Protective effect of ginsenoside Rd on military aviation noise-induced cochlear hair cell damage in guinea pigs

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Abstract

Noise pollution has become one of the important social hazards that endanger the auditory system of residents, causing noise-induced hearing loss (NIHL). Oxidative stress has a significant role in the pathogenesis of NIHL, in which the silent information regulator 1 (SIRT1)/proliferator-activated receptor-gamma coactivator 1α (PGC-1α) signaling pathway is closely engaged. Ginsenoside Rd (GSRd), a main monomer extract from ginseng plants, has been confirmed to suppress oxidative stress. Therefore, the hypothesis that GSRd may attenuate noise-induced cochlear hair cell loss seemed promising. Forty-eight male guinea pigs were randomly divided into four groups: control, noise exposure, GSRd treatment (30 mg/kg Rd for 10 days + noise), and experimental control (30 mg/kg glycerol + noise). The experimental groups received military helicopter noise exposure at 115 dB (A) for 4 h daily for five consecutive days. Hair cell damage was evaluated by using inner ear basilar membrane preparation and scanning electron microscopy. Terminal dUTP nick end labeling (TUNEL) and immunofluorescence staining were conducted. Changes in the SIRT1/PGC-1α signaling pathway and other apoptosis-related markers in the cochleae, as well as oxidative stress parameters, were used as readouts. Loss of outer hair cells, more disordered cilia, prominent apoptosis, and elevated free radical levels were observed in the experimental groups. GSRd treatment markedly mitigated hearing threshold shifts, ameliorated outer hair cell loss and lodging or loss of cilia, and improved apoptosis through decreasing Bcl-2 associated X protein (Bax) expression and increasing Bcl-2 expression. In addition, GSRd alleviated the noise-induced cochlear redox injury by upregulating superoxide dismutase (SOD) and glutathione peroxidase (GSH-Px) levels, decreasing malondialdehyde (MDA) levels, and enhancing the activity of SIRT1 and PGC-1α messenger ribonucleic acid (mRNA) and protein expression. In conclusion, GSRd can improve structural and oxidative damage to the cochleae caused by noise. The underlying mechanisms may be associated with the SIRT1/PGC-1α signaling pathway.

Keywords Noise pollution · Ginsenoside Rd · Noise-induced hearing loss · Oxidative stress · Apoptosis · SIRT1 · PGC-1α

Introduction

With the development of modern industry, transportation and urban construction, environmental noise pollution has become one of the important social hazards that endanger the health and peace of residents. Among them, the most influential noise sources for residents are road traffic noise (Fredianelli et al. 2022; Halonen et al. 2015; Munzel et al. 2020), railway noise (Petri et al. 2021; Smith et al. 2017), airport noise (Stansfeld 2013), and port activities noise (Murphy and King 2014). Military service members are often exposed to high-intensity noise produced by military weapons and equipment during training and combat activities, whose prevalence of hearing loss and tinnitus is much higher than the general population. The hazardous noise sources in a military operating scenario are primarily the engines of aircraft, tanks, warships and other weapons, and the means of delivery such as the firing of guns and gunpowder explosion (Moore 2020). Hence, the detriment of military noise should not be underestimated.

Noise-induced hearing loss (NIHL) is a type of sensorineural hearing loss caused by overexposure to occupational or recreational noise. It can seriously affect a
person’s quality of life and results in immense economic loss (Ding et al. 2019). Noise damage may also manifest as mental disorder, digestive disorder or cardiovascular diseases (Hahad et al. 2019; Rabiei et al. 2021). Considerable efforts exist to elucidate the pathogenesis of NIHL. However, the mechanisms underlying NIHL are not completely understood. The current belief is that the causes of NIHL include environmental and genetic factors. Environmental factors include noise intensity, spectrum characteristics, and the length of time of exposure to noise (Chen et al. 2022). Genetic factors primarily refer to NIHL-related susceptibility genes such as CDH23 (Kowalski et al. 2014), CAT (Yang et al. 2015), KCNQ4 (Pawelczyk et al. 2009), PON2 (Li et al. 2016), GRM7 (Yu et al. 2018), and GJB2 (Pawelczyk et al. 2009). Four main theories related to mechanical damage, metabolic damage, vascular changes and immunoinflammation damages explain the pathogenesis of NIHL (Basta et al. 2018; Bielefeld 2015; Imam and Hannan 2017). Increasing evidence indicates the possible role of dysregulation of free radicals and oxidative stress (Henderson et al. 2006; Honkura et al. 2016; Tuerdi et al. 2017). Noise exposure makes hair cells highly active, requiring a large amount of oxygen supply and mitochondrial production, which produces large amounts of superoxide, and further produce reactive oxygen species (ROS) and reactive nitrogen species (RNS). Oxidative stress is an imbalance between cell damage and cell adaptive changes that result from these products. The excessive production of ROS and RNS may lead to lipid peroxidation and accelerate hair cells apoptosis, and more ROS and RNS were induced by the positive feedback pathway (Yamashita et al. 2004). Besides, noise exposure results in the instant increase of free Ca\(^{2+}\) content in cochlear hair cells (Fridberger et al. 1998), and Ca\(^{2+}\) overload leads to the loss of the mitochondrial membrane potential and increased membrane permeability (Esterberg et al. 2014). Subsequently, ROS in quantity may be released to the cytoplasm and damage cellular components irreversibly through chemical reactions with many cell surface receptors and membrane lipid molecules, which ultimately leading to impaired cell function and death (Droge 2002). Accordingly, antioxidants have been proved to exert protective effects against NIHL (Le et al. 2017).

Critical to the homeostasis of redox systems is silent information regulator 1 (SIRT1), a nicotinamide adenine dinucleotide (NAD)-dependent histone deacetylase and member of the SIRT family. SIRT1 deacetylates certain substrates, including proliferator-activated receptor-gamma coactivator 1α (PGC-1α), class O of fork head box (FoxO), p53, and Nrf2 (Maillet and Pervaz 2012; Mercken et al. 2014; Mitchell et al. 2014; Nemoto et al. 2005; Singh et al. 2018). PGC-1α is a main regulator influencing the expression of several mitochondrial genes, thereby making a valuable contribution toward mitochondrial biogenesis (Andres et al. 2015). Mitochondrial dysfunction may result in oxidative stress, which contributes to the pathogenesis of NIHL and possibly influences SIRT1 and numerous cellular antioxidant defense mechanisms (Kudryavtseva et al. 2016; Le et al. 2017). Previous studies have demonstrated that the inhibition of SIRT1 expression in vitro increased the apoptosis rate of House Ear Institute-Organ of Corti 1 (HEI-OC1) cells, and that SIRT1 had a protective role against cochlear injury in age-related hearing loss (AHL) by activating downstream PGC-1α signaling (Xiong et al. 2014, 2015; Xue et al. 2016), which is another type of sensorineural hearing loss that has a similar metabolic pathogenesis as NIHL. Besides, in response to oxidative stress, SIRT1 is redistributed at the chromatin level, and causes deregulation at the transcription level (Oberdoerffer et al. 2008).

Ginsenosides (GSs) are the main active components of Panax ginseng, Panax quinquefolium, and Panax notoginseng. More than 50 types of GS monomers have been isolated and extracted from ginseng plants (Yu et al. 2019). The separation of effective monomers from GS is helpful in clarifying its pharmacological effects. Among all GS monomers, Rd, Rb1, Rb2, Rc, Re, and Rg1 account for more than 80% of the total saponins, and are thus termed the main saponins. The contents of Rg3, Rg5, Rg2, Rh1, Rh2, Rh3, Rh4, and CK are rare, while their pharmacological efficacy are better than those of the main saponins (Song et al. 2022). Ginsenoside Rd (GSRd) is a main monomer extract of total saponins, which is low in Panax ginseng and high in Panax notoginseng (Karikura et al. 1992). Belonging to the protopanaxadiol (PPD) type, its molecular formula is C\(_{48}\)H\(_{82}\)O\(_{18}\)·3H\(_2\)O and its molecular weight is 1001 Da. GSRd has high fat solubility, easy diffusion, and permeation through biofilm, but has poor water solubility (Liu et al. 2003). The two glycosyl groups at C-3 position is the pharmacological basis of GSRd antioxidant activity (Yang et al. 2007). Gastrointestinal microbiota and metabolic enzymes are involved in the biotransformation of the main saponins Rb1, Rb2, and Rc into Rd (Ku et al. 2016; Song et al. 2022); therefore, Rd is one of the main forms of absorption and utilization of main saponins in the intestine after metabolism (Kim et al. 2013; Ye et al. 2008). Among the more than 50 types of GS, GSRd is the main active ingredient in broad-spectrum pharmacological actions of Panax ginseng. GSRd has the effect of anti-excitatory amino acid toxicity and inhibiting Ca\(^{2+}\) influx (Zhang et al. 2013). GSRd has also been shown to suppress oxidative stress, which shows distinct protective effects on neurodegenerative diseases (Kim et al. 2014; Yan et al. 2017; Zhang et al. 2016), cardiovascular diseases (Cai et al. 2009; Zhang et al. 2019), renal diseases (Jung et al. 2021), autoimmune diseases (Ren et al. 2021; Zhu et al. 2014), and tumors such as colorectal cancer (Phil et al. 2019), hepatocellular carcinoma (Yoon et al. 2012), and cervical.
cancer (Yang et al. 2006). Notably, Ye et al. (2009) speculated that the antioxidant effect of GSRd may be through the direct scavenging of ROS.

Previous publication from our laboratory has indicated that GSRd could ameliorate auditory cortex damage of the central auditory system associated with military aviation noise-induced hearing loss by activating the SIRT1/PGC-1α signaling pathway (Chen et al. 2020). However, the potential pharmacological effect of GSRd and its mechanisms on noise induced cochlear damage remain virgin lands. Hence, in this study, further exploration was conducted to determine whether GSRd supplementation could attenuate cochlear hair cell damage via the regulation of oxidative stress and apoptosis, and whether the SIRT1/PGC-1α signaling pathway is involved in.

Methods

Functional enrichment analyses of GSRd and SIRT1 via bioinformatics analyses

The initial hypothesis of the involvement of SIRT1 in the otoprotection effects of GSRd was enlightened by bioinformatics analyses. Essential information of GSRd was retrieved from the Encyclopedia of Traditional Chinese Medicine (ETCM, http://www.tcmip.cn/ETCM/index.php/Home/Index/) (Xu et al. 2019). The gene ontology (GO) enrichment analysis of GSRd pharmacological effects and the single gene enrichment analysis of SIRT1 were conducted by using the “Search Tool for Interacting Chemicals” (STITCH) database (http://stitch.embl.de) (Szklarczyk et al. 2016), which is an integrated and easy-to-use platform for searching or predicting potential interactions between proteins and chemicals, and viewing binding affinities of chemicals in the interaction network.

Animal groups

Forty-eight male guinea pigs, weighing approximately 250–300 g, were purchased from the Experimental Animal Center of Air Force Medical University (Xi’an, China). All animals had a sensitive pinna reflex, normal tympanic membrane, and no history of noise exposure. They were housed in an environment with natural light, room temperature at 20–25 °C, air relative humidity of 60–65%, and free access to food and water. Adaptive feeding was administered for 5 d before the start of the experiment. All experimental procedures were approved by the Animal Ethics Committee of Air Force Medical University (No. 20220331). Ginsenoside Rd (Tai-He Biopharmaceutical, Guangzhou, China) was kindly provided by the Department of Neurology in Xijing Hospital (Xi’an, China).

Guinea pigs were randomly assigned to one of four groups, each containing 12 animals: the control group (Con), which received no noise exposure nor treatment; the noise group (NE), which was exposed to military helicopter noise but did not receive drug treatment; the GSRd treatment group (Rd), which was exposed to military helicopter noise and injected intraperitoneally with 30 mg/kg GSRd dissolved in glycerol; and the experimental control group (Vehl), which was exposed to military helicopter noise and injected intraperitoneally with just glycerol at 30 mg/kg. GSRd/glycerol was injected 5 days before noise exposure to the end of noise exposure for a total of 10 days. The intervention dose (30 mg/kg) was determined according to the pre-experiment results (data not shown). The experimental flow was presented in Fig. 1a.

Noise exposure and procedures

The ambient noise of a military helicopter was collected and input to a speaker (Soundtop SF-12; Jia-sheng Audio Equipment, Co., Ltd., Guangzhou, China) through a power amplifier (Soundtop QA-700; Jia-sheng Audio Equipment) for cyclic playback. Noise exposure was conducted in a soundproof room with an air-conditioned fan to moisten the air and strengthen local ventilation. The guinea pigs from the experimental groups were placed in a rat cage on which a speaker was placed. The noise intensity was measured by an A-weighted sound level (Heng-sheng Electronics, Jiaxing, China) to ensure that the difference in the sound pressure level in the activity range of guinea pigs was less than 3 dB. The animals were exposed to 115 dB (A) noise exposure for 4 h daily for 5 consecutive days (Chen et al. 2019). The Con group was not exposed to noise exposure. The background noise in the cage was less than 20 dB, and the other conditions were the same as those in the experimental groups.

Auditory brainstem response (ABR)

To evaluate hearing levels, the hearing thresholds of the guinea pigs were detected before, 1 h, 1 day, 3 days, and 5 days after noise exposure. The guinea pigs were anesthetized by intraperitoneal injection of 4% pentobarbital with the dosage of 0.3 mL for every 100 g of body weight. The testing was conducted in a sound-proof chamber, and a heating pad was utilized to maintain animals’ temperature. ABR was recorded using a system (Otometrics, Taastrup, Denmark) and needle electrodes. The recording electrode was inserted subcutaneously at the vertex, the reference electrode was inserted subcutaneously at the pinna of the tested ear, and the grounding electrode was inserted into the other ear. Tone bursts (2, 4, and 8 kHz) and click sound were presented to the tested ear. The signal started at 90 decibel sound pressure level (dB SPL) and decreased successively by 10 dB.
degrees, which was finally determined at 5-dB steps. The threshold was defined as the lowest sound intensity with an identifiable and repeatable wave III.

**Tissue preparation**

On the day 5 after ABR measurement, the animals were euthanized. The bilateral temporal bones of guinea pigs were removed, and the bilateral cochleae were separated immediately. There were 24 cochlear specimens in each group of guinea pigs.

The cochlear tissues used in the surface preparation of the basilar membrane and section staining were removed and then soaked in 4% paraformaldehyde, and those used for scanning electron microscopy (SEM) were soaked in 2.5% glutaraldehyde. The stapes were removed with microscopic forceps under an anatomical microscope. A small hole was created at the tip of the cochlea by a syringe needle. The corresponding fixed fluid was slowly perfused by drilling of cochlear apex and the oval window more than three times. The specimens were then transferred into an Eppendorf tube containing the fixed solution overnight at 4 °C. Beginning
on the second day, the 10% ethylene diamine tetraacetic acid (EDTA) solution was used for decalcification for 14 days. The freshly decalcified solution was replaced every day until the cochlear bone softened. The decalcified cochlear tissues were used for surface preparation of the basilar membrane or were embedded in paraffin to prepare 5 μm sections parallel to the direction of the modiolus for terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling assay (TUNEL) and immunofluorescence staining. The rest of the specimens were stored at −80 °C.

Phalloidin staining of basilar membrane preparation

Phalloidin staining was conducted as described by Qi and colleagues (Qi et al. 2018). The bony shell and spiral liga-

Phalloidin staining was conducted as described by Qi and colleagues (Qi et al. 2018). The bony shell and spiral liga-
mament were removed with microscopic forceps under an anatomical microscope. The basilar membrane was separated from the tip to the bottom. The removed basilar membranes were rinsed three times with 0.1 M phosphate-buffered saline (PBS) for 1 min each time. A 100 μL of 1% Triton X-100 (MP Biomedicals, Solon, OH, USA) was subsequently added to each hole for 10 min. Specimens were rinsed three times with 0.1 M PBS for 5 min each time. A phalloidin dilution (1:1000; ab176753; Abcam, Cambridge, MA, USA) was added to each well for 30 min without light. The specimens were rinsed three times with 0.1 M PBS and 4,6-diamino-2-phenylindole (DAPI) (1:1000; ab104139; Abcam) and staining was conducted for 8 min without light to display the nucleus. The specimens were then rinsed with 0.1 M PBS three times for 5 min each time. The basilar membrane was drawn onto the glass slide by using a pipette. The positive and negative sides of the basilar membrane were observed under a microscope (SMZ745; Nikon Corporation, Tokyo, Japan). The slides were carefully covered with an 80% glycerin seal to prevent fluorescence quenching. A confocal microscope (LSM 800; Zeiss, Oberkochen, Germany) was used to observe the experimental results.

SEM observation

The experimental steps of SEM corresponded to those proposed in articles by Sung et al. (2018) and Santi et al. (2016). The basilar membrane was soaked overnight in 2.5% glutaraldehyde at 4 °C. The specimens were rinsed three times with 0.1 M PBS for 15 min each, and then postfixed in 1% osmium tetroxide for 2 h at a room temperature of 20 °C–25 °C. The specimens were then dehydrated with 30%, 50%, 70%, 80%, and 90% gradient ethanol and anhy-
drous ethanol; critical-point-dried by using liquid carbon dioxide; and then sputter-coated with gold–palladium for 30 s. The results were observed under a scanning electron microscope (S-3400; Hitachi, Tokyo, Japan).

TUNEL staining

TUNEL staining, as described by Liu and colleagues (Liu et al. 2017), was carried out on sections of the cochlea by using the In Situ Cell Death Detection Kit (11,684,795,910; Roche, Basel, Switzerland), based on the manufacturer’s instructions. Sections of the cochlea were deparaffinized using xylene, hydrated with anhydrous, 90%, 80%, and 70% gradient ethanol. After rinsing with distilled water, the sections were covered with proteinase K solution for 15 min. They were subsequently rinsed with 0.1 M PBS and distilled water for 6 min each and fixed with 3% methanol-hydrogen peroxide solution for 20 min. Following three rinses with 0.1 M PBS for 6 min each, the sections were covered with 3% bovine serum albumin-PBS solution for blocking, followed by incubation with the TUNEL reaction mixture in the dark for 1 h at a room temperature of 20 °C–25 °C. Finally, the sections were rinsed with PBS-Tween (PBST) buffer solution and 0.1 M PBS for 6 min each. The results were examined manually and photographed under fluorescence microscopy (DP71; Olympus, Tokyo, Japan).

Immunofluorescence staining for 4-hydroxy-4-hydroxynonenal and 3-nitrotyrosine

Immunofluorescence staining was carried out, as described by Tian and colleagues (Tian et al. 2018). Sections of cochlea were deparaffinized using xylene; hydrated with anhydrous, 90%, 80%, and 70% gradient ethanol; and then rinsed three times with 0.1 M PBS for 10 min each. A 1% Triton X-100 (MP Biomedicals) was added for 10 min. Three rinses were carried out using 0.1 M PBS. The sections were incubated with 5% BSA (MP Biomedicals) for 1 h. The antibodies used for immunofluorescence staining included rabbit polyclonal anti-4-hydroxy-4-hydroxynonenal (anti-4-HNE) (1:1000; ab46545; Abcam) and mouse monoclonal anti-3-nitrotyrosine (anti-3-NT) (1:1000; ab61392; Abcam). Antibodies were diluted and were added to the sections. The sections were placed in a black wet box and stored overnight in a refrigerator at 4 °C. They were removed on the second day and reheated at a room temperature of 20 °C–25 °C for 1 h before the three washes with 0.1 M PBS. The secondary antibodies of cy3-labeled goat anti-rabbit immunoglobulin G (IgG) (red, 1:1000; Zhuangzhi Biotechnology, Xi’an, China) and 488-labeled goat anti-mouse IgG (green, 1:1000; Zhuangzhi Biotechnology) were added and the sections were incubated at room temperature at 20 °C–25 °C without light for 2 h. Another three rinses with 0.1 M PBS were applied and 8 min of DAPI (1:1000; ab104139; Abcam) staining was applied without light to display the nucleus. Glycerin (80%) was used to prevent fluorescence quenching. A confocal microscope (LSM 800; Zeiss) was used for observation.
Quantitative real-time polymerase chain reaction analysis

The protocols used in this study were conducted as described by Chen et al. (2020). RNAiso (TaKaRa, Kyoto, Japan) was added to the homogenized cochlear specimens to extract total ribonucleic acid (RNA), based on the manufacturer’s instructions. The extracted RNA was diluted to 300–500 ng/μL, and then combined with 2 μL 5×Primer Script RT Master Mix (TaKaRa). The 10 μL reverse transcription reaction system was filled with diethyl pyrocarbonate water. The mixture was placed in a real-time system (Applied Biosystems, Waltham, MA, USA) at 37 °C for 15 min and then at 85 °C for 5 s to synthesize the complementary DNA (cDNA) template. Two microliters of the cDNA template were mixed with 20 μL of SYBR Premix Ex Taq™ II (2×) (TaKaRa), 14 μL of diethyl pyrocarbonate water, and 2 μL of the forward and reverse primers (Table 1; Sangon, Shanghai, China). The configured reaction system was fully blown and mixed and then was added to an octagonal tube with 9 μL per hole. The parameters were set as follows: 95 °C for 30 s, 40 cycles of 95 °C for 5 s and 60 °C for 34 s, and 65 °C for 15 s. Glyceraldehyde-3-phosphate dehydrogenase was used as an internal control and the $2^{-\Delta\Delta C_T}$ method was used to calculate the relative expression of the target gene.

Western blot analysis

The protocols used in this study were conducted as described by Su et al. (2019). The left and right cochlea of the same guinea pig were placed together in a tissue homogenizer. A 200 μL protein extraction reagent (78,505; Thermo Scientific, Waltham, MA, USA) containing 2 mM phenylmethylsulfonyl fluoride was used as the protein lysate to extract

| Table 1 | Sequences of primers used in this study |
|---------|----------------------------------------|
| Gene name | Forward (5’-3’) | Reverse (5’-3’) |
| GAPDH | GGAAGCTTGCTGCGCTGATGG | TTCTCAGGCGGCAGGTCAG |
| Bax | TCGCTGAGGGAACATCCAAGGG | GGCCTCGGGGAGAATCTAG |
| Bcl-2 | CCAAGACTTCACTGAGATGTCAG | GGAGTTGCTACACAGGAGG |
| SIRT1 | CGTTGGAAACAGTGCCAGAATCC | TCTCCGACAGCTCAGTCACAC |
| PGC-1α | GACACAACACGGACAGAACTGAGG | GCATACAGGTGTAACGGTAGGTG |

| Table 2 | Single gene enrichment analysis of GSRd |
|---------|----------------------------------------|
| #Pathway ID | Pathway description | False discovery rate | Matching proteins in your network (labels) |
| GO.0034599 | Cellular response to oxidative stress | 0.00000000727 | EP300, FoxO1, FoxO3, KAT2B, SIRT1, PGC-1α, TP53 |
| GO.0071453 | Cellular response to oxygen levels | 0.000124 | EP300, FoxO1, SIRT1, PGC-1α |
| GO.0042981 | Regulation of apoptotic process | 0.000201 | EP300, FoxO1, FoxO3, KIAA1967, SIRT1, PGC-1α, TP53 |
| GO.1901701 | Cellular response to oxygen-containing compound | 0.000201 | EP300, FoxO1, FoxO4, KAT2B, SIRT1, PGC-1α |
| GO.0009719 | Response to endogenous stimulus | 0.000216 | FoxO1, FoxO3, FoxO4, KAT2B, SIRT1, PGC-1α, TP53 |
| GO.0043066 | Negative regulation of apoptotic process | 0.000243 | EP300, FoxO1, KIAA1967, SIRT1, PGC-1α, TP53 |
| GO.0042127 | Regulation of cell proliferation | 0.00026 | FoxO1, FoxO3, FoxO4, KAT2B, SIRT1, PGC-1α, TP53 |
| GO.1901214 | Regulation of neuron death | 0.000731 | FoxO3, SIRT1, PGC-1α, TP53 |
| GO.0010468 | Regulation of gene expression | 0.00577 | EP300, FoxO3, KAT2B, KIAA1967, SIRT1, PGC-1α, TP53, XRCC6 |
| GO.0032922 | Circadian regulation of gene expression | 0.0194 | SIRT1, PGC-1α |

EP300, E1A binding protein p300; FoxO, Forkhead box O; KAT2, K(lysine) acetyltransferase 2B; PGC-1α, Peroxisome proliferator-activated receptor gamma, coactivator 1 alpha; XRCC6, X-ray repair complementing defective repair in Chinese hamster cells 6
the total cochlear proteins. The total protein concentration was calculated using the BCA Protein Assay Kit (23,250; Thermo Scientific). A total of 30 μg of each protein sample was denatured, separated on 4–12% Bis–Tris PAGE gels, and then transferred to polyvinylidene fluoride membranes (0.45 μm; Millipore, Darmstadt, Germany). The membranes were blocked in 5% fat-free milk powder for 2 h at a room temperature of 20–25 °C and were then incubated with rabbit polyclonal antibody against Bax (1:500; WL01637; Wanleibio, Shenyang, China), Bcl-2 (1:500; WL01556; Wanleibio, China), SIRT1 (1:500; WL02995; Wanleibio), PGC-1α (1:500; WL02123; Wanleibio), or β-actin (1:1000; WL01845; Wanleibio) overnight at 4 °C. After six rinses with PBST for 5 min each, the membranes were incubated in PBST with horseradish peroxidase-conjugated goat anti-rabbit IgG (1:5000; WLA023; Wanleibio) for 1 h at a room temperature of 20–25 °C and were detected using enhanced chemiluminescence detection reagents (Millipore) in a Gel Image Analyzing System (Tanon Science & Technology, Shanghai, China). The band intensity was measured using ImageJ v1.51 (National Institutes of Health, Bethesda, MD, USA), and the values were normalized to β-actin.

Detection of superoxide dismutase activity, malondialdehyde level, and glutathione peroxidase level

The left and right cochlea of the same guinea pig were placed in the same Eppendorf tube, and the samples were prepared with 0.9% normal saline as a 10% homogenate. Superoxide dismutase (SOD) assay kit (WST-1 method; A001-3; Jiancheng Biotechnology, Nanjing, China), malondialdehyde (MDA) assay kit (TBA method; A003-1; Jiancheng Biotechnology), and glutathione peroxidase (GSH-Px) assay kit (colorimetric method; A005-1; Jiancheng Biotechnology) were used to detect SOD activity, MDA levels, and GSH-Px levels, based on the manufacturer’s instructions. Each group’s chromaticity was assessed using a microplate reader (Thermo Fisher) at 450 nm for SOD activity, 532 nm for the MDA level, and 412 nm for the GSH-Px level.

Statistical analysis

Statistical analyses were performed using GraphPad Prism 7.0 (GraphPad Software, San Diego, CA, USA) and SPSS 23.0 (IBM Corporation, Armonk, NY, USA). Immunofluorescence intensity determination was performed using Image J v1.8.0 (National Institutes of Health, Bethesda, MD, USA). The results are presented as means ± the standard error (SE). The hearing thresholds level; immunofluorescence staining results; mRNA and protein expression; and SOD, MDA, and GSH-Px activities were statistically analyzed using one-way analysis of variance. Dunnett’s t-test was used to compare the experimental and control groups. Statistical significance was set at $p < 0.05$.

Results

Bioinformatics analyses predicted that GSRd might improve noise-induced hearing loss through the SIRT1/PGC-1α signaling pathway

The retrieval results in the ETCM database suggest that the diseases treated with GSRd (chemical formula is shown in Fig. 1b) include sensorineural hearing impairment, progressive sensorineural hearing impairment, and dysregulation of apoptosis (http://www.tcmip.cn/ETCM/index.php/Home/Index/cf_details.html?id=4640). Therefore, the initial hypothesis that GSRd may be a candidate drug for hearing impairment was brought out. In addition, GO enrichment analysis showed that the pharmacological effects of GSRd are primarily the regulation of oxidative stress and apoptosis, and involvement in mitochondrial activity (Fig. 1c). NIHL is a type of illness characterized by oxidative stress damage and apoptosis progression. The results of the GSRd GO enrichment analysis focused our attention on a molecule associated with the regulation of mitochondrial activity and oxidative stress, which is SIRT1 (Table 2). Furthermore, SIRT1 single gene enrichment analysis demonstrated that the SIRT1/PGC-1α signaling pathway is more prominently involved in gene expression, oxidative stress, and apoptosis regulation.

GSRd mitigated hearing threshold shifts of guinea pigs after noise exposure

ABR measurements were conducted to evaluate the hearing levels of guinea pigs after noise exposure and the representative results of ABR thresholds are shown in Fig. 2. There was no significant difference in baseline hearing thresholds of the four groups before noise exposure (data not shown). One hour after noise exposure of 115 dB (A) for 5 consecutive days, obvious elevated hearing thresholds of click, 2 k, 4 k, and 8 k of the noise group (NE, $p = 0.0001$ for all tested frequencies), the GSRd supplement group (Rd, $p = 0.0001$ for all tested frequencies), and the experimental control group (Vehl, $p = 0.0001$ for all tested frequencies) were observed compared to the control group (Con). Besides, the Rd group manifested a significant decrease of hearing threshold compared to the NE ($p = 0.0001$ for all tested frequencies) and Vehl group ($p = 0.0001$ for all tested frequencies). After noise exposure for 1 day, 3 days, and 5 days, the hearing thresholds recovered gradually. Of note are the hearing thresholds of the Rd group at all tested frequencies, which were significantly lowered than those of the NE group ($p < 0.05$) and the Vehl group counterparts ($p < 0.05$) with the extension of time.
GSRd ameliorated noise-induced damage to cochlear hair cells of guinea pigs

Basilar membrane phalloidin staining (Fig. 3a) revealed the microfilament cytoskeleton and manifested the distribution of actin in the cochleae. In the Con group, the outer hair cells of the guinea pigs were intact and neatly arranged, and the cilia were clearly visible and arranged in a “V” shape. The outer hair cells of the NE group and the Vehl group showed damage with different degrees of dot deletion. The degree of outer hair cell loss in the Rd group was less than that of the NE and Vehl groups.

Fig. 2 The ABR hearing thresholds of the four groups 1 h (a), 1d (b), 3d (c), and 5d (d) after noise exposure with click, 2 k, 4 k, and 8 k frequencies. The values are presented as the means ± SE. n = 24 in each group. *p < 0.05, **p < 0.01, ***p < 0.001
SEM images (Fig. 3d) showed the structural damage to the cilia and supporting cells of inner ear hair cells. The SEM images of the Con group verified the results of phalloidin staining. In the NE and Vehl groups, the polarity of the outer hair cells disappeared, and the arrangement was disordered with lodging, fusion, and even falling off. Among them, the third layer of outer hair cells was most seriously damaged. Following Rd treatment, the outer hair cells in the Rd group showed slight lodging and loss of cilia.

GSRd reduced the expression of apoptotic factors in the cochleae of guinea pigs after noise exposure

TUNEL staining exploits the biochemical hallmarks of apoptosis by labeling the 3'-OH terminal with fluorescently labeled UTP by virtue of TdT, thereby enabling visualization of fragmented DNA. The results of the TUNEL staining showed no TUNEL-positive cells in the cochleae of guinea pigs in the Con group. The NE and Vehl groups had TUNEL-positive cells, primarily distributed in the organ of Corti, vascular stria, and spiral ligament. The number of TUNEL-positive cells in the Rd group was lower than that in the NE and Vehl groups. The results of TUNEL staining are shown in Fig. 3b. The statistical results of TUNEL positive cell number are shown in Fig. 3c.

The results of quantitative real-time PCR (RT-qPCR) and western blot analysis showed that the levels of B-cell lymphoma-2 (Bcl-2)-associated X protein (Bax) messenger ribonucleic acid (mRNA) (Fig. 4a) and protein (Figs. 4c and 4d) in the NE group (p = 0.0003 and p = 0.0001, respectively), Rd group (p = 0.0274 and p = 0.0002, respectively), and Vehl group (both, p = 0.0001) were significantly higher than those in the Con group, whereas Bcl-2 mRNA (Fig. 4a) and protein (Figs. 4c and 4e) in the NE group (p = 0.0002 and p = 0.0001, respectively), Rd group (p = 0.0130 and p = 0.0152, respectively), and Vehl group (p = 0.0002 and p = 0.0183, respectively) was significantly lower than that in the Con group. The levels of Bax mRNA, Bax protein, Bcl-2 mRNA, and Bcl-2 protein content in the Rd group were significantly lower than those of the NE group (p = 0.0157, p = 0.0256, p = 0.0240 and p = 0.0205, respectively) and Vehl group (p = 0.0037, p = 0.0187, p = 0.0181, and p = 0.0106, respectively).

**Fig. 3** Preparations of hair cells (a), TUNEL staining (b, c), and SEM observation (d) of the basilar membrane of the four groups after noise exposure. **a** The “*” symbol indicates the loss of outer hair cells. Scale bar = 50 μm. **b** oC: organ of Corti; BM: basilar membrane; SV: stria vascularis; SL: spiral ligament. Scale bar = 100 μm. **c** Summarized data of TUNEL positive cell number. The values are presented as the means ± SE. n = 6 in each group. ****p < 0.0001. **d** Scale bar (left) = 20 μm. Scale bar (right) = 10 μm.
GSRd may exert its otoprotective effects via the activation of SIRT1/PGC-1α signaling pathway

The mRNA content (Fig. 4b) and protein content (Figs. 4f and 4g) of SIRT1 in the NE group (p = 0.0003 and p = 0.0001, respectively), Rd group (p = 0.0192 and p = 0.0015, respectively), and Vehl group (p = 0.0003 and p = 0.0001, respectively) were significantly lower than those of the Con group, whereas they were significantly higher in the Rd group than in the NE group (p = 0.0255 and p = 0.0111, respectively) and Vehl group (p = 0.0221 and p = 0.0173, respectively). The mRNA content (Fig. 4b) and protein content (Figs. 4f and 4h) of PGC-1α likewise showed a similar relationship between groups:

NE group (p = 0.0004 and p = 0.0001, respectively), Rd group (p = 0.0272 and p = 0.0003, respectively), and Vehl group (p = 0.0006 and p = 0.0001, respectively), compared to the Con group; NE group (p = 0.0246 and p = 0.0101, respectively) and Vehl group (p = 0.0454 and 0.0137, respectively), compared to the Rd group.

GSRd reduced the expression of free radicals in the cochleae of guinea pigs after noise exposure

The common indices of the free radical level test are 4-hydroxy-4-hydroxynonenal (4-HNE) (i.e., an ROS marker) and 3-nitrotyrosine (3-NT) (i.e., an RNS marker). In this experiment, 4-HNE was displayed with red fluorescence, 3-NT was displayed with green fluorescence,
4,6-diamino-2-phenylindole (DAPI) staining nucleus was displayed with blue fluorescence, and merge was synthesized in the three colors (Fig. 5a). Only a small amount of 4-HNE and 3-NT was expressed in the Con group. Following the noise exposure, the expression of 4-HNE (Fig. 5b) and 3-NT (Fig. 5c) was significantly increased in the NE group \((p = 0.0001)\) and Vehl group \((p = 0.0001)\) and was primarily located in the organ of Corti. The immunofluorescence expression of 4-HNE and 3-NT was decreased significantly in the Rd group \((p = 0.0002)\).

**GSRd downregulated the level of oxidative stress in the cochleae of guinea pigs**

SOD and GSH-Px are antioxidant enzymes which scavenge ROS, while MDA is a widely examined oxidative damage marker representing lipid peroxidation. The SOD levels in the NE, Rd, and Vehl groups, compared to the Con group, were decreased by 46.25\% \((p = 0.0001)\), 22.71\% \((p = 0.0122)\), and 47.35\% \((p = 0.0001)\), respectively (Fig. 5d). The MDA levels in the NE, Rd, and Vehl groups were increased by 3.30 \((p = 0.0009)\), 1.60 \((p = 0.0466)\), and 3.53 \((p = 0.0006)\) times, respectively (Fig. 5e). The GSH-Px levels in the NE, Rd, and Vehl groups were downregulated by 72.06\% \((p = 0.0001)\), 39.67\% \((p = 0.0027)\), and 72.64\% \((p = 0.0001)\), respectively (Fig. 5f). Compared to the NE and the Vehl groups, the Rd group had significantly increased SOD levels \((p = 0.0099\) and \(p = 0.0076\), respectively) and GSH-Px levels \((p = 0.0090\) and \(p = 0.0082\), respectively), and had significantly decreased MDA levels following noise exposure \((p = 0.0430\) and \(p = 0.0232\) respectively).

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**Fig. 5** Immunofluorescence staining of 4-HNE (a, b) and 3-NT (a, c), and influence of GSRd supplement on the level of oxidative stress (d–f) in the cochleae of the four groups after noise exposure. a Original images of immunofluorescence staining of 4-HNE (red) and 3-NT (green) in the cochleae of the four groups after noise exposure. Scale bar = 200 μm. DAPI staining (blue) indicates the location of the nucleus. b, c Summarized data of IF staining of 4-HNE and 3-NT in the cochleae of the four groups after noise exposure. d–f SOD activity (d), MDA (e), and GSH-Px levels (f) in the cochleae of guinea pigs of the four groups after noise exposure. The values are presented as the means ± SE. \(n = 3\) in each group or colorimetric assay. * \(p < 0.05\), ** \(p < 0.01\), *** \(p < 0.001\).
Discussion

In this study, the hypothesis that GSRd supplementation would attenuate NIHL via regulating oxidative stress and apoptosis, mediated by the SIRT1/PGC-1α signaling pathway was tested. Our results suggested that GSRd can alleviate hair cell loss and free radical production in the cochleae of guinea pigs. The possible mechanism may involve the activation of the SIRT1/PGC-1α signaling pathway, the upregulation of SIRT1/PGC-1α expression, increased SOD and GSH-Px activity, and decreased MDA level, which enhances the antioxidant capacity of the auditory system and hence reduces peripheral auditory system damage. The schematic diagram is shown in Fig. 6.

As the main active component of ginseng, GSRd can pass through the blood–brain barrier and cell membrane even in the case of energy deficiency, owing to its high lipophilicity. At present, GSRd has become the first-class drug candidate for the treatment of ischemic stroke in China (Ye et al. 2013). Zeng et al. (2010) designed a randomized, open-label, 3-way crossover clinical trial to assess the pharmacodynamics and safety of GSRd in healthy Chinese volunteers. There were no dose-related adverse events in the trial, suggesting that GSRd has good tolerance and safety profile. As a potential neuroprotective agent, our previous research has indicated that noise-induced neuronal apoptosis in the auditory cortex was significantly corrected by GSRd treatment. As a continuous work, our experimental results showed that GSRd treatment may have otoprotective effects by regulating the apoptosis pathway and oxidative stress. Following noise exposure, the peripheral auditory system is first damaged, including mechanical damage to the inner ear and a series of metabolic damage. Relevant studies have illustrated that the expression of activated Caspase-3 and Bax in the cochlea increases significantly after noise exposure, whereas the expression level of Bcl-2 decreases significantly, thereby resulting in a significant decrease in the Bcl-2/Bax ratio (Jia et al. 2020). Besides, Mungan Durankaya et al. (2021) gavaged the rats with Korean red ginseng, enriched in GSRd, with a dosage of 200 mg/kg/day for 10 days, and found that TUNEL positive cells were less visible in the organ of Corti and spiral ganglion. These findings are consistent with our results that GSRd reduced the expression of apoptotic factors and TUNEL positive cells in the cochleae of guinea pigs after noise exposure.

After noise exposure, excessive ROS and lipid peroxidation were stimulated in the inner ears. The aldehyde products are thus produced and may continue to contribute to the destruction of the normal physiological activities of hair cells, including 4-HNE, which form cross-links with proteins and nucleic acids (Choi et al. 2011). The 4-HNE level is closely associated with oxidative stress, apoptosis, and inflammation, and is considered a marker of ROS. In addition, excessive RNS stimulated by noise nitrify the free tyrosine or tyrosine residues in the protein, thereby resulting in the formation of 3-NT (Han et al. 2018). It attacks the normal structure of the protein, affects its normal physiological function, and causes cell damage, which is a marker of RNS. Research has confirmed that once excessive oxidative stress under hazardous noise conditions overwhelms the beneficial potential of autophagy, the hair cell apoptosis and necrosis may be exacerbated (Yuan et al. 2015). In the present study, the change of 4-HNE and 3-NT in the cochleae of guinea pigs before and after noise exposure was quantitatively analyzed. Besides, a significant increase of these free radical markers was observed in the NE group compared with that of the Con.

Fig. 6 Schematic diagram of protective effects of GSRd on military aviation noise-induced cochlear hair cell damage via SIRT1/PGC-1α signaling pathway in guinea pigs. GSRd supplement reduced the expression of apoptotic factors and production of free radicals, and downregulated oxidative stress, and hence reduces peripheral auditory system damage caused by noise exposure. The possible mechanisms were thought to be the activation of SIRT1/PGC-1α signaling pathway.
group. GSRd treatment downregulated the expression of Bax, 4-HNE and 3-NT, and upregulated the expression of Bcl-2, thereby reducing the level of apoptosis and oxidative stress in the inner ear. Considering the pathogenic mechanisms underlying NIHL, which is associated with excessive oxidative stress, the aforementioned findings suggest that GSRd has protective therapeutic effects against NIHL by regulating the level of oxidative stress in the cochlea.

In this experiment, the damage to the third-row outer hair cells (OHC3 in Fig. 3) was the most severe, whereas the damage to inner hair cells was relatively mild, and this result may be attributable to the location (Ren 2002). The third-row outer hair cells are in the center of the basilar membrane where the largest vibration amplitude occurs. However, the inner hair cells are on the edge of the bony spiral lamina, which is affected by less vibration from the basilar membrane. SEM revealed that GSRd may improve the lodging and loss of the cilia of hair cells, but the results of basilar membrane preparation were not significantly different when compared to the NE and Vehl groups. It may come to the conclusion that the abnormal morphology of the cilia and ultrastructural changes in the hair cells generally occurred after noise exposure. Only when the damage factors reached a certain degree did death and loss of hair cells occur and manifest in the basilar membrane preparation (Ding 2010).

According to our bioinformatics analyses, SIRT1 and PGC-1α are remarkably involved in cellular response to oxidative stress. Notably, the definite relationship between SIRT1 and ROS was evidenced by previous researches, whose results showed that the inhibition of SIRT1 with siRNA or other pharmacological agents might lead to an elevation of ROS levels (Hori et al. 2011). On the other hand, resveratrol, an SIRT1 activator, was validated to exert a protective effect against NIHL in various animal models (Seidman et al. 2003; Xiong et al. 2017), ultimately leading to deacetylation and activation of PGC-1α, and improved mitochondrial function (Tennen et al. 2012). Moreover, SIRT1 activation negatively regulates apoptosis via its effects on Bax (Yang et al. 2021). Xiong et al. (2014) explored SIRT1 expression in the cochlea and found that it was primarily expressed in the spiral ganglion neurons, outer hair cells, inner hair cells, and some supporting cells. A small amount of SIRT1 was expressed in the vascular stria of the lateral wall of the cochlea. The expression of SIRT1 was less pronounced in the outer hair cells than in the inner hair cells (Takumida et al. 2016). Besides, GO enrichment analysis showed that GSRd are primarily involved in the regulation of oxidative stress, apoptosis, and mitochondrial activity, which is correspond to the pathogenesis of NIHL. Thus, the hypothesis that GSRd acts as an SIRT1 activator for protecting against noise-induced cochlear damage seemed promising. Our results showed that the mRNA and protein levels of SIRT1/PGC-1α in the cochlea of guinea pigs were downregulated after noise exposure, which indicated that they may be involved in the noise-induced damage in the inner ear. Furthermore, GSRd can upregulate the SIRT1/PGC-1α signaling pathway, which helps to maintain the oxidative balance of the peripheral auditory system, thereby exerting its otoprotective effects. Besides, GSRd treatment significantly alleviated the noise-induced cochlear redox injury in guinea pigs by increasing antioxidant enzymes, such as SOD and GSH-Px, and significantly reducing the levels of MDA, which is an end product of lipid hydrogen peroxide. Taken together, as a natural saponin, GSRd should be developed as a new potential candidate to prevent or reduce the noise induced cochlear damage.

There are some limitations of our study. Firstly, a single given strength and frequency was used in this study. However, the dose–response curve of GSRd in the treatment of NIHL and the therapeutic effect of GSRd after prolonged noise exposure or other different types of auditory stimuli remain obscure. Secondly, because of the failure to illustrate whether GSRd directly regulates oxidative stress levels by regulating SOD and MDA activity, or indirectly regulates these parameters through other signaling pathways, further investigations were commanded. Finally, there are still gaps between laboratory animal pharmacological experiments and clinical application of GSRd on NIHL protection. With in-depth exploration into the physiological and pathological changes of hair cells in the whole process of noise-induced injury and GSRd treatment, the translation of GSRd from bench to bedside is just around the corner.

**Conclusion**

To the best of our knowledge, our laboratory made first attempt to explore the otoprotective effects of ginsenoside Rd (GSRd) on noise-induced hearing loss (NIHL) and the potential involvement of the silent information regulator 1 (SIRT1)/proliferator-activated receptor-gamma coactivator 1α (PGC-1α) signaling pathway. The salient finding of this study was that GSRd manifested potent effects to ameliorate damage to the peripheral auditory system of guinea pigs caused by military helicopter noise. After noise exposure, GSRd supplement mitigated hearing threshold shifts, ameliorated outer hair cell loss and lodging or loss of cilia, reduced the expression of apoptotic factors and production of free radicals, and downregulated oxidative stress. The possible mechanisms were thought to be the activation of SIRT1/PGC-1α signaling pathway. Further investigations are recommended concerning the dose–response curve of GSRd in the treatment of NIHL and the therapeutic effect with other types of auditory stimuli.
Abbreviations 3-NT: 3-Nitrotyrosine; 4-HNE: 4-Hydroxy-4-hydroxynonenal; ABR: Auditory brainstem response; AHI: Age-related hearing loss; Bcl-2: B-cell lymphoma-2; Bas: Bcl-2 associated X protein; DAPI: 4,6-Diamidino-2-phenylindole; GO: Gene ontology; GSH-Px: Glutathione peroxidase; GSRd: Ginsenoside Rd; IgG: Immunoglobulin G; MDA: Malondialdehyde; NHH: Noise-induced hearing loss; PBS: Phosphate-buffered saline; PBST: Phosphate-buffered saline-Tween; PGC-1α: Proliferator-activated receptor-gamma coactivator 1α; RNA: Ribonucleic acid; ROS: Reactive oxygen species; SEM: Scanning electron microscopy; SIRT1: Silent information regulator 1; SOD: Superoxide dismutase; TUNEL: Terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling assay

Acknowledgements Our deepest gratitude goes to colleagues from Senior Department of Otolaryngology & Head and Neck Surgery, Chinese PLA General Hospital, led by Prof. Shi-ming Yang and Department of Otolaryngology, Xijing Hospital, led by Prof. Jian-hua Qiu for their valuable technical support.

Author contribution XMC, YHL, YMC, and XCW conceived and designed the experiment methods. XMC and YHL drafted the manuscript. SFJ performed bioinformatic analysis. XMC and LLW performed phallolidin staining, SEM observation RT-qPCR, and western blot analysis. SFJ and XMX performed TUNEL staining. XMC and SFJ revised the manuscript. XCW generated phalloidin staining, SEM observation RT-qPCR, and western blot analysis. SFJ and XMX performed TUNEL staining. YHL and SFJ conducted the statistics. XMC and SFJ revised the manuscript. XCW drafted the manuscript. YHL, YMC, and XCW conceived and designed the experiment methods.

Funding This work was supported by grants from the Major Military Project (grant number AWS14L009) and Key Researcher and Development Plan in Shaanxi (grant number 2018SF-252).

Data availability All data generated or analyzed during this study are available from the corresponding author on reasonable request. All materials are commercially available.

Declarations

Ethics approval and consent to participate All experimental procedures were approved by the Animal Ethics Committee of Air Force Medical University (No. 20220331).

Consent for publication Not applicable.

Competing interests The authors declare no competing interests.

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