

RESEARCH ARTICLE

Histopathology and Tumor Necrosis Factor- α Expression in The Kidney of an Asphyxial Cardiac Arrest Rat Model

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Abstract

Multiple organ injuries in patients with post cardiac arrest syndrome (PCAS) after cardiac arrest (CA) is associated with mortality. Among multiple organ injuries after return of spontaneous circulation (ROSC), renal dysfunction can lead to acute kidney injury, which is known to be associated with high mortality. This renal injury is associated with a systemic inflammatory response syndrome mediated by ischemia reperfusion by ROSC following CA. However, the mechanism remains unclear. Therefore, the objective of this study was to determine, the relationship between the expression of tumor necrosis factor- α (TNF- α), a pro-inflammatory cytokine, and renal injury in PCAS. In the present study, asphyxial CA was induced in Sprague-Dawley rats with normothermia and the survival rate was measured at two days after ROSC. The rats in each group (n=6) were sacrificed at 6 h, 12 h, 1 day, and 2 days after ROSC. Renal injury was analyzed with by Masson's trichrome stain, TNF- α immunohistochemistry (IHC) and western blot. The mortality was 72% at 12 h after ROSC and the survival rate of rats was decreased to 24% 2 days after ROSC. Histopathological score in the renal tissue after CA showed a significant increase at 6 h than sham group. The expression level of TNF- α in the renal cortex tissue was also increased at 6 h after CA based on both IHC and western blot results. After CA, the renal histopathological injury was significantly increased at 6 h after ROSC with a proportional increase of TNF- α expression in the kidney tissue leading to rapid injury to the kidney.

Keywords: Post cardiac arrest syndrome, Asphyxial cardiac arrest, kidney, Histopathology, TNF- α

Asfiksiyal Kardiyak Arrestli Rat Modelinde Böbrek Histopatolojisi ve Tümör Nekrozis Faktör- α Ekspresyonu

Öz

Kardiyak arrest (KA) sonrası post-kardiyak arrest sendromu (PKAS) olan hastalarda çoklu organ hasarı mortalite ile ilişkilidir. Spontan dolaşıma dönüş (SDD) sonrası çoklu organ hasarları arasında böbrek fonksiyon bozukluğu, yüksek mortalite ile ilişkili olduğu bilinen akut böbrek hasarına yol açabilir. Bu böbrek hasarı, KA'yı takiben SDD tarafından iskemi-reperfüzyonun aracılık ettiği sistemik bir inflamatuvar yanıt sendromu ile ilişkilidir. Ancak, bunun mekanizması belirsizliğini korumaktadır. Bu nedenle, bu çalışmanın amacı, bir proinflamatuvar sitokin olan tümör nekrozis faktör- α (TNF- α) ekspresyonu ile PKAS'ta böbrek hasarı arasındaki ilişkiyi belirlemektir. Çalışmada, normotermik Sprague-Dawley ratlarda asfiksiyal KA indüklendi ve ratların hayatta kalma oranı SDD'den iki gün sonra ölçüldü. Her gruptan ratlar (n=6), SDD'den 6 saat, 12 saat, 1 gün ve 2 gün sonra sakrifiye edildi. Böbrek hasarı Masson trikrom boyama, TNF- α immünohistokimyasal (IHC) yöntem ve western blot ile analiz edildi. SDD'den 12 saat sonra mortalite %72 iken, SDD'den 2 gün sonra ratlarda hayatta kalma oranı %24'e düştü. KA sonrası böbrek dokusundaki histopatolojik skor, kontrol grubuna göre 6. saatte anlamlı bir artış gösterdi. Renal korteks dokusundaki TNF- α ekspresyon seviyesi, hem IHC hem de western blot analiz sonuçlarına göre KA'dan 6 saat sonra arttı. KA'dan sonra, böbrek histopatolojik hasarı, böbrek dokusunda orantılı bir TNF- α ekspresyon artışı ile birlikte böbrekte hızlı hasara yol açan SDD'den 6 saat sonra önemli ölçüde arttı.

Anahtar sözcükler: Post cardiac arrest syndrome, Asphyxial cardiac arrest, kidney, Histopathology, TNF- α

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INTRODUCTION

Cardiac arrest (CA) is a sudden stop of normal blood flow. It is a major cause of death in Europe and United States [1]. Over the last few decades, many researchers have conventionally tried to improve the rate of return of spontaneous circulation (ROSC) for CA, achieving remarkable outcomes [2]. Even after ROSC, mortality rates are still high. Overall survival rates after CA have been reported to be less than 1% worldwide [3]. The poor prognosis after ROSC is explained by a pathophysiologic process called post cardiac arrest syndrome (PCAS). PCAS is clinically manifested as brain injury, myocardial dysfunction, ischemia/reperfusion (IR) injury and persistent precipitating pathology [4]. According to Roberts et al. [5], dysfunction of multiple organs is common after ROSC following CA and that is associated with PCAS.

Chronic kidney disease and poor renal function with heart failure have been associated with poor outcomes and high mortality [6]. Even small changes in serum creatinine (Cr) can be associated with high systemic mortality rates [7]. Acute kidney injury (AKI) after ROSC following CA is quite common, with an incidence of over 40% [8]. However, most recent studies have focused on brain or heart injury after ROSC. Despite extensive renal impairments, research has been barely conducted on kidney injury after CA. The PCAS causes systemic IR-mediated injury, inducing systemic inflammatory response syndrome. While inflammatory reactions are usually caused by certain infections, "sterile inflammation" occurs during ischemic insults [9]. As systemic inflammatory response syndrome, IR injury after CA can triggers inflammation. Several studies have reported that tumor necrosis factor- α (TNF- α) is increased in the heart, lung and brain after CA in animal models, although the exact mechanisms have not been clearly understood [10-12]. Previous studies have reported that AKI by IR is associated with pro-inflammatory cytokines such as TNF- α [13]. However, there is no previous study on the CA-induced kidney injury.

The present study hypothesized that IR after ROSC causes a sterile inflammatory response in the kidney, leading to renal injury and production of pro-inflammatory cytokines. We attempted to investigate the relationship between low survival rate and renal injury in the early an inflammatory stage after ROSC. The aim of present study was to understand the mechanism of renal injury by the changes of TNF- α as an inflammatory cytokine in the asphyxial CA rat model.

MATERIAL AND METHODS

Animals and Ethical Approval

Male Sprague-Dawley rats (9 weeks, 280~310g) were obtained from the Experimental Animal Center of Jeonbuk

National University (Iksan, South Korea). Rats were housed in a rat isolator with a 12 h light/dark cycle and maintained on standard laboratory chow *ad libitum*. All experimental animals used in this study were maintained under the protocol approved by the Institutional Animal Care and Use Committee (Approval no. JBNU 2019-005) of Jeonbuk National University. The rats were randomly divided into two groups as follows: Sham group (n=6), which was not subjected to CA operation, and CA-operated group (n=24), which was subjected to CA. The rats in each group were sacrificed at 6 h (CA-6 h, n=6), 12 h (CA-12 h, n=6), 1 d (CA-1 d, n=6), and 2 d (CA-2 d, n=6), respectively, after ROSC.

Induction of CA, and Cardiopulmonary Resuscitation (CPR)

CA induction and CPR were performed according to published protocols [14]. In brief, the rats were anesthetized with 2% to 3% isoflurane and mechanically ventilated with a rodent ventilator (Harvard device, Massachusetts Holly Stern material, USA). We monitored peripheral oxygen saturation (SpO₂) using an oxygen saturation probe (Nonin Medical, Plymouth, MN, USA) attached to the left hind foot of each rat. The electrocardiographic probes (GE Healthcare, Milwaukee, WI, USA) were attached to limbs, and the electrocardiogram (ECG) was recorded ceaselessly. The left femoral artery was cannulated under monitoring the mean arterial pressure (MAP) (MLT 1050/D, AD Instruments, Bella Vista, Austria), and the right femoral vein was cannulated for injection. After 5 min of the stabilization period, we administered 2 mg/kg of vecuronium bromide (GensiaSicor Pharmaceuticals, Irvine, CA, USA) intravenously, and stopped anesthesia and mechanical ventilation. About 3-4 min after vecuronium injection, CA was defined when MAP was less than 25 mmHg and pulseless electrical activity occurred. CPR was initiated at 5 min after CA by a bolus injection of epinephrine (0.005 mg/kg; Yeongdeungpo-gu, Seoul, Korea) and sodium bicarbonate (1 mEq/kg; Sungnam, Kyunggi-do, Korea). Mechanical ventilation (VentElite; Harvard apparatus, Holiston, MA, USA) with 100% oxygen was followed. Mechanical chest compression was given at a rate of 300/min until the MAP reached 60 mmHg, as well as electrocardiographic activity, was observed. When the animals were hemodynamically stable and spontaneously breathable they were extubated 2 h after resuscitation and monitored for outcome evaluation.

Measurement of Serum Blood Urea Nitrogen (BUN), Creatinine (Cr)

Rats were anesthetized with 30% urethane (1.5 g/kg, i.p.; Daejung, Gyeonggi-do, Korea) and 3 mL blood was collected from inferior vena cava. Thereafter, it was centrifuged to 4000 rpm for 10 min and serum was obtained. The serum was used for the analysis of BUN and Cr with Automatic Analyzer 7020 (Hitachi, Japan).

Histopathologic Assessment

The kidneys were collected and fixed with 4% paraformaldehyde (PFA) at room temperature for 24 h, and paraffin-embedded. Tissues were cut into 7 μ m thick sections, which were stained with Masson's trichrome (Scytek, West Logan, UT, USA) at room temperature and visualized under a light microscope at \times 400 magnifications (Leica DM 2500; Leica Microsystems GmbH, Germany). Masson's trichromic method was used for defining tubular injury, with tubular dilatation, tubular atrophy, tubular cast formation, vacuolization, degeneration and sloughing of tubular epithelial cells, or thickening of the tubular basement membrane. In brief, only cortical tubules were included in the following scoring system in all cortex regions each sample: 0 = no tubular injury; 1 \leq 9% of tubules injured; 2 = 10-25% of tubules injured; 3 = 26-50% of tubules injured; 4 = 51-75% of tubules injured; 5 \geq 76% of tubules injured [15,16].

Immunohistochemistry (IHC)

The expression and localization of TNF- α in renal tissue were detected immunohistochemically with a rabbit polyclonal antibody against TNF- α (Abcam Incorporated, Cambridge, MA, USA). After deparaffinization, tissue sections were treated using a microwave antigen-retrieval procedure in 10 mM sodium citrate buffer pH6.0 (Sigma-Aldrich, Sigma Aldrich, Burlington, MA, USA) After blocking endogenous peroxidases, sections were incubated with a non-immune serum to block non-specific staining. To assess alterations of TNF- α immunoactivity levels, tissue sections were incubated with anti-TNF- α (diluted 1:500, Abcam, Cambridge, UK), anti-rabbit secondary antibody (biotinylated anti-rabbit IgG(H+L); Vector Laboratories, Burlingame, CA, USA), and using 3,3'-diaminobenzidine (DAB; Sigma Aldrich, Burlington, MA, USA). After DAB the sample slide counterstained the hematoxylin. A light microscope was used to make images at fixed \times 400 magnifications. For quantitative analysis of densities of TNF- α immunoreactivities, relative optical density percentage (ROD %) was measured using image-J threshold analysis software [IJ172-win-Java1.8.0, Bethesda, MD, USA].

Western Blot Analysis

To examine change in the level of TNF- α protein in the kidney after CA, western blot analysis was performed according to a previously published method [17]. Kidney tissues were homogenized with proteinase and phosphatase inhibitors in a protein extraction solution (Pro-Prep; Intron). The homogenates, which contained 20 μ g of protein, were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene fluoride (PVDF) membranes. The blot was probed with primary antibody against TNF- α (diluted 1:500, Abcam, Cambridge, UK), and β -actin (diluted 1:1000, Cell Signaling Technology, Danvers, MA). Horseradish per-

oxidase-conjugated anti-rabbit immunoglobulin G (Santa Cruz Biotechnology, CA, USA) was used as a secondary antibody. The band images were detected with a Las-4000 imager (GE Healthcare Life Science, Pittsburgh, PA, USA).

Statistical Analysis

All data were entered into Graph Pad Prism 5.0 and presented as means \pm standard error of the mean (S.E.M.). Survival was analyzed using Kaplan-Meier statistics and log-rank test. Statistical analyses were used one-way analysis of variance (ANOVA) and Masson's trichrome staining was analyzed Kruskal Wallis analysis followed by Bonferroni's multiple comparison tests. Differences were considered significant when P-value was less than 0.05.

RESULTS

Physiological Variables and Survival Rate

We estimated the survival rate 2 d after CA. Survival rate of CA group was significantly reduced (Fig. 1). A mortality rate of 75% occurred at 12 h after ROSC and the survival rate of rats decreased to 24% 2 days after ROSC ($P < 0.05$). Before CA operation, all the rats in the sham and CA-operated groups showed physiological values of body weight, body temperature, and heart rate (Table 1). There was no significant ($P > 0.05$) difference among groups for baseline characteristics, including body weights, body temperature, and heart rate. The induction of CA was 2-3 min after the intravenous injection of vecuronium bromide (2 mg/kg). CA was confirmed with isoelectric electrocardiogram (ECG), SpO₂, and MAP. ECG, SpO₂ and MAP were changed as expected according to the experimental protocol. Body temperature was the same as that at the baseline or after ROSC.

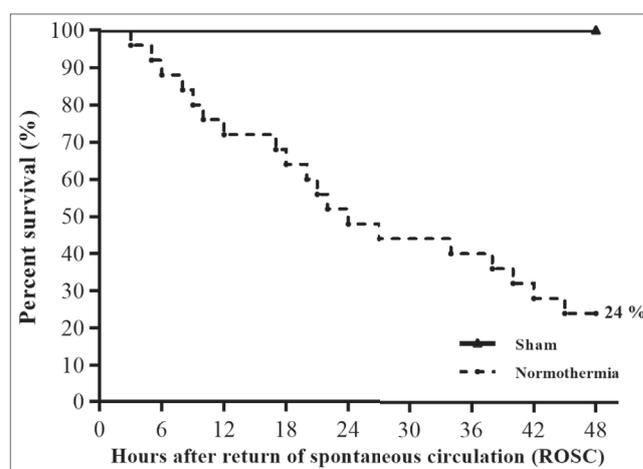


Fig 1. Survival rate. Cumulative survival rate analyzed by a Kaplan-Meier analysis in the sham- and cardiac arrest (CA) groups 2 days after return of spontaneous circulation (ROSC). The survival of rats in the CA group had significantly different from that in the sham-CA group (log-rank test, $P < 0.05$)

Table 1. Physiological variables in the sham and CA groups. Schematic representation of the asphyxial CA model in rats and measurements obtained at animal stabilization period (baseline), induction of CA, CPR time, Heart rate, and sacrifice time

Parameters	Sham	CA-6h	CA-12h	CA-1d	CA-2d
Body temperature (°C)	36.88±0.04	37.06±0.05	36.75±0.06	36.60±0.10	36.33±0.03
Asphyxial time to CA (second)	-	163.2±4.66	163.75±5.54	170.00±5.40	154.75±4.92
CPR time (minute)	-	1.23±0.06	1.26±0.07	1.22±0.09	1.26±0.07
Heart rate (beat/min)	337.83±2.09	347.83±3.06	342.67±2.95	336.17±2.91	343.67±2.23

CA: Cardiac arrest, CPR: Cardiopulmonary resuscitation

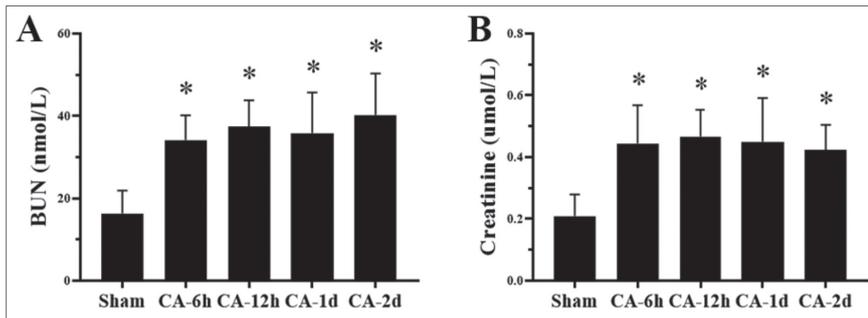


Fig 2. Blood urine nitrogen (BUN) and creatinine (Cr). BUN (A) and Cr (B) were significantly higher in the CA groups compared to the sham group. Data are expressed as mean ± standard error means (SEM). * P<0.05 compared with the sham group and CA groups at 6 h, 12 h, 1 d, and 2 d groups respectively in a time-dependent manner

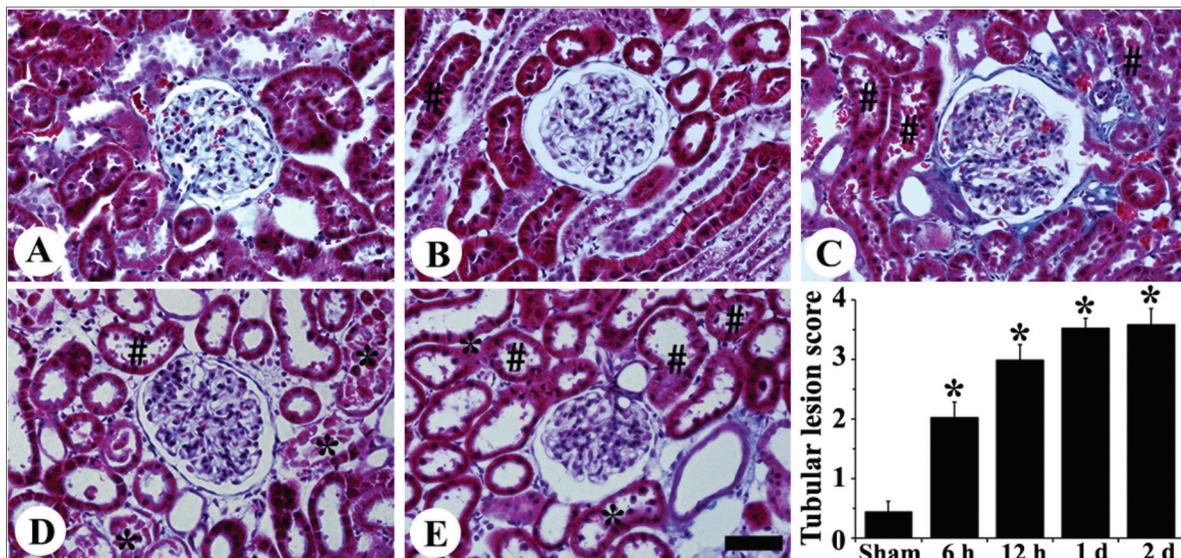


Fig 3. Masson's trichrome stain. Masson's trichrome staining of the sham group (A) has shown no tubular injury. In CA groups [B-6h, C-12h, D-1d, E-2d], the tubular injury score is markedly increased at 6 h (B) and maintained until 2 d (E). Renal tubules showed severe dilatation (star), loss of brush border with necrosis (hash). Data are expressed as mean ± standard error means (SEM). * P<0.05 compared with the sham and CA-operated groups at 6 h (B), 12 h (C), 1 d (D), and 2 d (E). Scale bar: 200 μm (400x)

Renal Function

The serum level of BUN and Cr was measured to assess the effect of CA in kidney (Fig. 2). One day after CA, serum BUN and Cr levels remarkably increased in CA group compared with the sham group (P<0.05). The results were maintained until 2 days after ROSC.

Renal Histopathological Changes

Masson's trichrome staining was performed to observe the extent of renal injury. It was shown that glomeruli and tubular injury was increased with the increase of time after

ROSC. As indicated in Fig. 3-A,B,C,D,E severe architectural disruptions of the kidney were triggered by CA, including glomerular capillaries dilation, brush border loss. Tubular lesion scores were significantly increased in the CA group (P<0.05) at 6 h after CA (Fig. 3-B). Tubular injury lesions at 6 h after ROSC were found to increase time-dependently. Glomeruli injury of the kidney increased at 2 d after ROSC as compared to that in the sham group (Fig. 3-A,E).

Immunohistochemical Analysis

Immunohistochemical analysis was performed to examine the expression and localization of TNF- α (Fig. 4). TNF-α

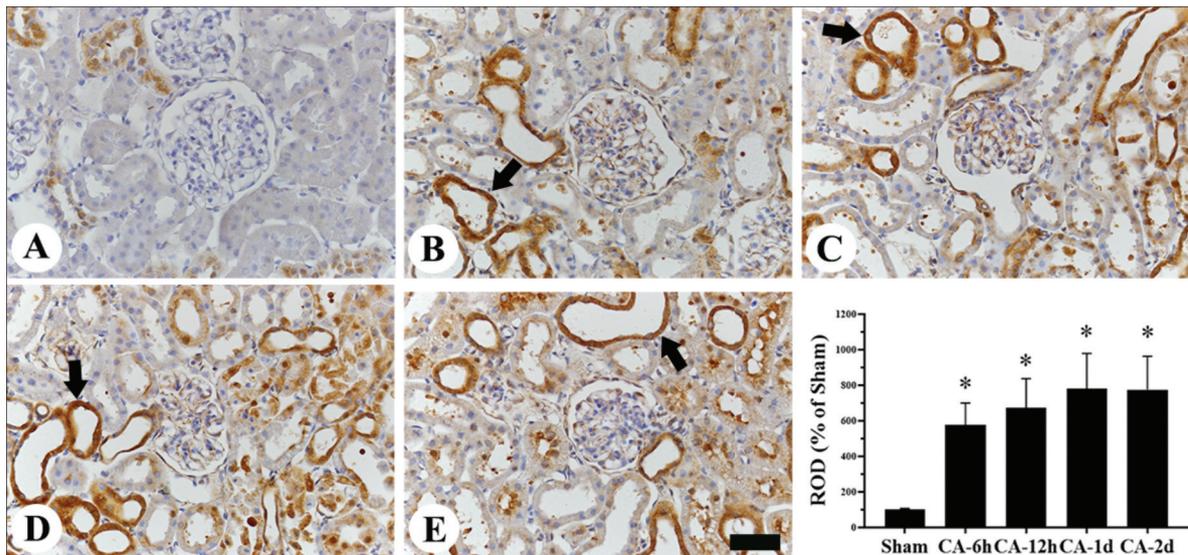


Fig 4. TNF- α IHC. IHC of TNF- α in the kidney of the sham (A) and cardiac arrest (CA) operated [B-6h, C-12h, D-1d, E-2d] groups. Relative optical density (ROD%) of TNF- α expression is significantly increased in proximal convoluted tubules (PCT) and some glomeruli at 6 h (B), 12 h (C), 1 d (D), and 2 d (E) after ROSC in CA-operated groups compared to the sham (arrow). Data are expressed as mean \pm standard error means (SEM). * $P < 0.05$ compared with the sham and CA groups at 6 h (B), 12 h (C), 1 d (D), and 2 d (E) groups. Scale bar: 200 μ m (400x)

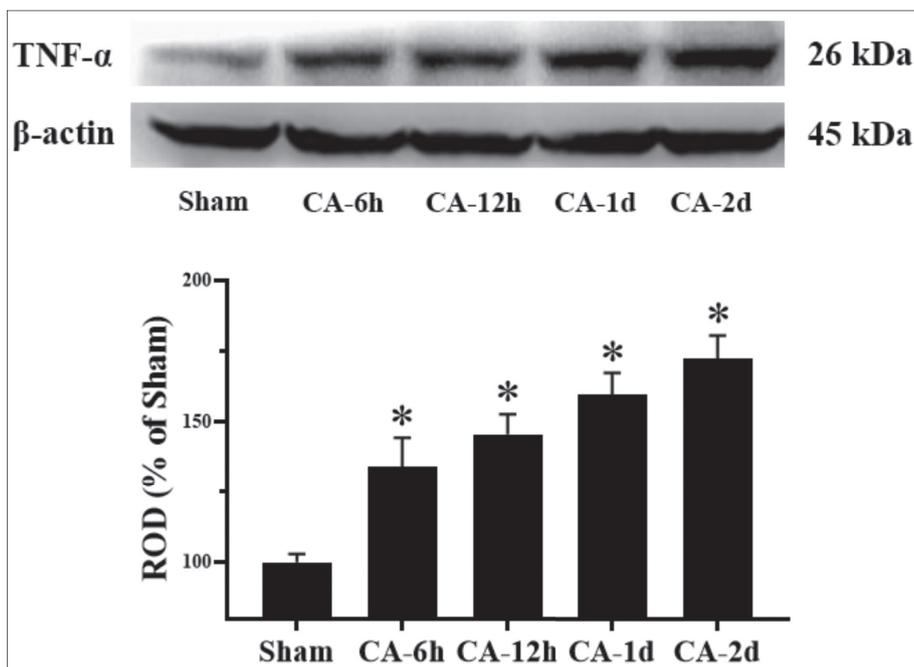


Fig 5. Western blot of TNF- α level. Western blot analysis of TNF- α in the renal cortex of the sham and cardiac arrest (CA) operated groups. TNF- α protein level was significantly increased at 6 h and increased by 2 d as compared to the sham. TNF- α protein levels were measured relative to β -actin. All the data are expressed as mean \pm standard error means (SEM)

expression significantly increased at 6 h after ROSC. TNF- α was highly expressed at 1 d in CA renal cortex sections compared with the sham group. These expressions appeared almost on the proximal convoluted tubules (PCT) and some expression was found in the glomeruli.

Western Blot Analysis

TNF- α levels in the kidney of CA-operated group changed in a time-dependent manner after ROSC (Fig. 5). TNF- α level significantly increased at 6 h after ROSC compared with the sham-operated group. Two days after ROSC, TNF-

α expression showed the peak level. TNF- α expression was upregulated in a time-dependent manner after 6 h ROSC, but it was not significant among the groups.

DISCUSSION

In the present study, we studied the time-dependent inflammatory injury of the kidney after ROSC in an asphyxial CA rat model. A cohort study of survival rates in patients with CA showed a survival rate of 18.6% after 1 d of ROSC [18] and Lei et al. [19] reported that survival rates

was 25% in asphyxial CA rat model at 48 h. In the present study, the survival rate after 2 d of ROSC was 11%. As such, present study showed similarly low survival rates after ROSC in CA patients and in previous CA model [19]. AKI caused by CA occurs in 12 to 81% of patients [20-22]. The amount of serum BUN and Cr in the present CA model increased significantly after 6 h of ROSC, and this result is related to renal injury. Six hours after ROSC, tubular epithelium cell degeneration and infiltration of inflammatory cells in the renal cortex were observed. This injury is similar to renal tubular necrosis and endothelial cell injury in AKI patients due to renal IR [23]. Therefore, the renal injury pattern caused by the asphyxiation CA in this study was similar to the injury pattern of AKI patients caused by ROSC, thus we thought that the present asphyxia CA model was suitable for AKI of PCAS.

ROSC following CA causes IR injury and it induce AKI, which is associated with inflammation of the renal tubular epithelial [23]. This was consistent with the PCT injury to the loss of tubular epithelial cells of the renal cortex, brush border loss and as shown by Masson's trichrome stain. This tubular injury is derived from an inflammatory response, and IR-induced inflammation is regulated by many pro-inflammatory cytokines [24-26]. TNF- α is one of pro-inflammatory cytokines known to play a role in inflammation. Interleukin (IL)-1b, IL-6, and IL-12 also belong to pro-inflammatory cytokines [27]. TNF- α is produced in a variety of cells, including macrophages, lymphocytes, fibroblasts, and keratinocytes, and is known to regulate inflammation by immunocytes [27,28]. In the present study, time-dependent changes in TNF- α in the kidneys were confirmed by IHC and western blot analysis. TNF- α expression was significantly increased in the tubular epithelial cells of PCT 6 h after ROSC in the renal cortex and this increase was at peak in 1 d and maintained until 2 d after ROSC. Based also on the western blot analysis, TNF- α expression in the renal cortex significantly increased at 6 h, being the highest at 2 d after ROSC. These results are similar to the increase in TNF- α mRNA expression in renal tissue qPCR in the rat renal IR injury model [29], and suggests that CA-induced IR injury can increase TNF- α in the kidney. In the present study, TNF- α expression in the kidney increased accordingly to the time after CA. Furthermore, Nagata et al.[30] reported inhibition of TNF- α reduced inflammation caused by renal IR injury in rats. Therefore, it is suggested that an increased TNF- α would be injury to the kidney through an inflammatory response.

After ROSC, PCAS causes a systemic inflammatory response and the production of pro-inflammatory cytokines, leading to an early inflammatory response that is closely related to the systemic inflammatory response [31]. In addition, an increase in TNF- α in vital organs is observed after CA [31,32]. TNF- α level was shown to be significantly increase at 6 h after ROSC in the CA1 region of the

hippocampus of the brain and maintained until 2 d after ROSC, however, brain injury was reported to be not observed histopathologically [32]. TNF- α was also shown significantly increase in the heart 12 h after ROSC and it was maintained until 2 d [10], moreover, Myocardial injury and inflammatory cells infiltration were also observed 12 h after ROSC [10]. Therefore, TNF- α expression increased in the brain and heart early of ROSC, it may be related to tissue injury through an inflammatory response. In the present study, TNF- α expression in the PCT region of the renal cortex significantly increased at 6 h after ROSC. Moreover, a significant cellular injury was observed at 6 h after ROSC. Although it is hard to directly compare heart and brain, these phenomena were faster than the brain and heart of a similar asphyxial CA model [10,32], thus we hypothesized that increasing TNF- α in the kidney induces a rapid inflammatory response and induces renal injury, which is associated with a low early survival rate. After CA, a time-dependent increase TNF- α was confirmed in the kidney however, the exact mechanism was not confirmed in the present study. These were the potential limitations of the present study. Expression levels of other pro-inflammatory cytokines in an asphyxial CA rat model also need to be evaluated in the future and it may be related to the high mortality in the early period after CA.

In conclusion, ROSC after CA increased TNF- α expression in renal tubular epithelial cells and induced inflammatory response. This leads to rapid injury to the renal tubules, which may be associated with low survival rates. Moreover, we suggest that TNF- α level in the kidney might be an indicator of early survival rate in the CA.

AVAILABILITY OF DATA AND MATERIALS

The datasets during and/or analyzed during the current study are available from the corresponding authors (JC Yoon and H Tae) on reasonable request.

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COMPETING INTEREST

The authors declare that they have no competing interest.

AUTHOR CONTRIBUTIONS

YJY, JHL and HJT were responsible for the experimental design, data acquisition, data analysis and manuscript writing. YJY, JCY, ISK, DHY, YH and JCY performed the experiments and data analysis. JCY, JHL, ISK, DHY and YH performed data analyses and made critical comments on the entire process of the study. All of the authors read and approved the final version of manuscript. JCY and HJT confirm the authenticity of the raw data.

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