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Protective Effects of Methane-Rich Saline on Renal Ischemic-Reperfusion Injury in a Mouse Model

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Background: Renal ischemic-reperfusion (RIR) injury remains a major cause of acute kidney injury, with increased in-hospital mortality and risks for chronic kidney disease. Previous studies have proposed that oxidative stress, inflammation, and renal apoptosis are the most common causes of injury, whereas recent research proved that methane, the simplest alkane generated by an enteric microorganism or accompanying the production of reactive oxygen species (ROS), can alleviate inflammation and oxidative stress and reduce apoptosis in different organs.





Material/Methods: In the present study, we analyzed the possible effects of methane-rich saline in RIR injury in a mouse model and analyzed its possible protective effects on inflammation, oxidative stress, and apoptosis.

Results: The results showed that treatment with methane significantly improved blood creatinine and blood urea nitrogen (BUN) levels and improved renal histology in RIR injury. Further experimentation proved that this protective effect was primarily manifested in decreased oxidative stress, less apoptosis, and reduced inflammation in renal tissues, as well as improved general responses.

Conclusions: Our present study proved the protective effects of methane in RIR injury and, together with previous research, confirmed the multi-organ protective effects. This may help to translate methane application and develop its use in organ ischemic-reperfusion injury.

MeSH Keywords: **Acute Kidney Injury • Anti-Inflammatory Agents • Oxidative Stress**

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Background

Renal ischemic-reperfusion (RIR) injury remains a major cause of acute kidney injury and leads to increased in-hospital mortality and increased risks for the development of chronic kidney disease [1–3]. Multiple conditions, including surgical intervention, trauma, and cardiac arrest, which all lead to local renal hypoperfusion, can result in RIR injury with multiple pathophysiological mechanisms involved [3–5]. In these mechanisms, after ischemia challenges, early reperfusion first increases the permeability and induces leukocyte recruitment and infiltration [1,6]. Then, together with the occurrence of local inflammation, enhanced oxidative stress that injures membrane structure [7], and, to some degree, induced apoptosis or necrosis of the endothelial system [7,8], further causes acute renal injury [2,7,9,10]. Although the specific pathophysiological process varies in different pathogenesis of RIR injury, it is established that oxidative stress, inflammation, and apoptosis are the most common causes of injury, and numerous treatments targeting these central processes have been developed [1,3,4,7,9,11,12].

Methane, a well-studied chemical that is generated from enteric microorganism or cellular responses to transient oxygen deprivation in aerobic cells that is accompanied by the abnormal production of reactive oxygen species (ROS), showed promising protective effects for ischemia and inflammation in different models [13]. Soon after the first description of the anti-inflammatory effects of methane in the intestine by Boros et al. [13], many researchers proved and advanced this phenomenon in treating myocardial ischemia [14], diabetes in an animal model [15], hepatic ischemia-reperfusion injury [16], hepatitis [17], and skin flaps [18]. These investigations demonstrated that the protective effects of methane were mainly via anti-oxidative, anti-inflammation, and anti-apoptosis, which strongly indicated the possible effects on RIR injury as a shared pathophysiological process [14–16,19].

To investigate the possible protective effects of methane in renal ischemia-reperfusion injury, we used a mouse RIR model [20,21] and determined extensive outcomes after methane intervention. Moreover, in addition to monitoring conventional parameters, we also showed more comprehensive histological evidence to analyze the effects of methane more directly.

Material and Methods

Animal preparation

Male C57BL/6 mice age 10–12 weeks old were purchased from Shanghai Research Center for Model Organisms and raised in specific pathogen-free (SPF) animal rooms of the Laboratory

of the Faculty of Anesthesiology, Changhai Hospital, Second Military Medical University. This study was approved by the Institutional Animal Care and Use Committee of the Second Military Medical University. The mice were anesthetized with sevoflurane inhalation and underwent bilateral renal pedicle clamping surgery according to previously reported research. After 45 min of clamping, the renal pedicles were released and the incisions were closed. The grouping of mice was as follows: Sham group: mice underwent laparotomy procedures without the occlusion of bilateral renal pedicles with 20 mL/kg normal saline after surgery; MS group: mice underwent laparotomy procedures without the occlusion of bilateral renal pedicles and then injected with 20 mL/kg methane-rich saline; RIR group: mice underwent laparotomy procedures with the occlusion of bilateral renal pedicles with 20 mL/kg normal saline; and RIR+MS group: mice underwent laparotomy procedures with the occlusion of bilateral renal pedicles and then injected with 20 mL/kg methane-rich saline. Each group contained at least 6 mice for analysis. The mice were allowed free access to food and water. Twenty-four hours later, the mice were anesthetized and blood was harvested through heat puncture. The mouse kidneys were then harvested.

Methane saline preparation

Methane was dissolved in normal saline and then pressurized at 0.4 MPa for 8 h using our professional apparatus (Shanghai Yangyuan Pressure Vessel Co., Ltd.). The MS was stored at 4°C and made freshly once a week.

Oxidative stress analysis

The kidneys were harvested and washed in cold normal saline. Then, 0.1 g of renal tissue was collected and homogenized immediately in 1 mL of normal saline at 4°C, and the homogenate was centrifuged at 12 000 rpm for 15 min. The protein concentration in the supernatant was determined using a bicinchoninic (BCA) assay (Thermo Fisher, Shanghai, China), and the malondialdehyde (MDA) concentration (nmol/mg protein), superoxide dismutase (SOD) activity (U/m), catalase (CAT) activity (U/mg protein), and myeloperoxidase (MPO) activity (U/mg protein) were measured using commercially available kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China).

Apoptosis analysis

The renal tissues from mice were fixed in 10% formalin, and the fixed specimens were processed to paraffin blocks, sectioned every 5 mm, and stained with hematoxylin-eosin (H&E) for histological analysis. The apoptosis of renal tissues was analyzed using deoxyuride-5'-triphosphate biotin nick-end labeling (TUNEL) methods. Briefly, the TUNEL and 4,6-diamino-2-phenylindole (DAPI), which were from Sigma (Shanghai, China),

were used to detect the apoptosis of cultured cells, and the apoptotic cells could be TUNEL-positive. Then, the number of TUNEL-positive renal tubular epithelial cells were counted under a Carl Zeiss microscope (Axio Observer A1, Jena, Germany).

Enzyme-linked immunosorbent assay (ELISA)

The mouse blood was centrifuged (3000 rpm, 10 min), and the supernatant serum was collected. Tumor necrosis factor- α (TNF- α), interleukin (IL)-6, IL-10, and IL-1 β levels in serum were measured using ELISA kits (eBioscience, Shanghai, China).

Flow cytometry analysis

Renal tissues were ground in a strainer. After red blood cell lysis and washing with phosphate-buffered saline (PBS) 3 times, cells were marked using an anti-mouse-F4/80-PE (eBioscience, Shanghai, China) antibody. All samples were washed 3 times after the antibodies staining, and then subjected to processing with a fluorescence-activated cell sorter (FACS) LSR II (BD Bioscience, USA), and further analyzed by FlowJo software (Tomy Digital Biology Co., Ltd., Japan). At least 3 duplicates were measured for each group.

Statistical analysis

Data are shown as the mean \pm standard error of the mean (SEM). The *t* test was performed for comparisons between 2 groups, and one-way analysis of variance (ANOVA) was employed for comparisons among several groups. *P* value <0.05 was considered to be statistically significant.

Results

Methane-rich saline attenuates renal ischemia-reperfusion injury

To first confirm the protective effect of methane-rich saline on the renal ischemia-reperfusion model of mice, we used a renal pedicle clamping model combined with intraperitoneal injection of methane-rich saline (RIR+MS) or normal saline (RIR) immediately after the surgery. The sham surgery group (sham) and the intraperitoneal methane injection without surgery group (MS) were compared as controls. Mouse blood and renal tissue were harvested for further analysis. As the results showed, compared with the RIR group, the blood urea nitrogen (BUN) and creatinine in the serum were significantly decreased in the RIR+MS group (Figure 1A, 1B). Pathology analysis also showed that the RIR+MS group had alleviated injury compared with the RIR group, especially in tubular regions, as the tube casts in the RIR group were significant, whereas no significant tube casts were observed in the RIR+MS group (Figure 1C).

Methane-rich saline decreased oxidative stress of renal ischemia-reperfusion injury

Since oxidative stress has been reported as one of the foremost pathophysiological changes during RIR injury [8,16], we sought to investigate the possible effects of methane on oxidative responses. We determined the activity of myeloperoxidase (MPO), catalase (CAT), superoxide dismutase (SOD), and the level of malondialdehyde (MDA). As previous studies reported, the elevated level of CAT and SOD, as well as the decreased level of MPO and MDA, should indicate the decreased oxidative stress [14,22,23]. After methane treatment, we found a significant increase in SOD and CAT, nearly to the level of the control groups, and a decrease in MDA and MPO (Figure 2A–2D), indicating reduced oxidative stress in the RIR+MS group. To further verify this finding, we also performed immunohistochemistry to analyze *in situ* oxidative stress presented by 8-hydroxyguanosine (8-OHdG). In accordance with the results of MDA and MPO, methane intervention significantly reduced the oxidative level in RIR injury (Figure 2E).

Methane-rich saline reduced apoptosis of RIR injury

After confirming the decrease of oxidative stress of RIR injury, we reasoned that this should lead to some improvement in apoptosis. Thus, we used TUNEL staining of immunohistochemistry to analyze the *in situ* alteration of apoptosis. As observed in Figure 3A, a significant reduction of the apoptosis region in the RIR+MS group was confirmed. We also noticed that the most significant apoptosis in the RIR group was in the tubular region, which is in accordance with previous research [24,25], and methane treatment reduced this pathological alteration. We further stained for caspase 3 of apoptosis to verify the results, and results showed a similar outcome, which proved the reduced apoptosis after methane intervention in the RIR+MS group (Figure 3B).

Methane-rich saline alleviated *in situ* and general inflammatory responses of RIR injury

Inflammatory responses after ischemia and reperfusion play an important role in renal injury and are possibly related to progression to chronic kidney disease (CKD) [3,7,11]. Therefore, inflammatory responses were also analyzed to evaluate the protective effects of methane. We found that general pro-inflammatory cytokines, including IL-6 and TNF- α , were suppressed, and the regulatory cytokine, IL-10, was elevated (Figure 4A–4C), indicating an alleviated general inflammation. Moreover, *in situ* inflammation, presented by F4/80⁺ macrophage staining (Figure 4D), was also alleviated in the RIR+MS group. We also ground renal tissue, marked it with F4/80⁺ antibody, and analyzed it using flow cytometry. The results also proved a reduction of F4/80⁺ macrophages in the RIR+MS

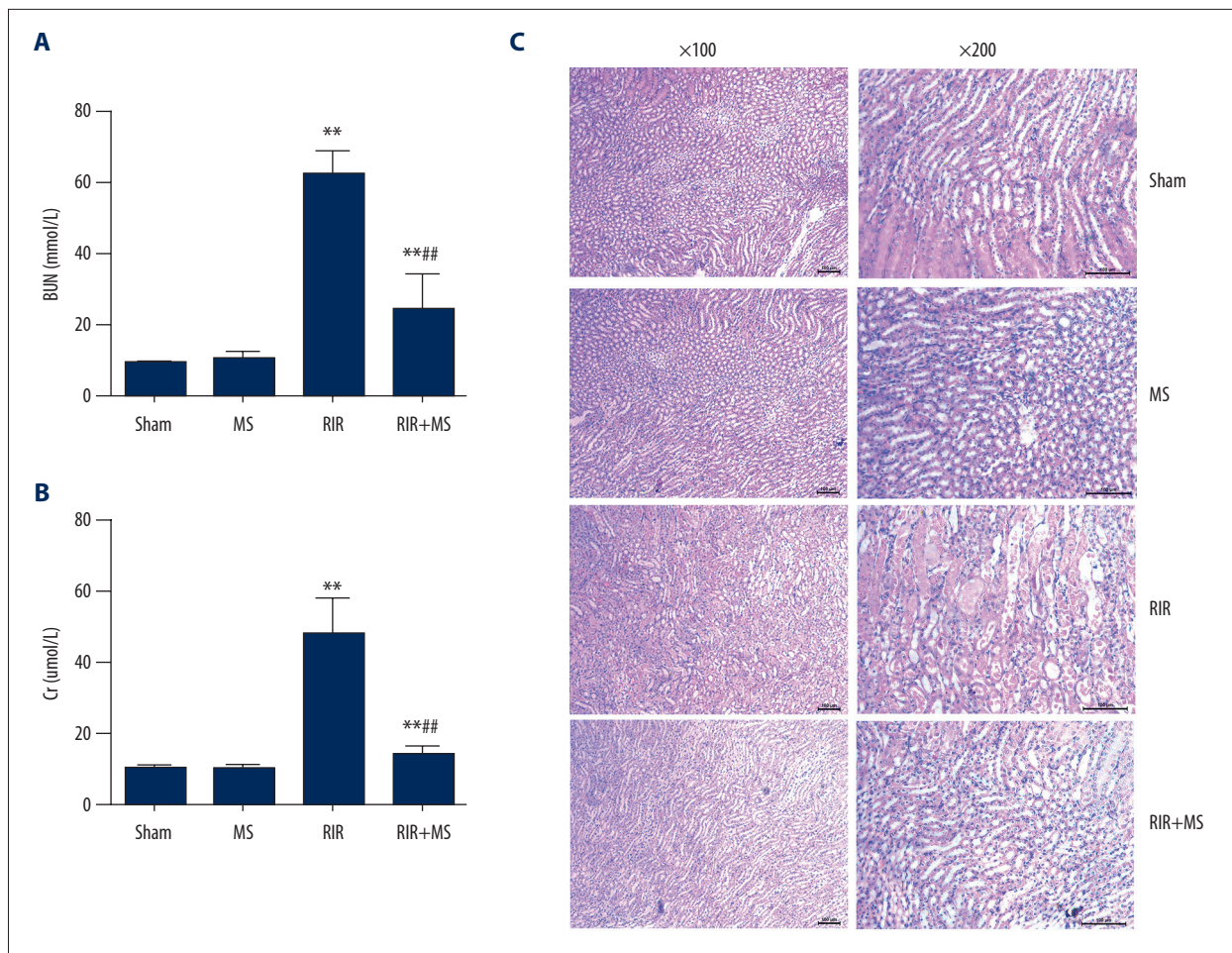


Figure 1. Methane-rich saline attenuates renal ischemia-reperfusion injury. (A, B) Serum blood urea nitrogen (BUN) (A) and creatinine (B) in the serum of the sham group (mice underwent surgery without renal pedicle clamping), the MS group (mice underwent intraperitoneal methane injection without surgery), the RIR group (mice underwent renal pedicle clamping surgery for 30 min and intraperitoneal saline injection), and the RIR+MS group (mice underwent renal pedicle clamping surgery for 30 min and intraperitoneal methane injection after the surgery) (n=6). (C) Hematoxylin-eosin staining of renal tissue of the sham, MS, RIR, and RIR+MS groups (bar=100 μ m) (n=6). The error bars represent \pm standard deviation (SD) (* $P<0.05$ and ** $P<0.01$ compared with sham; # $P<0.05$ and ## $P<0.01$ compared with RIR); n=biological replicates.

group (Figure 4E). Thus, our results proved the anti-inflammatory effects of methane in RIR injury.

Discussion

It was not too long ago when the commonly held notion of futile physiological effects of methane was challenged. Previous perspectives were that methane was generated only by methanogens in the colon, and then was circulated and excreted unchanged via the lungs or flatus [13,26]. However, subsequent research demonstrated not only non-microbial formation of methane in anoxic mitochondria confirmed by *in vitro* and *in vivo* studies, but also methane's bioactivity involving intestinal neuromuscular function, its protective effects on oxidative

stress and inflammation, reducing apoptosis, and even alleviating diabetic retinopathy [13–16]. Updated evidence proved the organ-protection effects of methane in different animal models, and further investigation showed possible therapeutic approaches through anti-oxidative, anti-apoptotic, and anti-inflammatory actions [14]. As all 3 of these approaches share the most common pathophysiological mechanism in the development of RIR injury [6,27,28], we sought to investigate its possible effects in RIR injury and acute renal injury.

Unlike previous research in hepatocytes or myocardium, RIR injury is much more common with complex systematic reaction and risks progressing to CKD [2,3,7,27]. Renal ischemia can have many causes, including hemorrhage, shock, trauma, or surgical interventions [3]. Most of these pathogenesis lead to

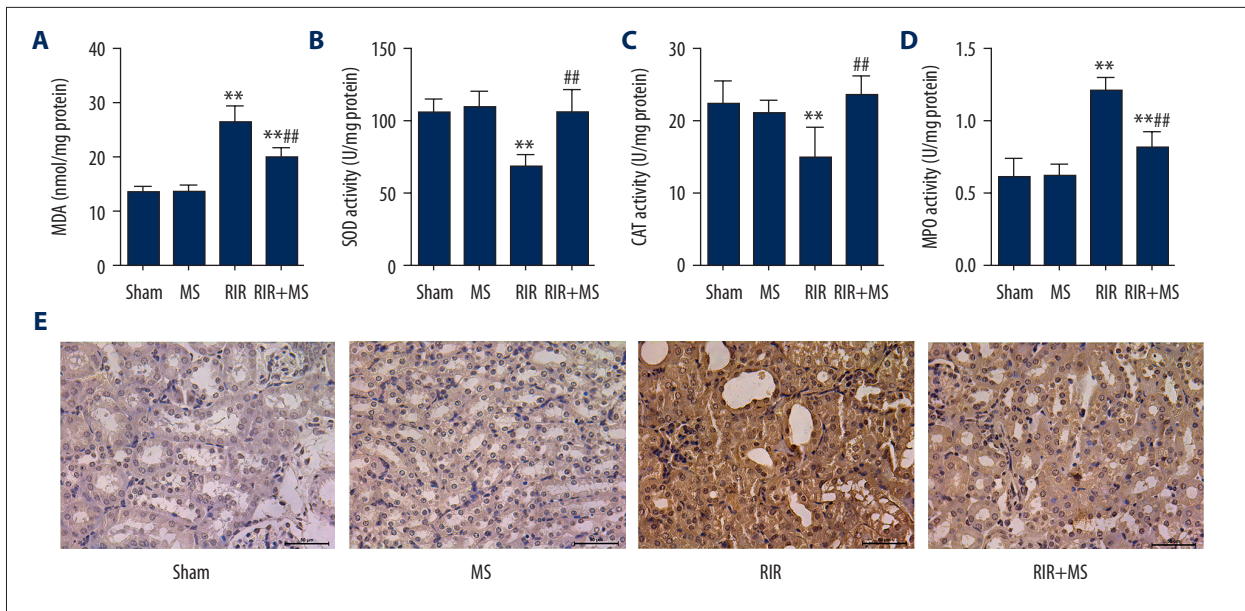


Figure 2. Methane-rich saline decreased oxidative stress of renal ischemia-reperfusion injury. (A–D) Renal homogenate level of malondialdehyde (MDA) (A), superoxide dismutase (SOD) (B), catalase (CAT) (C), and myeloperoxidase (MPO) (D) in different groups (n=6). (E) Immunohistochemistry staining of 8-hydroxyguanosine (8-OHdG) in different groups (bar=50 μ m). The error bars represent \pm standard deviation (SD) (* $P<0.05$ and ** $P<0.01$ compared with sham; # $P<0.05$ and ## $P<0.01$ compared with RIR); n – biological replicates.

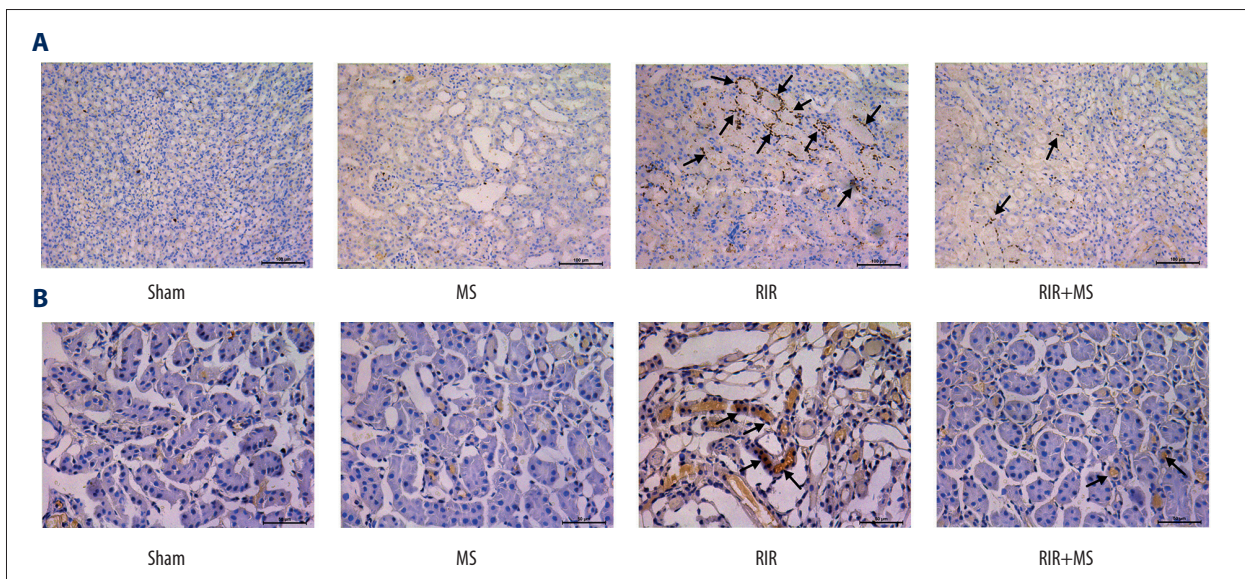


Figure 3. Methane-rich saline reduced apoptosis of renal ischemia-reperfusion injury. (A, B) Immunohistochemistry staining of deoxyuride-5'-triphosphate biotin nick-end labeling (TUNEL) (A) (bar=100 μ m) and caspase 3 (B) (bar=50 μ m) of renal tissues in different groups (n=6); n – biological replicates.

immediate vasoconstriction and reduced renal blood flow [8]. Although no sequential histological or cellular damage was universally accepted, endothelial dysfunction seemed to be an acknowledged as being involved in the course of RIR [3,8]. Early after reperfusion, given the compromised endothelial permeability and increased expression of chemokine, infiltration

of leukocytes and further inflammatory cascade are initiated, leading to extended cytokine production and monocyte-macrophage recruitment and, almost immediately, inflammatory and oxidative damage [4,7,27,28]. Given these responses, the time point of methane intervention varies among models. However, in our preliminary investigations, pre-treatment with methane

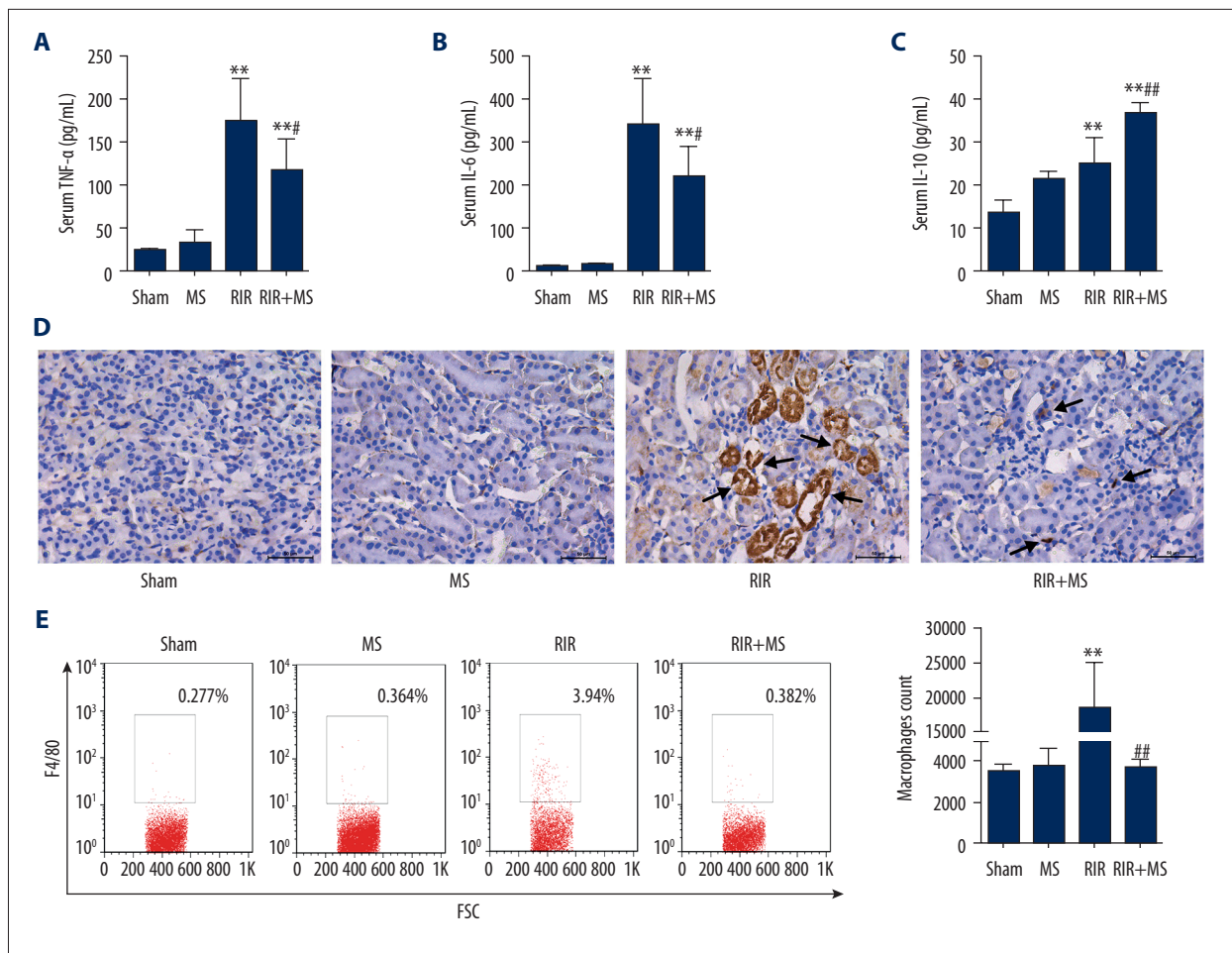


Figure 4. Methane-rich saline alleviated *in situ* and general inflammatory responses of renal ischemia-reperfusion injury. (A–C) Serum level of tumor necrosis factor-alpha (TNF-α) (A), interleukin (IL)-6 (B), and IL-10 (C) in different groups (n=6). (D) Immunohistochemistry staining of F4/80+ macrophages of renal tissues in different groups (arrow shows positive cells) (bar=50 μm) (n=6). (E, F) Dot plot (E) and cell count of positive cells (F) of F4/80+ macrophages in renal tissues of different groups. The error bars represent ± standard deviation (SD) (* P<0.05 and ** P<0.01 compared with sham; # P<0.05 and ## P<0.01 compared with RIR); n – biological replicates.

approximately 30 min before the surgery, did not show significant improvement of injury (data not shown). The methane intervention in our research, approximately 10 min after the reperfusion, showed the best therapeutic outcome. This might be due to the plasma concentration of methane reaching its peak at 10 min [14]. Also, as early macrophage influx on reperfusion of the post-ischemic kidney can facilitate a later inflammatory cascade [7], our results of decreased macrophage infiltration through histological staining and flow cytometry in part explain the attenuated injury. Thus, we can reason that the methane might be useful in the pre-inflammatory-response interposing the reaction of endothelial and leukocyte interaction and decreasing the foremost initiation of inflammatory and oxidative cascade, as significant inflammation and immune responses were reported much later [1]. Thus, it also indicated that methane-rich saline may be useful in emergency situations such as

an accidental surgical injury caused by renal pedicles, or in the case of suspected renal ischemia. The possible therapeutic effect of methane-rich saline discovered in our research may provide some degree of protection from renal injury.

Another phenomenon found in our research is the significant alteration in the renal tubular region with or without methane intervention. As previous studies proposed, renal tubules are more easily and severely injured in ischemia-reperfusion injury [24,25]. This may be because of the less abundant blood supply in renal tubules compared with the glomerulus, making them more susceptible to damage from ischemia. This is in accordance with our results in histology and immunohistochemistry. More important, the methane intervention improved the pathology of this region and attenuated injury, which partly explains the protective effects of methane.

We also noticed changes in serum IL-10 level, which increased even in the MS group and increased markedly in the RIR+MS group. This is a novel finding, showing that its anti-inflammatory phenomenon is mainly achieved through increased IL-10 level, which we have thoroughly investigated and reported in previous research [29]. However, although IL-10-dependent anti-inflammation was confirmed as a mechanism of methane's protective effect, given its significant relief in physiological parameters, histology, cytokine production, and inflammation, further verifying the specific target or central mechanism may be difficult. A multi-pathway or multi-process mechanistic model might be more reasonable. Theoretically, it is not difficult to understand the anti-oxidative and anti-inflammatory effects, since methane is an organic chemical with reducibility. Similar studies that focused on hydrogen also obtained results in inflammation reduction and reduced hydroxyl radicals and peroxynitrite anion (ONOO⁻) production, which have already been successfully applied in treating sepsis and RIR injury [5,30–32]. However, gas molecules such as methane and hydrogen may not have multi-action and multi-pathway-related effects. Thus, the anti-oxidative, anti-inflammatory, and anti-apoptotic effects may not share the same regulation initiated from a single signal or pathway. Future studies aiming to define the mechanism might use a more comprehensive analysis of molecular interaction induced by methane and, thus, acquire full-scale physiological information about this gas molecule.

Combined with research conducted in myocardium, liver, intestine, and other organs [13–16], the protective effects of methane against RIR injury demonstrate its important role in body redox homeostasis. Although acute respiratory distress syndrome caused by methane was also reported in extreme

dosages [33], it would be more appropriately described as being caused by anoxia rather than by one of methane's physiological effects. The safety of methane dissolved in saline in normal applications has been thoroughly investigated and, thus, possible hidden dangers regarding methane have been dismissed, ensuring the safety of further research. To conclude, we proved the effects of methane in a mouse RIR injury model and showed that methane intervention led to the relief of oxidative stress, inflammatory responses, and renal apoptosis. These results confirmed the protective effects of methane in organ ischemic-inflammatory protection and add further supportive evidence for its future translation and applications.

Conclusions

Our study proved the protective effects of methane in RIR injury through attenuating apoptosis, inflammation, and oxidative stress. This may help to translate methane application and develop its use in organ ischemia-reperfusion injury.

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Conflicts of interests

None.

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