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Ameliorative Effects of the Neng-Jing-Huo Essential Oil Blend on H_2O_2-induced Oxidative Stress Injury in NRK-52E Cells via the AMPK/Nrf2/HO-1 Signaling Pathway

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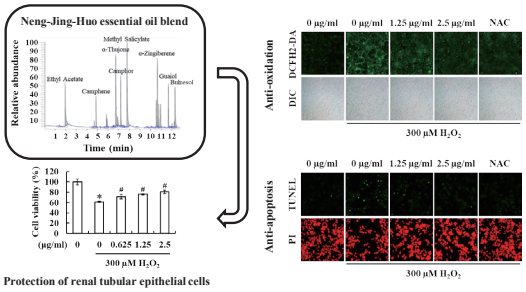
Abstract: Oxidative stress-induced renal tubular epithelial cell apoptosis or dysfunction promotes the progression of acute kidney injury and chronic kidney disease. Neng-Jing-Huo (NJH), an essential oil mixture of *Gaultheria procumbens*, *Zingiber officinale*, *Bulnesia sarmientoi*, *Artemisia vulgaris*, and *Styrax benzoin*, exhibits antioxidant potential. However, its specific action mechanisms remain unclear. Therefore, in this study, we aimed to investigate the protective effects of NJH against hydrogen peroxide (H_2O_2)-induced oxidative stress injury using NRK-52E renal tubular epithelial cells and assess the underlying mechanisms. Cell viability, reactive oxygen species level, mitochondrial membrane potential, apoptosis, and aging were evaluated via MTT assay, DCFH2-DA, JC-1, TUNEL, and red beta-D-galactopyranoside staining, respectively. Additionally, mRNA and protein expression levels were analyzed via semi-quantitative reverse transcription-PCR and western blotting, respectively. Notably, NJH alleviated H_2O_2-induced DNA damage and mitochondrial dysfunction and inhibited reactive oxygen species accumulation by activating the AMPK/Nrf2/HO-1 signaling pathway. Furthermore, NJH inhibited oxidative stress-induced apoptosis by upregulating the Bcl-2 levels and downregulating the Bax levels, thereby preventing the activation of caspase-9 and caspase-3. Senescence-associated β-galactosidase activity was significantly increased by H_2O_2; however, this effect was dose-dependently attenuated by NJH. The findings highlight NJH as a potential candidate for the prevention and treatment of oxidative stress injury-associated kidney disorders.



Key words: renal tubular epithelial cells, Neng-Jing-Huo essential oil blend, oxidative stress injury, apoptosis, renal aging

1 Introduction

Kidneys are the primary sites of metabolism in the human body that are responsible for maintaining the body fluid, electrolyte, osmotic pressure, and pH homeostases[1]. Acute kidney injury (AKI) is a clinical syndrome of acute kidney impairment characterized by the sudden cessation of normal kidney function, ranging from mild loss of renal function to complete renal failure, resulting in increased serum creatinine levels and diminished urine output[1]. AKI affects approximately 10–15% of hospitalized patients, accounting for up to 60% of deaths in intensive care units[2]. AKI increases the patient mortality and risk of chronic kidney disease (CKD) and end-stage renal disease[3]. AKI pathogenesis is multifactorial, including septic, toxic, and obstructive insults, ischemia–reperfusion injury, hypovolemia, rhabdomyolysis, and acute glomerulonephritis[1]. Recent histological and pathological studies have shown that AKI pathogenesis is primarily associated with oxidative stress, apoptosis, inflammation, and vasoconstriction[4]. Oxidative stress is a key factor involved in renal fibrosis,

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ultimately causing end-stage renal disease[5, 6]. Therefore, novel drugs blocking the associated signaling pathways can aid in AKI prevention and treatment.

Organisms have evolved an antioxidant system to combat the reactive oxygen species (ROS), including superoxide radicals ($O_2^{\cdot -}$), hydroxyl radicals ($OH^{\cdot}$), singlet oxygen ($^1O_2$), and hydrogen peroxide (H_2O_2), generated by endogenous and exogenous sources[7]. ROS are effectively eliminated by superoxide dismutase, glutathione peroxidase, and catalase under physiological conditions, thereby maintaining the intracellular redox equilibrium[7, 8]. However, when ROS production by cells is greater than their removal by the antioxidant system, oxidative stress is induced, which affects the structure and function of active molecules in the cell and causes DNA damage, lipid and protein oxidation, and mitochondrial dysfunction, ultimately resulting in cellular senescence and apoptosis[8]. Kidneys, which possess several mitochondria and receive approximately 25% of the total blood supply, are very susceptible to oxidative stress[9]. Inflammation, obesity, chronic hyperglycemia, and uremic toxins enhance the oxidative stress in renal tissues[10]. Increased oxidative stress causes energy metabolism disorders and morphological changes in renal tubular epithelial cells, ultimately leading to renal cell injury and aggravating AKI and CKD progression[6]. Therefore, controlling oxidative stress is a key strategy to treat and prevent AKI and CKD.

Natural products are used to treat and prevent various diseases and are sources of many beneficial compounds against oxidative stress, cell death, and inflammation[11]. Some compounds exerting antioxidant and anti-inflammatory effects, such as flavonoids, polyphenols, terpenes, alkaloids, saponins, and quinones, have been used to treat and prevent AKI in vivo and in vitro[12]. Neng-Jing-Huo (NJH) is an essential oil mixture of *Gaultheria procumbens*, *Zingiber officinale*, *Bulnesia sarmientoi*, *Artemisia vulgaris*, and *Styrax benzoin*. Essential oils and derivatives of these plants exhibit antioxidant and anti-inflammatory activities[13–17]. In this study, we used RK-52E renal tubular epithelial cells and H_2O_2 to establish an oxidative stress model in vitro[18] and explored the protective effects and action mechanisms of NJH against oxidative stress injury.

2 Materials and Methods

2.1 Reagents and NJH preparation

H_2O_2, *N*-acetyl-L-cysteine (NAC), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), dimethyl sulfoxide (DMSO), 2′,7′-dichlorofluorescein diacetate (DCFH2-DA), and propidium iodide (PI) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Xite red beta-D-galactopyranoside (red-β-D-Gal) and 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolylcarbocyanine iodide (JC-1) were purchased from (AAT Bioquest, CA, USA). TdT-mediated dUTP nick-end labeling (TUNEL) assay kit was obtained from Roche (Basel, Switzerland). NJH essential oil blend was prepared by mixing the *G. procumbens* (China), *Z. officinale* (China), *B. sarmientoi* (Paraguay), *A. vulgaris* (Nepal), and *S. benzoin* (Sumatra) oils purchased from Faressence Trade Co., Ltd. (Taichung, Taiwan) in a 9:9:7:7:4 proportion. The primary components of NJH identified by the Research and Development Office of the National Yang-Ming Chiao Tung University (Hsinchu, ROC) using a high-resolution gas chromatograph mass spectrometer (AccuTOF GCX; JEOL, USA) were methyl salicylate (20.30%), α-thujone (14.46%), bulnesol (9.98%), α-zingiberene (9.80%), camphor (8.03%), guaiol (6.63%), and others, shown in mole percent. NJH was prepared as a 10 mg/mL stock solution in DMSO and subsequently diluted with the complete culture medium to achieve a final DMSO concentration $<1\%$ for the in vitro experiments.

2.2 Cell culture and treatment

NRK-52E rat renal proximal tubular epithelial cells obtained from the Bioresource Collection and Research Center (Hsinchu, Taiwan) were cultured in the Dulbecco's modified Eagle's medium (Gibco BRL, NY, USA) supplemented with 5% bovine calf serum (Gibco BRL) and penicillin/streptomycin (Gibco BRL) in a humidified atmosphere with 5% CO_2 at 37℃.

2.3 Cell viability assay

The cells were seeded in a 96-well plate at a density of 2.5×10^4 cells/well with 100 μL medium for 16 h and subsequently treated with NJH and H_2O_2. After incubation for the indicated period, 10 μL of MTT solution (5 mg/mL) in the medium was added to each well and incubated at 37℃ for 4 h. Formazan crystals were dissolved in 50 μL DMSO, and absorbance at 550 nm was measured using a microplate reader (SpectraMax Plus 384; Molecular Devices, USA). Then, cell viability was calculated as a percentage of optical density relative to the control (100%). The experiments were performed in triplicate and repeated at least three times.

2.4 Intracellular ROS detection

After overnight culture on a 96-well plate or 15 mm coverslip (Assistant, Germany), the cells were pretreated with 0–2.5 μg/mL NJH for 3 h and then treated with 300 μM H_2O_2 for 3 h. After washing with phosphate-buffered saline, the cells were incubated with 20 μM DCFH2-DA solution for 30 min in the dark. Fluorescence was detected using the CLARIOstar microplate reader (BMG Labtech, Ortenberg, Germany) at 485 nm excitation and 535 nm emission wavelengths and observed under a fluorescence micro-

scope (ZEISS AXioskop2; Carl Zeiss). ROS levels were quantified as a percentage relative to the control (100%). The experiments were performed in triplicate and repeated at least three times.

2.5 JC-1 and red-β-D-Gal staining

Mitochondrial membrane potential (MMP) and senescence-associated β-galactosidase (SA-β-Gal) activity were analyzed using the JC-1 and red-β-D-Gal fluorescent dyes, respectively. NRK-52E cells were cultured on a 15 mm coverslip (Assistant, Germany) in a 12-well plate overnight. After reaching 100% confluency, the cells were pretreated with NJH (1.25 and 2.5 μg/mL) or NAC (10 mM) for 3 h and subsequently treated with 300 μM H_2O_2 for 3 h. Then, the supernatant was replaced with the complete culture medium and incubated for 0 and 24 h to establish a cell model to analyze the protective effects of NJH against oxidative damage. To measure MMP and SA-β-Gal activity, the supernatant was substituted with a medium containing JC-1 (10 μM) and red-β-D-Gal (5 μM), respectively, and incubated at 37℃ for 30 min in the dark. Fluorescent images were acquired using a fluorescence microscope (ZEISS AXioskop2; Carl Zeiss) and analyzed using the ImageJ 1.47t software (National Institutes of Health). The experiments were repeated three times.

2.6 Semi-quantitative reverse transcription-polymerase chain reaction (RT-PCR)

Semi-quantitative RT-PCR was used to measure the mRNA expression. Total RNA was extracted from the treated cells using TRIzol (Genepure, Taichung, Taiwan), according to the manufacturer's instructions. After reverse-transcription into cDNA using the HiSpec Reverse Transcriptase kit (Yeastern Biotech Co., Ltd., Taipei, Taiwan), the target genes were amplified using specific primers (Table 1) and 5×Taq PCR Master Mix (Biomate, Taipei, Taiwan). Then, PCR products were subjected to electrophoresis on a 2% agarose gel with ethidium bromide, and signal density was detected using a UV transilluminator (Alpha Imager HP, CA, USA). The results were quantified and normalized to β-actin levels using the ImageJ 1.47t software (National Institutes of Health). The experiments were performed three times.

2.7 Western blotting assay

Radioimmunoprecipitation assay buffer (Bio Basic Inc., Canada) containing a protease inhibitor cocktail (Amresco Inc., USA) and phosphatase inhibitor cocktail (Bionovas, Toronto, Canada) was used to extract the total proteins. Equal amounts of protein (20 μg/mL) were separated on 8–12.5% sodium dodecyl sulfate-polyacrylamide gels and transferred to 0.22-μm polyvinylidene difluoride membranes (FluoroTrans; PALL, Dreieich, Germany). Following blockade of the non-specific binding sites using 5% skim milk for 1 h, the membranes were incubated with primary antibodies against AMP-activated protein kinase (AMPK), caspase-9, and caspase-3 (1/250 dilution; Santa Cruz Biotechnology, TX, USA), *p*-AMPK, nuclear factor erythroid 2-related factor 2 (Nrf2), and *p*-Nrf2 (1/1000 dilution; Novus Biologicals, CO, USA), Bcl-2-associated X (Bax), Bcl-2, and β-actin (1/1000 dilution; iReal Biotechnology Co., Ltd., Hsinchu, Taiwan), and heme oxygenase (HO)-1 (1/1000 dilution; GeneTex, CA, USA) overnight at 4℃. After washing with 0.5% Tween-20 in Tris-buffered saline, the membranes were incubated with biotin-conjugated secondary antibodies (1/1500 dilution; Santa Cruz, CA, USA) for 2 h, followed by incubation with peroxidase-conjugated streptavidin (Jackson ImmunoResearch Inc., USA) for 1 h at 25℃. Protein signals were detected using an enhanced chemiluminescence reagent (T-Pro Biotechnology, Taipei, Taiwan) with the chemiluminescence imaging analyzer (GE LAS-4000; GE Healthcare Life Sciences, NJ, USA), quantified using the ImageJ 1.47t software (National Institutes of Health), and normalized by comparing with β-actin levels. The experiments were performed three times.

2.8 Immunofluorescence staining

Treated cells were fixed with 10% neutral-buffered formalin, permeabilized with 1% NP-40 in phosphate-buffered

Table 1 Primer sequences for Semi-quantitative RT-PCR.

Gene	Sequences
AMPK	Forward: 5′-CGGCAAAGTGAAGGTTGGCAAA-3′
	Reverse: 5′ -CAAATAGCTCTCCTCCTGAGAC-3′
Nrf2	Forward: 5′-TCTGACTCCGGCATTTCACT-3′
	Reverse: 5′ -GGCACTGTCTAGCTCTTCCA-3′
HO-1	Forward: 5′-CACGCATATACCCGCTACCT-3′
	Reverse: 5′ -CCAGAGTGTTCATTCGAGA-3′
β-actin	Forward: 5′-AAGTCCCTCACCCTCCCAAAAG-3′
	Reverse: 5′ -AAGCAATGCTGTCACCTTCCC-3′

saline, blocked with 5% bovine serum albumin (Sigma-Aldrich), and incubated with antibodies against HO-1 (1/200 dilution; GeneTex) and 8-hydroxy-2′-deoxyguanosine (8-OHdG; 1/200 dilution; Bioss, MA, USA) overnight at 4℃. After 2-h exposure to biotin-conjugated secondary antibodies (Santa Cruz, CA, USA), the cells were incubated with Alexa Fluor 488 streptavidin (Jackson ImmunoResearch Inc.) for 1 h at 25℃. Nuclei were stained with PI. Finally, images were acquired using a fluorescence microscope (ZEISS AXioskop2; Carl Zeiss) and quantified using ImageJ 1.47t software (National Institutes of Health). The experiments were repeated three times.

2.9 TUNEL assay

After fixing with 10% neutral-buffered formalin, the samples were exposed to 3% H_2O_2 in methanol and permeabilized with 0.1% Triton X-100 in 0.1% sodium citrate on ice. Subsequently, the cells were incubated with the TUNEL solution for 1 h at 37℃, according to manufacturer's instructions, counterstained with PI, and observed using a fluorescence microscope (ZEISS AXioskop2; Carl Zeiss). The assays were repeated three times.

2.10 Statistical analyses

Data are represented as the mean ± standard deviation (SD). Unpaired Student's *t*-test and one-way analysis of variance were used to analyze the differences between two and among multiple groups using Excel 2016 and SPSS v16.0, respectively. Statistical significance was set at $p < 0.05$. All experiments were repeated at least three times in duplicate or triplicate.

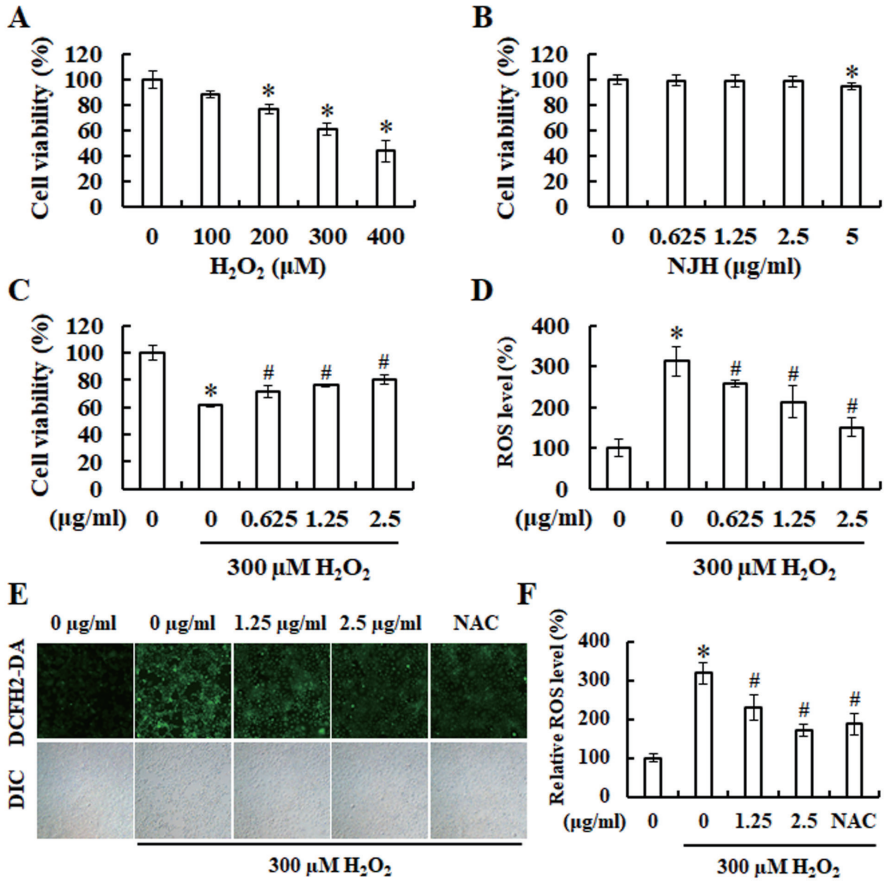

Fig. 1 Effects of Neng-Jing-Huo (NJH) and hydrogen peroxide (H_2O_2) on cell viability and ROS levels in NRK-52E cells. (A and B) After treatment with H_2O_2 (100, 200, 300, and 400 μM) for 3 h and NJH (0.625, 1.25, 2.5, and 5 μg/mL) for 24 h, cell viability was analyzed via MTT assay. (C and D) Cell viability and intracellular ROS levels in NRK-52E cells pretreated with NJH for 3 h and subsequently treated with 300 μM H_2O_2 for 3 h were assessed via MTT and DCFH2-DA assays, respectively. (E and F) Green fluorescence was analyzed under a fluorescence microscope and quantified as a percentage relative to the control. Data are represented as the mean ± standard deviation (SD) of three independent experiments. *$p < 0.05$ vs. control; #$p < 0.05$ vs. H_2O_2 alone. NAC, *N*-acetyl-L-cysteine; DIC, differential interference contrast.

3 Results

3.1 NJH enhances cell viability and decreases ROS levels in H_2O_2-treated NRK-52E cells

H_2O_2 is a common oxidant used to induce oxidative stress[18]. In this study, H_2O_2-treated NRK-52E cells were used to investigate the effects of NJH on oxidative stress-induced injury in renal tubular epithelial cells. Optimal dose of H_2O_2 was determined to establish an oxidative stress model. NRK-52E cells were exposed to increasing H_2O_2 concentrations (100, 200, 300, and 400 μM) for 3 h, and cell viability was analyzed via MTT assay. As shown in Fig. 1A, cell viability decreased with increasing H_2O_2 concentration and was 61.34% at 300 μM H_2O_2, indicating significant oxidative stress. However, no significant change was observed in the viability of cells treated with NJH (< 2.5 μg/mL) for 24 h compared to that of the control (Fig. 1B). Therefore, the tested concentrations of H_2O_2 (300 μM) and NJH (0.625, 1.25, and 2.5 μg/mL) were selected for subsequent experiments. After pretreatment with NJH (0.625, 1.25, and 2.5 μg/mL) for 3 h and treatment with H_2O_2 (300 μM) for 3 h, cell survival and intracellular ROS levels were analyzed. NJH pretreatment dose-dependently enhanced the cell survival, decreased the ROS levels, and reduced the number of DCFH2-DA-positive H_2O_2-treated cells (green fluorescence; Figs. 1C–1F). Overall, NJH enhanced cell survival and alleviated oxidative stress in H_2O_2-treated NRK-52E cells in a dose-dependent manner.

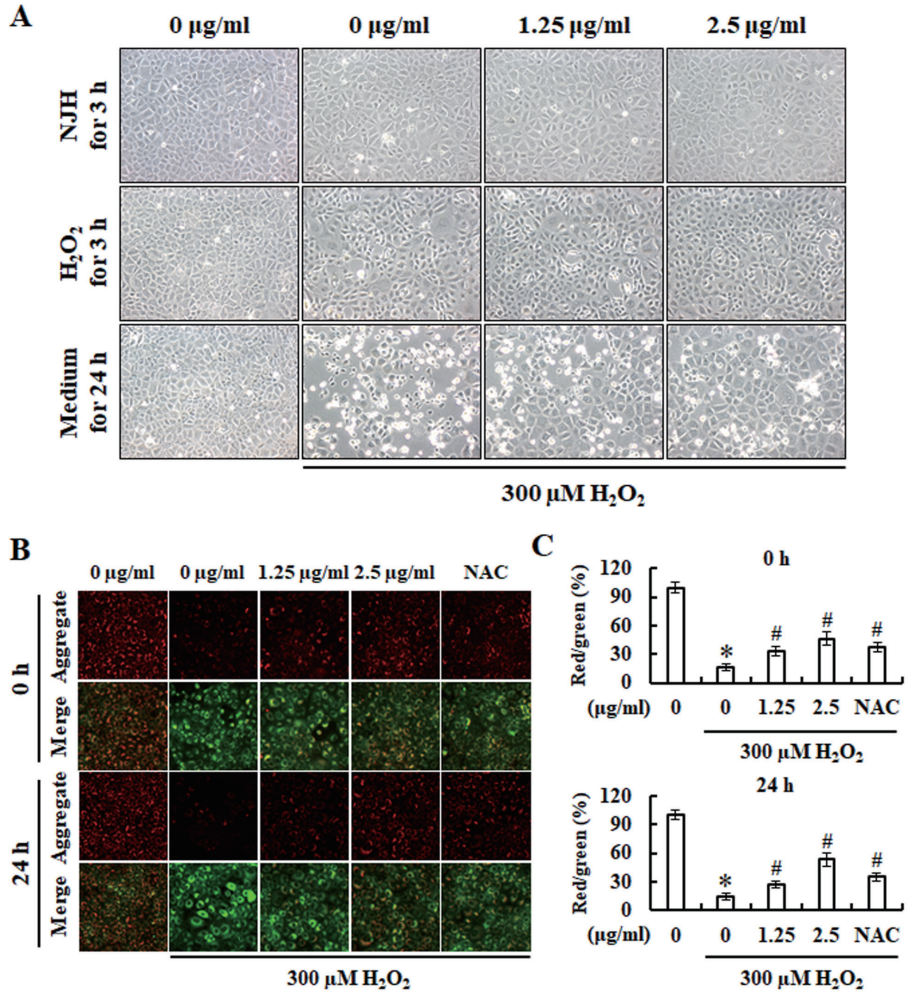

Fig. 2 Effects of NJH and H_2O_2 on cell morphology and mitochondrial membrane potential (MMP) in NRK-52E cells. After reaching 100% confluency, the cells were pretreated with 1.25 and 2.5 μg/mL NJH for 3 h and exposed to 300 μM H_2O_2 for 3 h. Then, the supernatant was substituted with the complete culture medium and incubated for 0 and 24 h. Cell morphology (A) and red (aggregate)/green (monomer) fluorescence of JC-1 (B and C) were observed and photographed. Data are represented as the mean ± SD. *$p < 0.05$ vs. control; #$p < 0.05$ vs. H_2O_2 alone. NAC, *N*-acetyl-L-cysteine.

3.2 NJH maintains MMP and protects NRK-52E cells against oxidative damage

After reaching 100% confluency, the cells were pretreated with NJH (1.25 and 2.5 μg/mL) or NAC (10 mM) for 3 h, followed by exposure to 300 μM H_2O_2 for 3 h. The supernatant was replaced with the complete culture medium and incubated for 0 and 24 h to establish an oxidative stress injury model. After 3-h H_2O_2 treatment, NRK-52E cell morphology changed from a typical epithelial cuboidal shape to an elongated shape, along with the decrease in cell–cell interactions (Fig. 2A). Interestingly, upon 24-h culture after H_2O_2 removal, the cells were still injured by oxidative stress, resulting in cell shrinkage, detachment, and death. However, NJH pretreatment improved the cell morphology in a dose-dependent manner. Mitochondrial respiratory chain is a major ROS source critical for various cellular processes, such as energy production, metabolism, redox regulation, and programmed cell death[7]. To determine the impact of NJH on the oxidative damage model in vitro, its effect on MMP were measured using the JC-1 dye. As shown in Figs. 2B and 2C, the ratio of aggregates (red fluorescence) to monomers (green fluorescence) increased with NJH and NAC pretreatment in a dose-dependent manner compared to that with H_2O_2 alone, indicating that NJH ameliorated H_2O_2-induced oxidative damage via the mitochondrial pathway.

3.3 NJH alleviates oxidative damage by upregulating AMPK/Nrf2/HO-1 signaling

AMPK/Nrf2/HO-1 signaling pathway is closely associated with scavenging free radicals, mitigating mitochondrial damage and mediating apoptosis and other biological activities; it is indispensable for oxidative stress regulation[19]. Therefore, we explored the effects of NJH on the AMPK/Nrf2/HO-1 pathway in H_2O_2-treated NRK-52E cells via semiquantitative RT-PCR and western blotting. Notably, mRNA and phosphorylated protein levels of AMPK and Nrf2 were decreased in H_2O_2-treated cells; however, this effect was inhibited by NJH pretreatment (Fig. 3). Similar changes were observed in HO-1 levels (Fig. 3), which were further confirmed via immunofluorescence staining (Figs. 4A and 4B). NJH pretreatment also decreased the levels of 8-OHdG, an oxidative DNA damage marker, in H_2O_2-treated cells (Figs. 4C and 4D). Overall, NJH protected NRK-52E cells against H_2O_2-induced oxidative damage via the AMPK/Nrf2/HO-1 signaling pathway.

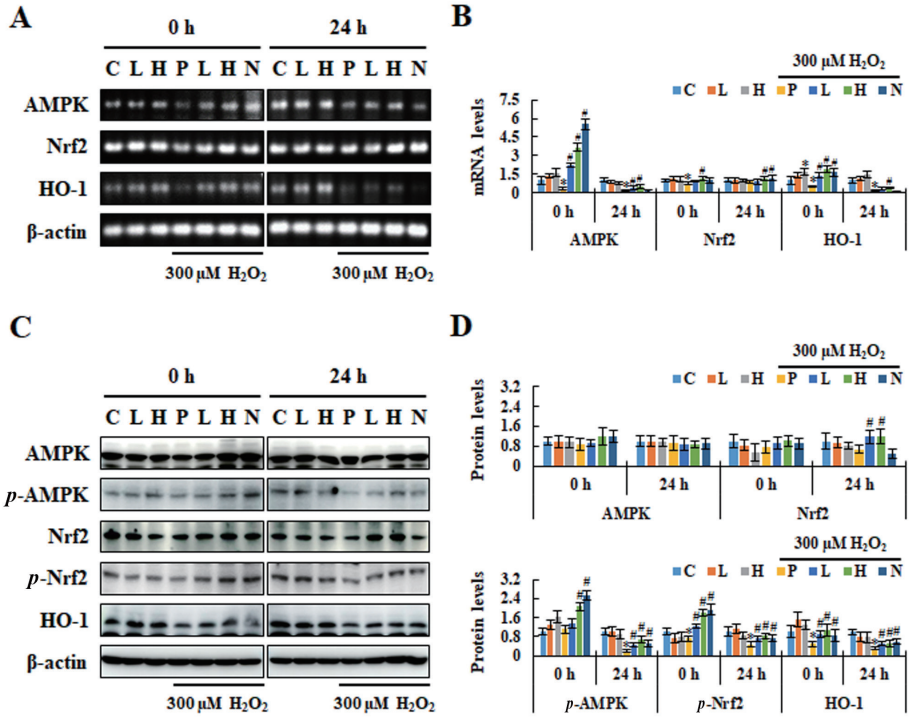

Fig. 3 Effect of NJH on AMPK/Nrf2/HO-1 signaling in H_2O_2-treated NRK-52E cells. (A and B) Semi-quantitative reverse transcription-polymerase chain reaction (RT-PCR) was used to determine the mRNA levels of *AMPK*, *Nrf2*, and *HO-1* in NRK-52E cells treated with NJH and H_2O_2. (C and D) Western blotting analysis of AMPK, *p*-AMPK, Nrf2, *p*-Nrf2, and HO-1 protein levels in NJH-pretreated cells after H_2O_2 treatment. Quantitative values are expressed relative to β-actin levels. Data are represented as the mean ± SD. *$p<0.05$ vs. control; $^{\#}p<0.05$ vs. H_2O_2 alone. C, control; L, low-dose NJH (1.25 μg/mL); H, high-dose NJH (2.5 μg/mL); P, H_2O_2 only; N, *N*-acetyl-L-cysteine.

3.4 NJH inhibits oxidative stress-induced NRK-52E cell apoptosis

Increased ROS levels is strongly associated with cell apoptosis under oxidative stress[8]. Therefore, we investigated the effect of NJH on H_2O_2-induced NRK-52E cell apoptosis. TUNEL staining showed that, compared to those in the control (5.02 ± 1.38%), H_2O_2 treatment significantly enhanced the apoptosis rate (32.41 ± 7.31%), whereas NJH pretreatment (1.25 and 2.5 μg/mL) dose-dependently decreased the apoptosis rate (17.97 ± 2.45 and 9.19 ± 1.27%) in NRK-52E cells (Figs. 5A and 5B). Proteins associated with the apoptotic pathway were assessed via western blotting. Compared to those in the control, Bax, cleaved caspase-9, and caspase-3 levels were markedly increased, whereas Bcl-2 levels were decreased in NRK-52E cells exposed to 300 μM H_2O_2 for 3 h (Figs. 5C and 5D). However, NJH pretreatment reduced the Bax/Bcl-2 ratio and cleaved caspase-9 and caspase-3 levels in H_2O_2-treated cells. These results indicate that NJH prevented H_2O_2-induced NRK-52E cell death by inhibiting oxidative stress-induced apoptosis.

3.5 NJH inhibits SA-β-Gal activity in H_2O_2-treated NRK-52E cells

Oxidative stress, characterized by ROS accumulation, is a well-known inducer of renal cell senescence[8]. To investigate the effect of NJH pretreatment on oxidative stress-induced senescence in vitro, activity of SA-β-Gal, a widely used senescence marker[20], was analyzed via red-β-D-Gal staining. Notably, number of SA-β-Gal-positive cells was not significantly changed after H_2O_2 treatment for 3 h and culture medium incubation for 0 h (Figs. 6A and 6B). The number of SA-β-Gal-positive cells gradually increased 24-h post-incubation; however, NJH significantly inhibited this

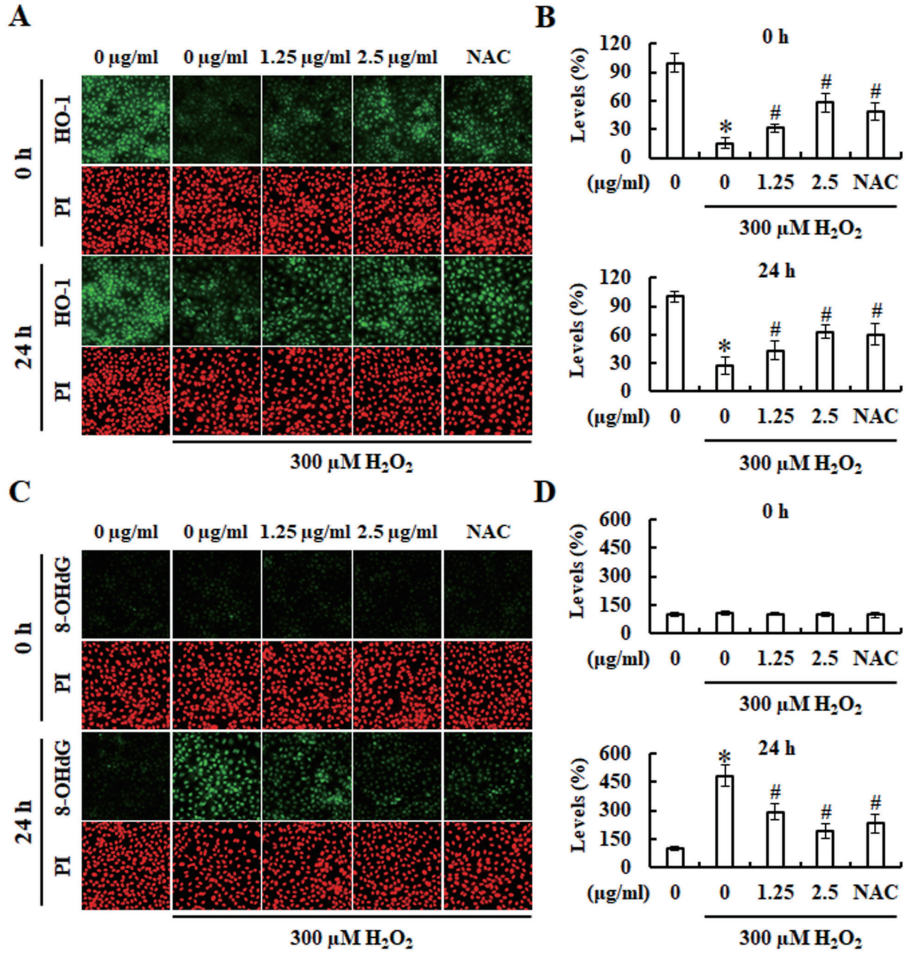

Fig. 4 Effects of NJH on HO-1 and 8-OHdG expression levels in H_2O_2-treated NRK-52E cells. NRK-52E cells were pretreated with 1.25 and 2.5 μg/mL NJH for 3 h and exposed to 300 μM H_2O_2 for 3 h, followed by incubation in a medium lacking NJH and H_2O_2 for 0 and 24 h. Protein expression levels of HO-1 (A and B) and 8-OHdG (C and D) were determined via immunofluorescence staining and quantified using the ImageJ 1.47 t software. Data are represented as the mean ± SD. *$p < 0.05$ vs. control; #$p < 0.05$ vs. H_2O_2 alone.

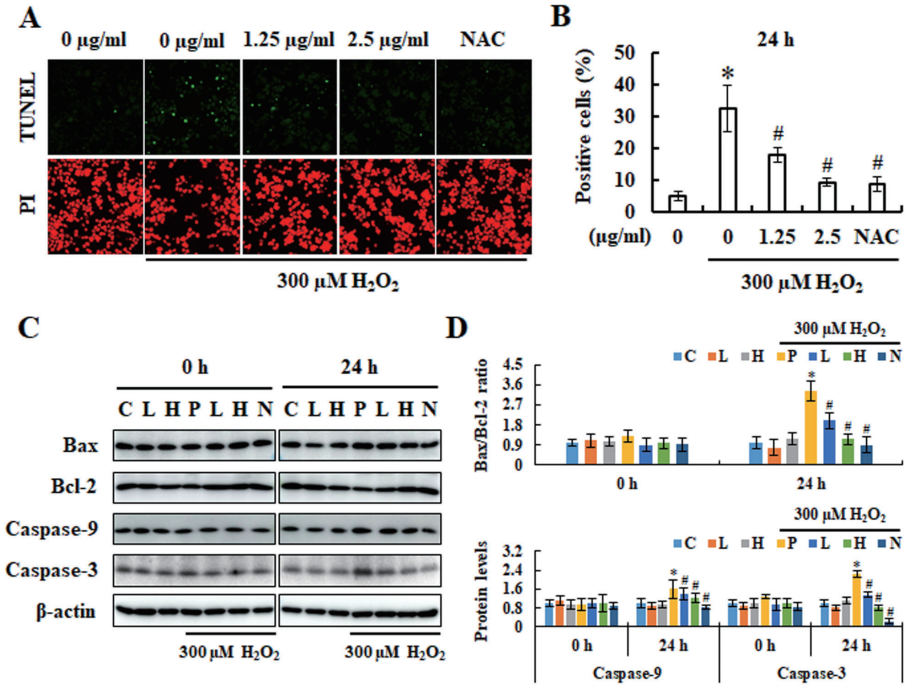

Fig. 5 Effect of NJH on oxidative stress-induced apoptosis of NRK-52E cells. (A) NRK-52E cell apoptosis was assessed via TUNEL assay and observed via fluorescence microscopy. (B) Quantitative evaluation of TUNEL-positive cells. (C) Expression levels of Bax, Bcl-2, caspase-9, caspase-3, and β-actin in cells were determined via western blotting. (D) Bax/Bcl-2 ratio and each band density were quantified and analyzed. Data are represented as the mean ± SD. *$p < 0.05$ vs. control; #$p < 0.05$ vs. H_2O_2 alone. C, control; L, low-dose NJH (1.25 μg/mL); H, high-dose NJH (2.5 μg/mL); P, H_2O_2 only; N, *N*-acetyl-L-cysteine.

phenomenon in a dose-dependent manner. These results further confirmed that NJH inhibits oxidative stress-induced senescence of renal tubular epithelial cells.

4 Discussion

Oxidative stress is a common characteristic of AKI and CKD. ROS production is significantly elevated in damaged kidneys and patients with renal failure[5, 6]. In this study, NRK-52E renal proximal tubular epithelial cells were exposed to H_2O_2 in vitro to mimic the oxidative stress in kidneys. NJH pretreatment enhanced the cell viability, decreased the intracellular ROS levels, and inhibited ROS-induced mitochondrial damage and MMP depolarization. NJH decreased ROS levels in the in vitro H_2O_2 model and protected NRK-52E cells from oxidative stress-induced cellular injury. AMPK is a serine/threonine protein kinase that acts as a sensor of energy status, preserving the cellular energy balance[21]. AMPK activation upregulates the signaling of Nrf2[22], a key transcription factor for antioxidant protein and detoxifying enzyme expression[23]. Under normal physiological conditions, Nrf2 resides in the cytoplasm and forms a heterocomplex with Kelch-like ECH-associated protein 1[23]. However, upon detection of excess ROS in the body, phosphorylation of a series of protein kinases leads to the dissociation of Nrf2 from Kelch-like ECH-associated protein 1. Phosphorylated Nrf2 translocates to the nucleus and binds to antioxidant response elements, thereby regulating the transcription and expression of antioxidant enzyme genes, such as *HO-1*, glutathione, quinone oxidoreductase-1, superoxide dismutase, and catalase[24]. Subsequently, these enzymes eliminate excess ROS, thereby alleviating the oxidative stress-induced cell damage. Considering the critical role of the AMPK/Nrf2/HO-1 pathway in combating oxidative stress[19], we investigated the effect of NJH on this signaling pathway. mRNA levels of *AMPK*, *Nrf2*, and *HO-1* were increased by NJH pretreatment in H_2O_2-treated cells. Moreover, NJH pretreatment upregulated the protein levels of *p*-AMPK, *p*-Nrf2, and HO-1 under oxidative stress. After exposure to NJH and H_2O_2, the increased expressions of these mRNAs or proteins were more obvious in cells incubated with complete medium for 0 h compared with those incubated for 24 h. It suggested that the antioxidant effect of NJH emerges rapidly and diminishes over time. Overall, NJH protected NRK-52E cells from H_2O_2-induced oxidative stress injury by activating the AMPK/Nrf2/HO-1 pathway.

Apoptosis is a form of programmed cell death essential for the development and maintenance of cellular homeo-

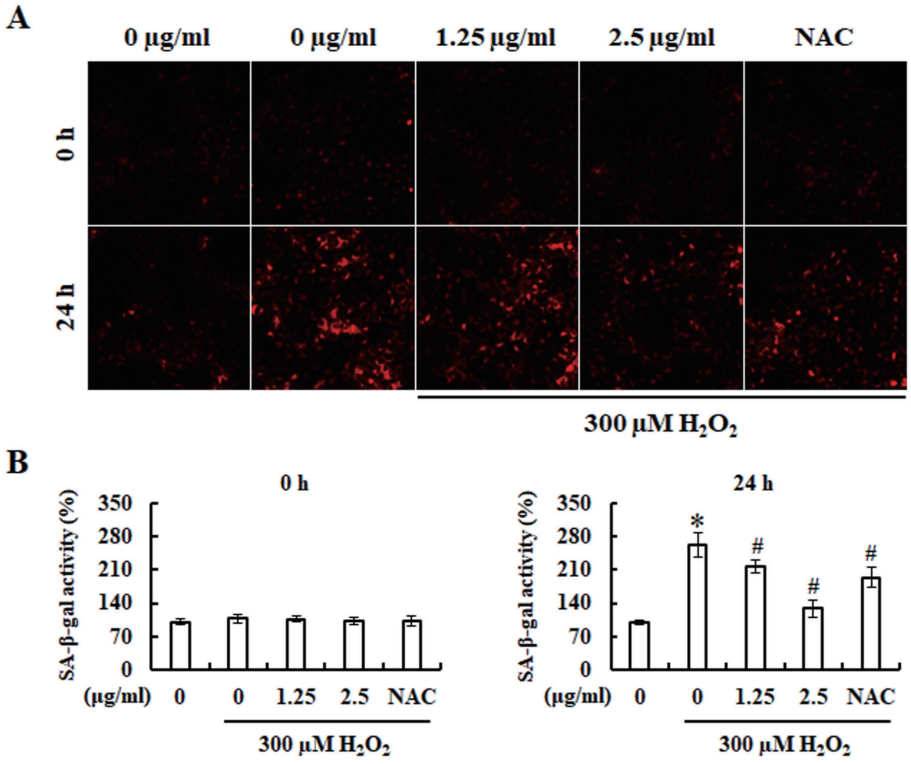

Fig. 6 Effect of NJH on oxidative stress-induced senescence of NRK-52E cells. NRK-52E cells were pretreated with or without NJH (1.25 and 2.5 μg/mL) for 3 h and subsequently treated with 300 μM H_2O_2 for 3 h. After replacing with the complete culture medium and incubating for 0 and 24 h, senescence-associated beta-galactosidase activity of cells was analyzed via red-β-D-Gal staining. Characteristic fluorescence photographs (A) and quantification (B) revealed cellular senescence. Data are represented as the mean ± SD. *$p < 0.05$ vs. control; #$p < 0.05$ vs. H_2O_2 alone.

stasis. It is triggered by multiple signaling pathways and characterized by shrinkage of the cell and nucleus, chromatin condensation, DNA fragmentation, and formation of apoptotic bodies[25]. Kidneys are high oxygen-consuming organs susceptible to oxidative damage by ROS, which results in apoptosis and is a critical factor for the development and progression of renal disorders[9]. Excess ROS target DNA, trigger DNA breaks, and activate p53 phosphorylation, resulting in the transcription of pro-apoptotic genes such as Bax[26]. Elevating the Bax/Bcl-2 ratio can boost mitochondrial membrane permeability and facilitate the release of cytochrome C, a mitochondria-derived pro-apoptotic factor[27]. Cytochrome c released into the cytoplasm binds to caspase-9 to form an apoptosome complex, which initiates the caspase cascade and activates downstream effector caspases, such as caspase-3, ultimately leading to apoptosis[27]. In this study, NRK-52E cells were significantly highly susceptible to oxidative DNA damage following H_2O_2 exposure, which resulted in apoptosis. However, this effect was dose-dependently reversed by NJH. Furthermore, 8-OHdG expression, Bax/Bcl-2 ratio, and cleaved caspase-9 and caspase-3 levels were increased in cells treated with H_2O_2, especially after 24 h of incubation. It is speculated that before inducing cell apoptosis, oxidative stimulation must accumulate to a significant degree of DNA damage. However, NJH significantly modulated the expression of these proteins, indicating that accumulative oxidative damage and mitochondria-mediated apoptosis were blocked by NJH treatment.

Aging is a dynamic ongoing process characterized by the gradual deterioration of organs, tissues, cells, and molecules, ultimately leading to the loss of function. Kidney aging is a risk factor for AKI and CKD[28]. A recent study revealed that specific characteristics of CKD become prevalent with aging, suggesting CKD as a clinical manifestation of premature aging[29]. Furthermore, the study indicated that CKD incidence in both sexes increases from 5.1 and 7.4% in individuals aged 18–39 years to 18.5 and 24.2%, respectively, in those aged >70 years[30]. Although CKD incidence varies among countries, it is positively correlated with aging[31]. Symptoms of renal aging include tubular atrophy, thickening of the glomerular basement membrane, glomerulosclerosis, and decreased glomerular filtration rate[32]. Kidneys are very susceptible to oxidative stress damage owing to their composition, including abundant long-chain unsaturated fatty acids[33]. Aging leads to the

progressive imbalance between oxidation and antioxidant mechanisms, resulting in oxidative lipid, protein, and DNA damage, eventually causing inflammation, apoptosis, and tissue damage[8]. Nrf2 expression progressively decreases with age, making the cells more sensitive to oxidative stress and aggravating the detrimental effects of ROS on cells[34]. These findings suggest that oxidative stress and aging promote renal disease progression. Therefore, preserving antioxidant capacity and minimizing oxidative damage are important strategies to delay cellular aging and inhibit disease progression. In this study, H_2O_2 significantly elevated the ROS levels, disrupted MMP, triggered oxidative DNA damage, and decreased the *p*-AMPK, *p*-Nrf2, and HO-1 protein levels. However, NJH reversed these effects in H_2O_2-treated renal tubular epithelial cells by enhancing their antioxidant capacity. Additionally, NJH markedly decreased the SA-β-Gal activity. This study successfully demonstrated the antioxidant effects of NJH, providing a new avenue to study the anti-aging effects of natural compounds. However, the specific mechanisms by which NJH regulates aging warrant further investigation.

The components of NJH that were identified using a high-resolution gas chromatograph mass spectrometer were methyl salicylate (20.30%), α-thujone (14.46%), bulnesol (9.98%), α-zingiberene (9.80%), camphor (8.03%), guaiol (6.63%). Methyl salicylate, a naturally occurring compound in fruits, functions as a signaling molecule in plant stress responses and can be transformed to salicylic acid[35]. The storage quality and antioxidant system of fruits have been improved through the pre-harvest application of methyl salicylate treatment[35]. Additionally, methyl salicylate has the ability to regulate the lipophilic and hydrophilic antioxidant potential of apricots via influencing phenolic metabolism and carotenoid production[36]. α-Thujone is a common monoterpene derived from the essential oil of sage and wormwood species and exhibits antioxidant activity[37]. A previous study indicated that bulnesol, a type of sesquiterpenoid, binds the antioxidant target protein xanthine oxidase, shows a glide docking score of −8.013 kcal/mol and forms hydrogen bonding interactions, suggesting it may have a potential against oxidation[38]. α-Zingiberene is a monocyclic sesquiterpene and the main antioxidant component in ginger essential oil[39]. Camphor, a terpenoid natural compound, has been shown to have antidiabetic effects in alloxan-induced diabetic rats[40]. Treatment with camphor increased antioxidant capacity and reduced oxidative stress markers in liver, pancreas, and kidney tissues, possibly due to its antioxidant properties[40]. Plant essential oil comprises elevated levels of oxygenated sesquiterpenes, such as guaiol, which have antioxidant properties against thiobarbituric-acid-reactive species[41]. In summary, all the above compounds in NJH, accounting for approximately 70% of components, have shown antioxidant potential, rendering NJH a beneficial antioxidant. This suggests that NJH could be a natural resource for preventing oxidative stress-related diseases.

It was determined that NJH has ameliorative effects on H_2O_2-induced oxidative stress injury in NRK-52E cells through the AMPK/Nrf2/HO-1 signaling pathway. However, this study has some limitations that require further exploration in future research. For example, real-time PCR analysis will be required in the future to support the mRNA quantitative results using semi-quantitative RT-PCR in this study. Moreover, animal experiments will be performed to validate the kidney-protective effects, mechanisms, and toxicity of NJH in vivo.

5 Conclusions

In conclusion, this study demonstrated the protective effects of NJH against H_2O_2-induced increasing ROS levels, mitochondrial dysfunction, oxidative DNA damage, mitochondrial-mediated apoptosis, and SA-β-Gal activity in a renal epithelial cell model of oxidative stress-induced injury. Our findings suggest that NJH exhibits specific mechanisms supporting its antioxidant activity, highlighting it as a potential therapeutic candidate for oxidative damage-associated disorders.

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Author Contribution Statement

Conceptualization: N.-M.T.; Data curation: N.-M.T. and K.-F.C.; Formal analysis: K.-F.C.; Funding acquisition: N.-M. T. and C.-Y.H.; Methodology: C.-Y.H.; Software: K.-F.C.; Writing - original draft: K.-F.C.; Writing - review & editing: N.-M.T. and C.-Y.H.

Conflict of Interest

The authors declare there are no conflicts of interest.

Data Availability

The data used and analyzed in this study are available from the corresponding author on reasonable request.

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