Pathophysiological and Histopathological Ailments in Asphyxial Cardiac Arrest Induced Ischemic Renal Injury


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ABSTRACT
Cardiac arrest (CA) is a sudden interruption in the effective blood flow due to heart failure. The current research aimed to conduct the pathophysiological and histopathological analysis in the kidney in asphyxial cardiac arrest rat model. Cardiac arrest was induced by intravenous injection of vecuronium bromide (2 mg/kg), following stop of mechanical ventilation. Rats were kept on the CA condition for 5 minutes. After that, cardiopulmonary resuscitation (CPR) was done to achieve return of spontaneous circulation (ROSC) following intravenous injection of epinephrine bolus (0.005 mg/kg), sodium bicarbonate (1 mEq/kg) and turn on mechanical ventilation. Then Rats were sacrificed after cardiopulmonary resuscitation (CPR) following asphyxial CA at 6 hrs, 12 hrs, 1 day, 2 days, and 5 days. The intensity of renal injury measured by the serum levels of blood urea nitrogen (BUN), creatinine (Crtn). Moreover, Hematoxylin & cosin, and Periodic Acid Schiff staining in the kidney was done for evaluating the renal histopathological changes. Furthermore, COX-2 immunoreactivity and western analysis were performed in the kidney. Survival rate declined following ROSC compared to the sham group, it showed 80% at 6 hrs and decreased time-dependently to 8% at 5 days. In this study, serum BUN and Crtn levels and renal histopathological scores significantly increased after ROSC in CA. Moreover, COX-2 expression also increased after ROSC in comparison to the sham group with its peak level at 5 days following CA. Renal histological damage score and COX-2 expression were upregulated after ROSC following CA. These results direct that COX-2 takes part in the asphyxial CA-induced ischemic renal injury.

INTRODUCTION
Cardiac arrest (CA) also known as a circulatory arrest is an abrupt stop in the effective blood flow due to heart failure and 60% of all deaths from cardiovascular diseases belong to out-of-hospital sudden cardiac death (SCD) (Engdahl et al., 2002; Chugh et al., 2008), globally, the survival rate of sudden CA is lower than 1% and close to
5% in the USA (Chugh et al., 2004; Mokdad et al., 2004). Following the resume of spontaneous circulation (ROSC) the systemic ischemia/reperfusion (I/R) reaction can lead to various organ dysfunctions which are called post-CA syndrome (PCAS) (Roberts et al., 2013). First 3 days after ROSC considered a critical phase, while organ damages and inflammation occurred (Nolan et al., 2008). Although many studies showed multiple organ damages in PCAS after ROSC (Roberts et al., 2013) however, the majority of the researches concentrated on brain and heart injuries; kidney, liver and lung dysfunction after ROSC in CA was rare (Madl and Holzer, 2004). Particularly acute kidney injury (AKI) after ROSC was also related with poor outcome in CA patients (Tujjar et al., 2015), moreover Chou et al. (2014) reported that AKI can affect neurological recovery in the rodent model. Thus, studying AKI after ROSC is similarly essential in CA.

AKI is characterized by a potent inflammatory response (Bonventre and Zük, 2004; Sharfuddin and Mollitoris, 2011). Transient or prolonged renal hypoperfusion (I/R injury) is the most common reason for AKI in patients (Schrier et al., 2004; Lameire et al., 2005) and AKI in CA patient is an instance of whole-body I/R injury after ROSC characterized by various-organ dysfunction (Roberts et al., 2013). Inflammatory reactions activate during the I/R injury process which consequences to produce inflammatory cytokines, for example, interleukin-1, metabolites of arachidonic acid and tumor necrosis factor-alpha. Particularly, arachidonic acid (AA) is responsible for the production of prostaglandins which are lipid autacoids and both play role for maintaining homeostatic activity, mediating the inflammatory response (Ricciotti and FitzGerald, 2011). Cyclooxygenase-2 (COX-2) known that responsible for catalyzing the early crucial steps of an enzyme in the arachidonic acid metabolism (Matsuyama et al., 2005; Nørregaard et al., 2015).

In the recent study, we hypothesized that a strong relationship may exist between COX-2 and asphyxial CA-induced ischemic renal injury following ROSC. However, there is a limited study of COX-2 and its representation in the renal tissues after ROSC in CA. Moreover, evaluation of changes of BUN, Crtn and histopathological parameters in the kidney after asphyxia cardiac arrest following CPR is rare. Thus, the goal of this current study was to assess the BUN, Crtn, physiological, and histological study of the kidney after ROSC in CA. Furthermore, we also performed western blot and immunohistochemical studies to evaluate the expression in the kidney tissue for knowing whether or how the renal injury was associated with COX-2 expression after ROSC in asphyxial CA rat model.

**MATERIALS AND METHODS**

**Experimental animals and groups:** We got male rats (Sprague-Dawley) from Jeonbuk National University’s Experimental Animal Center. We provided them open access to water and food. All the rats were placed in the cages with controlled 12 hrs light/ 12 hrs dark cycle and maintained the temperature(23±2°C) and humidity (60±10%). The whole experimental procedure was passed by the Animal Care Institution and use committee (approval no. CBNU 2018-00358)-Jeonbuk National University. The following groups were randomly divided from the experimental animals: A). Sham group (n=6), not subjected to CA methods but handled the same as CA group; and B). CA groups (n=6 for each specific time examined), subject to CA procedures. Then rats were sacrificed at each group time points (6 hrs, 12 hrs, 1 day, 2 days and 5 days).

**Induction of cardiac arrest and cardiopulmonary resuscitation:** According to the existing protocol CA and cardiopulmonary resuscitation (CPR) were conducted (Drabek et al., 2014; Lee et al., 2019). In short, 2-3% isoflurane was used to anaesthetize the rats and rodent mechanical ventilator (Harvard Apparatus, Holliston, MA, USA) was used to keep the respiration. An oxygen saturation probe of pulse oximetry was connected to the left foot for monitoring peripheral oxygen saturation (SpO₂).

Thermal pad was used to monitor body temperature at 37±0.5°C before and after the ROSC except CA period. Electrocardiogram (ECG) was monitored continuously. Left femoral artery cannulation was performed to monitor the mean arterial pressure (MAP) and right femoral vein cannulation was done to inject vucuronium bromide (2 mg/kg), epinephrine bolus (0.005 mg/kg) and sodium bicarbonate (1 mEq/kg) intravenously. Following 5 min of the stabilization phase, anesthesia and motorized aeration was blocked. MAP less than 25 mm Hg and following pulseless electrical movement were applied to confirm CA. After 2.5-3 minutes following vucuronium bromide injection CA was defined. After 5 minutes of CA, epinephrine bolus (0.005 mg/kg) and sodium bicarbonate (1 mEq/kg) were injected intravenously with CPR followed by motorized aeration with 100% oxygen and automatic chest compression at a pace of 300/minutes until MAP exceeded 60 mm Hg and there was observed electrocardiographic action. Usually, one hour after ROSC spontaneous breathing occurred and they were extubated and monitored for evaluating the outcome.

**Serum blood urea nitrogen (BUN), creatinine (Crtn) detection:** At the specific time points after ROSC, rats were sacrificed for collecting the sample. Whereas rats were anesthetized with 30% urethane, and 5 ml blood collection was done from inferior vena cava. Cenrifugation of the blood samples was performed to collect serum to determine blood urea nitrogen (BUN) and creatinine (Crtn) which were detected by the use of standard techniques with an Olympus AU 2700 Analyzer (PT10V comprehensive Plus 17V kit, Samsung, Suwon, Korea, Wow animal hospital).

**Hematoxylin-eosin (H-E) and Periodic Acid-Schiff (PAS) staining:** Briefly, rats were anesthetized with 30% urethane (intraperitoneal administration) then perfusion was performed with 4% paraformaldehyde (PFA). The kidney tissues were separated from the body, fixed in 10% NBF, and cut sagittally into two slices, embedded in paraffin, and thereafter sectioned (6 µm). To investigate pathologic alterations in kidney, H-E and PAS staining was done. H-E staining was conducted according to the published protocol (Park et al., 2018). In short, sections are fitted on gelatin-coated microscope slides, stained with H-
E and dehydrated with sequential ethyl alcohol solutions and thereafter, mounted with Canada balsam (Kanto Chemical, Tokyo, Japan). As recommended by the kit manufacturer, PAS and Masson trichrome stain were performed. Sections were imaged at fixed 400 × magnifications applying a Leica DM 2500 microscope (Leica Microsystems, Wetzlar, Germany). Ten specific areas in each group were captured. Histopathologic analysis of renal lesions was evaluated by published techniques (Acikgoz et al., 2014).

**Immunohistochemistry and western analysis:** According to our published protocol COX-2 immunohistochemistry and western analysis were performed (Akanda et al., 2019). For immunohistochemistry, paraffin sections have been deparaffinized in xylene and dehydrated in ethanol. Normal serum was used to block tissue. Anti-rabbit monoclonal COX-2 antibody incubated tissue overnight at 4°C (dilution 1:500). Sections incubated for 2 hours, at room temperature, with biotinylated secondary antibody. Until a brown color development, tissue antibody was incubated with diaminobenzidine (DAB) in the dark. After counterstain, sections were dehydrated, cleared, and fitted on a glass slide. Sections were imaged at fixed 400 magnifications using a Leica DM 2500 microscope (Leica Microsystems, Wetzlar, Germany). Ten specific areas have been captured in each group. Relative optical density percentage (ROD%) was measured using image-J threshold analysis software. For western analysis, total protein concentration of lysate renal tissues was evaluated with a bicinchoninic acid (BCA) protein assay kit. An equivalent volume of protein was isolated and transferred with a polyacrylamide gel electrophoresis (SDS-PAGE) to a nitrocellulose membrane. Serum BUN and Creatinine was measured using a single and/or bidirectional variance analysis (ANOVA). For all analyses the lowest statistical significance was found to be P<0.05.

**RESULTS**

**Physical and behavioral parameters of rats:** There was no important distinction (P<0.05) among the sham group and CA run groups in separate physiological parameters. (Table 1). Isoelectric ECG and SpO₂ were used for confirming CA. The survival rate was 80% at 6 hrs after CA following ROSC; the survival rate declined time-dependently with the time passed and it was approximately 55% at 12 hrs, 43% at 1 day, 20% at 2 days and 8% at 5 days.

**Serum BUN and Crtn:** Serum BUN and Creatinine was measured for evaluating kidney health. BUN and Crtn were markedly (P<0.05) increased at 12 hrs, 1-day, 2 days and 5 days post-CA groups compared to the sham-operated group. Moreover, BUN was also significantly increased after 6 hrs post-CA, however, Crtn did not show significant increase at 6 hrs after CA and the peak point of BUN and Crtn was in 12 hrs post-CA group (Fig. 1).

Five percent bovine serum albumin (BSA) incubated the membrane for 2 hrs and then overnight in primary antibodies. After washing and incubating with secondary antibodies bands images were taken by a LAS-400 image system, (GE Healthcare, Little Chalfont, UK).

**Statistical analysis:** Graph Pad Prism 5.0 was used for analyzing the data and showed as means ± standard error means (SEM). Survival figures were examined by using Kaplan-Meier statistics and log-rank tests. Groups were measured using a single and/or bidirectional variance analysis (ANOVA). For all analyses the lowest statistical significance was found to be P<0.05.

![Fig. 1: Blood urea nitrogen (BUN) and Creatinine (Crtn) were detected at 6 hrs, 12 hrs, 1 day, 2 days and 5 days following ROSC. BUN and Crtn levels significantly increased in normothermia groups as contrasted to the sham group. Data has displayed as mean ± standard error means (SEM). *P<0.05 compared with the sham and normothermia groups.](image-url)

**Table 1: Physical and behavioral parameters of rats**

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Baseline</th>
<th>CA</th>
<th>Nor-6 hrs</th>
<th>Nor-12 hrs</th>
<th>Nor-1 day</th>
<th>Nor-2 days</th>
<th>Nor-5 days</th>
</tr>
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<tbody>
<tr>
<td>Body weight (g)</td>
<td>365.4±17.33</td>
<td>372.3±16.72</td>
<td>367.8±13.17</td>
<td>374±17.04</td>
<td>374.4±12.06</td>
<td>378.6±13.02</td>
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<tr>
<td>Temperature (°C)</td>
<td>36.8±0.11</td>
<td>36.8±0.19</td>
<td>36.4±0.20</td>
<td>36.3±0.07</td>
<td>36.2±0.11</td>
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<tr>
<td>Asphyxial time to CA (S)</td>
<td>159.3±13.29</td>
<td>159.1±14.31</td>
<td>164.1±16.36</td>
<td>153.8±8.79</td>
<td>163.3±16.32</td>
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</tr>
<tr>
<td>CPR time (S)</td>
<td>75.8±10.56</td>
<td>75.3±10.61</td>
<td>71.1±12.49</td>
<td>73.5±11.07</td>
<td>73.5±11.66</td>
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<tr>
<td>Heart rate (beat/min)</td>
<td>335.1±8.51</td>
<td>350.4±6.99</td>
<td>346±11.01</td>
<td>336.1±6.51</td>
<td>342.5±5.77</td>
<td>329.5±10.04</td>
<td></td>
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<tr>
<td>Survival rate (%)</td>
<td>80</td>
<td>55</td>
<td>43</td>
<td>20</td>
<td>8</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Room Temperature (°C)</td>
<td>24.4±0.53</td>
<td>24.1±0.93</td>
<td>24.4±0.39</td>
<td>24.7±0.87</td>
<td>24.6±0.59</td>
<td>24.7±0.77</td>
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</table>

CA, cardiac arrest; CPR, cardiopulmonary resuscitation.
Fig. 2: Periodic Acid Schiff (PAS) stain in the renal tissue of the sham group (A) and normothermia cardiac arrest (CA) operated groups (B-F). Glomerular capillaries have dilated (arrows) in normothermia groups as compared to sham. Original magnification × 400 and mean ± standard error means (SEM) was used to express the data with the scale bar of 50 µm. *P<0.05 compared with the sham and normothermia groups.

Fig. 3: Hematoxylin-eosin (HE) staining of the kidney of the sham operated group (A) and normothermia cardiac arrest (CA) operated groups (B-F). Severe erosion of brush borders (asterisk) and irregular brush borders (sharp) in the renal tubules found in the normothermia groups as compared to the sham group. Scale bar = 50 µm. Original magnification × 400. Data has displayed as mean ± standard error means (SEM). Scale bar = 50 µm. *P<0.05 compared with the sham and normothermia groups.
**Fig. 4:** Immunohistochemistry of COX-2 in the kidney of the sham group (A) and normothermia cardiac arrest (CA) operated groups (B-F). Relative optical density (ROD) considerably increased in normothermia groups as compared to the sham group. Original magnification × 400. Data has displayed as mean ± standard error means (SEM). Scale bar = 50 µm. *P<0.05 compared with the sham and normothermia groups.

**Fig. 5:** Western blot analysis of COX-2 in the renal cortex of the sham and CA operated groups. Relative optical density (ROD) considerably risen in normothermia groups as compared to the sham group. All the data has displayed as mean ± standard error means (SEM). *P<0.05 compared with the sham and normothermia groups. #P<0.05 compared with the sham and normothermia groups.

**Renal histopathology:** In the CA run groups after ROSC, histopathology in the kidney was boosted in a significant manner (P<0.05) as compared to the sham group. The histopathological changes involved severe erosion of brush border of the proximal convoluted tubular epithelial cells, dilatation of glomerular capillaries with inflammatory cells but not severely and evidence of acute renal tubular necrosis and interstitial edema. The basis on the H-E stain score, damages of proximal and distal convoluted tubules of renal cortex area was significantly increased at 12 hrs post-CA. One day after CA, damages were more increased and keep up until 2 days and 5 days of post-CA (Fig. 3). PAS stain revealed that no serious injury in the glomerular basement membrane except for dilated glomerular capillaries and this damage score was significantly increased after 1 day, 2 days, and 5 days of ROSC as
compared to the sham. It also increased after 6 hrs and 12 hrs post-CA but not significantly (Fig. 2). Masson trichrome staining also carried out but there were no differences between groups and fibrosis was not detected (data not shown).

**COX-2 expression in renal cortex:** Western blot and immunohistochemistry analysis (Fig. 4 and Fig. 5) of the renal cortex showed that COX-2 expression was markedly (P<0.05) higher in the normothermia CA groups related with the sham group. The Relative Optical Density (ROD%) of COX-2 expression considerably boosted after 6 hrs, 12 hrs, 1 day, 2 days and 5 days post-CA as compared to the sham group. However, the peak time for COX-2 expression in the kidney was 5 days after ROSC.

**DISCUSSION**

In this present study, the survival rate reduced considerably after ROSC in a time dependent manner and it was 43% at 1 day and only 8% at 5 days. Several reasons could lead to these results, for instance ischemic time, experimental animal species, CA induction cause, and CA length. Histopathological renal injury scores significantly increased at 12 hrs after ROSC in CA, scores were noticeably elevated at 1 day and the scores remained the same until 5 days after ROSC. The most common histological features found on renal I/R injury was a loss of the apical brush border of proximal tubular cells, proximal tubular dilatation, distal tubular casts, and features of apoptosis in both proximal and distal tubular cells (Sharfuddin and Molitoris, 2011). In the present study histological study of the kidney also revealed renal glomerular capillary enlargement, severe damage of the brush border of proximal convoluted tubules, acute renal tubular necrosis, and interstitial edema. Moreover, rats of normothermia CA groups showed an increased level of BUN and Crtn as compared to the sham. However, the peak period of BUN and Crtn was at 12 hrs; after that, it was almost maintained until 5 days after ROSC in our recent study. Renal injury etiologies are multifactorial, however, I/R damage is one of the leading reasons in CA patients (Hoste et al., 2015). Besides, another significant factor contributing to the incidence of renal injury may be the renal ischemic length. Our findings are similar to traditional I/R kidney injury. In I/R injury, renal ischemia is 35 minutes or 45 minutes while ischemia was only 5 minutes in our asphyxial CA model. (Wang et al., 2015; Greite et al., 2018). The possible reason is that when the I/R injury happens after ROSC, the whole body can be used as an further stimulus for AKI when the I/R injury occurs simultaneously with harm in several organs, the detrimental product produced into circulation and aftershock scavenging function.

Inflammatory reactions are also significant variables that add to worsening renal function after I/R. COX-2, the main enzyme of prostanooid synthesis, increases the synthesis of pro-inflammatory factors, for example, prostaglandin (PG) E2 and PGI2 with its expression, which could further aggravate the response of inflammation. In normal conditions, COX-2 is expressed at low levels in the kidney however, it’s highly induced in the case of inflammation and kidney injury (Breyer and Harris 2001). Matsuyama et al. (2005) reported significantly increased expression of COX-2 in the kidney after 1.5 hrs, 3 hrs, 5 hrs, 12 hrs and 24 hrs of I/R injury; however, the peak period of COX-2 expression was at 3 hrs and 5 hrs. Moreover, in unilateral ureteral obstruction, selective COX-2 inhibition reduced the advancement of kidney parenchymal damage and interstitial fibrosis. The previous study also revealed that the increased expression of COX-2 is responsible for tubular damage of kidney and precise COX-2 pharmacological inhibition was related with the prevention of kidney damage (Rios et al., 2012; Kirkby et al., 2016). Therefore, it has been suggested that a strong relationship may exist between COX-2 and asphyxial CA-induced ischemic renal injury following ROSC of rats. Moreover, we had perceived a paucity of study about the expression of COX-2 at various time point in the kidney after ROSC in CA. In our recent study, the peak period of renal COX-2 expression was found at 5 days post-CA following ROSC. However, COX-2 expression in kidney markedly increased the early period after ROSC which aggravate the inflammation and could be related to the AKI as well as to the lower early survival rate after ROSC in CA.

In conclusion, renal histological damage score, and COX-2 expression was upregulated in asphyxial CA-induced renal ischemia injury of a rat model. The maximal histological damage was observed one day after ROSC following CA, which maintained until five days and the maximal COX-2 expression was found five days after ROSC in the kidney. These results direct that COX-2 takes part in the asphyxial CA-induced ischemic renal injury and it may be also related to the lower survival rate at the early stage after ROSC following CPR in CA.

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**Authors contribution:** JHC and AI contributed equally to this work. JHC and AI: methodology, data analysis and writing the original draft. JHL, YJY, SEK, AJ, WT, DA, BYP, KK, JHL and MRA review and editing. HJT and JCY: conceptualization, supervision, funding, review, and editing. All authors agreed the final version of document.

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