Original Paper

Enteral Baicalin, a Flavone Glycoside, Reduces Indicators of Cardiac Surgery-Associated Acute Kidney Injury in Rats

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Keywords

Baicalin · Cardiac surgery · Acute kidney injury · Cardiopulmonary bypass · Nrf2/HO-1 pathway

Abstract

Background/Aims: Cardiac surgery-associated acute kidney injury (CSA-AKI) is one of the most common postoperative complications in intensive care medicine. Baicalin has been shown to have anti-inflammatory and antioxidant roles in various disorders. We aimed to test the protective effects of baicalin on CSA-AKI using a rat model. **Methods:** Sprague-Dawley rats underwent 75 min of cardiopulmonary bypass (CPB) with 45 min of cardioplegic arrest (CA) to establish the AKI model. Baicalin was administered at different doses intragastrically 1 h before CPB. The control and treated rats were subjected to the evaluation of different kidney injury index and inflammation biomarkers. **Results:** Baicalin significantly attenuated CPB/CA-induced AKI in rats, as evidenced by the lower levels of serum creatinine, serum NGAL, and Kim1. Baicalin remarkably inhibited oxidative stress, reflected in the decreased malondialdehyde and myeloperoxidase activity, and enhanced superoxide dismutase activity and glutathione in renal tissue. Baicalin suppressed the expression of IL-18 and iNOS, and activated the Nrf2/HO-1 pathway. **Conclusion:** Our data indicated that baicalin mediated CPB/CA-induced AKI by decreasing the oxidative stress and inflammation in the renal tissues, and that baicalin possesses the potential to be developed as a therapeutic tool in clinical use for CSA-AKI.

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Introduction

Cardiac surgery-associated acute kidney injury (CSA-AKI) is one of the most common postoperative complications in adult patients who undergo cardiac surgery [1]. CSA-AKI is caused by multiple factors, which involve different injury pathways including metabolic abnormalities, ischemia and reperfusion injury, neurohormonal activation, inflammation, and oxidative stress [2]. Due to the differences in baseline characteristics and surgery types, studies of the epidemiology of CSA-AKI in recent years showed the range of CSA-AKI incidence is between 8.9 and 39% [3]. Renal impairment during cardiac surgery is often associated with renal hypoperfusion caused by the bleeding complications [4], while exposure of blood to the cardiopulmonary bypass (CPB) pump and circuit often activate different inflammatory pathways [5]. Previous research showed that the increase in inflammatory cytokines IL-6 and IL-10 in the plasma of adults after cardiac surgery is highly correlated with AKI and long-term mortality [6]. In addition, hemolysis during cardiac surgery results in multiple serious clinical impairment including renal tubular damage [7]. The release of catalytic iron and the production of free radicals during hemolysis following CPB cause oxidative stress, which also contributes to development of CSA-AKI [8]. In spite of the progress in the understanding of the pathophysiology of CSA-AKI, the complex framework remains incompletely known, and there is a massive gap between the awareness of this multifactorial risk and the effective interventions for AKI prevention [9]. Given the magnitude of this clinical concern, further discovery of potential pharmacological agents for the prevention and treatment of CSA-AKI holds a critical key in the fields of nephrology and intensive care medicine [10].

Baicalin is a glycoside that is isolated from the leaves of *Scutellaria galericulata*, and is reported to exert protective effects against hydrogen peroxide-induced oxidative stress, and to scavenge superoxide radicals [11, 12]. Baicalin protected against tissue damage in a rat periodontitis model, partially by inhibiting the expression of cyclooxygenase-2 and inducible nitric oxide synthase (iNOS) [13]. Baicalin significantly reduced the levels of proinflammatory cytokines but increased the level of anti-inflammatory cytokine in blood and peritoneum, and thus enhanced bacterial clearance and improved survival of septic mice [14]. It has also been reported that baicalin protects hepatocytes from the oxidative damage and inflammation caused by carbon tetrachloride [15] and suppresses the hemin-nitrite- H_2O_2 -induced liver injury caused by inhibitory oxidation and nitration in HepG2 cells [16]. Moreover, baicalin inhibits the activation of redox-sensitive nuclear factor- κB in kidney tissue from elderly rats [17] and attenuates renal ischemia-reperfusion injury by inhibiting proinflammatory responses and mitochondria-mediated apoptosis [18]. Intriguingly, it has been determined that less than 20% of baicalin were subjected to enterohepatic circulation [19], and treatment of baicalin at doses above 400 mg/kg/day induced significant kidney injury and fibrosis in rats, while doses at 200 mg/kg did not show significant alteration in any measured parameters [20].

In this study, we aimed to explore the protective effects of baicalin on kidney damage by using rat CSA-AKI model, and to evaluate its role in the inhibition of oxidative stress and inflammation as a possible molecular mechanism of the action.

Materials and Methods

Animals and Experimental Design

Male Sprague-Dawley rats (400–450 g, 14–16 weeks old) were purchased from Shanghai Laboratory Animal Center of the Chinese Academy of Sciences. The rats were housed in the animal facility with controlled temperature and at a 12-h light/dark cycle with free access to food and water. The animal protocol was approved by the Affiliated Hospital of Guizhou Medical University. All procedures were in accordance with

the guidelines of the National Institutes of Health for animal care. After 1 week of acclimatization period, the animals were divided randomly into five groups: Sham control group, CPB/CA control group, CPB/CA and baicalin (Sigma, St. Louis, MO, USA) at 50, 100, and 200 mg/kg groups [12]. Specifically, group 1 (Sham) was normal rats. Group 2 (CPB/CA) was male Sprague-Dawley rats that underwent 75 min of CPB with 45 min of cardioplegic arrest (CA). Groups 3, 4, and 5 (CPB/CA + baicalin) were administered baicalin at 50, 100, and 200 mg/kg intragastrically 1 h before CPB.

Cardiac Surgery with CPB/CA

The rat CPB model with CA was established based on the method described by de Lange et al. [21]. Briefly, fasted rats were anesthetized with 5% isoflurane in 50% O₂ in a plastic induction box and were cannulated for CPB. With ultrasound guidance, a 3.5-mm balloon angioplasty catheter was positioned via the right common carotid artery with its tip proximal to the aortic valve. Totally, rats underwent 75 min of CPB. To initiate CA, the balloon was inflated, and CA was accomplished by a commercial crystalloid solution with high potassium concentration (280 mmol•L⁻¹; Inzolen®, Köhler Chemie, Alsbach, Germany). After 45 min of CA, the balloon was deflated, ventilation resumed, and rats were weaned from CPB and recovered. Rats in the baicalin groups were administered baicalin at different doses before exposure to CPB/CA. Rats in the sham group were cannulated without exposure to CPB/CA and were sacrificed under 5% isoflurane. At 24 h postreperfusion, rats were reanesthetized, intubated, and mechanically ventilated for collecting the renal tissue and blood samples. Blood samples were stored at room temperature for 1 h and centrifuged at 3,000 rpm for 20 min at 4 °C to collect serum. The harvested renal tissue and serum samples were frozen in liquid nitrogen and stored at -80 °C for subsequent analysis.

Measurement of Serum Biochemical Parameters

The level of serum creatinine was measured by Creatinine Parameter Assay Kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) as described in the manufacturer's instructions. The concentration of serum neutrophil gelatinase-associated lipocalin (NGAL), a highly sensitive biomarker of AKI, was measured using the Rat NGAL ELISA Kit (BioPorto Diagnostics, Gentofte, Denmark) according to the manufacturer's protocol.

Quantitative Real-Time Polymerase Chain Reaction

Total RNA was extracted from frozen renal tissues using an RNeasy mini-kit (Qiagen, Valencia, CA, USA). The obtained RNA was reverse-transcribed into complementary DNA using Invitrogen SuperScript III First-Strand Synthesis, and the expression levels of rat kidney injury molecule-1 (Kim-1) and proinflammatory cytokine IL-18 were determined using TaqMan Real-Time PCR assays (ThermoFisher Scientific, Waltham, MA, USA) on an Applied Biosystems StepOnePlus Real Time PCR System. Kim-1 was detected using TaqMan Gene Expression Assays, Rn00597703_m1, and the specific primer sequences used for IL-18 were: forward, 5'-CGCAGTAATACGGAGCATAAATGAC-3' and reverse, 5'-GGTAGACATCCTTCCATCCTTCAC-3'. β-Actin was used as reference gene for the assays.

Oxidative Stress and Myeloperoxidase Activity Assay

The quantitation of malondialdehyde (MDA) and glutathione (GSH) and the activities of myeloperoxidase (MPO) and superoxide dismutase (SOD) were determined using biochemical kits (Nanjing Jiancheng) according to the instructions of the manufacturer.

Western Blot Analysis

To investigate the mechanisms of the inhibition of baicalin on the inflammation, the relative expression levels of iNOS, Nrf2, and HO-1 in the renal tissues were determined by Western blot. Briefly, equal amounts (20 µg per lane) of total proteins were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred onto polyvinylidene difluoride membranes. The membranes were blocked by 5% nonfat milk in TBST buffer for 2 h at room temperature, and incubated with primary antibodies against iNOS, Nrf2, HO-1, and anti-β-actin antibodies overnight at 4 °C. Membranes were washed five times with TBST and were incubated with secondary antibodies for 1 h and detected by ECL substrate (Pierce, Rockville, MD, USA). The density of each band was measured by Amersham Imager 600 (GE Healthcare, Little Chalfont, UK).

Fig. 1. Biochemical measurements of acute kidney injury markers. **a** Serum creatinine. **b** Serum neutrophil gelatinase-associated lipocalin (NGAL). **c** Messenger RNA (mRNA) expression of kidney injury molecule-1 (Kim-1) in renal tissues. $n = 8$ in each experimental group. Experiments were performed in triplicate. Data are presented as mean $\pm$ SD. * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ vs. sham group; # $p < 0.05$, ## $p < 0.01$ vs. CPB/CA group.

Statistical Analysis

The values are presented as mean $\pm$ standard deviation. The intergroup comparisons were made with one-way analysis of variance, followed by Tukey post hoc test to estimate the differences between individual groups. All data were analyzed using GraphPad Prism 6.0 software (GraphPad Software Inc., La Jolla, CA, USA). Statistical significance was defined as $p < 0.05$.

Results

Biochemical Measurements of AKI after CPB/CA Induction

Compared with sham, serum creatinine level at 24 h after termination of CPB/CA was significantly elevated in CPB ($p < 0.01$, Fig. 1a). The serum creatinine level in the CPB/CA rats treated with 100 mg/kg baicalin was significantly lower than that in the CPB/CA only rats ($p < 0.05$), and the serum creatinine levels in the 200 mg/kg baicalin group were further decreased ($p < 0.05$).

As shown in Figure 1b and c, serum NGAL level and mRNA expression of Kim-1 in renal tissues demonstrated similar changes among different rat groups. Specifically, the CPB/CA group showed higher serum NGAL levels than the sham group ($p < 0.001$). The NGAL levels in the baicalin-treated groups were significantly lower than those in the CPB/CA group ($p < 0.01$). Similarly, the CPB group had significantly higher expression level of Kim-1 compared with the sham group ($p < 0.001$), whereas baicalin treatment significantly lowered Kim-1 overexpression ($p < 0.01$). These results indicated that pretreatment with baicalin attenuated CPB/CA-induced kidney injury.

Baicalin Attenuated Inflammation and Oxidative Stress in CPB-Induced AKI

Further, we evaluated the effects of baicalin on the inflammation and oxidative stress in the kidney of CPB/CA. As shown in the Figure 2, the MDA level and MPO activity in the CPB/CA group were significantly higher than the ones of the sham group (Fig. 2a, b; $p < 0.001$). Notably, compared to the CPB/CA group, baicalin treatment significantly decreased the MDA level and MPO activity, and the effects were dose dependent. Likewise, baicalin inverted the renal tissue SOD activity and GSH level in the CPB/CA rats, especially at the 200 mg/kg dose;

Fig. 2. Baicalin suppressed oxidative stress in the renal tissues of rats undergoing cardiac surgery. The levels of MDA (**a**) and GSH (**d**) and activities of MPO (**b**) and SOD (**c**) in renal tissues. $n = 8$ in each experimental group. Experiments were performed in triplicate. Data are presented as mean $\pm$ SD. * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ vs. sham group; # $p < 0.05$, ## $p < 0.01$, and ### $p < 0.001$ vs. CPB/CA group.

Fig. 3. Baicalin inhibited inflammation in the renal tissues of rats undergoing cardiac surgery. The level of IL-18 in renal tissues measured by ELISA (**a**) and RT-PCR (**b**). $n = 8$ in each experimental group. Experiments were performed in triplicate. Data are presented as mean $\pm$ SD. * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ vs. sham group; # $p < 0.05$, ## $p < 0.01$ vs. CPB/CA group.

Fig. 4. Baicalin downregulated the expression of iNOS in the renal tissues of rats undergoing cardiac surgery. **a** Renal iNOS mRNA expression was detected by RT-PCR. **b** Renal iNOS protein expression was detected by Western blot. β -Actin was used as a loading control. Experiments were performed in triplicate. Data are presented as mean $\pm$ SD. * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ vs. sham group; # $p < 0.05$, ## $p < 0.01$, and ### $p < 0.001$ vs. CPB/CA group.

the SOD activity was restored to approximately 70% of the level in the sham group, and the GSH level was increased to about 80% of the level in the sham group (Fig. 2c, d).

Baicalin Downregulated the Expression of IL-18 in the Renal Tissues

We assessed the expression levels of IL-18 at both protein and mRNA levels in the renal tissues from different rat groups. As shown in Figure 3, protein and mRNA of renal tissue expression of IL-18 were significantly elevated in the CPB/CA rats when compared to the sham group ($p < 0.001$), and baicalin treatment significantly decreased the IL-18 expression in a dose-dependent manner ($p < 0.05$ and $p < 0.01$ for 100 mg/kg and 200 mg/kg baicalin, respectively).

Baicalin Suppressed the Expression of iNOS in the Renal Tissues

As shown in Figure 4, RT-PCR and Western blotting were used to evaluate the effects of baicalin treatment on the expression levels of iNOS, which is modulated by signaling pathways implicated in inflammation. Compared with sham control, the expressions of iNOS were significantly enhanced in the CPB/CA rats, and the levels were significantly downregulated by baicalin at 100 and 200 mg/kg ($p < 0.05$ and $p < 0.01$, respectively).

Baicalin Promoted the Activation of Nrf2/HO-1 Signaling Pathway

To investigate the mechanism of action of the attenuation of baicalin in the CPB/CA, we evaluated the expression levels of Nrf2 and HO-1 in the renal tissues treated by the surgery and baicalin by Western blot. As seen in Figure 5, compared with CPB/CA control, we noted that baicalin induced a remarkable increase in the Nrf2 and HO-1 protein expression in a dose-dependent manner (Fig. 5; $p < 0.05$ for 100 mg/kg, $p < 0.01$ for 200 mg/kg, respectively). However, we did not observe a significant difference between the CPB/CA and sham groups, indicating baicalin's independent modulation of the Nrf2/HO-1 signaling pathway, which played a role in protecting from kidney injury induced by CPB/CA.

Fig. 5. Baicalin activated the Nrf2/HO-1 pathway in the renal tissues of rats undergoing cardiac surgery. Renal Nrf2 (**a**) and HO-1 (**b**) protein levels were detected by Western blot. β -Actin was used as a loading control. Experiments were performed in triplicate. Data are presented as mean $\pm$ SD. * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ vs. sham group; # $p < 0.05$, ## $p < 0.01$, and ### $p < 0.001$ vs. CPB/CA group.

Discussion

Given the increasing population of patients who undergo cardiac surgery, which is approximately 2 million per year [22], continued efforts to improve myocardial protection are being made to minimize the organ injury including kidney damage. Due to the complicated logistic challenges and costs, appropriate animal models of cardiac surgery that include CPB induced kidney injury have not been developed [23]. Herein, we modified an in vivo survival model of CA and CPB in rats to establish a CPB/CA-induced AKI model [21] via adjusting primarily the time duration for CPB and CA. In intensive care medicine, risk factors for CSA-AKI are categorized as patient or procedure-related factors such as CPB time; cross-clamp time; on-pump versus off-pump etc. [23]. In this study, we ended up with using 75 min for the CPB time, followed by 45 min of CA. The results showed that the serum creatinine level in the CPB/CA group was 0.27 mg/dL, which was 1.5-fold the one in the sham group. Based on the Improving Global Outcomes (KDIGO) criteria [24], which can be used to diagnose and stage CSA-AKI, an increase in serum creatinine 1.5–1.9-fold the baseline level defines stage 1 of the disease. Therefore, our rat model met the minimum requirement for the definition of CSA-AKI. Of note, the serum biochemical markers were measured at 24 h after the surgery, which was within 48 h from the onset of the disease or after cardiac surgery.

Beyond the prediction of the creatinine-based method, other different biomarkers are needed to provide the indication of AKI at an earlier stage. NGAL is a small, 23-kDa protein that is an early biomarker for ischemic, septic, or nephrotoxic kidney injury [25]. In addition, urinary NGAL has been evaluated as an early biomarker of renal tubular damage in acute clinical settings, and is a highly sensitive biomarker for early detection of AKI [26]. Moreover, the level of NGAL also increases under inflammatory conditions. On the other hand, kidney injury molecule 1 (Kim1) is a transmembrane protein that is specifically upregulated in dedifferentiated proximal tubule cells after ischemic or nephrotoxic AKI and is a more sensitive biomarker for AKI with considerable predictive value, especially for cardiac surgery patients

[27]. In evaluating the renoprotective effects of baicalin on the CSA-AKI rat model, we found that rats that underwent CPB/CA surgery showed significantly higher kidney injury biomarkers in comparison to the sham group. Administration of baicalin remarkably reversed all of these changes in a dose-dependent manner, indicating its protective effect on CSA-induced kidney injury.

To further investigate the underlying mechanism of the renoprotective role of baicalin, we evaluated the index of oxidative stress caused by CPB, which may be an important mechanism in the development of CSA-AKI [28]. MDA is considered to be a lipid peroxidation product and thus used as an indicator for evaluating oxidative stress [29]. Our results showed that MDA level is significantly elevated in the CPB/CA rats compared to the sham group, and baicalin significantly lowered the MDA level in the renal tissue. MPO is a neutrophil-specific enzyme, which would increase during activation of neutrophils (infiltration) and is used as a surrogate marker for inflammation detection [30]. In our study, the rats in the CPB/CA group increased levels of MPO compared to sham control rats. We speculated that neutrophils start to infiltrate into the damaged renal tissue during surgery and stimulate proinflammatory markers, which would further promote neutrophil infiltration. Moreover, we also measured the activity of SOD and GSH, which are the endogenous antioxidants that counter the excess levels of free radicals. In comparison with the sham rats, the activity of SOD and GSH in renal tissues of CPB/CA rats were significantly decreased, while the baicalin treatment significantly upregulated the levels of these two oxidative stress indicators, which was in agreement with a previous report [31].

Since inflammation plays a critical role in the pathogenesis of CPB/CA-induced AKI, we evaluated the expression of IL-18, which is specific to renal tubules and is known to be upregulated in response to ischemia reperfusion injury (IRI) [32]. As expected, the IL-18 expression at both protein and mRNA levels in the CPB/CA rats was markedly higher than that in the sham group, while baicalin inverted these changes significantly in a dose-dependent way. Since oxidoinflammatory stress may be exacerbated by IRI during CPB, and the surgical injury to tissues and exposure of blood to the CPB pump and circuit activate multiple inflammatory pathways [23], the observed decreases in the levels of proinflammatory cytokine in the baicalin-treated rats specified the potential value in the amelioration of CSA-AKI.

Oxidative stress occurs when there is an imbalance between the generation of reactive oxygen and nitrogen species and the antioxidant defense systems in the body [28], and it has been previously reported that both NO antagonists and NOS inhibitors could act as the potential novel treatment for sepsis-induced AKI [33]. We also detected the expression of iNOS, which could aggravate the IRI in the kidney [34]. Our results suggested that the administration of baicalin attenuated the overexpression of iNOS in the CPB/CA model, proving its medical potential in AKI.

Accumulating evidence has proven that the activation of Nrf2 could effectively inhibit the development and progression of AKI [35]. In the present study, we demonstrated that the Nrf2 and HO-1 expression in the rats administered baicalin increased significantly in a dose-dependent manner. However, the relative expression levels of Nrf2 and HO-1 in CPB/CA rats did not differ from the sham group. Therefore, the ameliorative effects of baicalin on CPB/CA-related renal damage may be due to its activation function on the Nrf2/HO-1 pathway against oxidative stress.

Notably, in spite of the pharmacological interventions for CSA-AKI including the use of natriuretic peptides and dopamine agonists, there has been no clearly defined medicine for the treatment of this disorder [3]. In particular, a multisite trial in Japan showed that atrial natriuretic peptide did not significantly improve renal function in cardiac surgery patients with AKI [36]. Given that the cause of CSA-AKI is multifactorial, and we were not sure whether and how baicalin would affect CSA-AKI when designing the study, it was difficult to make the

right judgment for the selection of an ideal positive control. Thus, a drawback of our study was that we did not include a positive control group in the animal models. In the near future, baicalin will hopefully serve as positive control in other studies upon further exploration of its mechanism of action.

Taken together, baicalin attenuated CSA-AKI by suppressing the inflammation and oxidative stress induced by CPB/CA, and the Nrf2/HO-1 pathway could be the main target of baicalin for exerting the protective activity. In the future studies, we would evaluate the effects of baicalin on renal functions like the glomerular filtration rate and renal pump activity. In addition, further studies are required to explore the comprehensive signaling pathway framework behind the renoprotective activity of baicalin.

Conclusion

Our data indicated that baicalin mediated CPB/CA-induced AKI by decreasing the oxidative stress and inflammation in the renal tissues, and baicalin has the potential to be developed as a new therapeutic strategy for CSA-AKI.

Disclosure Statement

The authors declare that they have no conflict of interest.

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