Combined effects of the exposure to silver nanoparticles and noise on hearing function and cochlea structure in male rats

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Abstract

Objective: This study intended to investigate whether exposure to the combination of noise and Ag-NPs in rats induces cochlear damage, and hearing dysfunction.

Methods: A total of 24 Wistar rats were divided into four treatment groups and received /exposed to saline (IP), Ag-NPs (100mg/kg, 5 d/w for 28 days), 8 kHz narrowband noise (104 dB SPL, 6 hrs/day, 5 d/w for 4 weeks) and Ag-NPs plus noise. The DPOAE, signal-to-noise ratio, MDA and SOD levels in blood and changes in rats' body weight were assessed. The rat cochlea was further stained for investigating the mRNA expression (TL-6, NOX3, and TNF-), IHC (TUJ-1 and MHC7), and histological alterations. The Ag-NPs characteristics were also analyzed by SEM and XRD.

Results: The DPOAE values were remarkably reduced ($p < 0.05$) among the exposed groups. Furthermore, exposure to noise and Ag-NPs significantly increased MDA levels and decreased the SOD activity in the serum. The expression of IL-6, TNF-α and NOX3 was significantly increased in the Ag-NPs plus noise group compared with control group ($p < 0.05$). The body weight change in all groups, except for Ag-NPs plus noise group, significantly increased. IHC tests showed remarkable down regulation of beta tubulin (TUJ1) and myosin-7a (MYO7A). Morphological changes confirm these findings as well. The formation of Ag-NPs was confirmed by SEM and XRD patterns.

Conclusion: This study revealed that the combined exposure of noise and Ag-NPs damages the hair cells responsible for high-frequency perception, eventually leading to hearing deficits.

Keywords: Ag-NPs, noise, IHC, DPOAE, Cochlea.

Introduction

Noise-induced hearing loss (NIHL) is a serious occupational health issue on a global scale (1). The occupational exposure to the higher levels of noise also contribute to a tremendous financial and disease burden on both the workers (2) and the community (3). Additionally, exposure to the long-lasting and intense noise (130 dB sound pressure level (SPL) might disrupt the cellular connections between cochlear cells, decouple the organ of Corti from the basilar membrane, mix the endolymph and perilymph and injure the synapse within primary spiral ganglion neurons (SGNs) and IHCs (4-8).

A recent study linked NIHL etiology to cochlear ischemia-reperfusion damage caused by reduced blood flow and oxidative stress induced by exposure to the loud noise. (9). However, oxidative stress and free radical generation have been recognized as major contributions to NIHL and cochlear dysfunction.The

elevated levels of lipid peroxidation prompted by the exposure to the noise have been reported to be correlated to the increased products of free radicals in the cochlea (10). Acoustic injury to the cochlea also affects many genes related with immunity and inflammation (11-15).

Along with noise, exposure to several ototoxic chemical compounds have also been reported to adversely affect the morphology and functions of cochlea, the vestibule-cochlear system, the eighth cranial nerve and the associated neural pathways as well (16).

Additionally, the toxicological effects of consumer products containing engineered nanoparticles (NPs) on the environment and biological functioning of humans and animals, as well as the relative benefits and hazards of these products, are currently being debated (17).

Metallic silver nanoparticles (Ag-NPs) are one of the most widely used forms of nanomaterials worldwide and they have found a broader range of usage in antibacterial consumer, industrial, military, and medical products and services(18). Ag-NPs can find their way into the body through inhalation, ingestion, and even dermal contacts (19).

It is now well established that metallic nanoparticles principally contribute to toxicity via producing reactive oxygen species (ROS) and excessive releasing cytokines. (20). Previous research has shown that Ag-NPs can cause several forms of toxicity including genotoxicity in several cell lines and damage cochlea (21-25).

Accordingly, the results from the studies on cochlear inflammation have revealed that the appearance of TNF-a, IL-1b, IL-6, and MHCII are mainly correlated to monocyte infiltration (12, 26, 27). Moreover, ROS generation induced the inflammation and production of pro-inflammatory cytokines such as interleukin(IL)-1β (28), IL-6 (26, 29), tumor necrosis factor-α (TNF-α) (30) and expression of NOX3 (10).

It is unknown whether the mechanisms of destruction imposed on the inner ear cells are mediated by the altered gene expression or biochemical events (31). Additionally, the cochlea contains two distinct types of proteins (i.e., MYO7A and TUJ-1 isoforms), whose presence is interpreted as a possible reaction to the induced stress. While the latter of these is expressed throughout the HCs and is observed in the cytoplasm as well as the apical stereocilia (32), the former is shown to be particular to the nerve fibres and is expressed in spiral ganglion cells (33).

Until now, only a few studies have examined the combined health effects of noise and ototoxic chemicals (34) and the exact cellular mechanisms by which the damage to the cochlea occurs are still under investigation (35). Moreover, the toxicity of Ag-NPs and the effect of their physicochemical characteristics in animal models still need to be comprehensively investigated (36).

Considering the combined exposure to noise and nanomaterials, reliable information regarding the expression of genes involved in hearing loss should be investigated, as this knowledge might then be used to build novel and emerging molecular therapeutics or to identify candidate genes for gene therapy (37-40). The present study has therefore been conducted to investigate whether there is an additive or synergistic interaction for the combined exposure to Ag-NPS and intensive noise in an animal model.

2. METHODS AND MATERIALS

2.1. Animals

24 male albino Wistar rats (180–200 g) with normal Preyer's reflex were purchased from animal house of Tarbiat Modares University (TMU, Tehran, Iran). Having been acclimatized, these rats were randomly divided into 4 experimental groups (6 rats in each group) as: Group 1, untreated and had normal hearing (control group); group 2, received Ag-NPs alone (100 mg/kg body weight) through intraperitoneal (i.p) injection, 5 days/week for 4 weeks; group 3, exposed to noise (104 dBA) 6hrs/day, 5 days/week for 4 weeks (from 7:30 a.m. to 1:30 p.m); and group 4, exposed to noise plus Ag-NPs (at the same time set and dosage). During the study period, the rats were housed 4 per cage and kept in accordance with standard animal housing conditions (relative humidity 50%±10%, room temperature 23°C±2°C as well as 12 hours light/dark cycle. Animals were also weighed weekly by a pan-digital scale (model SF-400) with 0.1oz/or 1g accuracy during exposure period. The auditory functions of the rats were confirmed to be in the normal range using the DPOAE tests prior to the experiments. Echolab OAE-ABR device has been selected for such experiment.

All experiments were performed in the animal house of the TMU and the protocols of the study followed the guidelines from the Animal Ethics Committee of TMU (IR.MODARES.REC.1400. 100). The statement of Helsinki guidelines was also attentively considered in this study.

2.2. Preparation of the Ag-NPs

The Ag-NPs were sonicated for 3 hrs in deionized water, using a Branson sonifier 450 (Branson Ultrasonics Corp, Danbury, CT, USA). This was done to disperse the Ag-NPs in the solution. The Ag-NPs were vibrated for 2 min,

immediately before being administered to the animals. All specimens were made under sterile conditions.

2.3. Physicochemical characterization of Ag-NPs

The Ag-NPs with a diameter of 30-50 nm (US Research) were employed in this study. Before in vivo administration, in order to investigate the shape, and crystal structure of these particles, they were observed by scanning electron microscopy (SEM) (Nikon, Japan) and, X-ray powder diffraction (XRD) techniques.

2.4. Noise exposure procedure

Exposure to the noise was performed inside a reverberant chamber made from Plexiglas (580 × 490 × 300 mm) with ventilation holes and a centrifugal fan on both sides of the wall for airflow. Six rats were located in individual wire mesh cages (15 × 13 × 17 cm^3) and the water or food was unavailable during the exposure to prevent piling. The noise was generated with 104 dB SPL (Sound Pressure Level) and centred at 8 kHz octave band using Tone Generator PRO software. Cool edit Pro 2.1 software along with an amplifier (Rock Jw-s317) and 4 loudspeakers (SPEAKER YASHIAO YD103-1) were also employed to play the noise. The loudspeakers were installed at a distance of 15 cm above the animal cages to ensure the uniformity of the noise level with a variation of ± 1 dB SPL inside the chamber. The locations of the rats' cages were rotationally changed per day to maintain the same exposure for all rats.

A precision handheld noise level meter (CEL-450 type 1D, Casella CEL) equipped with an analyser was used to continuously measure and monitor the noise level within every side of the chamber. The control and Ag-NPs groups were kept in a separate and fairly quiet room with baseline SPLs of 40-50 dB.

2.5. Distortion product otoacoustic emissions (DPOAEs)

Distortion product otoacoustic emissions (DPOAEs) is an objective as well as sensitive test, which allows for the exploration of the outer hair cell system function in the cochlea. This test is commonly used as a tool to quickly screen the animals for inner ear function assessment (41).

For the study the cochlear function of each rat, an asymmetric DPOAE test along with unequal initial tone stimulus intensity (L1= 60 dB, L2= 50 dB) with f1 and f2 frequencies were used to elicit the responses. The variation between

the levels of L1 and L2 was maintained at 10 dB SPL. The two tones in the peak f2/f1 ratio were fixed at 1.21 to receive the most significant responses (42). To prevent the failures caused by the creation of the standing waves in the external meatus, the investigated DPOAE data were limited to the frequency range 4.0 to 10 kHz.

One-day post-exposure, DPOAE tests were performed only on the left ear of each rat. The DPOAE amplitudes were recorded three times or more and the average value of the SNR (Signal to Noise Ratio) calculation was used for the data analysis (43). SNR values equal to 3 dB and higher were regarded as positive. The SNR values of all groups collected during the DPOAE analysis were compared within and between the groups as well.

All DPOAE tests were conducted using an infant-size hearing assembly probe coupled to the external auditory canal of the rat at five frequencies: 4840, 6050, 7260, 8470, and 9680 Hz (44). The DPOAE measurements were carried out in a soundproof room following general anaesthesia (Ketamine 30 mg/kg, Xylazine 6 mg /kg) and prior to Ag-NPs administration and, exposure to noise. The body temperature of the anaesthetised rats was kept at 37 ± 1 0C with a heating blanket throughout the DPOAE examination. The second DPOAE measurement was conducted after the end of the experiments (post-exposure, on 29th day). Accordingly, permanent level variations were recognized one-day post-exposure to, noise, Ag-NPs, and both noise and Ag-NPs. The timeline is shown in Fig. 1.

2.6. Biomarkers of oxidative stress analysis

Blood samples were obtained and the serum specimens were stored at - 80 0C for biochemical analysis. The serum concentrations of MDA and SOD activity were determined with a commercial assay kit (Zell Bio, Germany) following the manufacturer's protocol.

2.7. Gene expression

Immediately after the last day of exposure (day 29), all animals were sacrificed using CO2. They were then beheaded and their skulls underwent the surgery operation along the sagittal midline. The brains were exposed and extracted and the inner ears were separated from the temporal bones. The bony capsule of the cochlea was carefully removed under a dissection microscope. Each cochlea was then used to measure the TNF-α, IL-6 and, NOX3 gene expression levels

(45). Total RNA of cochlear tissue collected from all animals was also extracted using Trizol reagent (Kiazist, Iran) according to the instructions of the manufacturer. Thereafter, cDNA was synthesized with the easy cDNA Synthesis Kit (Parstous, Iran). GAPDH was used as an internal control. The housekeeping gene GAPDH operated as an internal reference to normalize gene expression levels of pro-inflammatory cytokines and oxidative stress mediators. The gene sequences are displayed in Table 1.

A major mixture of PCR-reactive components at final concentrations was shown as follows: 3 µl water, 0.5 µl forward primer (Sinaclon, Iran, 10 µM), 0.5 µl reverse primer (Sinaclon, Iran, 10 µM), and 5.0 µl SYBR Green I real-time PCR MasterMix (addbio, Korea). The total volume for reverse transcription-quantitative PCR (q-PCR) analysis was 10 µl, consisting of 9 µl of the master mix and 1 µl of cDNA, was added which were filled into glass capillaries and prepared for analysis. The thermocycling conditions were executed according to the device program: initial denaturation 95˚C for 5 min, followed by amplification 40 cycles at 95˚C for 15 sec, 60 ˚C for 15-20 sec and 72 ˚C for 15-30 sec. subsequently, a melt curve was conducted to investigate the products generated, which started at 95˚C for 15 sec, then 65˚C for 1min, and finally a cooling step to 30˚C for 20 sec. The relative gene expression level was measured using the previous method (46).

2.8. Histological analysis of the cochlear tissues

Having performed the DPOAE measurements, the cochleae of the rats were immediately harvested from the temporal bone and fixed in 10% formalin for 24 hrs. These cochleae were then decalcified (10% nitric acid solution (2-37-7697| Sigma); and the decalcification solution was washed out with water. The tissues were fixed and embedded in paraffin wax. The samples were then sliced into semi-thin sections of 5 µm using a rotary microtome. These were then transferred to a glass slide, and stained in hematoxylin and eosin (H&E). The sections were mounted by Entellan (07961.1- Sigma) for the morphological assessment. Finally, each sample was inspected and photographed by who made use of a light microscope (LABOMED). All of the pathologists were unaware of the grouping of samples.

2.9. Immunohistochemistry (IHC)

the expression of myosin7a (MHC7), isoform and beta tubulin (TUJ1) were assessed by IHC. The cochleae of the rats were immediately extracted and samples were fixed with 4% paraformaldehyde in PBS (P4417-Sigma) at 4 ◦C. Afterwards, the cochleae were decalcified in 10% nitric acid solution, and excess bone was cut away using scalpel and steel forceps. After rinsing in water, the soft tissue of the cochleae was dehydrated and embedded in paraffin block as mentioned previously (47). The cochleae were sectioned serially at a thickness of 5 µm and mounted on silane-covered slides. Immunochemistry staining was carried out under the guided procedure. Briefly as follows; after dewaxing, and rehydrating, in order to execute the antigen retrieval sections, slides were immersed in TBS 1 X solution (Sigma-T5912) and stored inside a microwave oven. upon reaching the boiling point, the microwave oven was switched off the and the samples remained there for 20 minutes. 0.3% Triton (T8787-Sigma) was added to the samples to permeabilize the membrane for 30 minutes followed by using10% goat serum (G9023-Sigma) to block the non-specific binding sites. Next, the sections were incubated by anti-MHC7 and anti-tuj1antibodies. Alex Fluor 488- labeled secondary antibodies (Secondary: orb688924) was used for 60 min at 37 C in a dark room. Eventually, samples were counterstained with 0.1µg/ml blue-fluorescent 4, 6-diamidino-2-phenylindole (blue fluorescence, 1:500, DAPI, D9542-Sigma) for 20 min in the dark at room temperature. Finally, All samples were photographed, and the expression of TUJ1 and MHC7 (green fluorescence) were identified using a fluorescent microscope (Olympus Corporation, Tokyo, Japan). Then positive areas were randomly taken and investigated using Image-Pro Plus 6.0 software.

2.10. Statistical Analysis

SPSS software (version 19) was used to analyse the DPOAE data. The normality of the data was checked using Shapiro-Wilk test for each group and frequency. In order to simultaneously compare the groups and times, in frequencies where there was no significant difference among groups before the intervention, we used the repeated measures ANOVA and BONFERRONI post hoc test. However, for the last frequency, where there was a significant difference among groups before the intervention, ANCOVA was used along with BONFERRONI post hoc test; so that the differences among the values were controlled. Paired t-test was employed to compare the pre and post mean differences in each frequency and each group. The same test was also employed to compare the difference among the values of mean body weight before and

after the exposures. A p-value of less than 0.05 ($p < 0.05$) was accepted at a statistically remarkable level. The rest of the data was analysed using Graphpad Prism software version 8.

3. Results

3.1. Animals physical appearance and body weight

During the exposure and treatment periods, no signs of depression, irritation, hyper-responsiveness, as well as other physical symptoms were observed among the rats. Moreover, no mortality of the subjects was seen during this research.

Fig. 2 shows the comparison of the difference between the initial mean weight and the final mean weight of the rats in the control group (81.25±14.63, P <0.001) the Ag-NPs group (38.16 ± 27.13, p = .018), the noise group (7 ± 7.4, p = .026) and the Ag-NPs plus noise group (22.33±17.44, P = .068).

3.2. Characterization of the Ag-NPs

Two methods of analysis (SEM and XRD) were used to study the size, shape, phases, crystallite size as well as the size distribution of the Ag-NPs. The results from SEM (a) and XRD (b) analyses are presented in Fig.3.

3.3. DPOAE Recordings

Tables 2 to 6 did not show any significant changes in the mean DPOAE level one day before exposure in all frequencies and groups, except at frequency 9680. Nonetheless, three days after the last encounters, significant changes in DPOAE levels were observed between groups in all frequencies. In all frequencies and groups except the control group, the comparison of the groups before and after the intervention revealed a significant difference in each frequency. Except for the frequency 4840, the most significant effect was observed in the groups 4, 2, and 3, respectively, at all frequencies. The noise exposed group, the Ag-NPs group and the combined Ag-NPs plus noise group showed a significant difference in all frequencies than the control group. Comparing the Ag-NPs group and the Ag-NPs plus noise showed a significant difference in the frequencies of 4840 Hz and 7260 Hz.

3.4. Measurement of MDA and SOD

As illustrated in Fig. 4 (A, B) oxidative stress markers including MDA, and SOD levels were measured in serum after the treatment for 4 weeks. The serum MDA levels in the Ag-NPs group (P = 0.0450), the noise group (P=0.0108), and the noise plus Ag-NPs group (P = 0.0010) were significantly higher than the control group. The serum SOD levels in the Ag-NPs (P = 0.0425), noise (P = 0.0089) and noise plus Ag-NPs (P = 0.001) group showed a significant decrease than the control group. The combined effects of noise plus Ag-NPs were found to be more severe and toxic than the other groups.

3.5. Expression of inflammatory genes and oxidative genes in the cochlea

The relative expression of cochlear inflammatory and stress genes, which was determined by RT-PCR 4 weeks following the treatment, was remarkably enhanced in the noise plus Ag-NPs group compared to the control group ($p < 0.05$). The results from mRNA expression (Fig. 5.A-C) analyses demonstrated an upregulation of NOX3, TNF-α, and IL-6 gene expression in the experimental groups as compared to the control group.

3.6. Morphological observations

Histological photomicrographs of the inner ear cochlear duct were gained using H&E staining under a light microscope (Fig. 6A-D) Based on the obtained results from the control group, it was found that the inner hair cells (IHCs) are in one row and the outer hair cells (OHCs) are in three rows, covered by the tectorial membrane (TM) and are regularly located on both sides of the Corti tunnel. In addition, the inner pillar cells (IPC) and outer pillar cells (OPC) that make up the inner and outer edges of the tunnel were in place in a regular manner. The nerve fibres (NF) exiting the OHCs were seen by passing through the tunnel canal and joining NF of the IHCs as a nerve at the end of the HCs. On the other hand, IHCs and OHCs ' supporting cells were well visible beneath the IHCs and OHCs. The basilar membrane (BM) was visible as a thin layer beneath the HCs and supporting cells and the TM was visible in contact with the HCs (Fig.5A). In the noise exposure group, the images showed the destruction of HCs, in addition to the unregularly arrangement of the HCs, and the HCs were located on the support cells and completely lost (Fig.5B). In the group that received Ag-NPs, NF extruding from the ends of OHCs were visible inside the tunnel. However, due to the apoptosis of supporting cells of the HCs, the internal cavity of the Corti tunnel was larger. The morphology of the tunnel was also in disarray. Moreover, the relationship between supporting cells and IHCs

and OHCs were reduced. However, the relationship between the cilia of IHCs and TM was established (Fig.6C).

In the noise and Ag-NPs group, an obvious disruption was observed in IHCs and OHCs. The rate of destruction of IHCs was higher than that of OHCs, and also, the IHCs and supporting cells could not be distinguished visibly. The BM was thick and damaged in some areas relative to the control group. The cortical tunnel and the cells of the tunnel wall were destroyed and the inner cavity of the tunnel degenerated. No association was observed between TM and HCs (Fig.5D).

3.7. IHC observations

The results from applying IHC method on the rats' cochlea showed that sub-chronic co-exposure to the noise and Ag-NPs contributes to the hair cell damage and degeneration of ganglion cells 30 days after the treatment. In order to investigate these lesions, the expression of proteins in hair cells (myosin 7 (MHC7)) and ganglion cells (betatubulin (TUJ-1)) in the cochlea was tested.

In compared with the control group, the IHC testes indicated that the expression of MHC7 (green) protein in hair cells and TUJ1 (green) in ganglion cells were significantly decreased in the noise, Ag-NPs and noise plus Ag-NPs groups.

Fluorescent stain, 4',6-diamidino-2-phenylindole (DAPI) was employed for staining the nucleus of cells (blue) and evaluating the results. The number of green cells was reported as a percentage of the total number of blue cells. Alterations in MHC7 protein expression occur following the loss of HCs, and eventually, degeneration of ganglion cells in the cochlea is followed by loss of HCs leads to expression of TUJ1 protein. As shown in Fig. 6A-D, in the noise, Ag-NPs group, as well as in the noise plus Ag-NPs group, the colour intensity of the images decreased, which is expressed in quantitative analyses in the graphs (Fig. B and D).

Images from IHC tests on the noise, Ag-NPs and noise plus Ag-NPs groups showed that the percentage of protein expression in HCs (MHC VII) and neurons (TUJ-1) were significantly decreased ($P < 0.001$). For noise plus Ag-NPs group, the expression rate showed a greater decrease than the other groups and the colour intensity of the images was lower. The data in the graphs are expressed as standard deviation ± mean.

4. Discussion

The present study for the first time reported that an exposure to noise and Ag-NPs either alone or concomitantly contribute to biochemical changes in serum as well as functional, morphological and IHC alterations in cochlear cells of the rats. Our results provide solid in vivo evidence about the toxic effects of Ag-NPs and noise on cochlear cells. The combined effects of noise and Ag-NPs cause more severe damage to the hair and nerve cells responsible for perceiving the higher noise frequencies; thus leading to permanent hearing loss.

4.1. Body weight changes

We found a significant increase in the body weight in all groups, except in the Ag-NPs plus noise group. However, compared to the control group, weight gain was observed to a lesser extent in the noise plus Ag-NPs group. These findings would therefore indicate that combined exposure to noise and Ag-NPs is a strong stressor as being approved by the effect on the weight, and are in consistent with a research that showed chronic exposure to noise and dexamethasone-treated rats induce decreased body weight gain and food intake (48). Yin et al also reported that Ag-NPs induced a significant reduced body weight (49).

4.2. DPOAE test

Despite the large amount of experimental and clinical studies performed on the contributing factors of hearing loss, and ototoxicants (50), well-grounded knowledge about the risk of exposure to nanomaterials (51) and the effects of toxicity on the inner ear of the animals and humans is still lacking; and sufficient data is unavailable to assess the risks of combined exposure (52). Therefore, this study investigated the impacts of administration of Ag-NPs and exposure to intense noise on the structure and function of cochlea in the rat.

Our findings showed that compounds, which are not inherently ototoxic might expand the risk of developing NIHL when combine with exposure to intense noise levels. Other studies indicate that co-exposure to white noise (102 dB SPL, 8–10 h a day) centred at 8 kHz for 10 days and cigarettes smoke, resulted in decreasing the DPOAE amplitudes only 1-day post combined exposure. Despite the fact that the noise or smoking alone reduced DPOAE amplitudes, the combination of the two contributed to a long-term reduction (44).

In the present study the DPOAE recordings of the rats exposed to the noise pressure level of 104 dB centred at 8 kHz frequency were determined at 5 frequencies. The results showed that at 8 kHz frequency there was a slight increase in hearing loss. Having observed the alterations in the hearing levels of the animals, it is also discernible that the impacts of the noise intensity is more dominant than that of effects of frequency; that is, it has a more prominent role in causing damage than sound frequency.

We also emphasize that the reduction of the relationship between the frequency bands of the intense noise and the hearing loss frequency does not deny the achievement of a significant practical plan to control the frequency bands that bear the various intense noise levels.

These findings are confirmed by the study of Escher Boger et al., (2009) regarding the effects of noise spectrum on the prevalence of hearing loss. They showed that intense noise, regardless of frequency range, is a major risk factor for inducing hearing loss (53). Another similar study was performed on the auditory system of truck drivers who were exposed to intense sound with low-frequency. The results from this study showed that drivers experienced hearing loss similar to workers in industrial settings and this indicated the importance of intense noise relative to the frequency band(54).

The results from the DPOAE recordings showed that the noise plus Ag-NPs group had a greater decrease in DPOAE levels and permanent hearing loss; and DPOAE changes were more evident in the frequency of 8 kHz. We found considerable changes in DPOAE levels in the Ag-NPs plus noise group, noise group and Ag-NPs in all frequencies except the frequency 4840 Hz. In a similar study, mice exposed to 145 dB of impulsive noise showed reduced DPOAE amplitude 30 minutes after exposure to the noise. Four weeks later, the DPOAE amplitude returned to normal at the higher frequency range (8 to 32 kHz). At lower frequencies, a small degree of PTS remained(55). Another study showed that long-term exposure, with a sound in the range of 65 to 70 dB and a frequency of 16 to 20 kHz, affected the cochlea and central auditory system of male Sprague-Dawley rats, which was evident in the DPOAE range as well (56). Additionally, Nasiri et al. (2016) showed that noise levels of 65 dB, 85 dB, 95 dB and 105 dB for 3 hours and 8 hours per day showed a negative correlation between SNR values and noise intensity, i.e. whatever the higher the volume, the lower the SNR value obtained(57). Exposure to chemical

compounds such as polychlorinated biphenyls (PCBs DPOAE levels (58).

4.3. Changes in biochemical markers

Furthermore, enzyme measurements play a fundamental role in toxicological assessment (59). A previous study on textile workers reported that exposure to noise elevated MDA concentration as well as decreased SOD levels, which could be due to excessive oxidative stress in the cochlea of the rats (60). It has also been revealed that animals exposed to Lead acetate (gavage, 4 mg/kg) and noise (105 dB, 4 kHz) exposure for 30 days showed a significant increase in MDA level and significantly reduced TAC level in the serum (61). A study by Sabah Ansar et al (2017) showed that MDA level significantly enhanced and SOD activity, glutathione (GSH), catalase (CAT), and glutathione peroxidase (GPx) levels decreased in serum in the Ag-NPs (5 mg/kg/b.w, i.p)-treated rats. Consistent with these findings, our data showed that the rats were treated with Ag-NPs, and exposed to noise exhibited increase in MDA and decrease in SOD than the control group. These changes may be due to the increased levels of free radical formation and consequentially expresses the risk of injury to the cells and tissues. Extensive evidence illustrates that the inner ear is an active immune member, not a "specific immunological organ" that was earlier commonly accepted(62).

4.4. Changes of mRNA expression of inflammatory and oxidative

Our findings also showed that following exposure to high-intensity noise and Ag-NPs treatment could lead to the generation of ROS and an inflammatory response that is identified by mRNA expression TNF-α, IL-6, and NOX3 in the overstimulated cochlea would occur. These studies suggest that the expression of these genes and oxidative stress plays a fundamental role in the pathogenesis of noise and Ag-NPs that induce cochlear damage and hearing dysfunction. These findings are almost compatible with other research which had found that exposure to loud noise would lead to pro-inflammatory cytokines response such as formation of TNF-a, IL-1b, and IL-6 in cochlea (29). A previous study reported that the absence of NOX3 in the inner ear enhanced the susceptibility to noise damage (45). It has been proposed, meanwhile that cisplatin caused ototoxicity and NOX3 has been described as an inducing agent for other genes that sequentially contribute to the cochlear injury(45).

4.5. Morphological changes

Previous studies on Wistar rats indicated that overexposure to noise (0.5–32 kHz, 118 dB SPL) for 4 h/day in 4 consecutive days resulted in long-lasting hearing damage and peripheral, HCs loss, reactive glia, and central inflammatory reactions (63). Similarly, exposure to continuous noise (100 dB, 10 kHz, 1h) during ten days resulted in OHC loss in the basal turns and middle ear, changes in the integrity of SGN, and stereocilia destruction among rats. In another study, the toxic effect of Ag-NPs (20 μg/ml, 4000 μg/ml) on the inner, middle, and external ear canal of the rats were investigated after transtympanic injection and BALB/c 3T3 cell line showed dose-dependent functional and structure alternations. Moreover, the BALB/c 3T3 cell line was found to be more sensitive than the in vivo researches (64).

Alternatively, the noise exposure and Ag-NPs treatment model in the present study is different from the one applied in the aforementioned studies, as its adverse effects on the structure and function of cochlear cells are in line with the former reports. Comparing the morphological changes between the Ag-NPs-treated and noise-exposed groups; the destructive effects of noise were more evident, but these two groups maintained their cochlear structure better than the noise plus Ag-NPs group. Our findings confirm the results from the various morphological investigations, which have pointed out the probability of inflammatory alterations in the cochleae overstimulated by the noise (26, 29, 65, 66). The effects of exposure to excessive noise are usually considered as irreversible injuries and sensory HCs and SGCs loss in the cochlea that in turn contributes to the PTS (67).

4.6. IHCs test

The current study has highlighted MHC7 and Tuj1 downregulation in both hair cells and ganglion cells in the noise, Ag-NPs and noise plus Ag-NPs groups. Furthermore, the IHCs analysis revealed that extremely weak staining existed in the group exposed to noise, Ag-NPs, noise plus Ag-NPs. Such a decrease has been indicated to change the cochlear structure resulting in a permanent change in the auditory function as well.

Conclusion

Our findings demonstrate that an exposure to noise and Ag-NPs nanoparticles caused oxidative stress in the cochlea and led to the hearing loss of the rats. This was particularly evident at higher frequencies of the noise (above 4 kHz). The results from the SNR ratios of DPOAE test from the exposed groups (either

independently or simultaneously) showed that compared to day zero (baseline), a measure of permanent hearing loss had occurred three days after exposure. Exposure to noise exerted more adverse impacts on the auditory system compared to Ag-NPs at all frequencies.

The results also highlighted the fact that the combined exposure to Ag-NPs and noise contributed to a greater decline in SNR ratios in comparison to the other three groups. The largest decline was observed in the combined exposure and the frequency of 8470 Hz (40.66 ± 4.32), which can be attributed to the effect of the frequency of 8 kHz contact. However, at higher noise levels (104 dB) the effects of the frequency are less predominant as the hearing loss is observable for the other frequencies. Ag-NPs with a size range of 30-50 nm are more likely to have an additive impact on the effects of noise on the auditory system. Both types of exposure (alone as well as combined) to Ag-NPs and noise reduce the DPOAE levels. In addition, such exposures induce hair and nerve cells disturbing the perceiving of the high-frequency sound. Also, the effects of combined exposure exert more severe effects on the cochlea at higher frequencies and lead to permanent hearing loss, which confirms the results from morphological, gene expression, immunohistochemistry and biochemistry findings. However, beyond the extent of this paper, it is essential to note that future study would be required to study the expression of more proteins and genes, examination structural and functional in the cochlea of different animal models to further identify genes prone to damage for gene therapy studies.

Moreover, in order to prevent the hearing loss among the industrial workers, further studies should investigate the possible effects of the combined exposures on the hearing system.

Author contributions

All authors participated in all parts of the study.

Acknowledgments

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Conflict of Interest

The authors declare that they have no conflicting interest to the research, authorship, and/or publication of this paper.

Ethical standards

The Ethical Committee on Animal Research of Tarbiat Modares University evaluated and approved all experimental protocols. (Number: IR.MODARES.REC.1400.100).

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Fig.1. timeline of the experimental procedures

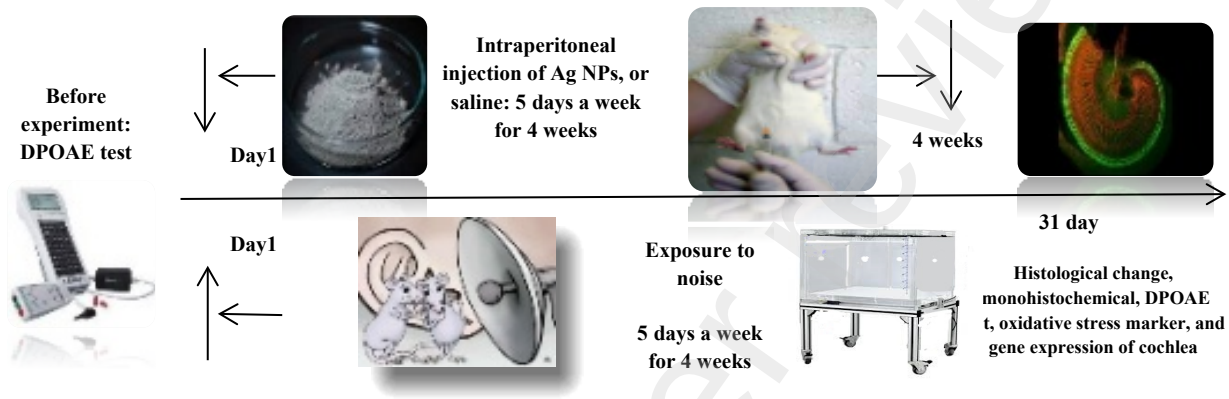

Table1. Forward (FW) and reverse (RW) primer sequence, used in reverse transcription quantitative PCR (q-PCR)

primer name	5'-3' primer sequence	Accession Number	Location	Amplicon length (bp)
IL-6	FW: AGGCAGAGTCATTCAGAGC RW: CATTGGTAGTTGGGGTAGGA	NM-012589.2	478 578	101
TNF-α	FW:GAGATGTGGAAATGGCAGAGGA RW:GAGAAGATGATGTGAGTGTGAGG	NM-012675.3	167 397	231
NOX_3	FW:AAGGCATTTGGAGCAGAGGGA RW: ACCCGGCAGATCCAGTAGAAG	NM_001004216.1	1093 1334	242
GAPDH	FW: AGGTCGGTGTGAACGGATTTG RW: TGTAGACCATGTAGTTGAGGTCA	NM-017008.4	83 205	123

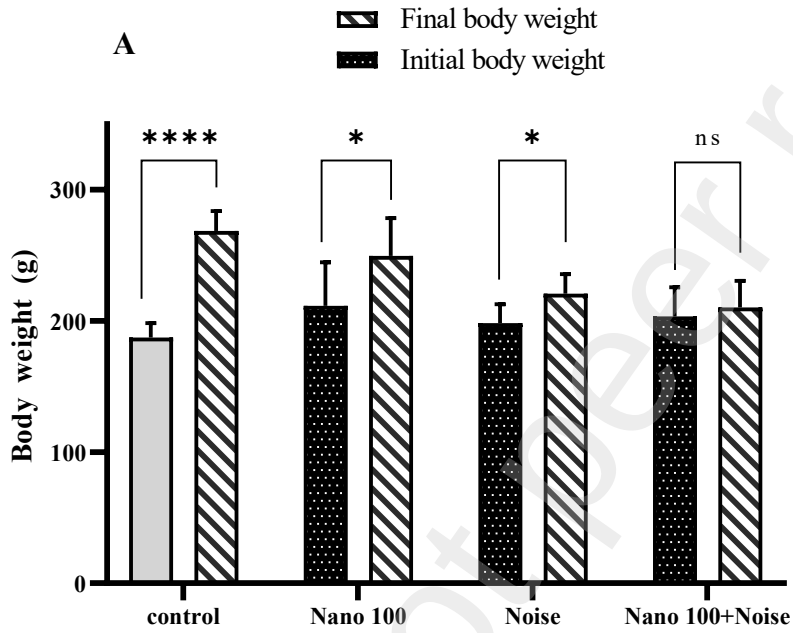

Fig2.Effects of Ag-NPs and noise on initial and final BW of the rats .Only the rats in the control and noise groups showed significant increase in the body weight. Data are expressed as mean ± SD (n=6). **** P < 0.001, *p=.026 and *p=.018.

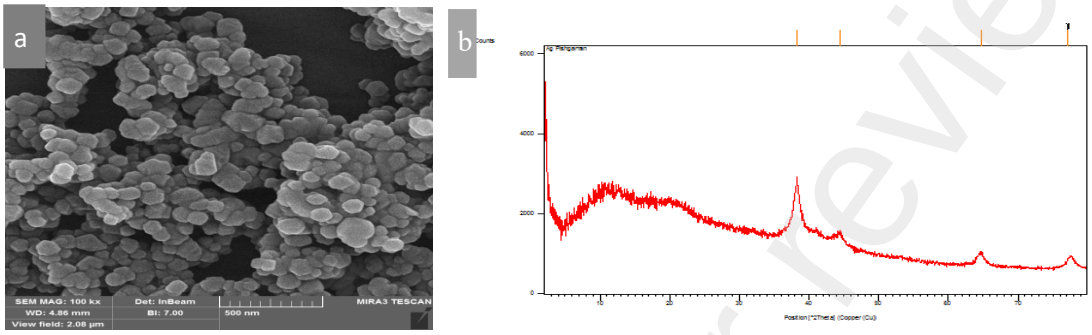

Fig3. SEM (a), and XRD (b) images of Ag-NPs 30-50

Table2: Mean and SD values of the DPOAE recordings at 4840 Hz in all groups. Bold italic values represent statistical differences.

4840 Hz	Groups	Pre exposure (Mean ± SD)	Post exposure (Mean ± SD)	Difference Mean±SD	P_value (Paired-Ttest) For comparing mean of DPOAE before and after intervention	**Multiple comparisons**
Control(C) Noise(N) Ag-NPs 100mg/kg (N100) Noise + Ag NP s(N+N100)	I II III IV	30.50±4.28 31.00±1.79 27.83±8.26 28.67±3.33	29.00±2.10 12.67±2.34 11.33±3.50 2.67±1.37	-1.50 ± 4.7 -18.33 ± 2.65 -16.50 ± 9.24 -26.00 ± 2.68	0.472 **<0.001** **0.007** **0.001**	**C and N(P<0.001)** **C and N100(P=0.001)** **C and N100+N(P<0.001)** N and N100(P=1.000) N and N100+N(P=0.156) **N100 and N100+N(P=0.045)**
P value for between-group		0.664	**<0.001**			

Table3: Mean and SD values of the DPOAE recordings at 6050 Hz in all groups. Bold italic values represent statistical differences.

6050 Hz	Groups	Pre exposure (Mean ± SD)	Post exposure (Mean ± SD)	Difference Mean±SD	P_value (Paired-Ttest) For comparing mean of DPOAE before and after intervention	**Multiple comparisons**
Control(C) Noise(N) Ag-NPs 100mg/kg (N100) Noise + Ag NP s(N+N100	I II III IV	37.00±5.62 41.00±2.28 40.00±5.21 34.00±4.14	37.00±4.19 8.83±2.78 11.00±1.78 2.50±2.94	0.00 ± 3.63 -32.16 ± 2.31 -29.33 ± 7.96 -31.50 ± 6.37	1.000 **<0.001** **<0.001** **<0.001**	**C and N(P<0.001)** **C and N100(P<0.001)** **C and N100+N(P<0.001)** N and N100(P=1.000) N and N100+N(P=1.000) N100 and N100+N(P=1.000)
P value for between-group		0.057	**<0.001**			

Table 4: Mean and SD values of the DPOAE recordings at 7260 Hz in all groups. Bold italic values represent statistical differences.

7260 Hz	Groups	Pre exposure (Mean ± SD)	Post exposure (Mean ± SD)	Difference Mean±SD	P_value (Paired-Ttest) For comparing mean of DPOAE before and after intervention	**Multiple comparisons**
Control(C) Noise(N) Ag-NPs 100mg/kg (N100) Noise + Ag NP s(N+N100	I II III IV	41.33±5.31 44.00±4.60 42.66±8.52 41.16±2.22	40.33±4.13 7.33±2.73 13.33±2.50 2.83±1.94	-1.00 ± 3.34 -36.66 ± 4.27 -29.33 ± 7.96 -38.33 ± 1.63	0.497 **<0.001** **<0.001** **<0.001**	**C and N(P<0.001)** **C and N100(P<0.001)** **C and N100+N(P<0.001)** N and N100(P=0.103) N and N100+N(P=1.000) **N100 and N100+N(P=0.028)**
P value for between-group		0.804	**<0.001**			

Table 5. Mean and SD values of the DPOAE recordings at 8470 Hz in all groups. Bold italic values represent statistical differences.

8470 Hz	Groups	Pre exposure (Mean ± SD)	Post exposure (Mean ± SD)	Difference Mean±SD	P_value (Paired-Ttest) For comparing mean of DPOAE before and after intervention	**Multiple comparisons**
Control(C) Noise(N) Ag-NPs 100mg/kg (N100) Noise + Ag NP s(N+N100)	I II III IV	44.50±3.33 43.66±6.80 44.66±8.23 42.33±3.88	43.50±2.42 4.50±2.25 13.00±3.40 1.66±0.81	-1.00 ± 4.60 -39.16 ± 7.90 -31.66 ± 8.35 -40.66 ± 4.32	0.618 **<0.001** **<0.001** **<0.001**	**C and N(P<0.001)** **C and N100(P<0.001)** **C and N100+N(P<0.001)** N and N100(P=0.370) N and N100+N(P=1.000) N100 and N100+N(P=0.166)
P value for between-group		0.899	**<0.001**			

Table 6. Mean and SD values of the DPOAE recordings at 9680 Hz in all groups. Bold italic values represent statistical differences.

9680 Hz	Groups	Pre exposure (Mean ± SD)	Post exposure (Mean ± SD)	Difference Mean±SD	P_value (Paired-Ttest) For comparing mean of DPOAE before and after intervention	**Multiple comparisons**
Control(C) Noise(N) Ag-NPs 100mg/kg (N100) Noise + Ag NP s(N+N100	I II III IV	42.66±7.20 39.33±2.58 45.83±2.56 38.50±2.07	42.00±6.92 6.33±2.50 13.50±3.88 3.00±1.26	-0.66 ± 1.21 -33.00 ± 3.46 -32.33 ± 3.93 -35.50 ± 1.76	0.235 **<0.001** **<0.001** **<0.001**	**C and N(P<0.001)** **C and N100(P<0.001)** **C and N100+N(P<0.001)** N and N100(P=1.000) N and N100+N(P=0.850) N100 and N100+N(P=0.401)
P value for between-group		**0.024**	**<0.001**			

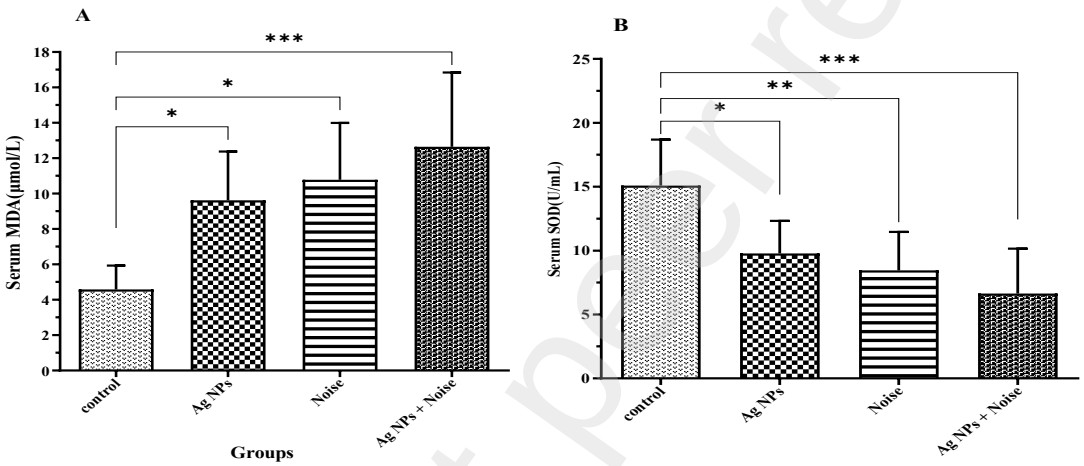

Fig.4. MDA (A) levels and SOD (B) activities of the control group (1) as well as the groups exposed to 100 mg/kg of Ag-NPs, noise and their combination (groups 2, 3 and 4, respectively). The values are expressed as means ± SD. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ vs. control group

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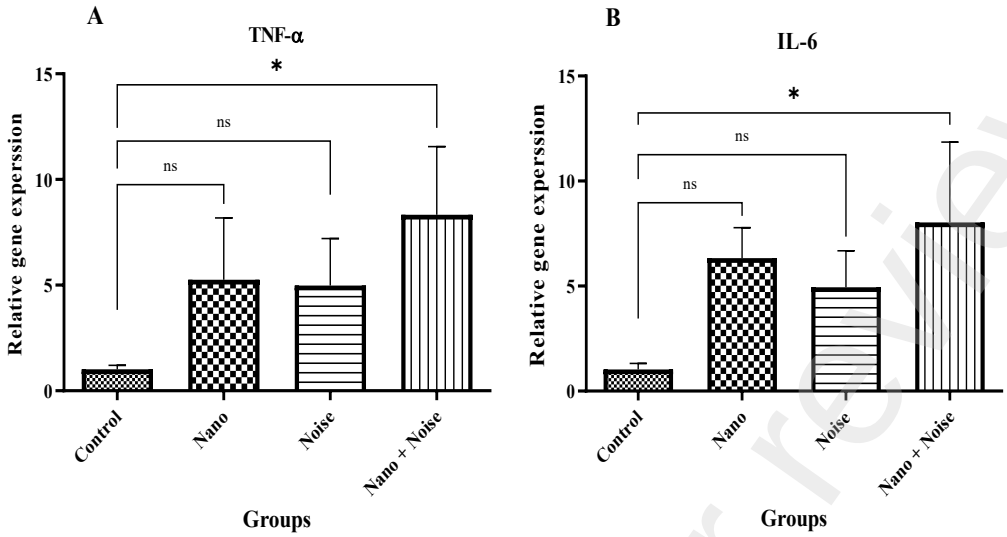

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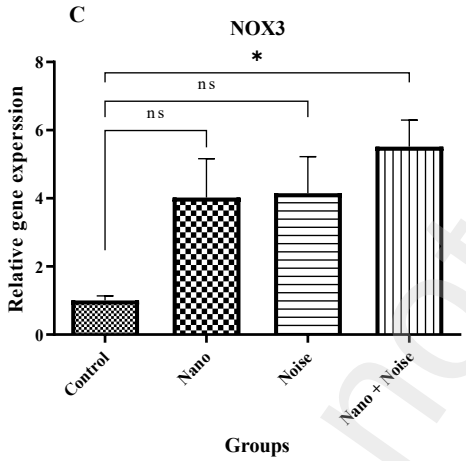

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982 Fig5. A-C. The effects of Ag-NPs 30-50 nm, noise and both noise plus Ag-NPs on proinflammatory cytokine
983 (A= TNF-α, B= IL-6) and stress gene expression (C= NOX3) in Cochlea rat. Data are shown as mean ± SD.
984 Significance was set at *P < 0.05 vs. control group.

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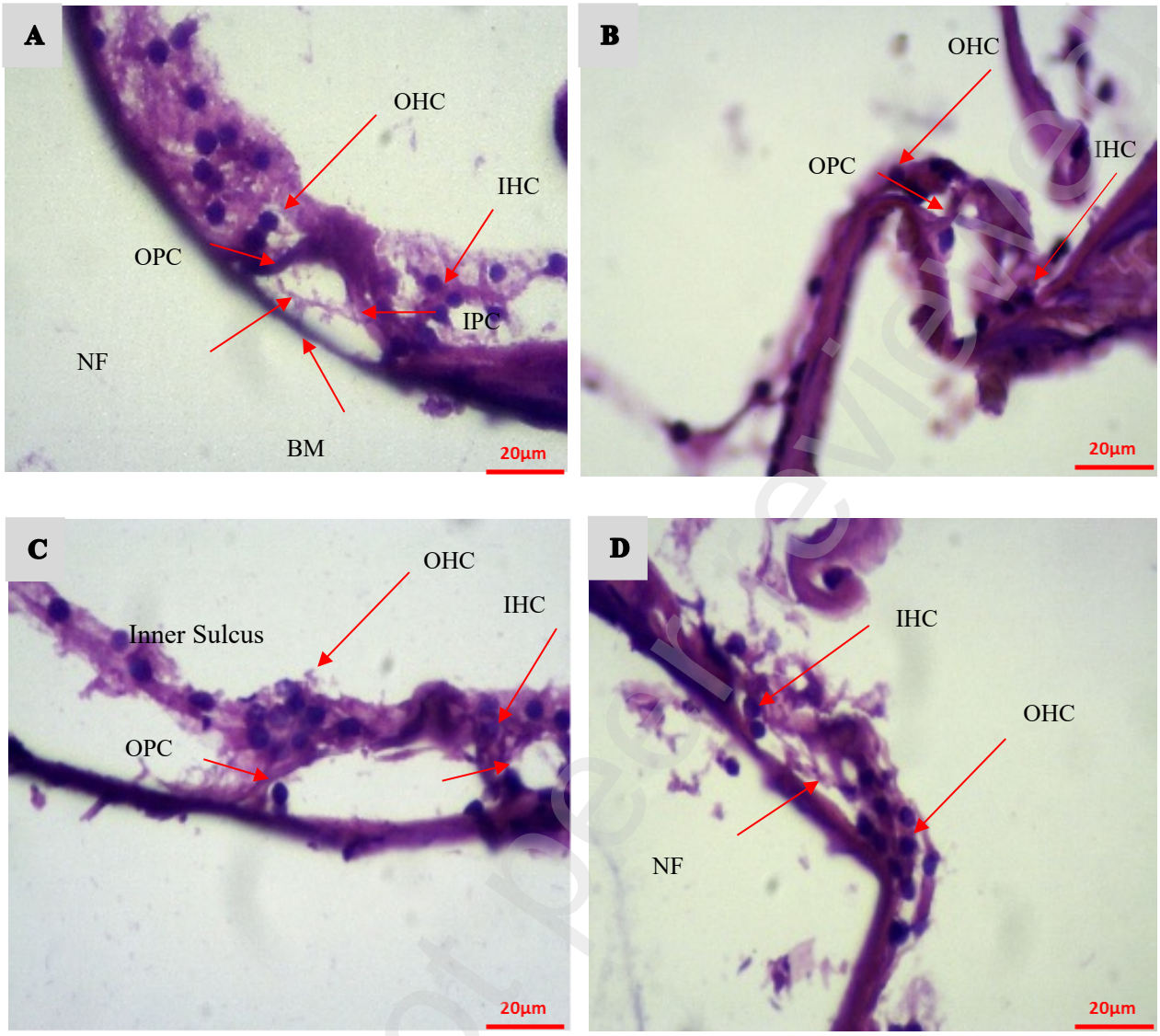
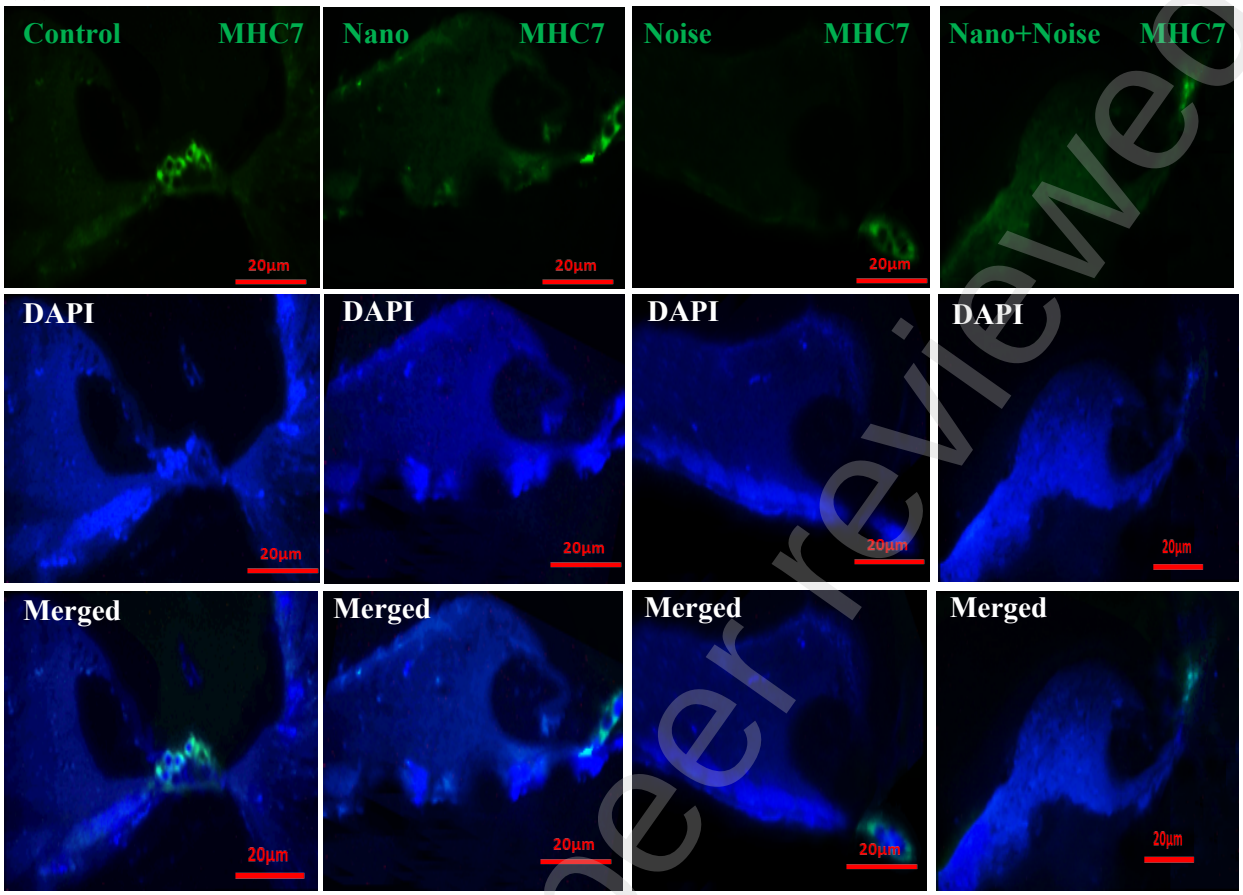
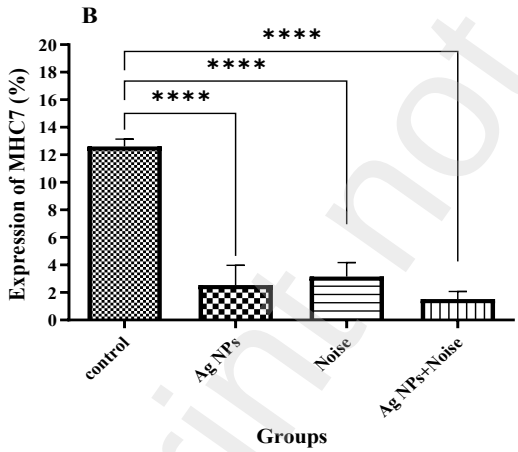


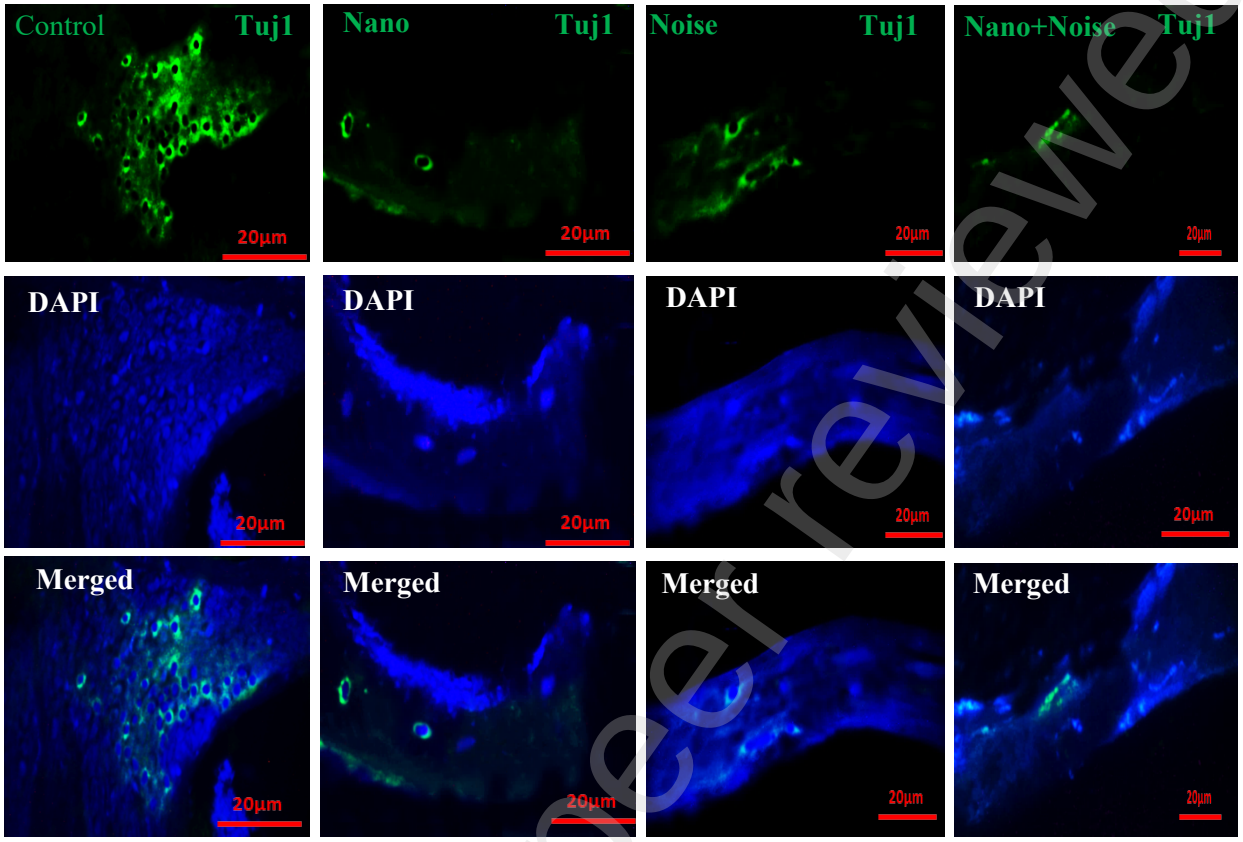
Fig.6A-D. Histopathological examination of the inner ear cochlear in Rat. A) Control group, B) Noise, C) Ag-NPs, D) Noise + Ag-NPs groups. The control group exhibited normal architecture. In the noise exposure, the group and Ag-NPs treated group showed disorganized structure in OHCs, IHCs, OPC, IPC, and NF. Both Noise plus Ag-NPs treated group showing damage was more severe than other groups (red Arrow). Scale bar represents 20 µm

(A)





(C)



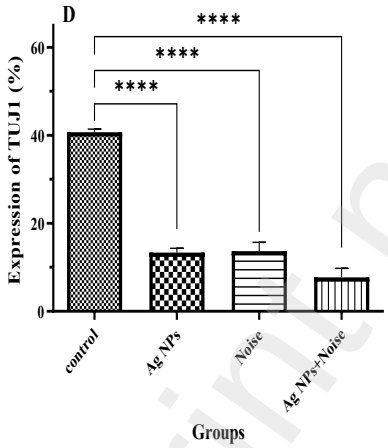


Fig.7 A-D. Effects of Noise, Ag-NPs, and both noise plus Ag-NPs on MHC7 and TUJ1 protein expression. A, C) MHC7 and TUJ1 were stained with green color, cell nucleus was labelled by DAPI with blue color and then merged. Images indicated the positions of MHC7 and TUJ1/DAPI/merged. Scale bars = 20 μm. B, D) Analysis of MHC7 and TUJ1 protein expression. The MHC7 and TUJ1 protein expression level was down-regulated as compared to the control group. ****P < 0.001. Data are shown as mean ± SD.