necrosis factor
IFN-γ: Interferon gamma
TH: Total hydroperoxide
NOx: Nitric oxide metabolites
hours from 3 to 6 h after CLP. The remaining 14 were sham, of which 7 underwent ECC. Serum interleukin (IL)-6, IL-10, tumor necrosis factor (TNF), interferon gamma (IFN-γ), total hydroperoxide (TH), and nitric oxide metabolites (NOx) were measured at pre-CLP and at 3, 6, and 9 h in the CLP groups, and continued in the sham groups at 12, 15, 18, and 24 h. The CLP group with ECCs compared to the CLP group without it showed higher IL-6, IL-10, and NOx at 6 h and higher TH at 6 and 9 h. The sham group with ECCs compared to the one without it showed higher IL-6 and IL-10 at 12, 15, and 18 h, TH at 6 and 9 h, TNF at 6 h, and IFN-γ at 9 h.

Applying ECCs provoked a window of cytokines and free radicals elevation, which could be hazardous in critically ill newborns, especially after abdominal surgeries complicated with sepsis.

Key words: newborn, infection, cytokine, free Radicals, circulation, shock

INTRODUCTION

The use of extracorporeal circuits (ECCs) in critically ill patients is frequent in adult and neonatal intensive care units for direct hemofiltration, direct hemoperfusion, and extracorporeal membrane oxygenation (ECMO). The contact between the blood and the various artificial surfaces of the extracorporeal system leads to the so-called postperfusion syndrome (or postpump syndrome), which can escalate in severe cases into the systemic inflammatory response syndrome (SIRS), acute lung failure (ARDS: adults respiratory distress syndrome), and sepsis, or even multiple organ failure (MOF).

It has been reported in an in vivo study in adult patients, and in an in vitro study using adult human blood, that applying ECCs would induce some degree of imbalance between oxidants and antioxidants resulting in oxidative stress. On the other hand, in non-septic pediatric patients undergoing cardiopulmonary bypass, ECC application was shown to decrease circulating oxidants with a latent increase in inflammatory mediators.

During Gram-negative bacterial sepsis syndrome, the stimulation of macrophages and monocytes by the endotoxin results in the acute release of early inflammatory mediators such as tumor necrosis factor (TNF) and interleukin (IL)-1, and later interferon (IFN)-γ.

IL-6, which has been regarded as a proinflammatory cytokine is now considered to be both proinflammatory and anti-inflammatory. On the other hand, IL-10, a well known anti-inflammatory cytokine, plays an important role in the development of a syndrome known as compensatory anti-inflammatory response syndrome (CARS). Furthermore, it has been reported that reactive oxygen species (ROS) and nitric oxide (NO) play a significant role in the pathogenesis of neonatal sepsis and its complications. The total hydroperoxide (TH) represents a measure of overall oxidative damage; it includes the intermediate oxidative of lipids, peptide, and amino acids.
The immunological response to infection in neonates differs compared to elder children and adults\textsuperscript{12}, and therefore the neonatal immune response when exposed to an immunological stimulant may be different or manifest in another manner than what is observed in elder children or in adults.

This study was to evaluate the changes in the immunological response after applying an ECC to a characterized neonatal sepsis model cecal ligation and perforation (CLP)\textsuperscript{13} and its sham.

MATERIAL AND METHODS

Animal preparation:

The experiments were performed in adherence to National Institute of Health guidelines on the use of experimental animals, and the protocol was approved by the Ethics Committee of the Nagoya City University Graduate School of Medical Sciences. Twenty-eight (7 in each of four groups: CLP with ECCs, CLP without ECCs, sham with ECCs, and sham without ECCs) mixed-strain newborn piglets were obtained on their third day of life from a local farmer. They were transported on the day of the procedure. The mean body weight was 1690 ± 62.7 g (Mean ± SEM) in the CLP group with ECCs, 1760 ± 35.6 g in the CLP group without ECCs, 1775 ± 184 g in the sham group with ECCs, and 1715 ± 91.4 g in the sham group without ECCs. The piglets were premedicated with ketamine chloride (10 mg/kg intramuscularly), after which anesthesia was induced with pentobarbital sodium (20 mg/kg intravenously) and maintained by a continuous infusion of pentobarbital sodium (5 mg/kg/h) in 5% glucose solution via a peripheral line at a rate of 5 ml/kg/h throughout the study to avoid hypovolemia. All surgical procedures were performed under sterile conditions. Each piglet underwent a tracheotomy and was intubated with an endotracheal tube (internal diameter 4.0 mm) and ventilated with an infant ventilator (model IV-100, Sechrist, Anaheim, CA, USA). The inspiration/expiration pressures were initially set at 14/4 cm H\textsubscript{2}O with an inspiration time of 0.5 sec under room air. A cut-down procedure was used to insert a 3-Fr polyvinyl catheter in the left femoral artery for blood sampling. Body temperature was maintained by a thermal pad and monitored by a rectal probe. Modified cecal ligation and perforation (cecal devascularization and perforation) was performed on the two CLP groups as previously explained\textsuperscript{13}. Briefly, a paramedian incision approximately 4 cm long was made, sufficient to expose the cecum and terminal ileum. The ileocecal artery was identified ligated near the cecum, resulting in a devascularization of the distal end of the cecum. A 1 cm incision was made in the antimesenteric side. The cecum was gently milked to extrude feces into the peritoneal cavity. Then the abdominal incision was closed in two layers.

Animals in the sham groups underwent the paramedian incision and not the CLP procedure.

The ECCs application:

The ECCs were formed of polyvinylchloride (AP chamber set 15 m, Niporo Corp, Tokyo, Japan) and a peristaltic pump (Iwaki PST-110, Asahi Techno Glass Corp, Tokyo, Japan) between the right femoral artery and the left external jugular vein (arteriovenous ECCs). The ECCs were filled
with 20 cc of blood from a donor piglet. Cross matching was done before the CLP procedure. Blood flow rate through this circuit was 3 ml/kg/min. The ECCs application started after the 3 h sampling for 3 hours until the 6 h sampling time. Low-molecular-weight heparin was applied as an anticoagulant just before the ECCs were started.

**Survival and Experimental protocol:**

The study was continued to the time of spontaneous death in the two CLP groups and continued in the two sham groups until 24 hours when they were sacrificed with a lethal dose of pentobarbital sodium. The survival time in the CLP with ECCs was shorter than in the CLP without ECCs \(9.4 \pm 0.4\) h vs. \(11.6 \pm 1\) h, \(P<0.05\), and both were shorter than in the two sham groups.

In each group, blood samples for IL-6, IL-10, TNF, IFN-\(\gamma\), NOx, and TH assays were aseptically collected from the femoral arterial catheter. Blood samples were taken pre-CLP and at 3, 6, and 9 h after CLP in the two CLP groups and were continued in the sham groups at 12, 15, 18, and 24 h. Each sample was placed into pyrogen-free sterilized tubes.

**Measurements:**

Serum IL-6, IL-10 and TNF were measured using an immunoassay kit specific for porcine IL-6, IL-10 and TNF, respectively (GT, Minneapolis, MN, USA). Serum IFN-\(\gamma\) was measured using ELISA kit specific for porcine IFN-\(\gamma\) (BioSource International, Camarillo, Calif, USA). Duplicate measurements were performed for each sample. The concentrations were calculated based on the obtained standard curves. Nitric oxide metabolites were evaluated by measurement of serum concentration of NO2-+NO3- (NOx). NOx concentration was measured using PFA-310 NO (F.I.A instruments Co., Tokyo, Japan).

The intra- and inter-assay coefficients of variation (CV) of IL-6, IL-10, TNF, IFN-\(\gamma\) and NOx were presented in table 1.

TH production was measured with the free radical analytic system (FRAS), using the reactive oxygen metabolites (d-ROMs) kit (Diacron srl, Italy), as previously described.

It has been reported that replicate measures of TH on the same serum sample showed a within-assay CV of less than 0.5% and a between-assay CV of less than 2.9%.

**Statistical analysis:**

The mean of the four groups (inter-groups) at the same time point until 9 h were compared using the analysis of variance (ANOVA) for repeated measures, followed by the Tukey-Kramer post hoc test. After 9 h the Mann-Whitney test was used to compare between the two sham groups. Data are reported as mean ± SEM. Probability values of less than 0.05 were considered significant. Differences in survival between the two groups were calculated using the Kaplan-Meier test and were compared using the Kruskal-Wallis test followed by the Mann-Whitney test. All data analyses were performed with commercially available statistical analysis software package SPSS (Statistical Package for Social Sciences, Chicago, Illinois, USA).
RESULTS

There were no differences between the four groups in their cytokines, NOx, or TH levels at pre-CLP. There were none between the two CLP groups or between the two sham groups in their serum levels of cytokines, NOx, or TH until 6 h samples were taken.

**Effects on IL-6 and IL-10:**

After the ECCs were applied, the serum IL-6 and IL-10 levels became higher in the CLP group with ECCs than in the CLP group without them at 6 h (Fig. 1-A, B). The mean IL-6 and IL-10 serum levels remained higher at 9 h in the CLP group with ECCs, but these differences did not reach significance (Fig. 1-A, B).

IL-6 serum levels in both CLP groups became higher than in both sham groups at 3, 6, and 9 h. In the sham group with ECCs, IL-6 became higher than in the sham group without ECCs at 12, 15, and 18 h. At 24 h, serum IL-6 in the sham group with ECCs returned to near the levels in the sham group without them (Fig. 1-A).

Serum IL-10 levels in the sham group with ECCs became higher than in the sham group without them at 6, 12, 15, 18, and 24 h (Fig. 1-B).

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**Table 1.** The intra- and inter-assay coefficients of variation (CV) of interleukin (IL)-6, IL-10, tumor necrosis factor (TNF), interferon (IFN)-γ and nitric oxide metabolite (NOx).

<table>
<thead>
<tr>
<th>Sample</th>
<th>Intra-assay coefficients of variation</th>
<th>Inter-assay coefficients of variation</th>
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<tr>
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</tr>
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<td>NOx</td>
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</tbody>
</table>

* The CV% presented are from the manufactory pamphlets of IL-6, IL-10, TNF, IFN-γ and NOx ELISA kits, respectively.
Serial serum measurements of \( \text{IL-6} \) and \( \text{IL-10} \) in CLP neonatal sepsis model \((n = 7)\), CLP with ECCs \((n = 7)\), sham \((n = 7)\), and sham with ECCs \((n = 7)\) until 9 h. The measurements continued in the two sham groups until 24 h. *3 h is the time of starting the extracorporeal circuit; **6 h is the time of stopping the extracorporeal circuit. † The difference between the CLP groups \((†p < 0.05)\).

‡The difference between the CLP and sham groups without ECCs \((‡p < 0.05)\). ¶The difference between the CLP and sham groups with ECCs \((¶p < 0.05)\). *The difference between the sham groups \((⁎p < 0.05)\). The error bar represents the SEM.
Effects on TNF and IFN-γ:

Serum levels of TNF and IFN-γ showed no differences between the two CLP groups, one with and one without ECCs, throughout the study.

TNF serum levels in both CLP groups were higher than in both sham groups with and without ECC at 3, 6, and 9 h. The mean TNF serum levels in the sham group with ECCs were higher than in the other sham group at 6 h and maintained higher levels until the 24 h samples, but a significant difference was detected between the two shams only at 6 h (Fig. 2-A).

The serum levels of IFN-γ showed no differences among all four groups until 9 h when serum IFN-γ levels were higher in both CLP groups than in both sham groups with and without ECCs. Later, at 12 h, the IFN-γ serum levels in the sham group with ECCs were higher than in the other sham group (Fig. 2-B).

Effects on serum NOx and TH:

At 3 h, the serum levels of NOx were higher in both CLP groups with and without ECCs compared to both sham groups. At 6 h, the serum levels of NOx were higher in the CLP group with ECCs than in the group without it. The NOx serum levels in the CLP group with ECCs were higher than in both sham groups at 6 h.

The mean NOx serum levels in the CLP group without ECCs were higher than in both sham groups at 6 h, but these differences were significant only compared to the levels in the sham group without ECCs. No differences were found between the two sham groups in their NOx serum levels throughout the study (Fig. 3-A).

Serum TH became higher in the CLP group with ECCs than in both CLP groups at 6 h and 9 h. The mean TH serum levels in the sham group with ECCs became higher than in both the CLP groups at 6 and 9 h, but the differences did not reach significance. This difference was maintained between the two sham groups with and without ECCs at 12, 15, 18, and 24 h. (Fig. 3-B).

DISCUSSION

In our study on a neonatal sepsis model, the proinflammatory mediators (TNF and IL-6) took the upper hand before we applied the ECC, but similar changes were not evident in serum levels of the anti-inflammatory cytokine IL-10. This immunological balance was due to the active response against the systemic infection induced by the CLP procedure, but after exposure to the ECCs, both the proinflammatory and the anti-inflammatory serum levels were elevated. Toft et al. detected that applying an extracorporeal circuit in a continuous venovenous hemofiltration did not increase the serum levels of the proinflammatory mediator IL-8 or the anti-inflammatory mediator IL-10 in a healthy porcine model, and not a critically ill newborn as in the present study. The main source of IL-6 and IL-10 production in human and murine models are CD4 Th2 cells, CD8 T cells, and monocytes/macrophages.
FIG. 2-A

Fig. 2-A Serial serum measurements of (A) TNF and (B) IFN-γ in CLP neonatal sepsis model \( n = 7 \), CLP with ECCs \( n = 7 \), sham \( n = 7 \), and sham with ECCs \( n = 7 \) until 9 h. The measurements continued in the two sham groups until 24 h. *3 h is the time of starting the extracorporeal circuit; **6 h is the time of stopping the extracorporeal circuit. †The difference between the CLP and sham groups without ECCs \( \%p < 0.05 \). ‡The difference between both the CLP and sham groups with ECCs \( \|p < 0.05 \). ††The difference between the sham groups \( \times p < 0.05 \). The error bar represents the SEM.
Fig. 3. Serial serum measurements of (A) NOx and (B) TH in CLP neonatal sepsis model (n = 7), CLP with ECCs (n = 7), sham (n = 7), and sham with ECCs (n = 7) until 9 h. The measurements continued in the two sham groups until 24 h. *3 h is the time of starting the extracorporeal circuit; **6 h is time of stopping the extracorporeal circuit. † The difference between the CLP groups (†p < 0.05). ‡ The difference between the CLP and sham groups without ECCs (‡p < 0.05). ¶ The difference between both the CLP and sham groups with ECCs (¶p < 0.05). ※ The difference between the sham groups (※p < 0.05). One Carr unit corresponds to the colour development caused by a H$_2$O$_2$ solution at a concentration of 0.08%. The error bar represents the SEM.
The CD4 cell clones can be classified into two populations on the basis of cytokine production, Th1 and Th2, with different cytokine pattern and immune functions. Th1 lymphocytes produce proinflammatory cytokines such as TNF. On the other hand, Th2 lymphocytes produce mainly IL-4, IL-6, IL-10, and IL-13. In our study an application of ECCs was associated with Th2 polarization, which was evident by the overproduction of IL-6 and IL-10.

The natural killer (NK) cells, which are important producers of IFN-γ, are known to be immature in neonates, and though changes in the NK cell count and activity were not directly evaluated in this study, IFN-γ changes may reflect the changes in NK cell count and activity.

It has been hypothesized that during the pathophysiology of sepsis, endotoxin and/or proinflammatory cytokines induce the overproduction of nitric oxide (NO), via inducible NO synthase. NO metabolites (NOx), nitrite and nitrate, have been used as indicators of NO production and have shown an elevation of serum NOx concentration in adult patients suffering from sepsis, and in neonatal sepsis. McDonagh et al., using adult blood in an in vitro study, showed that ECC increased the production of ROS. Our study showed similar results, but in an in vivo study, where NOx serum levels were higher in the CLP group with ECCs, and TH was also higher in both the CLP group and the sham group after the ECC applications. The changes in the inflammatory mediators together with the increase in free radicals resulting from ECC exposure may have resulted in a slightly shorter survival time in the CLP group with ECCs compared to the CLP group without them.

The application of an extracorporeal circuit provoked a window period of elevated serum cytokines and oxidative mediators in a neonatal sepsis model and its sham, which could be hazardous and should be considered during the treatment of critically ill newborns exposed to extracorporeal circuits, especially after abdominal surgeries complicated with sepsis.

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