• Basic Research •

N-acetylserotonin alleviates retinal ischemia-reperfusion injury *via* HMGB1/RAGE/NF-κB pathway in rats

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Abstract

● **AIM:** To observe the effects of N-acetylserotonin (NAS) administration on retinal ischemia-reperfusion (RIR) injury in rats and explore the underlying mechanisms involving the high mobility group box 1 (HMGB1)/receptor for advanced glycation end-products (RAGE)/nuclear factor-kappa B (NF-κB) signaling pathway.

● **METHODS:** A rat model of RIR was developed by increasing the pressure of the anterior chamber of the eye. Eighty male Sprague Dawley were randomly divided into five groups: sham group (*n*=8), RIR group (*n*=28), RIR+NAS group (*n*=28), RIR+FPS-ZM1 group (*n*=8) and RIR+NAS+FPS-ZM1 group (*n*=8). The therapeutic effects of NAS were examined by hematoxylin-eosin (H&E) staining, and retinal ganglion cells (RGCs) counting. The expression of interleukin 1 beta (IL-1β), HMGB1, RAGE, and nod-like receptor 3 (NLRP3) proteins and the phosphorylation of nuclear factor-kappa B (p-NF-κB) were analyzed by immunohistochemistry staining and Western blot analysis. The expression of HMGB1 protein was also detected by enzyme-linked immunosorbent assay (ELISA).

● **RESULTS:** H&E staining results showed that NAS significantly reduced retinal edema and increased the number of RGCs in RIR rats. With NAS therapy, the HMGB1 and RAGE expression decreased significantly, and the activation of the NF-κB/NLRP3 pathway was antagonized along with the inhibition of p-NF-κB and NLRP3 protein expression. Additionally, NAS exhibited an anti-inflammatory effect by reducing IL-1β expression. The inhibitory of RAGE binding to HMGB1 by RAGE inhibitor FPS-ZM1 led to a significant decrease of p-NF-κB and NLRP3 expression, so as to the IL-1β expression and retinal edema, accompanied by an increase of RGCs in RIR rats.

● **CONCLUSION:** NAS may exhibit a neuroprotective effect against RIR *via* the HMGB1/RAGE/NF-κB signaling pathway, which may be a useful therapeutic target for retinal disease.

● **KEYWORDS:** retinal diseases; retinal ischemia-reperfusion injury; N-acetylserotonin; high mobility group box 1; receptor for advanced glycation end-products; nuclear factor-κB; rats

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INTRODUCTION

Retinal ischemia-reperfusion (RIR) injury is a significant pathological process in common ophthalmic diseases, which may lead to blindness in the long term, including central retinal arteriovenous embolism, glaucoma, and diabetic retinopathy[1-2]. After ischemia, the retina's morphology and function change quickly. After reperfusion, many inflammatory factors are released from the retinal cells, which causes severe damage to the retina and threatens the quality of life among RIR patients[3-4]. Furthermore, there is currently a lack of effective treatment and an urgent need to develop effective therapeutics for RIR.

Inflammation is an important cause of RIR injury [5-6]. After RIR, high mobility group box 1 (HMGB1) was released from

retinal cells and bound to the receptor for advanced glycation end-products (RAGE)[7-8], which can promote the activation of nuclear factor-kappa B (NF-κB) protein and its translocation into the nucleus[9-10]. The phosphorylation of nuclear factor-kappa B (p-NF-κB) activates nod-like receptor 3 (NLRP3) and promotes the release of precursor interleukin-1β (pro-IL-1β)[11]. Pro-IL-1β can be transformed into IL-1β mediated by activated NLRP3. IL-1β, as a pro-inflammatory cytokine, can mediate inflammatory response and aggravate retinal tissue injury[11-12], suggesting that the downregulation of IL-1β *via* the HMGB1/RAGE/NF-κB pathway may ameliorate retinal injury in RIR rats.

N-acetylserotonin (NAS), the precursor of melatonin, can cross the blood-brain barrier easily. NAS can not only be converted into melatonin for its neuroprotective effects, but also exhibit antioxidant, anti-inflammatory and other effects[13-15]. NAS has a substantial protective effect against hepatic and intestinal ischemia-reperfusion injury[16-17]. NAS plays a neuroprotective role in ischemic encephalopathy and Alzheimer's disease by inhibiting NF-κB transcription and decreasing the expression of NLRP3[13,18]. Our previous study confirmed that NAS exerted a neuroprotective effect on the retina by reducing the expression of IL-1β, and the mechanism of NAS reducing the retinal injury might be related to the TLR4/NF-κB signaling pathway[19]. Our previous study also found that after the TLR4/NF-κB signaling pathway was antagonized by resatorvid (TAK-242, a selective TLR4 inhibitor), the expression of downstream NF-κB was not fully inhibited, and there still existed retinal damage in RIR rats[19], which indicated that there might exist other potential upstream regulatory mechanisms participating in the restoration of retinal injury after NAS administration and the possible mechanism still needs to be further illuminated. According to recent surveys, there existed some upstream signaling molecules that regulated the expression of inflammatory factors *via* NF-κB signaling pathway in rats after RIR[20]. So, we hypothesized that NAS administration might influence the expression of inflammatory factors through other potential upstream regulatory mechanisms.

Melatonin performs its protective function by inhibiting HMGB1 translocation out of the nucleus and RAGE and NF-κB expression in ischemic encephalopathy, nephropathy and liver diseases[21-24]. However, it remains to be further elucidated whether NAS can inhibit inflammation and alleviate retinal injury by regulating the HMGB1/RAGE/NF-κB pathway. Therefore, hematoxylin-eosin (H&E) staining was used to determine the effects of NAS on the retinal morphology and retinal injury in RIR rats, immunohistochemical (IHC) staining was performed to dynamically observe the effects of NAS administration on HMGB1 and RAGE protein expressions, IHC staining, Western blot analysis and enzyme-linked immunosorbent assay (ELISA) were conducted to detect the effect of NAS on IL-1β protein expression in RIR rats, thus explore the mechanism by HMGB1/RAGE/NF-κB pathway and offer scientific evidence for the application of NAS administration in RIR.

MATERIALS AND METHODS

Ethical Approval The Experimental Animal Ethics Committee of Weifang Medical University approved this study (Weifang, China, approval No.2021SDL182).

Animals Eighty adult healthy male Sprague Dawley (SD) rats, purchased from Jinan Pengyue Experimental Animal Breeding Co. Ltd (Jinan, China, license No. SCXK 20190003), the body weight from 200 to 220 g, were assigned into the sham (*n*=8), RIR (*n*=28), RIR+NAS (*n*=28), RIR+FPS-ZM1 (*n*=8), and RIR+NAS+FPS-ZM1 (*n*=8) groups according to the random number table. According to the time after RIR (6, 12, 24, and 72h and 7d after modeling), the RIR and RIR+NAS groups were further divided into five subgroups. The RIR model was established according to the following method. NAS (10 mg/kg•time; Millipore Sigma, Burlington, MA, USA) was injected intraperitoneally 30min before and after modeling in the RIR+NAS group[25]. The rats in the RIR+FPS-ZM1 group were intraperitoneally injected with FPS-ZM1 (RAGE inhibitor, 1 mg/kg•time; R&D system, Minneapolis, MN, USA)[26]. In the RIR+NAS+FPS-ZM1 group, FPS-ZM1 was intraperitoneally injected 15min before modeling, and NAS was injected 30min before and after modeling. In the sham group, the needle was simply inserted into the anterior chamber of the right eye. In the sham and RIR groups, the equal amount of normal saline was intraperitoneally injected 30min before and after modeling.

Retinal Ischemia-Reperfusion Injury Model An RIR model was established by anterior chamber ocular hypertension[27]. The rats were anesthetized with 1% pentobarbital sodium and locally anesthetized with cocaine hydrochloride eye drops. In brief, a 27G precision infusion needle was connected to a physiological saline bottle and introduced into the anterior chambers of the right eyes of rats. By raising the height of the normal saline bottle, the intraocular pressure was increased to 110 mm Hg. Direct ophthalmoscopy of the oculi fundus revealed an interruption of retinal blood flow. The intraocular pressure dropped to normal after 60min, and the RIR model was successfully established.

Tissue and Blood Sample Preparation Rats in each group were subjected to deep anesthesia. The eyeballs were removed and placed in the eyeball-fixing liquid (G1109, Servicebio, Wuhan, China) for over 24h. After the lens of each eyeball was removed, the eyeball tissue was embedded in the wax, and the waxed tissue was cut, with the paraffin slicer, into 3 μm

slices paralleling along the sagittal axis of the optic nerve. The paraffin sections were prepared for subsequent experiments.

At 24h after RIR, fresh retinal tissues were collected after deep anesthesia, and placed in an enzyme-free cryopreservation tube and refrigerated at -80℃ for the follow-up Western blot analysis. The blood samples of rats were collected by left ventricular injection and centrifuged at 3000 rpm for 15min, and the supernatant was acquired and stored in -80℃ freezers for ELISA.

Hematoxylin and Eosin Staining The paraffin sections were dewaxed with xylene and gradient hydrated with ethanol. The nuclei were stained with hematoxylin, and the cytoplasm was stained with eosin. After a thorough cleaning, the slices were sealed with neutral gum. Cellsens 1.6 software (Olympus, Tokyo, Japan) was used to measure the retinal inner layer thickness [the distance between the inner boundary membrane and the outer border of the inner nuclear layer (INL)], which was between 100-1000 μm on both sides of the optic nerve. The retinal ganglion cells (RGCs) were counted in the ganglion cells layer (GCL) of each group (cells/mm^2).

Immunohistochemistry The sections were deparaffinized and hydrated. Antigen retrieval was performed in citrate buffer by water bath heating. A 3% H_2O_2 solution was used to remove endogenous peroxidase, and 10% normal goat serum was used to block non-specific antigens for 1h at 37℃. The goat serum was discarded, and the slices were incubated with rabbit anti-HMGB1 (10829-1-AP, Proteintech Group, Wuhan, China) and mouse anti-RAGE (sc-365154, Santa Cruz Biotechnology, Dallas, USA), mouse anti-NF-κB (sc-136548, Santa Cruz Biotechnology, USA), rabbit anti-NLRP3 (A12694, ABclonal Technology, Beijing, China), rabbit anti-IL-1β (A1112, ABclonal Technology). The slices were placed in a 4℃ refrigerator overnight. Then sections were washed and incubated with the IHC secondary antibodies (ZSGB-BIO, Beijing, China). After washing, the positive cells were dyed brownish-yellow by DAB kits (ZSGB-BIO). The slices were sealed with neutral gum. Cellsens 1.6 software was used to count positive cells in the retina (cells/mm^2).

Western Blot The tissue lysate was added into the fresh retinal tissue, and the tissue was completely dissolved with an ultrasonic crusher, and centrifuged at 12 000 rpm for 15min (Eppendorf, Hamburg, Germany). The supernatant was removed from the lysate and the protein concentration was examined. The proteins were isolated by electrophoresis on 12% SDS-page gels and transferred onto a polyvinylidene fluoride (PVDF) membrane and incubated with a 5% blocking solution for 2h. The PVDF membrane was incubated with mouse anti-RAGE (sc-365154, Santa Cruz Biotechnology), rabbit anti-HMGB1 (10829-1-AP), rabbit anti-NF-κB (10745-1-AP), rabbit anti-NLRP3 (A12694), rabbit anti-IL-1β (A1112) or mouse anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH; 60004-1-Ig) in a 4℃ refrigerator overnight. The HMGB1, NF-κB, and GAPDH antibodies were from Proteintech Group Corporation, and NLRP3 and IL-1β antibodies are from ABclonal Technology Corporation. After three times of washing, the membrane was incubated with the secondary antibodies. The target protein was detected by a chemiluminescence imaging system, and the optical density (OD) of each protein blot was determined by Image J software. The relative concentration was calculated by the OD ratio of the target protein to GAPDH.

Enzyme-Linked Immunosorbent Assay HMGB1 ELISA Kit (ARG81310, Arigo Biolaboratories, Shanghai, China) was used to detect the HMGB1 expression in the serum of rats. The standard, sample, and control samples, about 100 μL, were added, and followed by 200 μL of HRP-conjugated antibody to each well. The plates were covered with Petri dishes and incubated at 4℃ overnight (without shaking). About 350 μL of cold washing buffer was added to each well. After washing, each well was added with 100 μL TMB and incubated for 10min in the dark. Then about 50 μL stopping solution was added. After the solution was shaken and stirred thoroughly, the solution color changed from blue to yellow. Then the OD value was read at 450 nm with a microplate reader immediately (Gen Company Limited, Hong Kong, China).

Statistical Analysis The results were statistically analyzed by the SPSS 22.0 software. All quantitative data were expressed as mean±standard deviation. One-way analysis of variance (ANOVA) and Newman-Keuls method were used for statistical analysis between groups. Q value is the test statistic. P value <0.05 was regarded as significant difference.

RESULTS

NAS Reduced Retinal Thickness and Loss of RGCs in RIR Rats The H&E staining results showed that the retinal morphological structure in the sham group was clear, each layer was well arranged, and no retinal edema was observed (75.20±5.75 μm). The retina of the RIR group was swelling and thicker (111.75±6.49 μm) than that of the sham group, the difference was statistically significant (P<0.001). The retinal thickness of rats in the RIR+NAS group (96.66±6.95 μm) was significantly thinner than that in the RIR group, but still thicker than that in the sham group (all P<0.001). FPS-ZM1 was used to inhibit the expression of RAGE, and the retinal thickness in the RIR+FPS-ZM1 group (91.17±7.75 μm) was thinner than that in the RIR group, but still thicker than that in the sham group (all P<0.001). However, no noticeable difference was observed between the RIR+NAS and RIR+FPS-ZM1 groups (P=0.181). The retinal thickness in RIR+NAS+FPS-ZM1 group (84.07±5.78 μm) was significantly thinner than that in the RIR (P<0.001), RIR+NAS (P<0.001), and RIR+FPS-ZM1

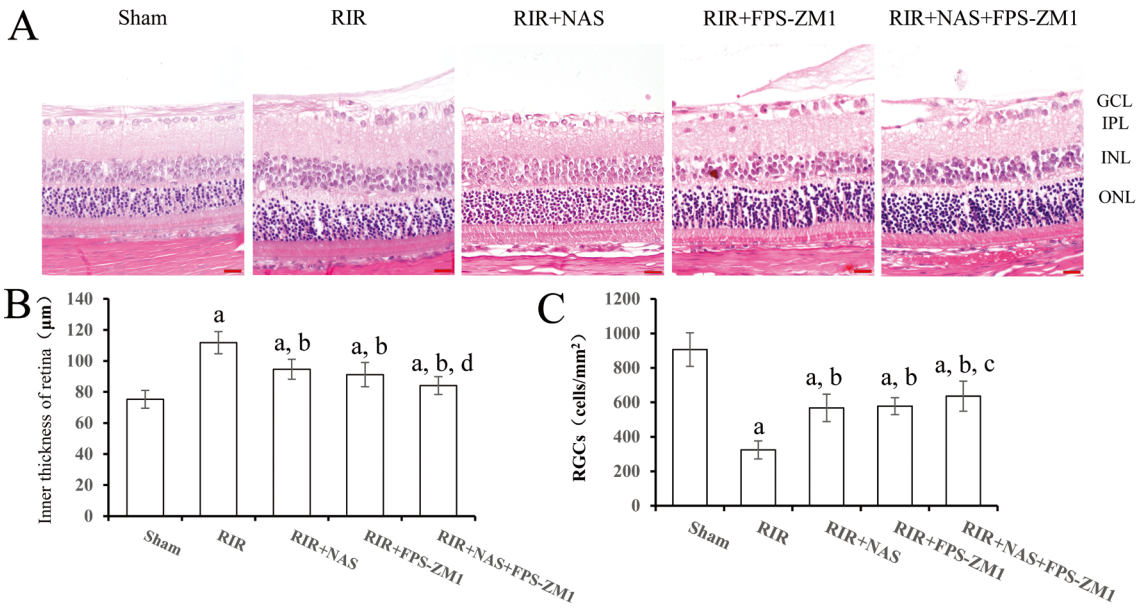

Figure 1 NAS administration improved the retinal morphology in RIR rats A: Retinal cross-sections H&E staining results (400×) 24h after RIR. Scale bar: 20 μm. B: Quantification of the retinal thickness. The data were shown as the mean±SD, ^{a}P<0.01 *vs* sham; ^{b}P<0.001 *vs* RIR; ^{d}P<0.001 *vs* RIR+NAS; *n*=5. C: Quantification of the RGCs number in the GCL. Bars are SD, ^{a}P<0.001 *vs* sham; ^{b}P<0.001 *vs* RIR; ^{c}P<0.05 *vs* RIR+NAS; *n*=5. NAS: N-acetylserotonin; GCL: Ganglion cell layer; IPL: Inner plexiform layer; INL: Inner nuclear layer; ONL: Outer nuclear layer; RGCs: Retinal ganglion cells; SD: Standard deviation; RIR: Retinal ischemia-reperfusion.

groups (*P*=0.01), but still thicker than that in the sham group (*P*=0.001; Figure 1A, 1B).

Many RGCs were observed in the sham group (906.40±97.02/mm^2). The number of RGCs of rats in the RIR group (323.84±51.72/mm^2) was obviously lower than that in the sham group (*P*<0.001). In the RIR+NAS group, the retinal cells in each layer were orderly arranged and the number of RGCs (568.04±79.51/mm^2) was obviously more than that in the RIR group, but still less than that in the sham group (all *P*<0.001). The number of RGCs in the RIR+FPS-ZM1 group (577.41±49.06/mm^2) was obviously more than that in the RIR group but lower than that in the sham group (all *P*<0.001). However, there was no significant difference in RGC numbers between the RIR+FPS-ZM1 and the RIR+NAS groups (*P*=0.732). The number of RGCs in the RIR+NAS+FPS-ZM1 group (635.71±87.09/mm^2) was obviously higher than that in the RIR (*P*<0.001), RIR+NAS (*P*=0.017) and RIR+FPS-ZM1 groups (*P*=0.033), but still less than that in the sham group (*P*<0.001; Figure 1A, 1C).

NAS Alleviated HMGB1 and RAGE Proteins Expression in RIR Rats HMGB1 is an amphoteric protein and generally exists in the nucleus. When tissue is damaged, HMGB1 is transferred from the nucleus to the cytoplasm and then released into the extracellular domain to activate downstream pathways through the pattern recognition receptor RAGE on the cell membrane surface and play an inflammatory role. Therefore, IHC staining and Western blot analysis were used to observe the effects of NAS on HMGB1 and RAGE proteins in the rat retina. The impact of NAS on HMGB1 levels in RIR rat serum was examined by ELISA.

IHC staining results showed that the cytoplasm of HMGB1$^+$ and RAGE$^+$ cells was stained brownish-yellow, and HMGB1$^+$ and RAGE$^+$ cells were mainly localized in the GCL and INL after RIR. HMGB1$^+$ and RAGE$^+$ cells were labeled with arrows in each figure respectively (Figure 2A). Few HMGB1$^+$ and RAGE$^+$ cells were observed in the retina of the sham group (238.50±30.71/mm^2, 258.98±37.45/mm^2). At 6h after RIR, the number of both HMGB1$^+$ and RAGE$^+$ cells in the retina of rats (489.93±78.29/mm^2, 500.20±48.50/mm^2) increased, which was substantially more than that in the sham group (all *P*<0.001). At 24h after RIR, the number of HMGB1$^+$ and RAGE$^+$ cells (796.85±63.09/mm^2, 732.17±81.32/mm^2) reached a higher level and then decreased gradually but still maintained a high level, which was higher than that in the sham group, as well as there was significant difference (all *P*<0.001). We observed HMGB1$^+$ and RAGE$^+$ cells at different time points after intraperitoneal injection of NAS. At 6h after RIR+NAS, the number of both HMGB1$^+$ and RAGE$^+$ cells (377.94±48.15/mm^2, 393.27±52.87/mm^2) increased, which were obviously less than that in the 6h after RIR (all *P*<0.001), but still higher than that in the sham group (all *P*<0.001). At 24h after RIR+NAS, the number of HMGB1$^+$ and RAGE$^+$ cells (467.79±66.37/mm^2, 415.48±57.75/mm^2) increased, but still less than that in the 24h after RIR (all *P*<0.001), but still higher than that in the sham group (all *P*<0.001; Figure 2B).

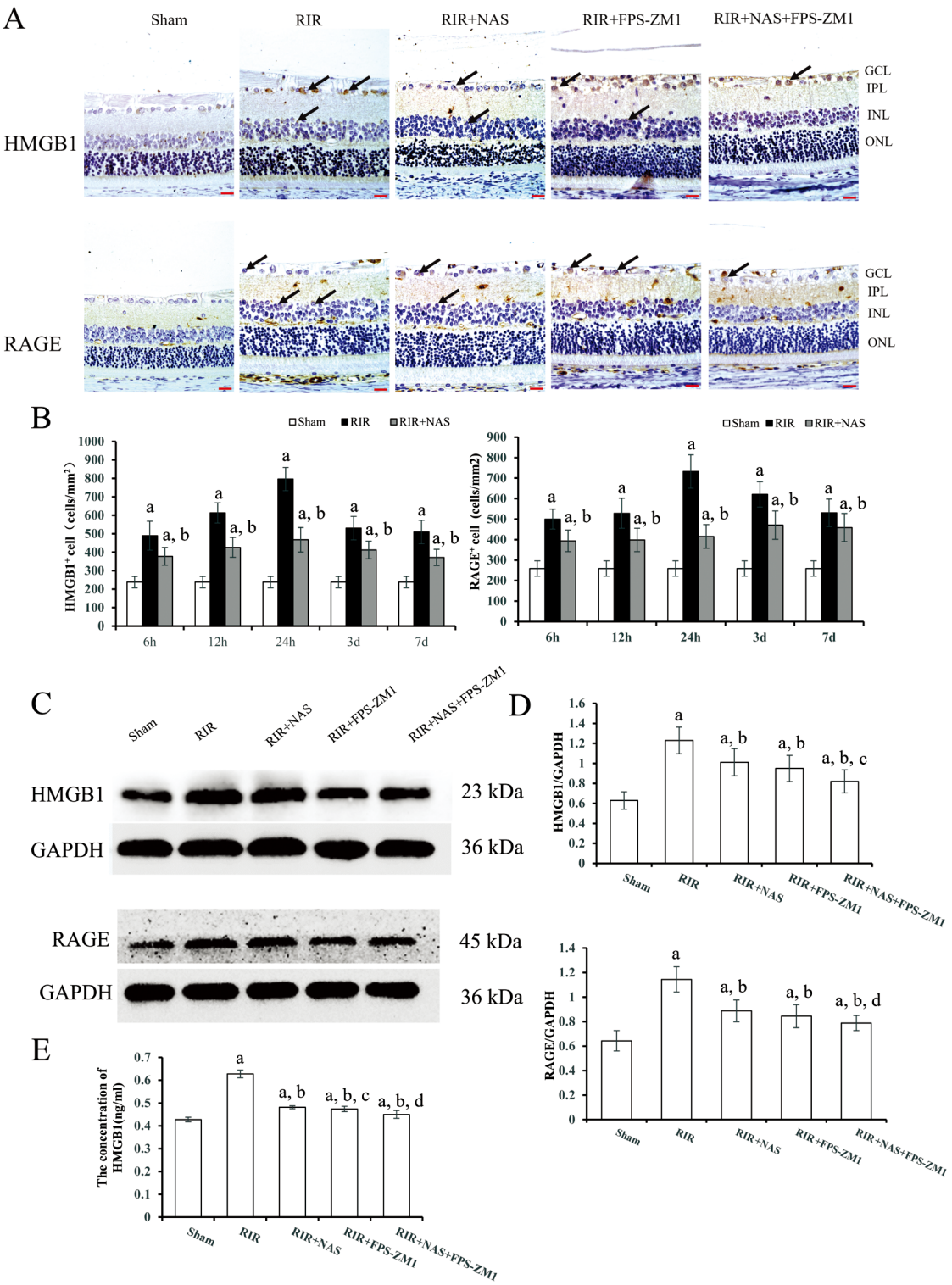

Figure 2 NAS administration alleviated HMGB1 and RAGE protein expression in the RIR rats A: The HMGB1 and RAGE expression as shown by IHC staining 24h after RIR (400×). Arrows indicate the HMGB1$^+$ and RAGE$^+$ cells in GCL and INL of the retina. Scale bar: 20 μm. B: Quantification of HMGB1$^+$ and RAGE$^+$ cells. Bars are SD, ^{a}*P*<0.001 *vs* sham; ^{b}*P*<0.01 *vs* RIR at each time point; *n*=5. C: Representative HMGB1, RAGE and GAPDH bands 24h after RIR; D: Quantification analysis of the HMGB1 and RAGE protein expression. Bars are SD, ^{a}*P*<0.001 *vs* sham; ^{b}*P*<0.001 *vs* RIR; ^{c}*P*<0.05 *vs* RIR+NAS, *n*=3. E: Quantification of the concentration of HMGB1 in each group. Bars are SD, ^{a}*P*<0.001 *vs* sham; ^{b}*P*<0.001 *vs* RIR; ^{d}*P*<0.001 *vs* RIR+NAS; *n*=3. HMGB1: High mobility group box 1; RAGE: Receptor for advanced glycation end-products. GAPDH: Glyceraldehyde-3-phosphate dehydrogenase; NAS: N-acetylserotonin; IHC: Immunohistochemical; GCL: Ganglion cell layer; IPL: Inner plexiform layer; INL: Inner nuclear layer; ONL: Outer nuclear layer; RGCs: Retinal ganglion cells; SD: Standard deviation; RIR: Retinal ischemia-reperfusion.

Western blot results showed that the expression of both HMGB1 and RAGE proteins were very low in the sham group (0.63±0.09, 0.64±0.08). At 24h after RIR, both HMGB1 and RAGE protein expression in the rat retina (1.23±0.13,

1.14±0.10) increased remarkably (all P<0.001). As compared with the RIR group, HMGB1 and RAGE protein levels decreased obviously after NAS administration (1.01±0.14, 0.89±0.09), but they were still higher than that in the sham group (all P<0.001). After administration of RAGE inhibitor FPS-ZM1, the expression of HMGB1 and RAGE proteins became lower than that in the RIR group (0.95±0.13, 0.84±0.09), but still higher than that in the sham group (all P<0.001). There was no obvious difference in the expression of HMGB1 and RAGE proteins between the RIR+FPS-ZM1 and RIR+NAS groups (all P>0.05, HMGB1: P=0.070, RAGE: P=0.082). The expression of both HMGB1 and RAGE proteins in the RIR+NAS+FPS-ZM1 group (0.82±0.12, 0.79±0.06) was obviously lower than that in the RIR (all P<0.001), RIR+NAS (all P<0.001) and RIR+FPS-ZM1 groups (all P<0.05, HMGB1: P<0.001, RAGE: P=0.025), and slightly higher than that in the sham group (all P<0.001; Figure 2C, 2D).

ELISA results showed that the concentration of serum HMGB1 in the sham group was deficient (0.43±0.01 ng/mg). At 24h after RIR, the concentration of serum HMGB1 was significantly higher than that in the sham group (0.63±0.02 ng/mg, P<0.001). Compared with the RIR group, the serum level of HMGB1 in the RIR+NAS, RIR+FPS-ZM1, and RIR+NAS+FPS-ZM1 groups (0.48±0.01 ng/mg; 0.47±0.01 ng/mg; and 0.45±0.02 ng/mg) respectively, was lower, but still higher than that in the sham group (all P<0.001). The serum HMGB1 concentration in RIR+NAS group and RIR+FPS-ZM1 group was similar (P=0.421). However, the serum HMGB1 concentration of the RIR+NAS+FPS-ZM1 group was significantly lower than that in the RIR (P<0.001), RIR+NAS (P=0.004), and RIR+FPS-ZM1 groups (P=0.021; Figure 2E).

NAS Suppressed Activation of NF-κB and NLRP3 in RIR Rats NF-κB, exists in the cytoplasm of normal tissues. When tissues are damaged, NF-κB is phosphorylated and transferred into the nucleus by upstream factors or inflammatory factors, which can activate the inflammasome NLRP3, promote the production of IL-1β, and exacerbate tissue inflammatory reaction.

IHC staining results showed that NF-κB$^+$ cells were dyed brownish-yellow in the nuclei and NLRP3$^+$ cells were stained brownish-yellow in the cytoplasm, both of the above positive cells were seen in the GCL and INL of the retina of RIR rats (Figure 3A). In the sham group, only a few p-NF-κB$^+$ and NLRP3$^+$ cells were seen (291.59±39.64/mm^2, 284.92±39.91/mm^2). There were more p-NF-κB$^+$ and NLRP3$^+$ cells in the RIR group (766.39±67.48/mm^2, 778.18±74.87/mm^2) than in the sham group (all P<0.001). After NAS administration, there were less p-NF-κB$^+$ and NLRP3$^+$ cells (421.76±50.49/mm^2, 462.04±64.46/mm^2) in the RIR+NAS group than in the RIR group, but still more positive cells than those in the sham group (all P<0.001). When the FPS-ZM1, the inhibitor of RAGE, was applied to prevent HMGB1 from binding to RAGE, there were obviously less p-NF-κB$^+$ and NLRP3$^+$ cells (403.40±48.67/mm^2, 442.11±52.10/mm^2) seen in the RIR+FPS-ZM1 group than in the RIR rats (all P<0.001), and still more than those in the sham group (all P<0.001). While there still existed no significant difference in both p-NF-κB$^+$ and NLRP3$^+$ cells between the RIR+FPS-ZM1 and RIR+NAS groups (P>0.05, p-NF-κB$^+$: P=0.385, NLRP3$^+$: P=0.349), more p-NF-κB$^+$ and NLRP3$^+$ cells were observed in the RIR+NAS +FPS-ZM1 group (348.74±62.60/mm^2, 361.02±44.45/mm^2) obviously than those in the sham group (all P<0.01, p-NF-κB$^+$: P=0.005, NLRP3$^+$: P<0.001) , and less than those in the other three groups (all P<0.01; Figure 3B).

Western blot results found that the expression of both p-NF-κB and NLRP3 protein (0.67±0.08, 0.62±0.09) was lower in the sham group. Twenty-four hours after RIR, the expression of both p-NF-κB and NLRP3 proteins (1.20±0.09, 1.19±0.11) was obviously higher than that in the sham group (all P<0.001). After NAS administration, the expression of both p-NF-κB and NLRP3 proteins (1.00±0.10, 0.96±0.14) decreased, but was still higher in the RIR+NAS group than in the sham group (all P<0.001). The p-NF-κB and NLRP3 protein expression was obviously lower in RIR+FPS-ZM1[RAGE's inhibitor FPS-ZM1-treated groups, (0.98±0.10, 0.91±0.11) than in the RIR group, but it is still higher than that in the sham group (all P<0.001), and there was no significant difference in the p-NF-κB and NLRP3 protein expression between the RIR+FPS-ZM1 and RIR+NAS groups (P>0.05, p-NF-κB: P=0.525, NLRP3: P=0.165). The levels of both p-NF-κB and NLRP3 proteins in RIR+NAS+FPS-ZM1 group (0.76±0.10, 0.79±0.10) was significantly lower than those in the RIR (all P<0.001), RIR+NAS (all P<0.001) and RIR+FPS-ZM1 (P<0.01, p-NF-κB: P<0.001, NLRP3: P=0.001) groups, but higher than those in the sham group (all P<0.01, p-NF-κB: P=0.001, NLRP3: P<0.001; Figure 3C, 3D).

NAS Attenuated IL-1β Expression after RIR IL-1β acts as an inflammatory factor, but also reverses the action of inactivated NF-κB to promote the phosphorylation of NF-κB, and aggravates tissue damage.

IHC staining results found that IL-1β positive cells were stained brown-yellow in the cytoplasm (Figure 4A), and there were only a few IL-1β$^+$ cells observed in the retinal GCL and INL in the sham group (330.58±42.53/mm^2). 24h after RIR, there were more significantly IL-1β$^+$ cells observed in the RIR group (1104.69±107.72/mm^2) than in sham group (P<0.001). There were less IL-1β$^+$ cells (599.85±64.11/mm^2) in the NAS group than in the RIR group, but was still more than those in the sham group (all P<0.001). There were less IL-1β$^+$ cells (586.30±77.33 mm^2) in the RIR+FPS-ZM1 group than

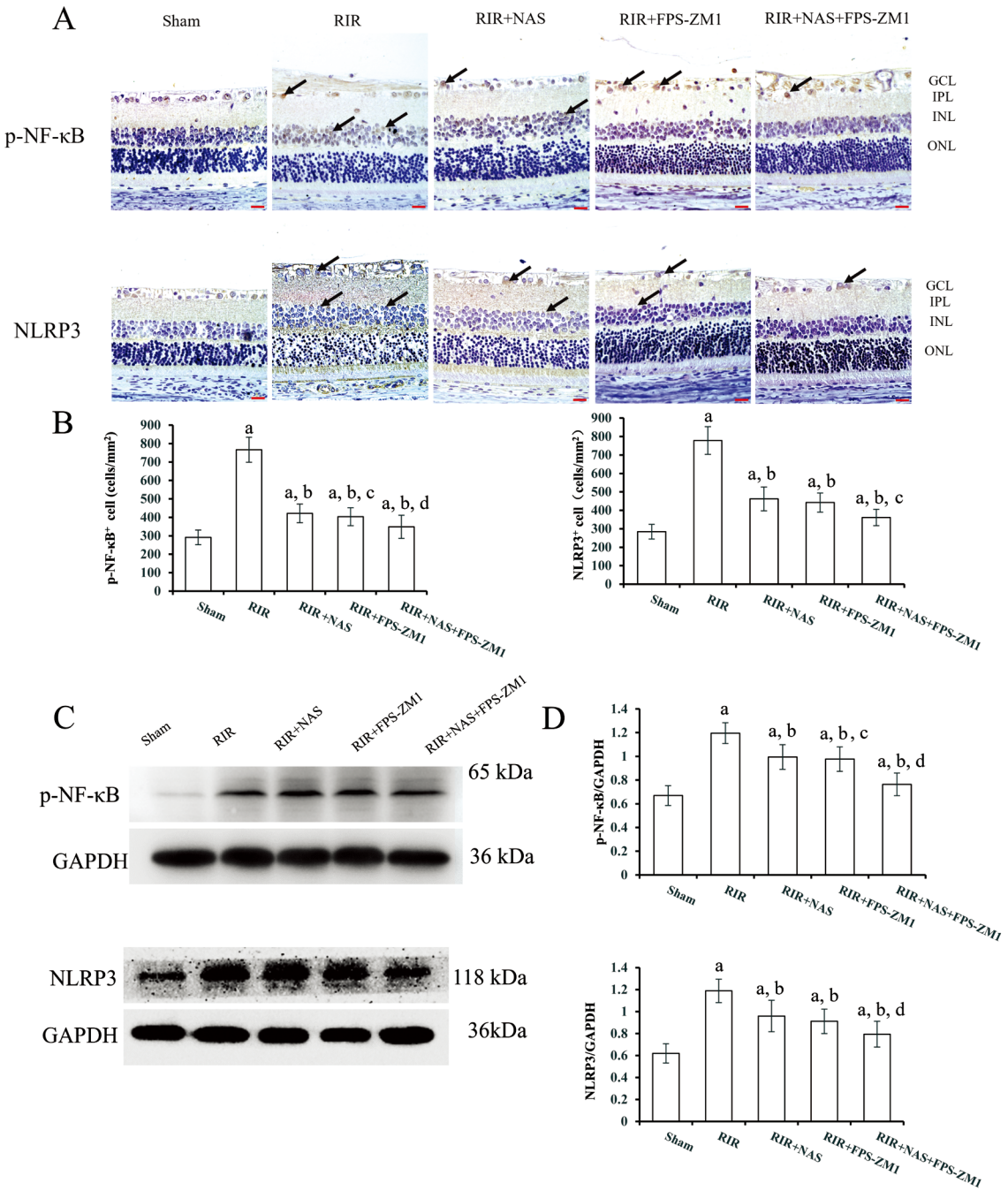

Figure 3 NAS administration inhibited the expression of p-NF-κB and RAGE proteins in RIR rats A: IHC staining of p-NF-κB and NLRP3 (400×) 24h after RIR. Both p-NF-κB$^+$ and NLRP3$^+$ cells were indicated by black arrows in the GCL and INL. Scale bar: 20 μm. B: Quantification of p-NF-κB$^+$ and NLRP3$^+$ cells. Bars are SD, ^{a}*P*<0.001 *vs* sham; ^{b}*P*<0.001 *vs* RIR; ^{c}*P*<0.05 *vs* RIR+NAS; *n*=5. C: Representative p-NF-κB, NLRP3, and GAPDH bands 24h after RIR; D: Quantification analysis of the protein expression of p-NF-κB and NLRP3. Bars are SD, ^{a}*P*<0.01 *vs* sham; ^{b}*P*<0.001 *vs* RIR; ^{d}*P*<0.001 *vs* RIR+NAS; *n*=3. RIR: Retinal ischemia-reperfusion; IHC: Immunohistochemical; p-NF-κB: Phosphorylation of nuclear factor-kappa B; NLRP3: Nod-like receptor 3; HMGB1: High mobility group box 1; RAGE: Receptor for advanced glycation end-products; GAPDH: Glyceraldehyde-3-phosphate dehydrogenase; NAS: N-acetylserotonin; GCL: Ganglion cell layer; IPL: Inner plexiform layer; INL: Inner nuclear layer; ONL: Outer nuclear layer; SD: Standard deviation.

those in the RIR group, but was still more positive cells than those in the sham group (all *P*<0.001). And no significant difference was observed in the number of IL-1β$^+$ cells between the RIR+FPS-ZM1 and RIR + NAS groups (*P*=0.647). The number of IL-1β$^+$ cells (475.68±70.26/mm^2) in the RIR+NAS+ FPS-ZM1 group was obviously lower than that in the RIR, RIR+NAS and RIR+FPS-ZM1 groups, but still higher than that in the sham group (all *P*<0.001; Figure 4B).

Western blot results showed the IL-1β protein level was very low in the sham group (0.63±0.09). At 24h after RIR, IL-1β protein level (1.14±0.10) increased in the RIR group than in the sham group (*P*<0.001), and the IL-1β protein level (1.01±0.12) in

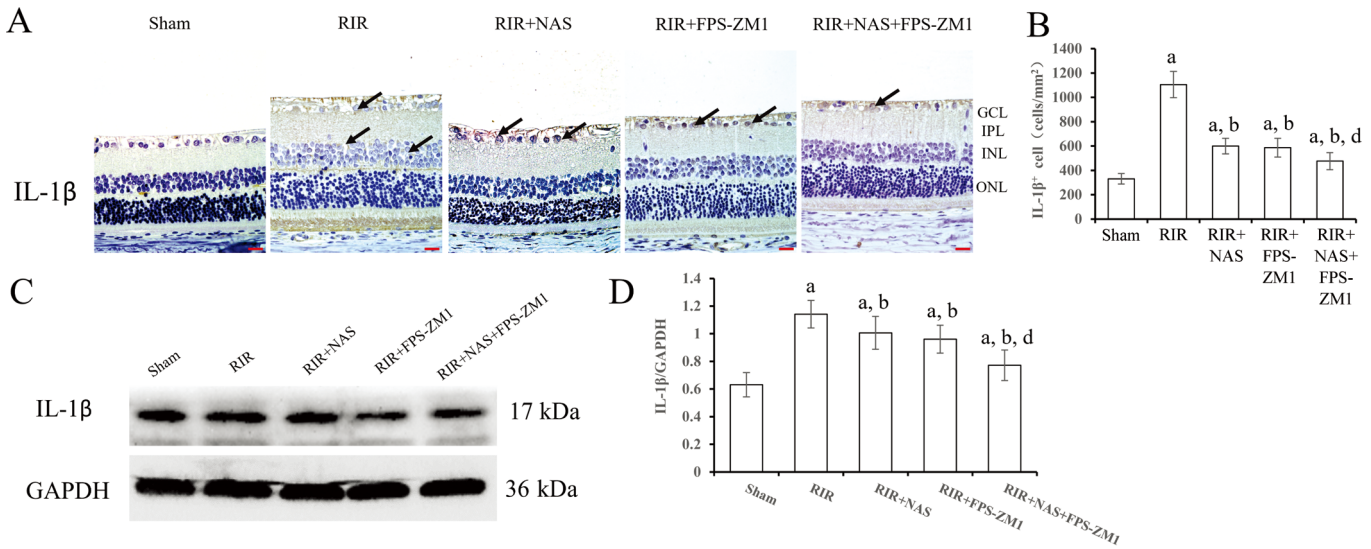

Figure 4 NAS administration attenuated IL-1β expression in RIR rats A: IHC staining of IL-1β (400×) 24h after RIR. IL-1β$^+$ cells were indicated by black arrows in the GCL and INL. Scale bar: 20 μm. B: Quantification of IL-1β$^+$ cells. Bars are SD, ^aP<0.001 *vs* sham; ^bP<0.001 *vs* RIR; ^dP<0.001 *vs* RIR+NAS; *n*=5. C: Representative IL-1β and GAPDH bands 24h after RIR. D: Quantification analysis of the protein expression of IL-1β and GAPDH. Bars are SD, ^aP<0.001 *vs* sham; ^bP<0.001 *vs* RIR; ^dP<0.001 *vs* RIR+NAS; *n*=3. IL-1β: Interleukin 1 beta; RIR: Retinal ischemia-reperfusion; IHC: Immunohistochemical; GAPDH: Glyceraldehyde-3-phosphate dehydrogenase; NAS: N-acetylserotonin; GCL: Ganglion cell layer; IPL: Inner plexiform layer; INL: Inner nuclear layer; ONL: Outer nuclear layer; SD: Standard deviation.

the NAS group decreased obviously, but still higher than in the sham group (P<0.001). And after the FPS-ZM1 management, the IL-1β protein level (0.96±0.10) in the RIR+FPS-ZM1 group was obviously lower than that in the RIR group, but still higher than that in the sham group (all P<0.001). There was no significant difference in protein levels between the RIR+FPS-ZM1 and RIR+NAS groups (P=0.110). And the IL-1β level (0.77±0.11) in the RIR+NAS+FPS-ZM1 group declined significantly as compared with that in the RIR, RIR+NAS and RIR+FPS-ZM1 groups, but it was higher in the RIR+NAS group than in the sham group (all P<0.001; Figure 4C, 4D).

DISCUSSION

The release of inflammatory mediators in large quantities during RIR can cause irreversible damage to retinal tissue[5], and the underlying mechanism of inflammation is still unclear. A previous study showed that resveratrol, a natural compound, reduces retinal cell death and apoptosis. Resveratrol exerts a protective effect by regulating the expression of ErbB2 and mousedouble minute 2 (MDM2) in RIR mice[28]. Nevertheless, the pathogenesis of ocular diseases is complex and can involve multiple signaling pathways and molecular mechanisms. Unfortunately, no effective treatment for RIR exists at present. As described in our previous reports[29-30], we also have successfully established the RIR rat model in this study, demonstrated by changes in retinal morphology and RGCs number, with disordered retinal layers and retinal edema, and a decrease of RGCs in RIR rats. Treatment with NAS was found to alleviate retinal edema and increase the number of RGCs, as well as reduce the IL-1β protein expression in the RGCs and INL of RIR rats. Endogenous melatonin is produced in various tissues, including the pineal gland and retina[31]. Arylalkylamine-N-acetyltransferase (AA-NAT) acetylates serotonin to produce NAS, and acetylserotonin-O-methyltransferase (ASMT) methylates NAS to form melatonin[32]. According to additional evidence, NAS not only acts as a melatonin precursor, but also has inhibitory effects on autophagy and apoptosis, antioxidant and anti-inflammatory characteristics, and acts as a tropomycin receptor kinase B (TrkB) agonist[33]. Some studies have pointed out that NAS has a stronger antioxidant effect than melatonin[34-35]. While NAS has a protective effect on RIR, the underlying mechanism remains unclear.

HMGB1, an amphoteric factor, is widely distributed in mammalian nuclei and abundant in full-layer retinal cells in rat retinas[36]. As a cofactor, HMGB1 regulates the transcription process. When tissue cells are stimulated by injury, HMGB1 is released extracellularly and binds to the V domain of the RAGE on the cell membrane as damage-associated molecular patterns (DAMPs), triggers a cascade reaction, stimulates cells to produce inflammatory mediators, and aggravates inflammatory response[8,37-38]. HMGB1/RAGE/NF-κB pathway is closely associated with the inflammatory response after limb ischemia-reperfusion injury and diabetic retinopathy[9-10]. Therefore, we hypothesize that the HMGB1/RAGE/NF-κB pathway may play a crucial role in RIR. IHC staining was used to detect the expression of HMGB1 and RAGE proteins dynamically in the retinas of rats in the RIR group at 6, 12, 24, and 72h and 7d after RIR. IHC staining results showed that

both the expression of HMGB1 and RAGE proteins reached a high level 24h after RIR; thus, we further explored the protective functions of NAS on the retina and its mechanism at 24h after RIR. Our data showed that after NAS administration, the HMGB1 and RAGE protein expression decreased 24h after RIR, which indicates that NAS administration inhibits the HMGB1 and RAGE expression.

The encoding gene of RAGE contains functional elements of NF-κB, which is able to activate the nuclear transfer of NF-κB and the downstream target cytokines[39]. Studies have shown that the combination of HMGB1 and RAGE promotes p-NF-κB, which regulates the expression of NLRP3, recruit its ligand and promote its activation, and promote pro-IL-1β production[10,37,40]. It can also reverse the action of RAGE to promote the formation of RAGE, forming a vicious circle[33]. p-NF-κB acts in reverse to promote the generation of RAGE, forming a vicious cycle. NLRP3 inflammasome also can stimulate the activation of the NF-κB pathway[41], and the latter can act on pro-IL-1β, and induce the production of IL-1β, thereby promoting inflammatory response and exacerbating retinal tissue damage[36]. NLRP3 inflammasome acts on damaged cells to release RAGE[11]. Our study found that NAS administration alleviates the p-NF-κB and NLRP3 expression in RIR rats, which indicating that NAS can decrease the nuclear translocation of p-NF-κB and the subsequent activation of NLRP3 in the retinal tissues of RIR rats. Furthermore, NAS reduces the IL-1β expression, alleviates retinal inflammation in RIR rats, and plays a neuroprotective role (Figure 5). However, whether NAS can reduce retinal injury in RIR rats due to the down-regulation of the HMGB1/RAGE/NF-κB pathway remains unclear. To further explore its mechanism, we added the RAGE's inhibitor, FPS-ZM1.

FPS-ZM1, a specific RAGE inhibitor, can competitively bind to the V domain of RAGE and block the interaction between HMGB1 and RAGE. FPS-ZM1 has no toxicity or side-effects on rats and can pass through the blood-brain barrier[26,42]. In this study, after intraperitoneal injection of FPS-ZM1, a RAGE antagonist, in RIR rats to prevent the binding of HMGB1 to RAGE, the p-NF-κB and NLRP3 expression in retinal tissue were reduced, which indicates that FPS-ZM1 can inhibit the p-NF-κB and NLRP3 expression. This finding is consistent with the results of previous experiments[43]. In addition, our study found that after injection of FPS-ZM1, the inflammatory factor IL-1β expression decreased, the lessened retinal and the increased RGCs in the retina in RIR rats, and the effect was consistent with that after NAS administration. Hence, the HMGB1/RAGE/NF-κB pathway plays an essential role in RIR rats, and NAS may exert a neuroprotective role through this pathway. The expression level of p-NF-κB, NLRP3 and

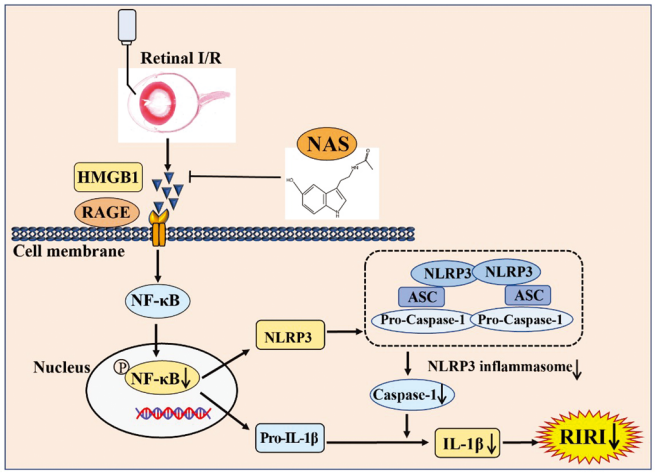

Figure 5 A summary diagram shows how NAS administration alleviates RIR by alleviating IL-1β protein expression *via* HMGB1/RAGE/NF-κB pathway NAS administration first attenuates the expression of HMGB1 and its receptor RAGE, and then suppresses the NF-κB activation, thus, inhibits the NLRP3 and IL-1β expression in the retina, attenuates retinal histopathological damage; thus, reduces RIR and has a neuroprotective effect on the injured retina in RIR rats. IL-1β: Interleukin 1 beta; NLRP3: Nod-like receptor 3; RIRI: Retinal ischemia-reperfusion injury; IHC: Immunohistochemical; p-NF-κB: Phosphorylation of nuclear factor-kappa B; NLRP3: Nod-like receptor 3; HMGB1: High mobility group box 1; RAGE: Receptor for advanced glycation end-products; NAS: N-acetylserotonin; ASC: Apoptosis-associated speck-like protein containing a CARD.

inflammatory factor IL-1β in the GCL and INL of the retina decreased obviously after administration with NAS and RAGE inhibitors. And the decreased retinal edema and increased RGCs also indicates that NAS and FPS-ZM1 administration had the same therapeutic effects. Hence, NAS administration may regulate the HMGB1/RAGE/NF-κB pathway, and thus reduce the IL-1β expression level. It also reduces retinal inflammation and damage in RIR rats and play a neuroprotective role. However, further studies are still needed to validate how NAS regulates NF-κB, and thus modulates inflammatory mechanisms in RIR rats. This study has limitations that need to be addressed. For instance, the half-life of NAS is very short *in vivo*, and more stable NAS derivatives should be developed, which may have better therapeutic effects on retinal diseases[44]. What's more, there still existed other mechanisms, by which NAS modulates inflammation after RIR, and the latter needs to be explored in further research. The present study investigates the mechanism of NAS in RIR disease, which will be useful for us to select more effective NAS-derived drugs and explore the mechanism in future.

In conclusion, our findings in this study indicate that NAS administration can alleviate RIR and increase RGCs survival *via* the HMGB/RAGE/NF-κB pathway, which proposes another alternative for RGCs neuroprotection.

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