Reno-protective effects of epigallocatechingallate in a small piglet model of extracorporeal circulation

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A R T I C L E   I N F O

Article history:
Received 4 September 2012
Received in revised form 16 October 2012
Accepted 16 October 2012

Keywords:
Cardiopulmonary bypass
Extracorporeal circulation
Renal impairment
Kidney
Epigallocatechingallate
Antioxidant
Anti-apoptotic
Nitrosative stress
Renoprotection

A B S T R A C T

Cardiopulmonary bypass still often is a necessary tool in cardiac surgery in particular in the correction of congenital heart defects in small infants. Nevertheless, among the complications linked to extracorporeal circulation (ECC) with cardiopulmonary bypass (CPB) in both infants and adults one of the most serious problems is renal impairment. Since this might be caused by ischemia/reperfusion injury and accumulation of free radicals, we used (−)-epigallocatechin-3-gallate (EGCG), a derivate from green tea, which is known to possess antioxidant, antiapoptotic and NO-scavenging properties in order to find out whether EGCG may protect the kidney.

23 four-week-old Angler Sattelschwein-piglets (8–15 kg) were divided into three groups: controlgroup (n = 7), ECC-group (n = 10), EGCG-group (n = 6). The ECC- and EGCG-group were thoracotomized and underwent CPB for 120 min followed by a 90-min recovery-time. The EGCG-group received 10 mg/kg EGCG before and after CPB.

Histology revealed that CPB led to widening of Bowman’s capsule, and to vaculolization of proximal tubular cells (p < 0.05) which could be prevented by EGCG (p < 0.05). Using immunohistology, we found significant nuclear translocation of hypoxia-inducible-factor-1-alpha (HIF-1-alpha) and increased nitrotyrosine formation in the ECC-group. Both were significantly (p < 0.05) inhibited by EGCG. ECC-induced loss of energy-rich phosphates was prevented by EGCG. In blood samples we found that CPB resulted in increases in creatinine and urea (in serum) and led to loss of total protein (p < 0.05), which all was not present in EGCG-treated animals.

We conclude that CPB causes damage in the kidney which can be attenuated by EGCG.

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1. Introduction

Cardiopulmonary bypass (CPB) greatly contributed to the development of heart surgery. Its first use took place in the Massachusetts General Hospital (Boston, MA, USA) in 1953 when Dr. John Gibbon Jr. – who invented and developed the CPB together with his wife Mary – performed a successful open heart surgery which included 45 min of CPB [1,2]. Although minimally invasive techniques are used today in many indications, CPB is still often necessary, in particular for the correction of inborn complex heart defects in small infants. However, there remain unsolved complications related to CPB which are attributed to the extracorporeal circulation (ECC). Among these complications renal impairment is one of the major problems.

Acute renal failure in children, who underwent cardiac surgery, occurs with a probability of up to 32.8% [3], acute renal injury even with a probability of up to 42% [4] within 48 h. It has been postulated that in fact there is a significantly elevated risk of developing renal failure after open heart surgery with CPB [5–7].

There is always an inflammatory response to CPB with variable degrees [8] which can harm different organs in multiple ways. Another pathologic change, which may also happen during CPB due to the temporary systemic hypoperfusion, is an ischemia/reperfusion injury to which the kidney as a highly vascularized organ is quite susceptible [9,10]. One characteristic of

Abbreviations: AEC, 3-amino-9-ethylcarbazol; BSA, bovine serum albumin; CPB, cardiopulmonary bypass; DAB, 3,3′-diaminobenzidine; ECC, extracorporeal circulation; EGCG, (−)-epigallocatechin-3-gallate; HE, hematoxylin eosin; HIF, hypoxia inducible factor; PBS, phosphate buffered saline; TCA, tyramide signal amplification.

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an ischemia/reperfusion injury is the generation of reactive oxygen species, which accumulate and surpass the body’s antioxidant mechanisms [11]. During the reperfusion phase, nitrosonogen free radicals, namely nitric oxide (NO) [12], and also superoxide anions [13] are produced in pathological amounts and may react to peroxynitrite, which can cause harm to the kidney.

However, the use of classical antioxidants like N-acetylcysteine has been investigated in earlier studies with negative results [14,15]. Therefore, we tried to find a substance which might combine antioxidant, anti-NO-releasing and anti-apoptotic effects. (–)-Epigallocatechin-3-gallate (EGCG) is a substance combining these effects, but has never been investigated for a possible reno-protective effect in CPB. Thus, anti-apoptotic and anti-NO-releasing effects have been reported for EGCG in brain cells exposed to quinolinic-acid [16]. El-Mowafy et al. [17] found that EGCG reduces the nephrotoxicity of cisplatin. Others [18] investigated if 45 min complete renal-artery-occlusion-induced ischemia/reperfusion injury in kidneys can be attenuated by EGCG and Itoh et al. [19] examined, whether EGCG is able to protect kidneys against urolithiasis, and both research groups reported beneficial effects. Moreover, EGCG can exert anti-inflammatory [17], anticancer [20], and immunostimulant [21] effects. EGCG has direct antioxidant properties, in particular via its radical scavenging effects (directed against both reactive oxygen and nitrogen species), and may also possess indirect antioxidant properties and anti-apoptotic effects [16,20,22]. On this background we chose EGCG for our study. EGCG is a natural element of green tea and constitutes 50% of the different catechins present in it [23].

2. Methods

2.1. Animals

The animals used for the experiments were 23 four-week-old Angler Sattelschwein-piglets with a body weight of 8–15 kg divided into three groups: control-group (n = 7; time control group without ECC), ECC-group (n = 10), EGCG-group (n = 6). The ECC- and EGCG-group were anesthetized, thoracotomized, and underwent CPB for 90 min (crossclamp time) followed by 30 min of reperfusion. Then, the CPB was stopped and the piglets stayed anesthetized another 90 min – the recovery time – until they were sacrificed through bleeding. Epigallocatechin-3-gallate was obtained from Sigma-Aldrich (Taufkirchen, Germany) and dissolved in 0.9% NaCl. The EGCG-group received two injections of EGCG (10 mg/kg i.v.); the first 15 min before connection to the CPB and the second directly after weaning. The pigs of the ECC-group received the injections of the solvent (0.9% NaCl). The control-group was also anesthetized and thoracotomized, but not connected to the CPB.

2.2. Medication and operation

For premedication atropine sulphate (0.02 mg/kg i.m.), midazolam (0.5 mg/kg i.m.) and ketamine hydrochloride (25 mg/kg i.m.) were used. Thereafter, the piglets were intubated and artificially ventilated with oxygen (1.5 l/min), air (1.5 l/min) and isoflurane (1.5–2%), and received an arterial and central venous access. During the whole experiment the piglets were closely monitored and every half hour blood gas analyses were performed.

For elimination of pain sufentanil-dihydrogenecitrat (3 μg/kg bolus initially followed by 1–2 μg/h/10 kg i.v.) was used. For stabilization of the circulatory system every piglet received 500 ml NaCl (0.9%) infusion in the course of the entire experiment according to the standard procedures as in pediatric cardiac surgery. If acidosis occurred, sodium hydrogencarbonate or trometamol were given as required. As a muscle relaxant pancuronium bromide (0.2 mg/kg i.v.) was used.

The piglets were thoracotomized and received heparin sodium (300 IU/kg) to avoid blood clotting before the CPB was connected through the cannula to the right atrial auricle and the aortic bow. The aorta was closed (crossclamp) and the 90-min-CPB-time started (CPB parameters: priming volume: 450 ml; flow: 100 ml/kg/min; mean systemic arterial pressure (femoral artery): >60 mmHg; system: SII pump, Stöckert, Munich, Germany; membrane oxygenator: D901 Lilliput, Dideco, Mirandola, Italy; pediatric complete tubing set: Sorin, Munich, Germany. We added 1000 IU heparin/450 ml to the priming volume of the heart–lung-machine. Thereafter, we controlled heparin therapy by measuring the activated clotting time and kept it at 400 s. At the end of the CPB we antagonized the 1000 IU heparin by protamine–HCl in 4 steps under control of the activated clotting time. This is according to the standard schemes in cardiac surgery. During CPB piglets were anesthetized with propofol. The cardiac arrest was initiated by 350 ml cold (5 ºC) saline cardioplegic solution (Custodiol®. Dr. Franz Köhler Chemie GmbH, Bensheim, Germany) given through the punctured aortic root. Since in cardiac pediatric surgery classically the body is cooled to achieve some anti-ischemic protection [2,7], in our experiments we used moderate hypothermia by cooling down the body temperature to 28 ºC according to the clinical protocols, and after 60 min warmed to 37 ºC again. At the end of the 90 min the aorta was re-opened and the 30-min-reperfusion-time started, during which CPB flow was progressively diminished, until the piglets could be weaned from the CPB. All piglets were successfully weaned from CPB. Subsequently, a 90-min-recovery-time followed, during which CPB flow was stopped. Then the piglets were sacrificed. During these last 120 min piglets received catecholamines (cumulative doses: 0.004 mg/kg norepinephrine and 0.029 mg/kg epinephrine) for stabilization of the circulation. Central venous pressure was controlled and was kept at 7–9 mmHg.

2.3. Sampling and sample preparation

At the beginning of the experiment (0 min), after the CPB-time (90 min), and directly before the scarification of the piglets (240 min) venous blood samples were taken. Prior to thoracotomy a biopsy was taken from the left kidney. Directly at the end of the experiment another kidney sample was taken from the same organ. The tissue samples were fixed in 4% formalin, embedded in paraffin, cut into slices, and placed on slides.

2.4. (Immuno-)histological stainings

2.4.1. Hematoxylin–eosin staining

For this routine staining 5 μm slides of the biopsies and the post-OP-samples were stained using hematoxylin–eosin according to classical protocols in order to get a histomorphological overview and to search for thrombi.

2.4.2. HIF-1-alpha–TSA staining

HIF-1-alpha is an intracellular transcription factor. In order to regularly eliminate it from the cell oxygen is needed. In a hypoxic situation HIF-1-alpha accumulates and HIF-1-alpha translocation to the nucleus is induced. In order to investigate if HIF-1alpha may be activated in the kidneys, 2 μm slides of post-OP-samples were stained immunohistochemically using 1% bovine serum albumin for blocking of unspecific binding sites and rabbit-anti-HIF-1-alpha primary antibody (sc-10798, Santa Cruz Biotechnology, Santa Cruz, CA, USA; 1:100) over night at 4 ºC. For detection we used goat-anti-rabbit IgG Horseradish Peroxidase conjugate (T-20921; Invitrogen, Eugene, OR, USA; 1:100, 2 h, room temperature) followed by tyramid signal amplification (TSA-Kit 11, T-20921; Invitrogen, Eugene,
OR, USA) using 3-amino-9-ethylcarbazol (AEC) according to the manufacturers protocol.

2.4.3. Nitrotyrosine-AEC staining

The amino acid nitrotyrosine is a well known biomarker for detection of cellular damage caused by nitrosative stress, i.e. by nitrosylation of tyrosine moieties caused by oxidant and nitrating agents such as peroxynitrite. 2 μm slides of post-OP-samples were stained immunohistologically for nitrotyrosine-positive structures using mouse-anti-3-nitrotyrosine monoclonal antibody (MC-374, Kamiya Biomedical Company, Seattle, WA, USA; 1:200 over night, 4 °C). As secondary antibody goat-anti-mouse- IgG2alpha-HRP (sc-2970, Santa Cruz Biotechnology, Santa Cruz, CA, USA; 1:100) was applied for 1 h. Staining reaction was performed with AEC so that nitrotyrosine positive cells stained in bright red.

2.4.4. Apoptosis-inducing factor (AIF) and poly-ADP-ribose (PAR) staining

2 μm thick sections were incubated were incubated with rabbit-anti-AIF (H-300) polyclonal IgG antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA; 1:100; 4 °C, over night) followed by goat-anti-rabbit IgG-horseradish-peroxidase conjugate (TSA-Kit, Invitrogen, Eugene, USA; 1:250; 1 h, room temperature) as secondary antibody. Subsequently, we used tyramide-amplification system for detection (TSA-Kit, Invitrogen, Eugene, USA) according to the manufacturers protocol. Using a similar protocol we stained sections for poly-ADP-ribose using a polyclonal rabbit anti-PAR-antibody (BD Biosciences, Heidelberg, Germany; 1:100, overnight, 4 °C), followed by 3,3′-diaminobenzidine (DAB) staining using the Envision Flex kit from DAKO (DAKO, Hamburg, Germany) according to the manufacturers protocol.

2.4.5. Evaluation of the stainings

After staining pictures were taken by a camera (Zeiss, AxioCam MR5, Zeiss, Jena, Germany) mounted on the microscope (AxioLab, Zeiss, Jena, Germany). First, an overview picture was taken with 100× magnifications. Thereafter, further pictures were taken at 400× magnification. The pictures were evaluated in a blinded fashion using axiovision vers.4.6 (Zeiss, Jena, Germany). For each histological parameter for each pig 10 pictures at 400× were evaluated, so that the mean given for a series is made from 60 to 100 slides. In H.E. stainings we measured the diameter of the glomeruli and the mean width of the gap between Bowman’s capsule and the capillary convolute, as well as the percentage of tubular cells with vacuoles and of tubular cells with pyknotic nuclei.

Regarding HIF-1-alpha we evaluated the percentage of HIF-1-alpha-positive nuclei related to the total number of nuclei separately in proximal and distal tubules, collecting ducts and glomeruli. The same was done for evaluation of AIF and PAR.

With regard to nitrotyrosine we measured the percentage of nitrotyrosine positive proximal and distal tubules, collecting ducts and glomeruli.

2.5. Blood parameters

Due to infusions and the volume management caused by CPB, some hemodilution may have occurred in the piglets. As a consequence, when evaluating the blood parameters mentioned below, the hematocrit always had to be taken into consideration and was used for normalization.

In order to determine if the kidney was able to function and filter properly, the total amount of proteins in the blood was measured. Moreover, serum creatinine and urea concentration were determined by classical methods of clinical chemistry.

2.6. High performance liquid chromatography (HPLC)

We used a HPLC protocol for additional investigation of the renal energy-rich phosphates ATP, ADP, and AMP as well as adenosine derived from [24]. Briefly, kidney tissue samples were taken at the end of the experiments and immediately quick-frozen. Tissue samples were homogenated at high-speed in 5 ml 0.4 M perchloric acid (10 min). We precipitated the extracts with 0.8 ml 0.2 M KOH on ice. After centrifugation (4 °C, 3000 × g) the supernatant was used for HPLC (Knauer, Berlin, Germany). 20 μl supernatant were injected; the mobile phase consisted of KH2PO4 (215 mM), tetra-butylammonium hydrogen sulphate (2.3 mM), acetonitrile (4%) and KOH (1 M, 0.4%); flow rate was 1 ml/min and temperature 25 °C. We used a RP18 (240×4, 5 μm) column (Merck, Darmstadt, Germany). We measured peak areas at 254 nm. Retention times were 5.03 min (AMP), 6.55 min (ADP), 6.75 min (adenosine), and 9.85 min (ATP). Each sample was injected three times, and the concentration was determined as the mean of these three detections. For calibration we injected three concentrations of AMP (6, 60 and 180 μg/ml), ADP (0.5, 5 and 15 μg/ml), ATP (2, 20 and 60 μg/ml), and adenosine (0.4, 4 and 12 μg/ml). These calibrations gave linear concentration-peak area relationships with good regression coefficients (R²: 0.96–1.0).

2.7. Statistics

All results are given as mean value and standard error of mean (SEM) of n experiments. In order to detect significances, ANOVA was performed and, if ANOVA indicated significant changes subsequently the Mann–Whitney U-test was used with a significance level of p < 0.05.

3. Results

3.1. Functional parameters

All piglets started at comparable functional parameters, in particular heart rate, blood pressure and arterial O2-saturation. During the experiment – similar to the clinical situation – we tried to keep these parameters within the physiological range for this species (Table 1).

3.2. (Immuno-)histological parameters

3.2.1. Hematoxylin–eosin staining

Histomorphologically we found that Bowman’s capsule was enlarged by ECC. Thus, the area and the width of the gap between Bowman’s capsule and the glomerular capillaries convolute increased significantly (p < 0.05) only in the ECC-group. In the EGCG-group, this increase could be significantly (p < 0.05) prevented by EGCG (Fig. 1A and B).

In the proximal tubules we found signs of injury such as loss of brush border, swelling or significantly increased numbers of cells showing diffuse intracellular vacuolization (p < 0.05; Fig. 1C) in the ECC-group. However, in the EGCG-group the percentage of vacuolated cells was significantly (p < 0.05) lower than the percentage in the ECC-group (Fig. 1C). The number of pyknotic nuclei was significantly (p < 0.05) increased post OP vs. pre OP in all three groups. This was most prominent in the ECC-group and could be slightly but not significantly antagonized by EGCG (Fig. 1D). Original HE stainings for all three groups are shown in Fig. 1E and F.

Moreover, it became obvious from these investigations that at the time of investigation there was no infiltration with inflammatory cells so far (Fig. 1E and F).

In addition, we did not find any macroscopically visible signs of vascular occlusion and did not observe thromboembolic occlusion
Table 1
Functional parameters. Systolic and diastolic arterial pressure; heart rate, arterial \(O_2\)-saturation at 0, 120 and 240 min of the experiment given as means± SEM for the animals of the control series (time controls), the animals receiving extracorporeal circulation (ECC), and the animals receiving extracorporeal circulation with additional epigallocatechin gallate treatment (EGCG).

<table>
<thead>
<tr>
<th>Groups</th>
<th>Systolic arterial pressure in mmHg</th>
<th>Diastolic arterial pressure in mmHg</th>
<th>Heart rate in bpm</th>
<th>Arterial (O_2)-saturation in %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control 0'</td>
<td>68.9 ± 2.8</td>
<td>34.3 ± 2.1</td>
<td>95.7 ± 4.9</td>
<td>99.9 ± 0.1</td>
</tr>
<tr>
<td>Control 120'</td>
<td>83.7 ± 4.8</td>
<td>40.3 ± 2.2</td>
<td>104.1 ± 4.5</td>
<td>98.9 ± 0.7</td>
</tr>
<tr>
<td>Control 240'</td>
<td>85.6 ± 3.8</td>
<td>38.5 ± 2.1</td>
<td>108.4 ± 5.0</td>
<td>99.7 ± 0.2</td>
</tr>
<tr>
<td>ECC 0'</td>
<td>74.3 ± 3.4</td>
<td>40.9 ± 1.4</td>
<td>107.5 ± 3.3</td>
<td>99.8 ± 0.1</td>
</tr>
<tr>
<td>ECC 120'</td>
<td>85.5 ± 5.3</td>
<td>34.2 ± 2.4</td>
<td>125.4 ± 8.1</td>
<td>98.1 ± 1.6</td>
</tr>
<tr>
<td>ECC 240'</td>
<td>76.9 ± 4.0</td>
<td>25.1 ± 1.9</td>
<td>150.5 ± 8.4</td>
<td>95.1 ± 3.1</td>
</tr>
<tr>
<td>EGCG 0'</td>
<td>73.5 ± 7.1</td>
<td>44.0 ± 4.7</td>
<td>111.5 ± 5.7</td>
<td>99.7 ± 0.3</td>
</tr>
<tr>
<td>EGCG 120'</td>
<td>83.0 ± 10.5</td>
<td>41.6 ± 5.0</td>
<td>128.0 ± 16.0</td>
<td>99.8 ± 0.2</td>
</tr>
<tr>
<td>EGCG 240'</td>
<td>72.3 ± 3.8</td>
<td>34.6 ± 2.0</td>
<td>139.0 ± 9.1</td>
<td>98.5 ± 1.0</td>
</tr>
</tbody>
</table>

1 Significance (\(p < 0.05\)) vs. 0 min.
2 Significance (\(p < 0.05\)) vs. the control-group.
3 Significance (\(p < 0.05\)) between EGCG and the ECC-group.

of vessels or microthrombi histopathologically by microscopic investigation (HE staining). Also, histologically (HE staining) we did not find necrotic zones in the kidneys, no acute tubular necrosis, and no myoglobin deposits in the nephrons. However, we observed induction of apoptosis (see below).

3.2.2. **HIF-1-alpha-TSA staining**

Nuclear translocation of HIF-1-alpha was only rarely seen in control samples, but was significantly enhanced in the ECC-group. The most prominent changes in this HIF-1-alpha translocation were found in the proximal tubules (Fig. 2B). The percentage of cell nuclei

![Figures](image URLs)

Fig. 1. (A) Area of the gap between Bowman’s capsule and the capillaries in the glomeruli in HE staining of all three groups given as means± SEM. Significance (\(p < 0.05\)) vs. pre OP is indicated by an asterisk (*), significance (\(p < 0.05\)) vs. the control-group by a hash tag (#), and significance (\(p < 0.05\)) vs. the ECC-group by a section sign (§). (B) Width of the gap between Bowman's capsule and the capillaries in the glomeruli in HE staining of all three groups given as means± SEM. The indicators for significance are the same as in (A). (C) Vacuolated cells in the proximal tubules in HE staining of all three groups given as means± SEM. The indicators for significance are the same as in (A). (D) Pyknotic nuclei in the proximal tubules in HE staining of all three groups given as means± SEM. The indicators for significance are the same as in (A). (E) Original HE staining for all three groups shown glomeruli. The arrow (→) indicates the gap between Bowman’s capsule and the capillaries. (F) Original HE staining for all three groups showing proximal tubules. The black arrow (→) indicates vacuolated cells, the blue arrow (→) pyknotic nuclei. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)
in the proximal tubules, which were positive for the HIF-1-alpha staining, was significantly \((p < 0.05)\) increased in the ECC-group, but could be significantly \((p < 0.05)\) attenuated by EGCG (Fig. 2A). In distal tubules, glomeruli and collecting ducts these changes were similar but less pronounced.

3.2.3. AIF and poly-ADP-ribose staining

As an early sign of apoptosis induction we observed in kidneys of the ECC-group significant nuclear translocation of AIF in a considerable percentage of cells. Its was most prominent in the glomeruli, but also in the distal and proximal tubules, as well as in the collecting ducts. Interestingly, this nuclear AIF translocation was reduced by treatment with EGCG in particular in the glomeruli and tubules (see Fig. 2C and D).

Similar findings were obtained regarding the production of poly-ADP-ribose in the nuclei of the cells. This highly energy-consuming repair mechanism was significantly enhanced in all parts of the nephron (in particular in glomeruli and tubules) of pigs undergoing ECC. This was significantly prevented by EGCG (Fig. 2E and F).

3.2.4. Nitrotyrosine-AEC staining

ECC resulted in significantly increased nitrotyrosine formation, which was predominantly found in distal tubules and in collecting ducts (Fig. 3A and B). The percentage of distal tubules and collecting ducts, which were nitrotyrosine-positive, differed significantly \((p < 0.05)\) by treatment: regarding the distal tubules the ECC-group showed the highest count, which was significantly \((p < 0.05)\) inhibited by EGCG (Fig. 3A). In the collecting ducts similar, but slightly attenuated changes were found (Fig. 3A).

3.3. Renal energy-rich phosphates

HPLC revealed that the sum of ATP, ADP, and AMP was significantly decreased in the ECC-group, while this decrease was completely prevented in the EGCG-group. Moreover, the phosphorylation-ratio given by the ratio ATP/(ADP + AMP) and by the ratio ATP/(ADP + AMP + adenosine) was significantly higher in the EGCG-group (Table 3). This indicates less de-phosphorylation of ATP in the EGCG-group.

3.4. Blood chemistry parameters

When evaluating the following three blood chemistry parameters, the hematocrit always has to be taken in consideration, because it influences these parameters and typically may slightly change during CPB (Table 2). Thus, we normalized all data to hematocrit, which is for clarity given in Table 2. The total amount of
Fig. 2. (A) Nuclei with HIF-1-alpha translocation in proximal tubules in all three groups given as means ± SEM. Significance (p < 0.05) vs. the control-group is indicated by a hash tag (#), significance (p < 0.05) vs. the ECC-group by a section sign (§). (B) Original HIF-1-alpha-TSA staining for all three groups. The arrow (→) indicates nuclei with HIF-1-alpha translocation. (C) Original AIF-staining showing the enhanced AIF-translocation (arrows) in the glomeruli of the ECC-group (middle panel) vs. control group (upper panel) and the inhibitory effect of EGCG on nuclear AIF translocation (lower panel). Nuclei, which are positive for AIF appears brown, while negative nuclei appear light-blue. (D) Quantitative data for nuclear AIF translocation in glomeruli, proximal and distal tubules as well as in collecting ducts in all three groups given as means ± SEM. The indicators for significance are the same as in (A). (E) Original poly-ADP-ribose (PAR) staining showing enhanced nuclear PAR in the ECC-group (middle panel). (F) Quantitative data for nuclear PAR in glomeruli, proximal and distal tubules as well as in collecting ducts in all three groups given as means ± SEM. The indicators for significance are the same as in (A). [For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.]
Fig. 2. (Continued).
Table 2
Hematocrit values for all groups at 0, 90 and 240 min of the experiment given as means ± SEM.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Hematocrit in % at 0 min</th>
<th>Hematocrit in % at 90 min</th>
<th>Hematocrit in % at 240 min</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>17.8 ± 1.31</td>
<td>19.49 ± 1.25</td>
<td>22.06 ± 2.19</td>
</tr>
<tr>
<td>ECC</td>
<td>22.69 ± 1.01*</td>
<td>20.12 ± 1.04</td>
<td>14.08 ± 0.93*</td>
</tr>
<tr>
<td>EGCG</td>
<td>19.55 ± 2.4</td>
<td>16.13 ± 0.94*</td>
<td>16.48 ± 1.85</td>
</tr>
</tbody>
</table>

* Significance (p < 0.05) vs. 0 min.
* Significance (p < 0.05) vs. the control-group.
§ Significance (p < 0.05) vs. the ECC-group.

Table 3
In the first column the concentration of the sum of the energy rich phosphates ATP, ADP and AMP is given for all groups at the end of the experiment. In the second column the phosphorylation ratio is given as ATP/(ADP + AMP) and in the third column the ratio for ATP/(ADP + AMP + adenosine). All values are given as means ± SEM.

<table>
<thead>
<tr>
<th>Groups</th>
<th>ATP, ADP and AMP (µg/mg)</th>
<th>ATP/(ADP + AMP) ratio</th>
<th>ATP/(ADP + AMP + adenosine) ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.973 ± 0.279</td>
<td>0.060 ± 0.018</td>
<td>0.060 ± 0.018</td>
</tr>
<tr>
<td>ECC</td>
<td>0.677 ± 0.139*</td>
<td>0.044 ± 0.033</td>
<td>0.047 ± 0.033</td>
</tr>
<tr>
<td>EGCG</td>
<td>1.154 ± 0.133*</td>
<td>0.135 ± 0.034*</td>
<td>0.134 ± 0.033*</td>
</tr>
</tbody>
</table>

* Significance (p < 0.05) vs. the control-group.
§ Significance (p < 0.05) vs. the ECC-group.

Fig. 4. Change in the amount of proteins in all three groups (240 min vs. 0 min) given as means ± SEM. The protein values are corrected for hematocrit, which was initially 23 ± 0.13. Significance (p < 0.05) vs. 0 min is indicated by an asterisk (*); significance (p < 0.05) vs. the control-group by a hash tag (#).

proteins decreased in all three groups during OP-time, but was significantly (p < 0.05) reduced only in the ECC-group at 90 and 240 min as compared to 0 min (Fig. 4). The values are given as total amount of proteins in g/l/hematocrit in %). There were no significant changes in serum proteins in the EGCG-group.

Serum creatinine and urea were measured as typical markers for renal function. In the ECC-group, both parameters were found to be increased. This increase in both creatinine and urea during 240 min was significantly (p < 0.05) higher in the ECC-group than in the control-group, and both increases could be significantly inhibited by EGCG (Fig. 5A and B).

4. Discussion

Open heart surgery in children – which often requires a CPB – is associated with several possible complications, which may increase the postoperative morbidity and mortality and the duration of the hospital stay. One of the major problems is renal impairment. This
occurs in children with a higher probability (up to 42% within 48 h [4]) than in adults (1–30% within 48 h [7,8,25]). Side effects of the CPB – the contact of blood with the machine’s surface, an inflammatory reaction, and the inadequate perfusion of the kidneys during the CPB-time – have been suggested to cause renal impairment [26,27]. Former studies have also shown that histological lesions are present in kidneys after CPB [28]. Moreover, the duration of the CPB correlates positively with the risk of developing renal malfunctions [3,4].

In our study, we were able to show that CPB caused renal damage as evident from (immuno-)histology, tissue ATP-status, and blood chemistry parameters, in particular creatinine and urea, indicating that the impairment of the kidney starts to be of initial functional relevance.

Our findings demonstrated widened glomeruli, proximal tubules showing loss of brush border and intracellular vacuolization, formation of nitrotyrosine, poly-ADP-ribose and nuclear translocation of HIF-1-α and AIF, together with increased plasma creatinine and urea levels. Taken together these changes indicate post-cardiopulmonary bypass acute kidney injury.

Interestingly, this renal damage could be attenuated by the administration of (−)-epigallocatechin-3-gallate, which has not been described yet. Of the different catechins present in green tea, EGCG is the one which predominates. Tea is one of the most frequently consumed beverages in the world, second to water. In particular in Asia, where green tea (Camellia sinensis) is common for thousands of years, beneficial effects for health have been repeatedly reported for it [29]. In this study, we focused on the renoprotective mechanisms of EGCG. The induction of nuclear activation of HIF-1-alpha indicates hypoxic damage to the kidneys. Interestingly, it is known that CPB can produce renal ischemia/reperfusion injury [30] and that during CPB reactive oxygen species accumulate in the blood, in particular during reperfusion [31]. The reason for this initial hypoxic/ischemic damage is still a matter of controversy and may involve too low output or too low blood pressure, thromboembolism, lipembolism or non-pulsatile flow. In our study all pigs showed the same blood pressure which is in the normal range for this species and age [32]. Oxygen saturation was also always in the normal range. With pathological and histopathological investigation we did not find any microthrombi or thromboembolism. Thus, our data, which indicate ischemia/reperfusion damage (HIF-1-alpha, nitrotyrosine), and the differences among the series both cannot be explained by low blood pressure or low oxygenation.

Reactive nitrogen species – in particular peroxynitrite which results from a reaction of nitric oxide and superoxide anions – are formed in large amounts in blood plasma during CPB [33]. Thus, oxidative respectively nitrosative stress is generated and may negatively act on the kidney’s cells as became obvious from histology. However, at present there is only little knowledge whether these effects indeed occur in the kidneys. Our new data support the view that also in the kidney there is hypoxic/ischemic damage and increased nitrosative stress as evident in our study from enhanced nitrotyrosine formation after CPB. Thus, nitrotyrosine, which has been considered to be “the footprint of peroxynitrite” [34], was excessively present in the cells of the ECC-group, which shows that the CPB caused nitrosative stress for the cells.

Efforts have yet been made to explore the protective effects of antioxidants against reactive oxidative species, e.g. in cardiac tissue during and after CPB – with a positive outcome [35]. However, regarding renal CPB-associated damage pure antioxidants like N-acetylcysteine failed to protect [14].

To the best of our knowledge there is nothing published on the effect of EGCG regarding the protection of the kidneys during CPB, in particular in pediatric cardiac surgery. In our study, we were able to demonstrate the occurrence of an ischemic/hypoxic situation as evident from nuclear HIF-1-alpha-translocation. During reperfusion typically increased free oxygen-derived radicals and NO-release should lead to the formation of peroxynitrite as was seen here by the increase in nitrotyrosine-formation.

In both, HIF-1-alpha- and nitrotyrosine-staining we found that EGCG was able to significantly (p < 0.05) protect the renal cells and to attenuate the adverse effects of the CPB. It was found by others that in cultured proximal tubular epithelial cells peroxynitrite could cause apoptosis following ATP-depletion [36]. In neurons the anti-apoptotic effect of EGCG has been related to its accumulation in the mitochondria, where it acts locally as a free radical scavenger [37]. Our own data show higher ATP-preservation in EGCG-treated animals, which might suggest that EGCG may act by a combination of antioxidant activity, NO-scavenging activity, anti-apoptotic activity and preservation of mitochondrial function. Thus, the number of pycnotic nuclei, which typically represent cells undergoing apoptosis was enhanced in the ECC-group, and partially prevented by EGCG. This was corroborated by the finding that in the ECC-group we found more nuclear AIF translocation and higher formation of poly-ADP-ribose (PAR) in the nuclei. Free radicals and peroxynitrite are known to cause DNA damage, which can lead to activation of poly-ADP-ribose polymerase leading to the formation of PAR.
a process which consumes high amounts of ATP and further will deplete ATP. PAR together with ATP-depletion is known to result in the release and nuclear translocation of AIIF, which is known to induce apoptosis. Thus, both parameters point to DNA impairment in the nephrons of the ECC-group. Interestingly, all related changes (PAR formation, AIIF translocation, loss of energy-rich phosphates, nitrotyrosine formation) were prevented by EGGC, which may be related to the antiapoptotic effects of this drug. Since PAR-formation leads to high energy consumption, this may partially contribute to or aggravate the observed loss of ATP in the ECC-group.

The considerations above are further supported by the standard histological HE staining, where several histomorphological lesions were to be seen in the ECC-group: the increased area and the width of the gap between Bowman’s capsule and the capillaries in the glomerulum, and the vacuolated cells in the proximal tubuli. These lesions could all be significantly (p < 0.05) attenuated by EGGC.

When looking at the kidney-specific blood chemistry parameters creatinine and urea, we could see the functional clinical correlate to the above findings: in our piglets, the values of both parameters rose significantly (p < 0.05) in the ECC-group which indicates reduced functioning of the kidney. Both creatinine- and urea-increase could be inhibited by EGGC indicating that the histochemoal changes may be of functional relevancy.

Another blood chemistry parameter that we investigated was the total amount of plasma proteins. We found a decrease in all three groups, which was significant (p < 0.05) only in the ECC-group. Although this shows, that the kidney seems to be affected by the CPB, the loss of proteins is not significant enough to indicate the presence of a complete acute renal failure. Possibly, the 120 min recovery time in our experiments was not long enough for a greater damage to develop, and our total amount of proteins only may indicate the beginning of it.

Another reason for the decrease of plasma proteins could be the catabolic situation, in which the piglet’s organism is brought due to the entire process of the operation and CPB.

It should be noted that in this study only one type of cardiopulgia was used, namely cold saline cardiopulgoic solution (Custodiol®. Dr. Franz Köhler Chemie, Bensheim, Germany). Thus, our data refer only to this type. However, the renal impairment probably is mainly depending on machine flow [38,39], flow type [40], temperature, and duration of CPB [41], while cardiac changes might be more related to the type of cardiopulgia [42].

In conclusion, our study demonstrated – to the best of our knowledge for the first time – that EGGC attenuates the adverse effects of CPB on renal nitrotyrosine formation, HIF-1α-alpha activation, PAR-formation, AIIF-translocation, loss of energy-rich phosphates and functional impairment of the kidneys, which may be explained by the antioxidant, NO-scavenging and anti-apoptotic activities of EGGC, but may also involve other properties, yet unknown. Further research on this promising substance seems reasonable.

Conflict of interest

The authors declare no competing interests.

Acknowledgement

This study has been supported by a grant given by ProCordis (Leipzig, Germany) to S.D.

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