Protective Effects of Pulsatile Flow During Cardiopulmonary Bypass

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Background. Children with congenital heart disease are often operated at a very young age. Cardiopulmonary bypass (CPB) has become indispensable for these sometimes very complex operations, but one cannot neglect a possible negative impact of CPB on organ function. Traditionally, CPB was developed with non-pulsatile flow but there are clinical observations that pulsatile flow might be superior with improved patient outcomes. Therefore, we attempted to elucidate whether CPB with pulsatile flow preserves organ integrity compared with nonpulsatile flow.

Methods. We studied 27 piglets of 4 weeks age and divided them into 3 experimental groups: control group (no CPB); non-pulsatile and pulsatile-CPB with 90-minutes CPB and 120-minutes recovery and reperfusion. Thereafter, histology of kidney, liver, and hippocampus was performed. Moreover, we measured adenosine triphosphate (ATP) content in these organs.

Results. Histologic evaluation revealed that laminar flow produced significant cellular edema in the kidney and hippocampus. Additionally, markers for hypoxia, apoptosis, and nitrosative stress were elevated but predominately in the hippocampus and proximal tubules of the kidney. Most of these alterations were reduced to or near control levels with pulsatile CPB. Moreover, ATP content of all 3 organs examined was higher and kidney and liver enzymes were lower in the pulsatile group compared with the non-pulsatile CPB. With regard to histologic changes, the liver seemed to be a less sensitive organ.

Conclusions. In our study during pulsatile CPB, organ damage was significantly attenuated as compared with non-pulsatile CPB. Therefore, in pediatric patients pulsatile CPB may improve clinical outcomes.

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Although cardiopulmonary bypass (CPB) has now become indispensable and allows very complex heart operations such as the correction of congenital cardiac diseases like tetralogy of Fallot or the hypoplastic left heart syndrome, the potential adverse effects of this technique on sensitive organs like the brain cannot be ignored. In children there are several reports on negative effects of CPB on the developing brain, with some of the cerebral areas including the hippocampus being especially sensitive to ischemia and reperfusion injury [1]. Moreover, there is solid evidence that the hippocampus is deeply involved in learning and memory processes and that lesions in this region might lead to cognitive impairments like learning disabilities, memory deficits, behavioral disorders, and hyperactivity [2]. There are several lines of evidence that the potential adverse effects of CPB on the developing brain are subtle and that neurologic deficits manifest themselves often years after successful surgical correction [3]. In fact, there are some clinical studies conducted on children with complex cardiac malformations operated in early childhood that showed that CPB might have negative implications on the later neurologic outcome [4, 5]. Due to auto-regulative processes (Bayliss effect) cerebral perfusion remains constant within a wide range (50 to 150 mm Hg), but nevertheless there is some evidence that a more physiologic pulsatile flow might be superior over the commonly used non-pulsatile flow during CPB [6]. Other organs like the kidney and liver are less tightly auto-regulated but also are clinically known to be possibly affected by CPB [7, 8]. Thus, the aim of our study was to evaluate in small piglets whether the hippocampus, kidney, and liver were affected by flow modality.

Material and Methods

The following procedures were approved by the Animal Care Committee of the German Regional Council Leipzig. We ensured humane treatment of all animals as indicated by the “Guide for the Care and Use of Laboratory Animals.” The Appendix can be viewed in the online version of this article [http://dx.doi.org/10.1016/j.athoracsur.2014.07.070] on http://www.annalsthoracicsurgery.org.
Animals” published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996). Anesthesia and perioperative management of the domestic pigs were carried out by veterinarians, thoracotomy and connection to the CPB by experienced heart surgeons, and operation of the heart-lung machine was done by cardio technicians. A detailed description of the experimental setup is outlined in Twal and colleagues [9] and in the online Appendix.

Briefly, 27 piglets of 4 weeks age (8 to 15 kg) were sedated, orally intubated, and mechanically ventilated with 50% oxygen and 50% air. Eighteen piglets underwent CPB (CPB group; moderate hypothermia, 28°C) with 90 minutes cross-clamp time followed by a 120 minutes reperfusion and recovery. Of the CPB group, 9 piglets were perfused with a non-pulsatile and 9 with a pulsatile flow (100 beats/minute; pump base flow 30%; pulsatile flow 70%; pump runtime 50%). Flow rate was set to 100 mL/kg body weight/minute for all animals. Nine control piglets (time control, normothermic) were also thoracotomized but not connected to the CPB.

**Histology**

Preparation and staining of the samples were done as previously published [10]. Briefly, specimens were embedded in paraffin and 2-μm sections were cut. Thereafter, the various stains were conducted. All specimens were analyzed using the Axiosmager M1 from Zeiss and the Zen Pro 2012 software (Carl Zeiss Microscopy GmbH, Jena, Germany). Pictures were taken at ×200 magnification and at least 10 pictures per piglet were evaluated by a blinded observer.

**Hematoxylin and Eosin (H&E) Staining**

The H&E staining was conducted following classical protocols. In the hippocampus at least 300 cells of the CA1 and also 300 cells of the CA3 region were counted and the percentage of edematous cells was evaluated separately for each region.

In the liver, cells of at least 10 liver lobules were examined for vacuoles or edematous swelling. In the kidney, the gap between Bowman capsule and the capillary convolute was measured and the percentage of tubular cells with vacuoles was determined (at least 10 glomeruli and 10 tubules were evaluated).

**Immunohistochemistry**

**HYPOXIA-INDUCIBLE FACTOR-1α (HIF-1α).** It is known that the transcription factor HIF-1α responds to changes in ambient oxygen. Thus, we aimed to evaluate translocation of this factor into the cell nucleus [9]. Again for the hippocampus, cells of the CA1 and CA3 region were counted and the percentage of positive (i.e., red) nuclei was calculated for each region separately.

In the liver, 10 lobules were evaluated and the results were expressed as positive stained cells per liver lobule. In the kidney, glomeruli and tubules were analyzed separately and the percentage of positive cells was related to the number of cells counted.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Control</th>
<th>CPB Non-Pulsatile Flow</th>
<th>CPB Pulsatile Flow</th>
<th>p Value CPB Non-Pulsatile Flow vs Control</th>
<th>p Value CPB Pulsatile Flow vs Control</th>
<th>p Value CPB Non-Pulsatile Flow vs CPB Pulsatile Flow</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lactate mmol/L</td>
<td>1.7 ± 0.17</td>
<td>6.4 ± 0.92&lt;sup&gt;a&lt;/sup&gt;</td>
<td>6.4 ± 0.92&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.001&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.05&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.001&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Mean arterial pressure mm Hg</td>
<td>90 ± 2.16</td>
<td>68 ± 2.4</td>
<td>64 ± 5.6</td>
<td>1.85 ± 2.1&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.89 ± 2.1&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.82 ± 2.1&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Brain ATP/ADP ratio</td>
<td>2.1 ± 0.16</td>
<td>1.6 ± 0.16</td>
<td>1.2 ± 0.13</td>
<td>0.65 ± 2.1&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.26 ± 2.1&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.56 ± 2.1&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Liver ATP/ADP ratio</td>
<td>1.9 ± 0.10</td>
<td>1.6 ± 0.10</td>
<td>1.6 ± 0.10</td>
<td>1.2 ± 0.13</td>
<td>0.65 ± 2.1&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.56 ± 2.1&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Kidney ATP/ADP ratio</td>
<td>1.8 ± 0.13</td>
<td>1.6 ± 0.13</td>
<td>1.6 ± 0.13</td>
<td>1.2 ± 0.13</td>
<td>0.65 ± 2.1&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.56 ± 2.1&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Creatinine mmol/L</td>
<td>1.9 ± 0.10</td>
<td>1.6 ± 0.10</td>
<td>1.6 ± 0.10</td>
<td>1.2 ± 0.13</td>
<td>0.65 ± 2.1&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.56 ± 2.1&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Urea mmol/L</td>
<td>0.7 ± 0.01</td>
<td>0.7 ± 0.01</td>
<td>0.7 ± 0.01</td>
<td>1.8 ± 0.13</td>
<td>0.65 ± 2.1&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.56 ± 2.1&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Glutamic oxaloacetic transaminase (GOT/GPT) ratio</td>
<td>2.6 ± 0.38</td>
<td>2.6 ± 0.38</td>
<td>2.6 ± 0.38</td>
<td>1.8 ± 0.13</td>
<td>0.65 ± 2.1&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.56 ± 2.1&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
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</table>

<sup>a</sup> Indicates significant differences to control. <sup>b</sup> Indicates significant differences to non-pulsatile CPB (p < 0.05).
apoPTOSIS-INDUCING FACTOR (AIF) AND (POLY-ADP-RIbOSE (PAR). The AIF triggers the caspase-independent pathway of apoptosis [11]. Moreover, as PAR is a molecule recently described to be also involved in cell death by stimulation of mitochondrial AIF-release we investigated PAR as well [12]. The immunohistologic staining and evaluation of hippocampus, liver, and kidney was carried out as described above.

NITROTYROSINE STAINING. Nitrosylation of tyrosine residues might result from reactive nitrogen species such as peroxynitrite. The resulting product nitrotyrosine might therefore serve as a biomarker for oxidative stress [13]. To analyze nitrotyrosine positivity we measured the percentage of nitrotyrosine positive cells in the CA1 and CA3 region of the hippocampus, in the glomeruli and tubules of the kidney, and additionally we evaluated the percentage of positive cells per liver lobule.

ADENOSINE TRIPHOSPHATE (ATP) MEASUREMENT BY HIGH-PRESSURE LIQUID CHROMATOGRAPHY (HPLC). Tissue ATP and adenosine diphosphate (ADP) levels were determined by HPLC (high-pressure liquid chromatography) as previously published [9]. Briefly, tissue samples were homogenized and centrifuged; 20 μL of the supernatant were injected onto a pre-equilibrated RP18 column. For detection of ATP and ADP an ultraviolet detector and a HPLC apparatus from Knauer (Berlin, Germany) was used. For calibration we injected 3 concentrations of ATP (2, 20, and 60 μg/mL) and ADP (0.5, 5, and 15 μg/mL). Each sample (standard and probe) was injected 3 times, and the concentration was determined as the mean of these 3 detections. For our data analysis we used the ATP/ADP ratio.

Statistical Analysis
For statistical analysis, analysis of variance was performed, and if analysis of variance indicated significant differences (p < 0.05), data were additionally analyzed with the post-hoc Tukey honest significant difference test. Statistical analysis was carried out using the software Systat for Windows, version 11 (Systat Inc, Evanston, IL). All data are given as means ± standard error of the mean of n = 7 experiments.

Results
All piglets were weaned successfully from CPB. Only rarely defibrillation was necessary (in each CPB group only 1 piglet). The other hearts came back right away.
Bradycardia was not observed. Lactate was significantly elevated in the CPB groups compared with control, with the non-pulsatile group having the highest lactate levels (Table 1). Mean arterial pressure was similar in all groups throughout the entire experiment (Table 1).

**Tissue ATP/ADP Levels and Blood Parameters**

In the hippocampus, liver and kidney ATP-levels significantly decreased in the non-pulsatile CPB-group, whereas pulsatile CPB better preserved ATP-content and did not result in a significant decrease in energy-rich phosphates in comparison with control. Moreover, kidney and liver enzymes indicative for organ damage significantly increased during both pulsatile and non-pulsatile CPB. However, experiments revealed that the non-pulsatile flow condition was even worse, resulting in the highest enzyme levels (Table 1).

**Cellular Edema**

The H&E-staining of the hippocampus revealed that non-pulsatile CPB resulted in a significant edematous cellular swelling of both regions analyzed (CA1 and CA3), whereas during pulsatile CPB a significant pericellular edema could not be observed. A compilation of the experiments is depicted in Figure 1A, original histological photographs in Figure 1C (upper panels).

In the kidney non-pulsatile CPB caused a significant increase in tubular vacuolization and glomerular edema with an enlargement of the gap between Bowman’s capsule and the glomerular capillary convolute as previously published [9]. During pulsatile CPB edematous glomerular swelling was significantly less, although control levels were not reached. In contrast, the amount of tubular vacuolization was not altered by pulsatile flow.

Analysis of liver lobules showed that hepatocytes did not exhibit an edematous swelling during both CPB protocols (Fig 1). A compilation of the experiments is depicted in Figure 1B, original histological photographs in Figure 1C (middle and lower panels).

**HIF-1α Staining**

In the hippocampus, HIF-1α positive cell nuclei, indicating translocation of this transcription factor into the nucleus was low under control conditions although the CA3 region showed more HIF-1α positive cell nuclei compared with the CA1 region. Non-pulsatile CPB enhanced the number of HIF-1α positive cell nuclei in the CA1 and CA3 regions (Fig 2A). In the kidney, HIF-1α staining was observed in the glomeruli and proximal tubules of control and non-pulsatile and pulsatile CPB groups (Fig 2B). Non-pulsatile and pulsatile CPB caused a significant increase in the number of HIF-1α positive cell nuclei in the kidney (Fig 2B). In the liver, HIF-1α staining was observed in the lobules and hepatocytes of control and non-pulsatile CPB groups (Fig 2C). Non-pulsatile CPB caused a significant increase in the number of HIF-1α positive cell nuclei in the liver (Fig 2C).

![HIF-1α Staining](image_url)
of positive cells in both CA-regions significantly by a factor of 2 to 3, whereas during pulsatile CPB the number of positive nuclei was not significantly increased compared with control. A compilation of the experiments is depicted in Figure 2A, original histological photographs in Figure 2C (upper panels).

In the renal tubules the number of HIF-1α positive cells was significantly enhanced, with the non-pulsatile CPB showing the highest values, and in the glomeruli HIF-1α was slightly but not significantly enhanced by both CPB protocols.

Again, in the liver no significant increase in HIF-1α positive cells could be detected; neither during non-pulsatile nor during pulsatile CPB. A compilation of the experiments is depicted in Figure 2B, original histological photographs in Figure 2C (middle and lower panels).

**Apoptosis-Inducing Factor and Poly-ADP-Ribose Staining**

Nuclear AIF translocation and PAR formation were significantly elevated during non-pulsatile CPB in the hippocampus. In contrast, the pulsatile CPB did not lead to a significant AIF translocation or nuclear PAR-formation. A compilation of the experiments is depicted in Figures 3A and 4A, original histological photographs in Figures 3C and 4C (upper panels).

In the kidney, AIF translocation into the nucleus was significantly enhanced within the tubules during non-pulsatile CPB. Pulsatile flow also increased AIF translocation but to a lesser extent. In the glomeruli, AIF translocation was not significantly elevated during CPB, although some AIF translocation within the nuclei of glomeruli could be detected during non-pulsatile as well as during pulsatile CPB. In contrast, PAR formation was increased by both CPB protocols within cells of glomeruli and tubules.

In the liver, no significant AIF translocation was induced by non-pulsatile or pulsatile CPB. Regarding PAR formation we could detect an increase in PAR positive cells during non-pulsatile CPB, which was absent when pulsatile CPB was used. Nevertheless, this increase in PAR during non-pulsatile CPB was only marginal. A compilation of the experiments is depicted in Figures 3B and 4B, original histological photographs in Figures 3C and 4C (middle and lower panels).

**Nitrotyrosine Staining**

Non-pulsatile CPB significantly increased the number of nitrotyrosine positive cells within the hippocampus by
about fourfold. If pulsatile CPB was used this increase in nitrotyrosine was significantly less in both regions (CA1 and CA3). A compilation of the experiments is depicted in Figure 5A, original histological photographs in Figure 5C (upper panels).

In the kidney (tubules) and liver, non-pulsatile CPB enhanced nitrotyrosine production and pulsatile flow prevented this increase. However, nitrotyrosine production in the liver, although significantly enhanced by non-pulsatile CPB, was low and in the glomeruli of the kidney nitrotyrosine positive cells could not be detected. A compilation of the experiments is depicted in Figure 5B, original histological photographs in Figure 5C (middle and lower panels).

**Comment**

**Kidney and Liver**

In our experiments we could corroborate and extend the results on a hypoxic or ischemic damage of the kidney accompanied by a significant edema formation (glomeruli and tubules) as previously published by our group [9]. This kidney damage resembling acute renal injury was also seen by Tiritomis and colleagues [7]. Additionally in our study, molecular markers of cellular damage were elevated in the non-pulsatile group indicating that cellular injury was more apparent in the proximal tubules which seem to be more sensitive to low-flow conditions compared with the glomeruli [14]. However, the pulsatile protocol in our study was not able to significantly reduce all biomarkers measured in the kidney. Only nitrotyrosine formation in the proximal tubules was significantly reduced to control levels during pulsatile CPB. Reactive nitrogen species occur during CPB and are thought to have a high impact on the development of the post-bypass systemic inflammatory response, which is involved in renal failure [15]. While total kidney perfusion was improved by pulsatile flow in the study by Undar and colleagues [16], in another study this was not the case [17]. Thus, the better biochemical outcome seen in our study (ATP and retention parameters) might be due to higher intermixing of cellular and noncellular blood components in pulsatile flow.

Contrary to the kidney, the liver seems to be only marginally affected by non-pulsatile CPB. In our study we could not detect severe damages in our liver specimen. Solely PAR and nitrotyrosine formation was slightly elevated. Peroxynitrite, which mediates tyrosine nitration also might induce DNA strand breakage with subsequent activation of poly-ADP ribose polymerase (PARP) and
PAR formation which has been shown to promote liver cell death [18]. Nitrotyrosine elevation was absent when pulsatile flow was used. Thus, it might be that pulsatile flow better preserves oxygen supply and is less associated with enhanced ATP consumption. This idea is also supported by a study of Talor and Ungar [19] who found that pulsatile CPB is able to enhance the hemodynamic energy and to improve the perfusion of microcirculation.

Interestingly in our study, AIF positive cells in the liver were slightly elevated (8 to 10 positive cells per liver lobule) without significant differences between the 3 groups. Despite all pigs had normal liver values before CPB start, especially glutamic oxaloacetic transaminase was increased after the reperfusion period. This increase was also apparent in the control group (without CPB). This might indicate a slight liver affection induced by the volatile agent isoflurane used in our anesthesia protocol. Elevation of liver enzymes by halogenated ether anesthetics has also been reported by Topal and colleagues [20].

**Hippocampus**

We found that non-pulsatile CPB had a significant negative impact on the neural cell band of the hippocampus with regard to ATP-levels, pericellular edema, HIF-1α, AIF nuclear translocation, PAR, and nitrotyrosine formation. These negative effects of the laminar flow were nearly reversed by the pulsatile protocol. Thus it seems that the hippocampus profited most from the pulsatile CPB.

Some clinical studies indicated that, especially in children, the harmful effects of CPB on the brain are sometimes associated with a negative outcome [21]. Therefore, several animal and human studies have been conducted to find better perfusion protocols with less unwanted effects. Among these, pulsatile perfusion during CPB might resemble the body’s own pulsating circulatory system better than the traditionally used laminar flow. However, study results are inconsistent; some authors found that pulsatile perfusion during CPB is superior to non-pulsatile as pulsatile flow was associated with an advantage for microcirculation, preservation of fibrinolytic balance, and with better clinical outcomes especially in pediatric patients [22–25], while others did not [26, 27]. However, as the brain is the most sensitive organ to low perfusion it seems reasonable to assume that a more physiologic pulsatile perfusion might be advantageous. Corresponding to our results, Yu and colleagues [28]
found in a chronic model of brain hypoxic ischemia that especially the CA1 region was susceptible to low oxygen and that cellular apoptosis occurs early after onset of ischemia and persists over several weeks. Furthermore, our results are in good accordance with another animal study, however on adult dogs, of hippocampal damage resulting from cardiac arrest and resuscitation showing diminished CA1 damage if resuscitation was combined with pulsatile flow [29]. In addition, another animal study could demonstrate that elevated levels of both PAR and nitrotyrosine were associated with a poor outcome [30]. Thus, it seems preferable to use pulsatile CPB more in depth with longer follow-ups.

## References