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2026 Volume 11, Issue 4
Advances in Precision Medicine
ISSN: 2424-9106 (Online)
ISSN: 2424-8592 (Print)

Effects of Sevoflurane on NGAL and MCP-1 Expression in the Kidneys of Diabetic Nephropathy Mice

Hongjing Tan, Hong'ai Yao, Bo Wang, Jiao Yang, Yi Zhao, Kai Luo
Zunyi First People's Hospital (The Third Affiliated Hospital of Zunyi Medical University), Zunyi 563000, Guizhou, China

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Abstract: *Objective:* To investigate whether AS1842856 can modulate the protective effect of sevoflurane on ischemia-reperfusion injury (IRI) in diabetic nephropathy (DN) mice. *Methods:* Forty DN mouse models were prepared and randomly divided into the sham operation group (Sham group), renal ischemia-reperfusion group (I group), sevoflurane group (S group), and AS1842856 group (AS group), with 10 mice in each group. The I group underwent IRI model establishment; the Sham group did not receive renal pedicle occlusion, the S group received sevoflurane pretreatment before IRI model establishment, and the AS group received AS1842856 solution via gastric administration prior to sevoflurane treatment and IRI model establishment. The conditions of all groups were compared. *Results:* Compared with the I group, mice in the AS group exhibited improved general condition, reduced serum creatinine and blood urea nitrogen levels ($P < 0.05$), milder renal injury as shown by hematoxylin-eosin staining, and decreased expression of neutrophil gelatinase-associated lipocalin (NGAL) and monocyte chemoattractant protein-1 (MCP-1) proteins and their mRNA levels ($P < 0.05$). *Conclusion:* Sevoflurane pretreatment abolishes its protective effect against IRI in DN mice, whereas AS1842856 can modulate the protective effect of sevoflurane on IRI in DN mice.

Keywords: Diabetic nephropathy; Ischemia-reperfusion injury; Sevoflurane; Forkhead box transcription factor O1 (FOXO1); AS1842856

Online publication: April 26, 2026

1. Introduction

Diabetic nephropathy (DN) is one of the common microvascular complications of diabetes mellitus, where hyperglycemia induces renal oxidative stress, leading to progressive deterioration of DN [1]. Sevoflurane is a widely used inhaled anesthetic with additional multi-organ protective effects; however, its stress protective efficacy is nullified in patients with concurrent diabetes mellitus [2]. Studies [3] suggest that the forkhead box transcription factor O1 (FOXO1) may serve as the central target underlying the loss of sevoflurane's ischemia-reperfusion protective effects in DN. AS1842856, a targeted inhibitor of FOXO1, specifically binds to phosphorylated FOXO1 to suppress the expression of downstream genes [4], potentially enhancing sevoflurane's protective effects against renal ischemia-reperfusion through mechanism remodeling. However, such studies remain limited. In this study, a renal ischemia-reperfusion model in DN mice was established to investigate the impact of sevoflurane pretreatment on renal function and whether AS1842856 can restore sevoflurane's protective effects against ischemia-reperfusion injury in DN mice. The findings are reported as follows.

2. Subjects and methods

2.1. Experimental animals

Healthy male C57BL/6 mice, clean-grade, aged 6–8 weeks, weighing 20–25 g, purchased from Shanghai Silek Laboratory Animal Co., Ltd. Post-purchase housing conditions included: temperature 18–22°C, relative humidity 55–60%, 12-hour/12-hour circadian rhythm, and standard feed with free access to water. Animal experiments were conducted in strict compliance with established experimental animal handling protocols and housing guidelines.

2.2. Main reagents, consumables, and instruments

Streptozotocin (STZ, Sigma Corporation, USA); Carboxymethylcellulose Sodium (CMC-Na, Sinopharm Chemical Reagents Co., Ltd.); AS1842856 (MCE Corporation, USA); Sevoflurane (Shanghai Hengrui Pharmaceutical Co., Ltd.); Serum Creatinine Kit (Abcam, UK); Serum Urea Nitrogen Kit (Shanghai Enzyme-linked Biotechnology Co., Ltd.); Electrochemiluminescence (ECL) Kit (Wuhan Biyuantian Institute of Biotechnology); Rabbit anti-mouse NGAL and MCP-1 polyclonal antibodies (Santa Cruz Corporation, USA); RNA Primer, Extraction, Reverse Transcription and Amplification Kit (TaKaRa, USA); HRP-labeled secondary antibodies and chromogenic reagents (Wuhan BoShide Bioengineering Co., Ltd.). The primers were synthesized by TOYOBO.

Small animal anesthesia apparatus (Revode Company); Fully automated biochemical analyzer (Model BK-400, Shandong Boke); Gel imaging system (Model iBright, Thermo Fisher Scientific); Real-time PCR detector (Model ABI-7300, ABI Corporation).

2.3. Methods

2.3.1. Construction of DN mouse model

After procurement, mice were fed adaptively for 3 days, followed by a high-fat, high-sugar diet (lard: sucrose: egg yolk: basal feed = 18:20:3:59) for 8 consecutive weeks. Starting from the first day of intervention, fasting was imposed for 12 hours (overnight fasting without water restriction). STZ was dissolved in citrate buffer (pH = 4.5) and administered via single intraperitoneal injection at a dose of 60 mg/kg. On the 8th day of intervention, blood glucose levels were measured by tail vein blood sampling; a blood glucose level > 16.7 mmol/L indicated successful establishment of the diabetic mouse model. For the DN model mice: after 4 weeks of STZ treatment, a successful DN model was confirmed when 24-hour urinary protein levels reached ≥ 30 mg/d [5].

2.3.2. Grouping and processing

Forty mice successfully constructed with the DN model were randomly divided into four groups using a random number table: the sham surgery group (Sham group), renal ischemia-reperfusion group (I group), sevoflurane group (S group), and AS1842856 group (AS group), with 10 mice in each group. (1) I group: On the day prior to inducing renal ischemia-reperfusion, 70 mg/kg of 1% CMC-Na solution was administered orally at 8:00 and 18:00, and again at 8:00 on the second day. Fasting was maintained after the initial administration until the end of the procedure. Mice were anesthetized via intraperitoneal injection of 10% chloral hydrate (400 mg/kg). A mid-abdominal incision was made, and the peritoneum was dissected layer by layer to expose the kidneys. The ureters were bluntly dissected, and the bilateral renal pedicles were exposed and clamped. Vascular clips were applied for 45 minutes; successful ischemia was indicated by the transition of kidney color from bright red to white. After 45 minutes of ischemia, the arterial clips were released to restore blood flow, and reperfusion was confirmed by the shift of kidney color from white to bright red [6]. The incision was closed layer by layer, and the mice were allowed free access to water and food. (2) Sham group: All procedures were performed as in the I group, except for the absence of clamping of the bilateral renal pedicles. (3) S group: The oral administration of CMC-Na solution followed the protocol of the I group. The mice were then placed in a sealed box with one end connected to an

animal anesthesia apparatus and the other to a gas detector. They were exposed to 2.5% sevoflurane at an oxygen flow rate of 1 L/min for 45 minutes, followed by a 10-minute transition to fresh air before undergoing ischemia-reperfusion (same protocol as the I group). Except for the oxygen flow without 2.5% sevoflurane, the oxygen treatment in the Sham and I groups was identical to that in the S group. (4) AS group: AS1842856 was dissolved in 1% CMC-Na to prepare a solution with a concentration of 5 mg/ml. Mice were administered the AS1842856 solution (70 mg/kg) via oral gavage at 8:00 and 18:00 on the day preceding renal ischemia-reperfusion induction, and again at 8:00 the following day. Sevoflurane anesthesia and ischemia-reperfusion procedures were identical to those in the S group.

2.4. Observation indicators

2.4.1. General information

Observe the mental status, fur color, and behavioral responses of mice in each group during the trial period, and record any cases of mortality.

2.4.2. Renal function

After 24 hours of ischemia-reperfusion treatment, 2 mL of peripheral blood was collected from each group of mice using the ocular blood sampling method. The blood was transferred to an anticoagulation tube, centrifuged at 4°C and 3,500 rpm for 10 minutes, and the supernatant was collected to measure serum creatinine (Scr) and blood urea nitrogen (BUN) levels.

2.4.3. Conventional pathology and immunohistochemistry

After blood collection, the bilateral kidneys of mice were extracted under anesthesia with 3% pentobarbital sodium (1.6 ml/kg administered intraperitoneally). The left kidney was placed in a cryovial and temporarily stored at -80°C for Western blot and PCR analysis; the right kidney was fixed in 4% polyformaldehyde solution, subjected to routine pathological processing, and its pathological changes were observed under light microscopy.

Renal tissue sections from each group of mice were routinely dewaxed, incubated with 3% H2O2 for 10 minutes, and subjected to microwave antigen retrieval for 15 minutes. Immunohistochemical staining was performed following the protocol specified for the neutrophil gelatinase-associated lipocalin (NGAL) and monocyte chemoattractant protein-1 (MCP-1) kits (PAS staining and Masson staining). After adding the primary antibodies against NGAL and MCP-1, sections were incubated at 4°C overnight. On the second day, sections were rewarmed for 30 minutes, followed by the addition of a polymer auxiliary agent and further incubation for 30 minutes. The sections were then incubated with HRP-labeled goat anti-rabbit IgG for 30 minutes, and developed using 3,3'-diaminobenzidine (DAB) for 0.5–2 minutes. Staining was visually assessed under a microscope, and sections were reimmersed and mounted. Ten interstitial regions were selected from each sample, and image acquisition and analysis were performed using the Image-Pro Plus 6.0 software. For each section, 10 randomly selected fields under high magnification (×400) were evaluated, with positive staining defined as brown-yellow or brown coloration.

2.4.4. Western blot detection of NGAL and MCP-1 protein expression in renal tissue

Weigh 50 mg of the preserved left kidney, cut it into fine fragments, and homogenize the renal tissue using RIPA lysis buffer with ultrasonication. Extract proteins using the tissue protein extraction kit (add 1% protease inhibitor and 1% phosphatase inhibitor first). Centrifuge at 4°C and 12,000 rpm for 10 minutes, then collect the supernatant. Use the BCA kit to detect NGAL and MCP-1 protein levels in renal tissues from each group. Load 45 μg of total protein per well, perform SDS-PAGE on a 4% sodium dodecyl sulfate-polyacrylamide gel, transfer the proteins to a polyvinylidene fluoride (PVDF) membrane, and block the membrane with 5% skim milk at room temperature for 1 hour. Incubate with NGAL antibody (1:300) and MCP-1 antibody (1:300) at 4°C overnight, then add horseradish peroxidase (HRP)-labeled goat anti-rabbit polyclonal antibody and incubate at room temperature for 1 hour. Develop the image using the ECL kit, use β-actin as the internal control, and analyze the bands by grayscale intensity measurement with ImageJ software.

2.4.5. qRT-PCR detection of NGAL and MCP-1 mRNA expression in renal tissue

We weighed 50 mg of the preserved left kidney, extracted total RNA, and determined its concentration and integrity using both colorimetric and agarose gel electrophoresis methods. Reverse transcription was performed to synthesize cDNA, and the expression levels of NGAL mRNA and MCP-1 mRNA in the samples were detected using a dual-blocked real-time quantitative PCR (qRT-PCR) kit.

NGAL upstream primer: 5’-CCGACACTGACTACGACCAG-3’; downstream primer: 5’-CAT TGGTCGGTGGGAACAGA-3’; fragment length: 20 bp.

MCP-1 upstream primer: 5’-ACA GACAGA GGC CAG CCC AG-3’; downstream primer: 5’-TCT CCAGCC GAC TCA TTG GGA-3’; fragment length: 215 bp.

Internal reference β-actin upstream primer: 5’-CTCTGGTCGTACCACTGGCATTG-3’; downstream primer: 5’-CCTGCTTGCTGATCCACATCTGC-3’; fragment length: 650 bp.

Take 2 μL of cDNA, add 2 μL of primers, 5 μL each of buffer and dNTP, and 1/8 μL of Taq polymerase, then supplement with double-distilled water to a total volume of 50 μL. Amplification conditions: 94°C pre-denaturation for 30 seconds, 55°C annealing for 30 seconds, and 72°C extension for 30 seconds; perform 30 cycles of PCR with fluorescence signal detection, each cycle repeated three times. The relative expression level of the target gene is expressed as $2^{-\Delta\Delta CT}$.

2.5. Statistical methods

Data analysis was performed using SPSS 26.0 software. Measurement data were expressed as mean ± standard deviation (SD). Single-factor analysis of variance (one-way ANOVA) was employed for comparisons among multiple groups, while the Dunnet *t*-test was used for pairwise comparisons. Comparisons of non-normally distributed data were conducted using the Mann-Whitney test. A *P*-value < 0.05 was considered statistically significant.

3. Results

3.1. General comparison

No deaths occurred in any group during the study period. Mice in the Sham group and AS group exhibited lethargy, dull yellowish fur without luster, and delayed responses; these manifestations were more pronounced in Groups I and S, with some mice showing raised fur, abdominal muscle twitching, tremors, and even tail necrosis, limb infections, and ulcers.

3.2. Comparison of serum Scr and BUN levels

After 24 hours of ischemia-reperfusion, the serum Scr and BUN levels in Group I and Group S were significantly higher than those in the Sham group and AS group ($P < 0.05$). However, no statistically significant differences were observed in serum Scr and BUN levels between Group I and Group S, or between Group AS and the Sham group ($P > 0.05$). See **Table 1**.

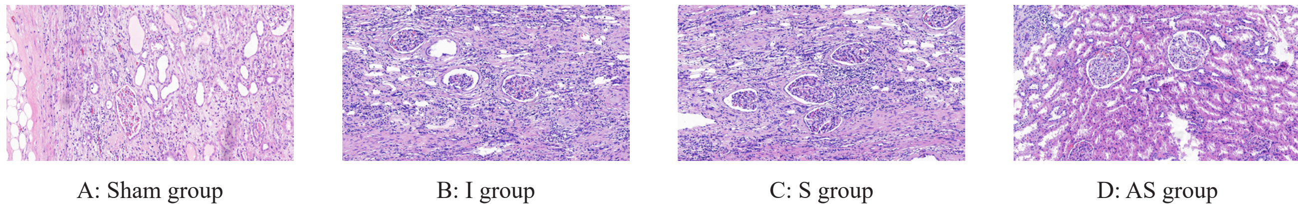
Table 1. Comparison of serum Scr and BUN among groups

Group	*n*	Scr (μmol/L)	BUN (mmol/L)
Sham group	10	49.57 ± 6.18	17.65 ± 3.52
I group	10	121.15 ± 30.72[1)]	53.60 ± 13.37[1)]
S group	10	119.07 ± 23.68[1)]	47.36 ± 11.89[1)]
AS group	10	53.15 ± 9.02[2) 3)]	21.55 ± 4.36[2) 3)]
F value		31.632	23.157
P value		0.003	0.006

Note: 1) $P < 0.05$ vs Sham group; 2) $P < 0.05$ vs I group; 3) $P < 0.05$ vs S group

3.3. Comparison of routine pathological and immunohistochemical results of renal tissue

Under light microscopy, the Sham group (**Figure 1A**) and AS group (**Figure 1D**) exhibited disordered renal architecture with interstitial edema, swelling of renal tubular epithelial cells, loss of nuclei, flattened epithelial cells, and shedding of brush-shaped cells in some tubules, along with tubular narrowing and inflammatory cell infiltration in the renal interstitium. In contrast, the I group (**Figure 1B**) and S group (**Figure 1C**) showed a significant reduction in intact tubular structures, a marked increase in necrotic cells, and extensive inflammatory cell infiltration.



A: Sham group B: I group C: S group D: AS group

Figure 1. HE staining for rat kidney ×400

PAS staining revealed that NGAL exhibited brown-yellow expression in the glomeruli, renal interstitium, and renal tubules, with abundant and intensely stained expression in Groups I and S, while showing lighter staining and reduced expression levels in the Sham and AS groups. Masson staining demonstrated that MCP-1 was expressed as brownish-red at the brush border of the proximal renal tubules in the renal cortex, with abundant and intensely stained expression in Groups I and S, and lighter staining and reduced expression levels in the Sham and AS groups.

3.4. Comparison of NGAL and MCP-1 protein expression in renal tissue

The expression levels of NGAL and MCP-1 in Group I and Group S were significantly higher than those in the Sham group and AS group ($P < 0.05$). However, no statistically significant differences were observed in NGAL and MCP-1 expression levels between Group I and Group S, or between Group AS and the Sham group ($P > 0.05$). See **Table 2**.

Table 2. Comparison of NGAL and MCP-1 protein expression among groups

Group	*n*	NGAL/β-actin	MCP-1/β-actin
Sham group	10	0.35 ± 0.13	0.37 ± 0.10
I group	10	0.56 ± 0.12$^{1)}$	0.50 ± 0.09$^{1)}$
S group	10	0.53 ± 0.15$^{1)}$	0.48 ± 0.10$^{1)}$
AS group	10	0.33 ± 0.10$^{2)\,3)}$	0.35 ± 0.08$^{2)\,3)}$
F value		25.511	28.271
P value		0.005	0.004

Note: 1) $P < 0.05$ vs Sham group; 2) $P < 0.05$ vs I group; 3) $P < 0.05$ vs S group

3.5. Comparison of NGAL and MCP-1 mRNA expression in renal tissue

The mRNA expression levels of NGAL and MCP-1 in Group I and Group S were significantly higher than those in the Sham group and AS group ($P < 0.05$). However, no statistically significant differences were observed in the mRNA expression levels of NGAL and MCP-1 between Group I and Group S, or between Group AS and the Sham group ($P > 0.05$). See **Table 3**.

Table 3. Comparison of NGAL and MCP-1 mRNA expression among groups

Group	*n*	NGAL mRNA	MCP-1mRNA
Sham group	10	5.26 ± 1.10	2.56 ± 0.73

Group	*n*	NGAL mRNA	MCP-1mRNA
I group	10	11.29 ± 2.53[1)]	7.88 ± 1.89[1)]
S group	10	11.07 ± 2.25[1)]	7.56 ± 1.80[1)]
AS group	10	5.10 ± 1.02[2) 3)]	2.43 ± 0.82[2) 3)]
F value		23.250	26.005
P value		0.006	0.005

Note: 1) $P < 0.05$ vs Sham group; 2) $P < 0.05$ vs I group; 3) $P < 0.05$ vs S group

4. Discussion

In recent years, the incidence of diabetes mellitus in adults in China has reached as high as 8.5%, with approximately 30%–40% of patients progressing to DN. In clinical practice, surgical intervention is frequently required for patients with DN, and these patients are more susceptible to ischemia-reperfusion injury (IRI) during renal surgery, which typically occurs within 48 hours postoperatively and is characterized by high incidence and mortality rates. Currently, anesthetic preconditioning-induced stress protection is a commonly employed clinical intervention, with sevoflurane being the most widely used agent. Studies have demonstrated that sevoflurane inhibits the translocation of glucose transporter 4 (GluT4) downstream of the insulin-regulated phosphatidylinositol 3-phosphokinase-protein kinase B (PI3K-Akt) signaling pathway in skeletal muscle into the cytoplasm, thereby suppressing glucose uptake. Consequently, sevoflurane attenuates insulin regulation and intracellular glucose metabolism, exerting protective and anti-inflammatory effects on the kidneys and helping to reduce the occurrence of IRI.

FOXO1 is a newly discovered subtype of transcription factors that exerts biological effects by activating or inhibiting gene transcription, participating in the regulation of critical processes such as the cell cycle, cell differentiation, metabolism, and apoptosis, as well as modulating the expression of downstream target genes. Recent studies [7] suggest that FOXO1 may be the central target responsible for the disappearance of sevoflurane's protective effects against DN ischemia-reperfusion injury. Lin *et al.* [8] demonstrated that downregulation of FOXO1 via N-acetylcysteine reversed the cardioprotective effects of sevoflurane in diabetic mice. AS1842856, a targeted inhibitor of FOXO1, specifically binds to phosphorylated FOXO1, thereby suppressing the expression of its downstream target genes. Huang *et al.* [9] found that a high-fat diet induces depression-like behaviors in mice, and AS1842856 reverses these behaviors. Li *et al.* [10] reported that AS1842856 binds to dephosphorylated FOXO1, blocking its interaction with downstream genes, thereby promoting glucose and pentose phosphate metabolism and maintaining the homeostasis of pancreatic β-cell function. These findings indicate that AS1842856 can modulate the effects of sevoflurane, thereby exerting a protective effect against renal ischemia-reperfusion injury. This study provides experimental validation of these findings.

The NGAL and MCP-1 selected for this study are proteins that are only minimally expressed in renal tissue under physiological conditions. For instance, mesangial cells secrete trace amounts of MCP-1, which chemotactically guide macrophages to phagocytose harmful substances, thereby fulfilling normal immune defense functions. However, during acute kidney injury, NGAL and MCP-1 are highly expressed in renal tubular cells at an early stage, making them promising biomarkers for assessing acute renal injury. The results of this study demonstrated that mice in Groups I and S exhibited poorer general conditions compared to the Sham and AS groups, with elevated serum levels of Scr and BUN ($P < 0.05$), indicating more severe acute kidney injury in these groups—a finding corroborated by HE staining results. Immunohistochemical, Western blot, and PCR analyses all revealed significantly higher expression levels of NGAL and MCP-1 proteins and mRNA in Groups I and S compared to the Sham and AS groups ($P < 0.05$), further supporting their more severe acute kidney injury.

5. Conclusion

In conclusion, under high-glucose conditions, the protective effect of sevoflurane pretreatment against IRI is abolished, while the FOXO1-targeted inhibitor AS1842856 can restore sevoflurane's protective effect against IRI in DN mice. These findings provide valuable insights for elucidating the underlying mechanisms and guiding the development of targeted therapies.

Funding

Guizhou Provincial Health Commission Research Project Number: Gzwkj2021-278

Disclosure statement

The authors declare no conflict of interest.

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