Establishment and assessment of a preclinical model of acute kidney injury induced by contrast media combined acute myocardial ischemia reperfusion surgery

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Abstract. Acute kidney injury (AKI) is a common complication after acute myocardial infarction (AMI) in clinical practice, and the majority of previous preclinical models were induced by a single factor. The objective of the present study was to establish a stable preclinic model of AKI induced by contrast media (CM) with acute myocardial ischemia reperfusion surgery and to identify the effect of oxidative stress on kidney injury. Rats were treated individually or with CM or myocardial ischemia reperfusion surgery. Renal baseline and AKI parameters, the level of oxidative stress and histopathological images were examined along with AKI biomarkers. Results showed the incidence of AKI in the CM group and ischemia reperfusion injury (IRI) group was 40%, χ^2 test ($P<0.05$ vs. CM-IRI) and 35%, χ^2 test ($P<0.05$ vs. CM-IRI) and the combination group had the highest incidence rate 75%. IRI surgery combined with CM diminished kidney function and induced oxidative stress by increasing creatinine, blood urea nitrogen and reactive oxygen species levels. Western blotting showed that the early AKI biomarker of NGAL and KIM-1 increased and that the combination group had the highest value. Pathology damage exhibited severe kidney damage in the combination group compared with other control groups. The present research established a reliable preclinic model of post-AMI AKI with a stable and high postoperative AKI rate. Additionally, CM was demonstrated to exacerbate AKI caused by acute myocardial infarction through oxidative stress and, thus, oxidative stress may be a potential therapeutic target.

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Introduction

Acute kidney injury (AKI) is a common complication in patients undergoing coronary angiography (CAG) or percutaneous coronary intervention (PCI), especially higher in acute myocardial infarction (AMI), with a prevalence ranging from 7.1 to 18.69% (1,2), and it raises the mortality risk of AMI patients by a factor of 2 to 20 times (3-6).

Current prevention strategy for post-AMI AKI patients mainly focused on minimizing the volume of iodinated radiocontrast media and optimizing periprocedural intravenous

hydration. Indeed, previous research has indicated that post-AMI AKI was related to various complicated pathophysiological changes, which include contrast media inducing hypoxic injury in renal tubular epithelial cells, oxidative stress inducing the increase of oxygen free radicals, and vasoconstriction (7). Preclinical models of post-AMI AKI are needed to better clarify the underlying mechanisms and may therefore help to find therapeutic targets.

The majority of previous animal models, such as cisplatin-induced AKI, bilateral nephrectomy or renal ischemia-reperfusion injury induced AKI, and contrast-induced AKI model, are insufficient to mimic the clinical characteristics of post-AMI AKI because they primarily focused on one single factor that causes kidney damage, ignoring the effect of myocardial injury influence on kidney (8). Thus, basis on previous models, we establish an animal model of post-AMI AKI by combining IRI surgery with CM that closely resembles the clinical situation. To investigate the reliability and stability of the CM-IRI model, we also constructed single factor model of CM or IRI surgery, compared the incidence rate of AKI in two definitions, as well as assessed the role of oxidative stress and the extent of kidney injury.

Materials and methods

Animals. Animal studies were carried out in accordance with China's National Guidelines for The Care and Use of Laboratory Animals and were approved by the Animal Ethics Committee of Guangdong Provincial People's Hospital (Ethics No. GDRECKY2020-266-01). Adult male Sprague-Dawley rats (200-250 g) were purchased from Guangdong Laboratory Animals Monitoring Institute [License No SYXK (Yue) 2021-0122] and were given at least a week to acclimate before the study began. The rats were maintained at 2 or 3 rats/cage, given unrestricted standard diet and sterile water, housed at a temperature at 25˚C as well as a 12:12 light-dark cycle. Meanwhile, the pad of rats was observed and replaced daily.

Study design and groups. The rats were divided into four groups, as shown in Fig. 1. (1) SHAM: sham operation + normal saline (NS, 15 ml/kg); (2) CM: sham operation + contrast media (ioprolamine, 15 ml/kg); (3) IRI: IRI surgery + normal saline (NS, 15 ml/kg); (4) CM-IRI: IRI surgery + contrast media (ioprolamine, 15 ml/kg). After fast and water restriction for 24 h before surgery, rats were weighed and anaesthetized using 50 mg/kg pentobarbital sodium and 4 mg/kg xylazine hydrochloride, intraperitoneally. Then rats received endotracheal intubation and monitored cardiac rhythm with a typical lead II electrocardiogram. After that, rats were given a 15 ml/kg injection of contrast media or normal saline through the tail vein. Myocardial ischemia-reperfusion surgery was performed as previously described (9). Briefly, a left thoracotomy was performed in the fourth intercostal space, and the pericardium was removed to expose the heart, after which the left anterior descending (LAD) coronary artery was ligated for 45 min and repercussed for 2 h. After 24 h of the operation, 1 ml blood was collected from the tail vein, then all the rats used in the experiment were euthanized by an overdose of sodium pentobarbital 100 and 8 mg/kg xylazine hydrochloride intraperitoneally and confirmed by the disappear of pupillary reflex to light, respiration and heartbeat. Finally, heart and kidneys were removed for the following detection and histological staining (Fig. 2).

Renal function analysis. Blood samples were collected in a sodium citrate tube and centrifuged at 2,500 g for 15 min to separate the serum. Serum creatinine (Scr) and blood urea nitrogen (BUN) levels were determined using an automatic biochemical analyzer. The level of eGFR (estimated glomerular filtration rate) was calculated based on equation (10). The diagnosis of AKI was defined as an absolute increase in the serum creatinine ≥0.5 mg/dl (44.2 μmol/l) or a relative increase ≥25% from the baseline within 72 h after contrast administration according to the European Society of Urogenital Radiology (ESUR) criteria (11). And Kidney Disease Improving Global Outcomes (KDIGO) defined AKI as an increase in Scr >0.3 mg/dl within 48 h or a 50% increase from the baseline within 7 days (12).

Hematoxylin and eosin staining. Kidney was excised and fixed in 4% formaldehyde for 48 h, then embedded in paraffin and each slide was cut into 4 μm thick sections. Dehydrated sections with gradient alcohol after rinsing with water for 30 min. Subsequently, continuous slices were stained with hematoxylin and eosin (HE) and determined under light microscopy (13).

Measurement of reactive oxygen species (ROS) level in kidney tissue. After the perfusion of kidney, half of left fresh kidney tissue was isolated and ground in a sample freeze grinding machine (LukyM-1). The abrasive solution was centrifuged for 10 min (4˚C, 5,000 rpm) to collect the supernatant. ROS level was determined by the ROS assay kit (Bestbio Assay BB-470512). 190 microliters of homogenate supernatant were added to the 96-well plate, and mixed with O13 reactive oxygen species probe then incubated the plate at 37˚C for 30 min. Finally, the fluorescein intensity was detected using excitation at 510 nm and emission at 610 nm wavelengths on a microplate reader.

Western Blotting Analysis. Total protein was extracted from left kidney tissues. A 12% separating gel and a 5% concentrated gel were configured separately based on the measured protein molecular weight. After electrophoresis, proteins were transferred onto the nitrate cellulose membrane. The primary anti-NGAL antibody (Abcam, ab216462) and anti-KIM-1 (Abcam, ab233720) antibody were incubated overnight, then the primary antibodies were washed away and the secondary antibodies were incubated. After 2 h, the secondary antibodies were washed away and the developer solution was added for development. Finally, the relative expression levels of proteins were obtained by comparing them with the grey levels of GAPDH internal reference proteins.

Immunohistochemistry Analysis. The tissues were fixed with 4% paraformaldehyde for 3-4 h before dehydration and sectioning. Waiting for the antigen repair solution to cool

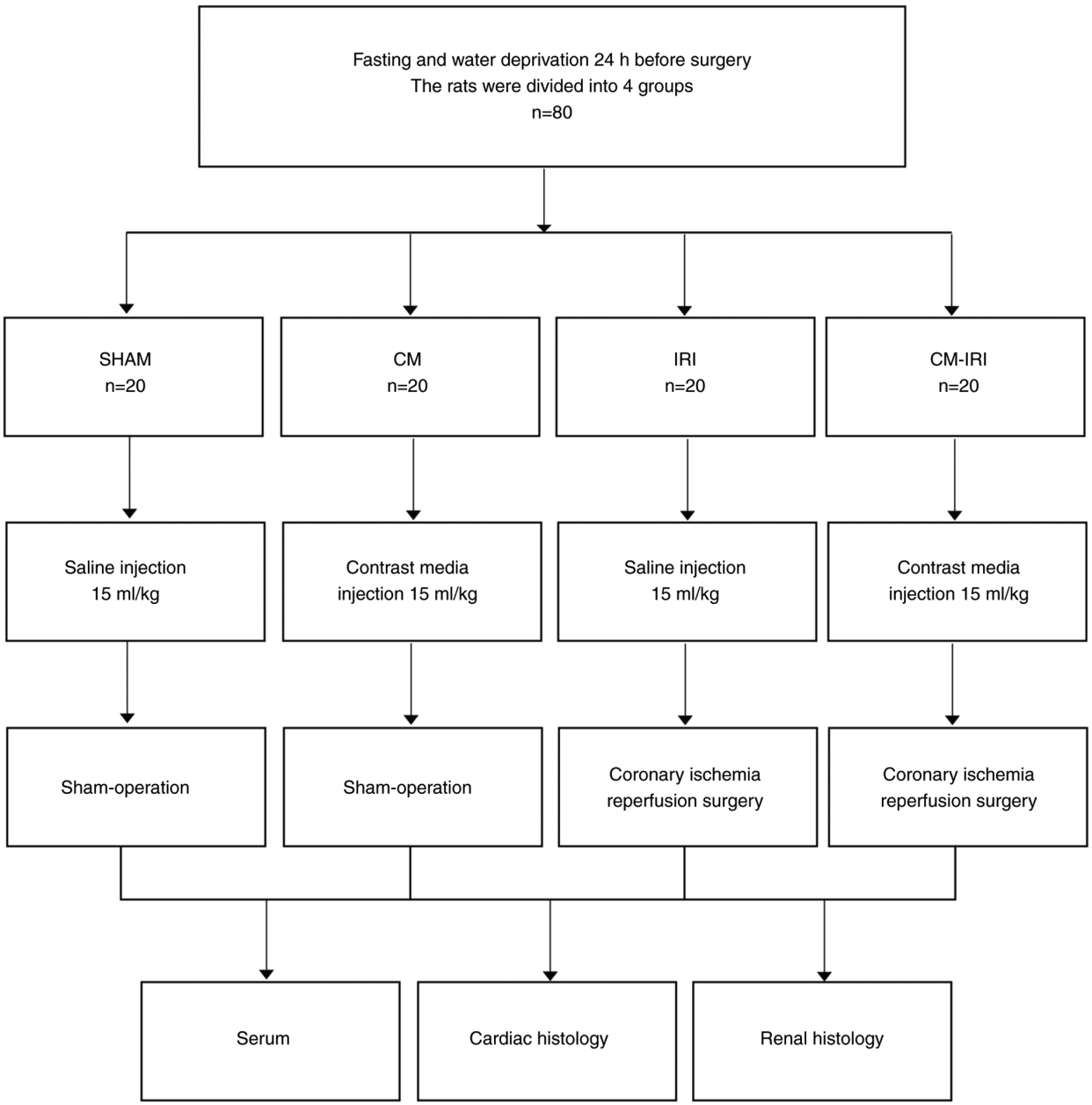

Figure 1. Workflow of the study. A total of 45 rats were divided into four groups: i) SHAM: NS (15 ml/kg) + sham operation; ii) CM: CM (15 ml/kg) + sham operation; iii) IRI: NS (15 ml/kg) + IRI surgery; and iv) CM-IRI: CM (15 ml/kg) + IRI surgery. Blood, heart and kidneys were removed for the following detection and histological staining. NS, normal saline; CM, contrast media; IRI, ischemia reperfusion injury.

naturally to room temperature and probed with anti-NGAL (Abcam, ab216462) at 4˚C overnight and labeled with secondary antibodies. The DAB staining solution was configured with the appropriate concentration according to the instructions. The stained tissue sections were observed and analyzed under a light microscope.

Data Analysis. All data are expressed as the mean ± standard deviation (SD). Statistical analysis was performed using Graph Pad Prism (GraphPad Software, CA, USA). Data were analyzed for normality using the Shapiro-Wilk test. One-way ANOVA followed by post hoc Tukey test was used. The enumeration data were expressed as rate and analyzed by the Chi-square test. P-value < 0.05 was considered statistically significant.

Results

Baseline and AKI parameters for each experimental group. As shown in Table I, no significant differences in baseline parameters were observed among the four groups. Compared with sham group, the level of BUN and Scr was elevated and eGFR was decreased in CM, IRI and CM-IRI groups (Fig. 3A-C).

The incidence of acute kidney injury. The sample size is primarily based on the prior study on the AKI model (14). At the same time, in the pre-experiment, we assessed the incidence of AKI in each group in general, so we selected a total of 80 rats to meet the statistical analysis requirement as well as to better compared the incidence of AKI in each group.

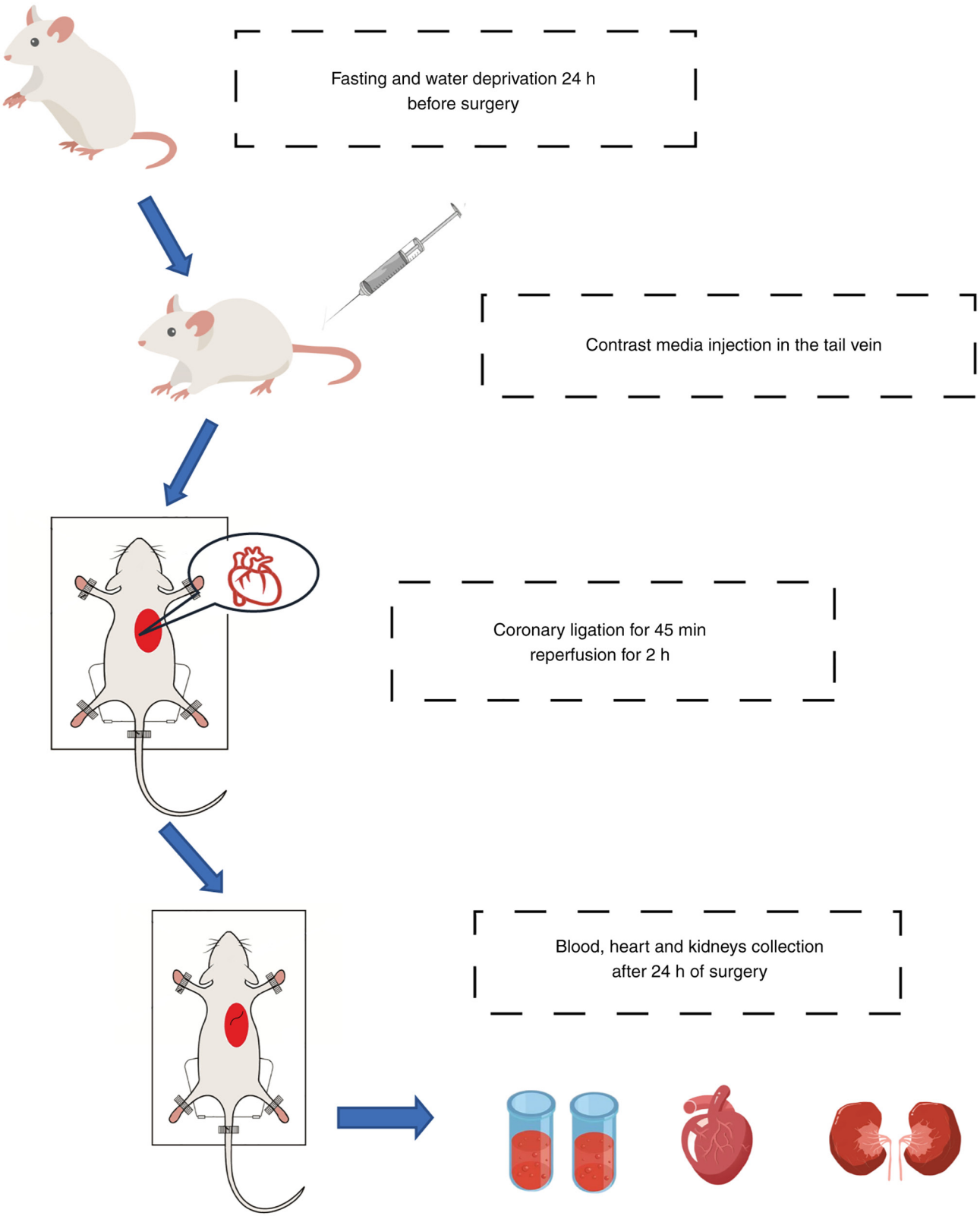

Figure 2. Detailed operation process of contrast media-ischemia reperfusion injury group.

Preoperative and postoperative levels of creatinine were used to determine the prevalence of AKI. As shown in Table II, the incidences of AKI rates were 40, 35 and 75% based on the ESUR criterion, and were 25, 25 and 55% in each group based on the KDIGO criterion, CM-IRI group has the highest AKI incidence rate.

Renal histopathological results. SHAM group showed a normal structure with complete glomerular structure and no proximal or distal renal tubule damage, no obvious interstitial edema, cell debris and presence of protein tubule (Fig. 4A, E). However, CM (Fig. 4B, F), IRI group (Fig. 4C, G) and CM-IRI group (Fig. 4D, H) displayed multiple kinds of renal tubular

Table I. AKI baseline parameters for each group.

Parameter	n	Scr (μmol/l)	BUN (mmol/l)	eGFR (μl/min)
SHAM	20	27.88±5.55	4.66±0.98	2,631.51±322.68
CM	20	33.63±6.89	5.02±1.19	2,213.95±394.25
IRI	20	34.00±16.39	4.74±1.60	2,436.47±885.52
CM-IRI	20	28.60±3.54	4.47±1.56	2,609.40±566.67

Data presenting mean ± standard deviation. Scr, Serum creatinine; BUN, Blood urea nitrogen; eGFR, estimated glomerular filtration rate.

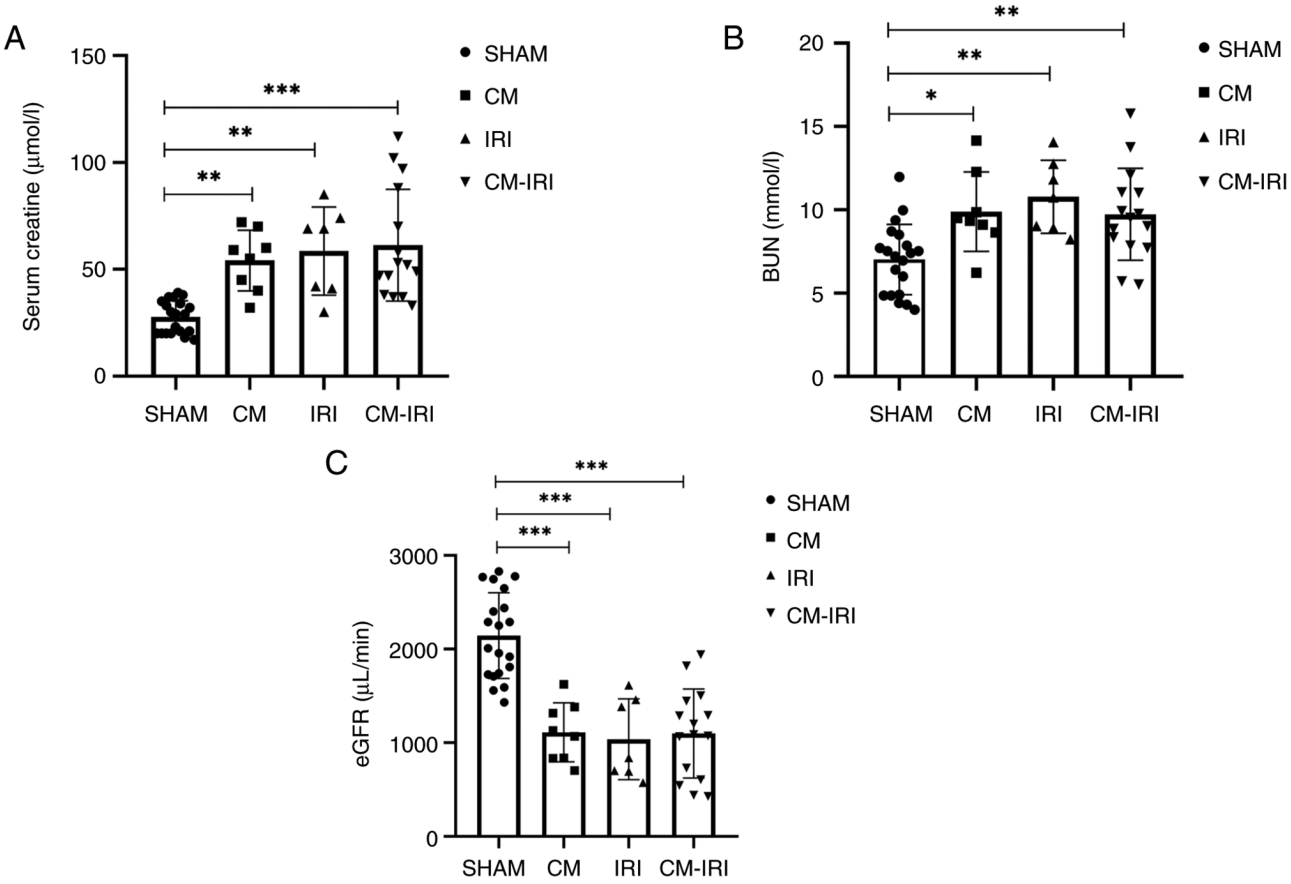

Figure 3. Level of postoperative renal function. (A) Representative the postoperative Scr level. (B) Representative the postoperative BUN level. (C) Representative the postoperative eGFR level. *P<0.05, **P<0.01, ***P<0.001. Scr, Serum creatinine; BUN, blood urea nitrogen; eGFR, estimated glomerular filtration rate; CM, contrast media; IRI, ischemia reperfusion injury.

injury, including glomerular structure destruction, interstitial edema, swelling of distal convoluted tubules, protein degeneration and formation of protein tubes.

The expression of AKI biomarkers and oxidative stress index. We next examined the expression of AKI biomarkers and levels of oxidative stress in renal tissues. Western blot analyses indicated NGAL and KIM-1 levels in the CM-IRI group increased significantly (Fig. 5A-C), with the CM-IRI group shows a statistically increased than CM and IRI group. IHC was used to detect the expression and distribution of NGAL in renal tissue. Compared with the SHAM group, the expression of NGAL significantly increased in the CM, IRI, and CM-IRI groups and the positive rate was the highest in the CM-IRI group (Fig. 5D-E). The level of oxidative stress was measured by ROS expression in kidney tissues. CM, IRI, and CM-IRI groups showed higher ROS levels than sham group, with the CM-IRI group shows statistically increased than CM and IRI group (Fig. 6).

Discussion

The present study aimed to establish a post-AMI AKI model and investigate the stability of model by assessing the expression of AKI biomarkers, level of renal injury and oxidative stress. Our study shows that CM combined IRI surgery has a higher postoperative AKI rate than single factor induced AKI, as well as more severe renal damage in pathology and

Table II. Incidence rate of AKI for each group.

Group	Cases of AKI, n (ESUR)	Incidence of AKI (ESUR, n %)	P-value[a]	Cases of AKI, n (KDIGO)	Incidence of AKI (KDIGO, n %)	P-value[a]
SHAM	-	-		-	-	
CM	8	40% (8/20)	0.055	5	25% (5/20)	0.1066
IRI	7	35% (7/20)[b]	<0.05	5	25% (5/20)	0.1066
CM-IRI	15	75% (15/20)		11	55% (11/20)	

[a]χ with Yates' continuity correction. [b]P<0.05 vs. CM-IRI. AKI, Acute kidney injury; ESUR, European society of urogenital radiology; KDIGO, kidney disease improving global outcomes; CM, contrast media; IRI, ischemia reperfusion injury.

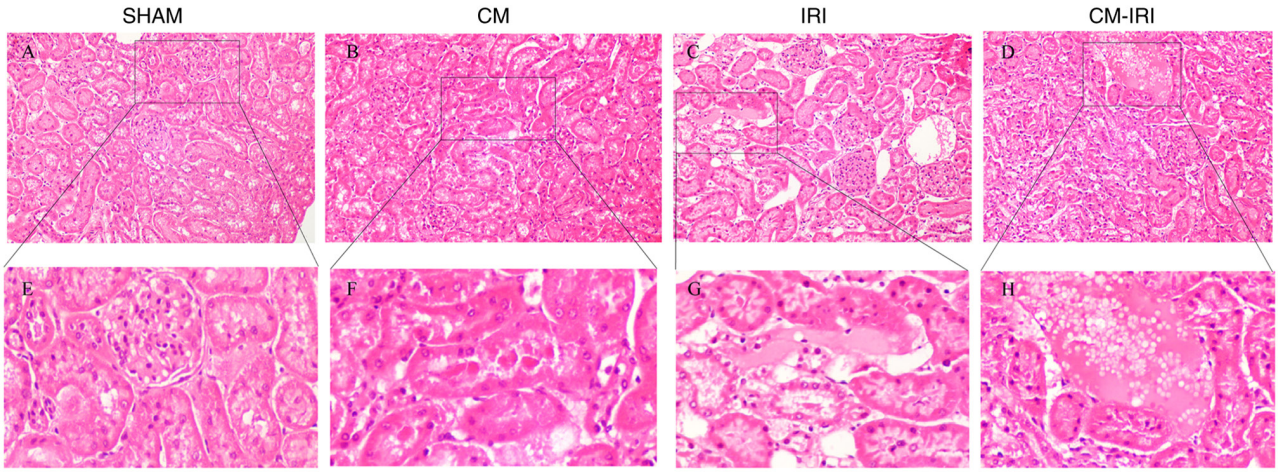

Figure 4. Representative photomicrographs of tubular cell injury in the SHAM, CM, IRI and CM-IRI groups. (A) SHAM group showed a normal structure with complete glomerular structure and no proximal or distal renal tubule damage. (B) CM group showed multiple renal tubular injury and protein degeneration. (C) IRI group showed multiple renal tubular injury including glomerular structure destruction and interstitial edema. (D) CM-IRI group showed multiple renal tubular injury including protein degeneration, swelling of distal convoluted tubules and interstitial edema. (E) SHAM groups show a micrograph of the normal and intact renal structures. (F) CM groups show the formation of protein tubes. (G) IRI groups show edema injury in the glomerulus. (H) CM-IRI groups shows indicate protein degeneration. Top row magnification, x40 (scale bar, 100 μm); bottom row magnification, x200 (scale bar, 20 μm). CM, contrast media; IRI, ischemia reperfusion injury.

a high level of oxidative stress in pathophysiology, indicating that oxidative stress injury may play a significant role in post-AMI AKI.

Various preoperative risk factors and mechanisms have been related to post-AMI AKI, including preoperative declining kidney function, and excessive use of intraoperative contrast media, in which contrast media induced renal hypoperfusion and hypovolemia are pivotal elements in pathophysiology (15). During the PCI surgery, heart vessels are exposed to a high dose of contrast media, which is later eliminated by the kidney. Firstly, contrast media in the circulation will be filtered through the glomeruli and as it progressively passed through the renal tubules, the concentration of contrast media in the distal convoluted tubules will gradually increase (16). Meanwhile, the viscosity of the fluid in the renal tubules increased due to the elevated concentration of CM, which prolonged kidney exposure to contrast media, resulting in congestion of the tubules and reduced kidney blood flow. This process may be affected by the amount of contrast media, the character of contrast media, and perioperative venous hydration. Previous studies show that physicochemical properties of contrast media can also cause renal vasoconstriction and hemodynamic change, causing renal hypoperfusion. Under high osmotic conditions, plasma water flows from vascular lumen to interstitium, resulting in enrichment of contrast media in vasa recta, thereby increasing local blood viscosity and local vascular resistance. Meanwhile, high osmosis can increase urate levels, resulting in renal tubular obstruction and poor drainage (17).

Furthermore, the state of basic renal function acts as a major contributor to the development of kidney injury. In the study of Cheng et.al, the CI-AKI model was constructed with furosemide combined contrast media demonstrated that dehydration 24 to 48 h before surgery or using diuretics prior to surgery can reduce basic renal function and more effectively induce AKI (18). In dehydrated individuals, most of the water is reabsorbed by the renal tubules during elimination of contrast media, leaving the high viscosity of contrast media that may cause damage to renal tubular epithelial cells. However, the AKI rate in the CM-induced AKI models is relatively low and solely concentrates on one organ. As a result, our research focuses on developing an animal model of a contrast agent associated with IRI surgery that is similar

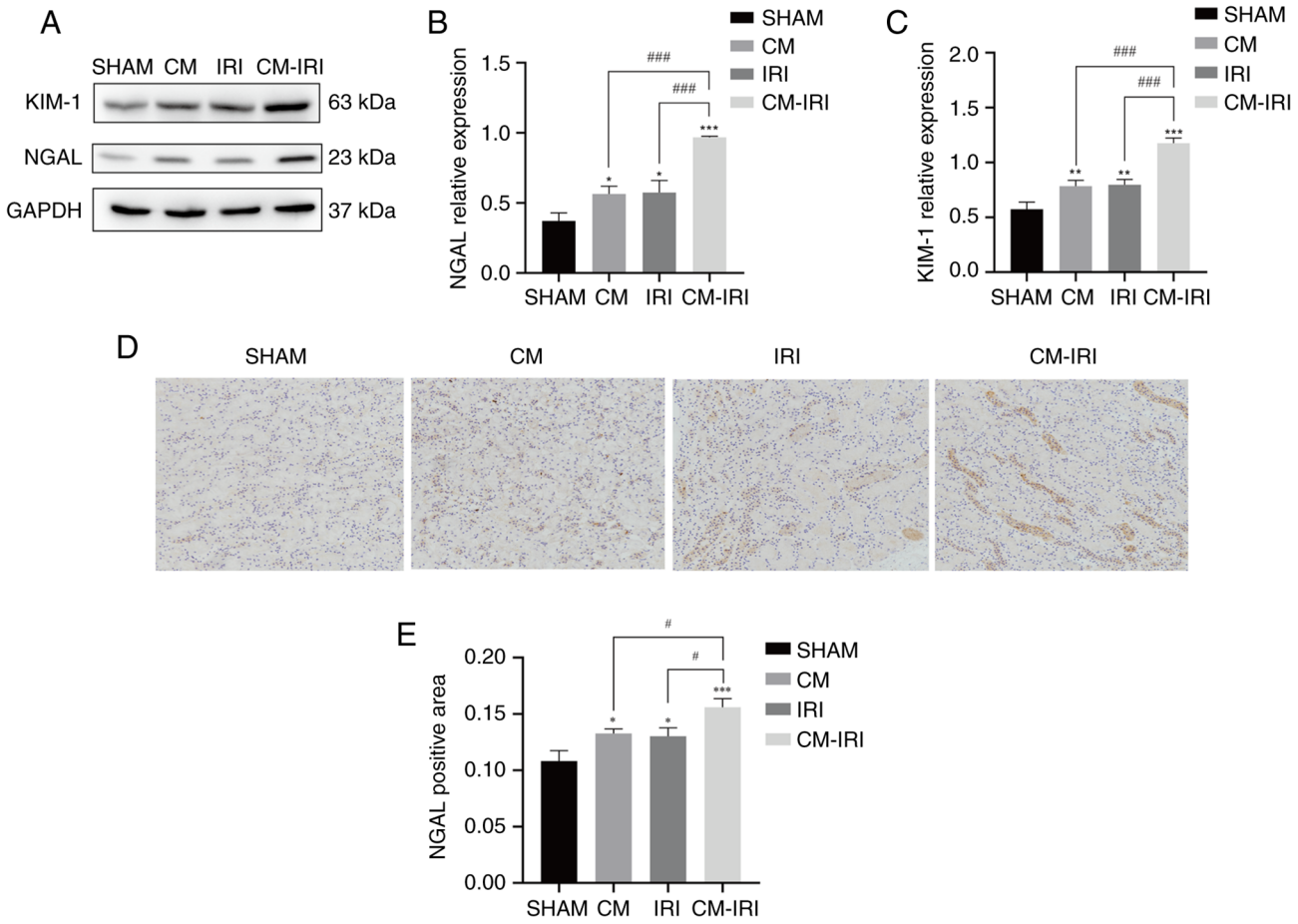

Figure 5. Western blotting and quantified analysis of renal injury biomarkers. (A) Representative blots of NGAL and KIM-1 protein expression. (B) Quantification on NGAL relative expression. (C) Quantification on KIM-1 relative expression. (D) The expression of NGAL was detected by immunohistochemistry. Row magnification, x100 (scale bar, 50 μm). (E) Quantification on the positive area. *P<0.05, **P<0.01, ***P<0.001 vs. SHAM group; $^{\#}$P<0.05, $^{\#\#\#}$P<0.001 vs. CM-IRI group). NGAL, neutrophil gelatinase-associated lipocalin; CM, contrast media; IRI, ischemia reperfusion injury.

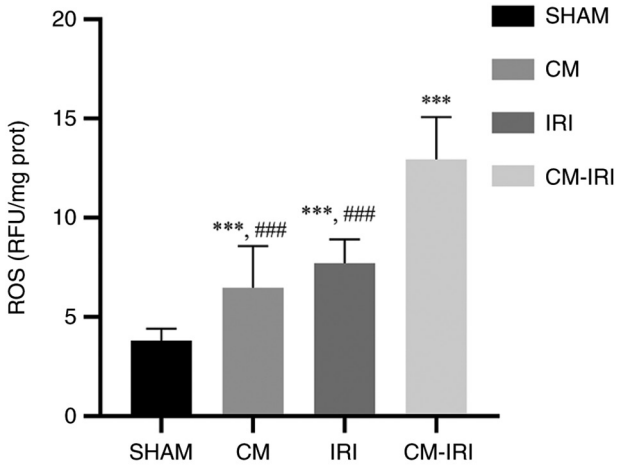

Figure 6. Level of oxidative stress in renal tissue. Representative the level of ROS on each group in renal tissue. ***P<0.001 vs. SHAM group; $^{\#\#\#}$P<0.001 vs. CM-IRI group. ROS, reactive oxygen species; CM, contrast media; IRI, ischemia reperfusion injury.

to the incidence of AKI in clinical patients following PCI or CAG and also better investigates the connection between the heart and kidney. Therefore, based on previous research on the AKI model, we used ioprolamine, a commonly used contrast media in clinical practice to construct our model. All the rats were dehydrated 24 h before surgery as a pre-treating procedure. Consistent with previous research, the model of contrast media after dehydration alone had a low postoperative AKI rate of around 30% (19). Given extended postoperative nursing time increases rat mortality, we collected blood, heart, and kidney 24 h following surgery. Compared with other traditional 72-h animal modeling methods, 24 h was sufficient for rats to meet AKI standards. In the meantime, the postoperative nursing time for the rats was reduced, which contributed to shortening the animal modeling cycle.

However, not all AKI observed after exposure to contrast media are caused by the contrast media itself, pathological processes that occur during coronary ischemia-reperfusion may also lead to kidney injury. During myocardial infarction, blood vessels of other organs contract to compensate for the decreased blood volume caused by the infarcted coronary artery (17). As an organ with a high blood volume, the kidney may reduce the effective blood volume to support and maintain the basis cardiac function. Besides, harmful substances are released into the circulation due to multiple organ injuries caused by myocardial ischemia-reperfusion, these harmful substances may cause an additional injury by the absorption of the kidney (20). As a result, we combine

the preoperative use of a contrast media with coronary ischemia reperfusion surgery to established the post-AMI AKI model. This model can better observe the effect of CM and IRI surgery on kidney injury, which is compatible with the disease background of post-AMI AKI. Under the double effects of CM and IRI surgery on kidney blood volume and kidney function, the incidence rate of AKI in the CM-IRI group is significantly higher than that of the other two groups.

Clinically, there are two main ways to define AKI patients: ESUR and KDIGO criteria and using clinical criteria can better compare the AKI modeling rate between groups. Based on the ESUR definition, 15 rats in the CM-IRI group met the criteria. And based on the KDIGO definition, only 11 rats in the CM-IRI group met the criteria. Because the KDIGO criterion standard is more stringent than the others, the number of AKI under this threshold is smaller. The CM-IRI group had the highest incidence of AKI in both definitions, indicating that contrast media combined with IRI operation produced more stimulation in rats.

Recent research has demonstrated the importance of oxidative stress in AKI (21,22). Excessive production of reactive oxygen species (ROS) causes oxidative damage to mitochondria and lipids (23). Quintavalle et al. demonstrated that CM may result in a dose-response increase in reactive oxygen species production, which activates Jun N-terminal kinases (JNK1/2) and p38 stress kinases (24). In our study, markers of oxidative stress such as ROS were significantly increased in the CM-IRI group. Similar results were observed in AKI biomarkers, NGAL and KIM-1 are biomarkers of early renal injury and are highly expressed in kidney tissue of the CM-IRI group. The results of creatinine and expression of biomarkers in the kidney were consistent with the increased expression of ROS suggesting contrast media can exacerbated acute kidney injury caused by IRI through oxidative stress. And elevated oxidative stress levels could be the cause of CM-IRI group had the highest number of postoperative AKI.

This model has some limitations. First, our rats were dehydrated for 24 h before surgery as a pre-treatment, and multiple dehydration time points or diuretic can be added to the model to further reduce renal function. Second, the occurrence of AMI in our models was determined by an elevated ST segment in an electrocardiogram. Thus, from the perspective of disease, the animal model of myocardial infarction in our study is more similar to STEMI patients in clinical practice. In our study, we used ROS as the primary indicator of oxidative stress. But some secondary indicators, like MDA or CAT, can also be used to investigate oxidative stress, and their importance shouldn't be overlooked. Therefore, the detection of these secondary indicators can be considered in subsequent experiments to comprehensively evaluate oxidative stress. Finally, based on the KDIGO standard, the number of AKI rats was small. As a consequence, animal research needs to be improved further, such as expanding sample size as well as including result analysis at different time points.

In conclusion, we established a reliable and stable animal model by combined CM with IRI surgery, the model has a high rate of postoperative AKI and our model can better simulate clinical features of post-AMI AKI. Meanwhile, we proved the important role of oxidative stress in our model by detecting ROS levels. Combined with the detection of kidney injury indicators such as NGAL and KIM-1, we demonstrated that contrast media can exacerbate acute kidney injury caused by IRI through oxidative stress and cause more severe kidney damage in rats. Our study provides an animal model basis for further exploring the pathogenesis of the disease. And this work emphasized that reducing oxidative stress levels may be a potential approach to preventing or treating the disease of post-AMI AKI.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

SY and JC conceived and designed the study. XD, WL and HL collected data and performed animal surgery. YX wrote the original draft and performed analysis and interpretation of data. YZ, KH and JLia analyzed data and the statistical analysis. JX, JLiu and YL made substantial contributions to the conception and acquisition of data. YL and JC confirm the authenticity of all the raw data. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The study protocol was approved by the Animal Ethics Committee of Guangdong Provincial People's Hospital (approval no. GDRECKY2020-266-01).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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