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Research Article
Otoprotective Effect of Nimesulide: Biochemical and Histopathologic Evaluation

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Abstract

Background and Objective: It is known that nimesulide reduces the toxic effects of Non-Steroidal Anti-Inflammatory Drugs (NSAID), eliminating their inhibitory effects on cyclooxygenase-1 (COX-1) enzyme. COX-1 enzyme inhibition is thought to be responsible for aspirin ototoxicity therefore current study aimed to investigate the effect of nimesulide on aspirin ototoxicity in rats. **Materials and Methods:** Nimesulide 100 mg kg^{-1} orally was administered to the nimesulide+aspirin group (NASA) and the same volume of distilled water as solvent was administered to the aspirin group (ASA) and the healthy (HG) group. Aspirin at a dose of 1000 mg kg^{-1} was administered orally to the NASA and ASA group one hour after the administration of nimesulide and solvent. This procedure was repeated once a day for 7 days. Then, the animals were euthanized with a high dose of anaesthesia (50 mg kg^{-1} thiopental sodium) and their cochlea and cochlear nerve tissues were removed. **Results:** Malondialdehyde (MDA) level was significantly higher and total glutathione (tGSH) and cyclooxygenase-1 COX-1 levels were significantly lower in the ASA group in which prominent histopathological damage was found in the cochlea and cochlear nerve, compared to the HG and NASA group. The COX-2 level was found to be almost the same in all the groups. This indicates that aspirin reduces the COX-1 and tGSH levels and increases the MDA level, causing ototoxicity. **Conclusion:** Aspirin significantly decreased the COX-1 activity in the cochlea and cochlear nerve tissue, compared to the healthy and nimesulide group. Current study concluded that the co-administration of nimesulide and aspirin will increase the therapeutic effect of aspirin and reduces its side-effects.

Key words: Ototoxicity, nimesulide aspirin, cochlea, otology, rat

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Nimesulide N- (4-nitro-2-phenoxy-phenyl) is a non-steroidal anti-inflammatory drug (NSAD) with molecular formula $C_{13}H_{12}N_2O_5S$ and analgesic and antipyretic properties[1,2]. Therefore, nimesulide is used in the treatment of diseases with pain, fever and inflammation[3]. After the 1990s, it was found that nimesulide is the selective inhibitor of cyclooxygenase-2 (COX-2)[4]. Then, it was found that nimesulide inhibits more selectively COX-2 enzyme than COX-1[5]. The study conducted by Demiryilmaz *et al.*[6] reported that nimesulide inhibits the decrease in COX-1 activity and the increase in COX-2 activity, protecting the liver tissue from ischemia perfusion injury[6]. Moreover, it has been shown that nimesulide prevents stomach damage associated with indomethacin, an NSAID drug[7]. The literature has revealed that NSAID toxicity is a rare but very serious side effect[8]. Drug ototoxicity limits the quality of life of patients after the treatment and causes serious consequences especially for the psychosocial development of children. Ototoxic drugs cause functional impairment or cellular degeneration of the tissues of the inner ear[9]. Ototoxic drugs include aminoglycosides, glycopeptide and macrolide antibiotics, platinum-based anticancer drugs, loop diuretics, quinine and salicylates[10].

Salicylates are the most commonly used drugs worldwide. However, salicylate treatment may cause permanent hearing loss[11]. In the literature, it has been reported that salicylates cause temporary hearing loss and tinnitus, however, some data suggest that long-term treatment may be neurotoxic[12]. As is known, tinnitus or sensorineural hearing impairment is associated with cochleotoxicity[9]. The mechanism of salicylate-related ototoxicity is unclear but abnormalities of the inner ear (cochlea) and eighth cranial nerves are considered as the cause of ototoxicity[13]. The reduced prostaglandin as a result of COX enzyme inhibition is also considered as a cause of salicylate-related ototoxicity[14]. COX-1 is responsible for the synthesis of prostaglandin in organs and tissues, while COX-2 is responsible for the synthesis of proinflammatory prostaglandin. In literature, the inhibition of COX-2 enzyme is associated with the therapeutic effects of NSAID, while the inhibition of COX-1 enzyme is associated with the side effects of NSAID[5].

Aspirin, which is the prototype of salicylates to be used to create experimental ototoxicity in animals in our study, is the salicylate ester of acetic acid[13]. Aspirin is the most commonly used drug as an analgesic, anti-inflammatory and antipyretic among salicylates, which are non-specific to COX-2[5,13]. Aspirin is more effective in inhibiting COX-1 than COX-2 and the inhibition is irreversible[5]. This information from the literature suggested that nimesulide can be effective in reducing aspirin-related ototoxicity. In the literature, it has also been reported that nimesulide protects the stomach tissue against indomethacin oxidative damage[7]. However, there is no research study on the protective effect of nimesulide against aspirin-related ototoxicity.

This study aims to investigate whether aspirin causes injury of the cochlea and cochlear nerve tissue in rats and the protective effect of nimesulide against cochlea and cochlear nerve tissue injury induced by aspirin.

MATERIALS AND METHODS

Study area: The study was carried out at Medical Experimental Application and Research Center of Ataturk University, form September-December, 2020.

Animals: The animals we used in this study were obtained from the Medical Experimental Application and Research Center of Ataturk University. A total of 18 Wistar albino male rats with a weight ranging from 290-308 g were used in the experiment. Before the experiment, the animals were kept and fed at normal room temperature (22°C) in a laboratory environment for one week. Ethics committee approval was received for this study from the Local Animal Ethics Committee of Ataturk University (Ethics Committee Number: 141, Dated: 30.09.2020).

Chemicals: The thiopental sodium was obtained from IE Ulagay-Turkey, nimesulide (100 mg tablet) from Biofarmallac Sanayi veTicaret A.S. Turkey and aspirin (ecopirin 500 mg tablet) from Abdi Ibrahim Ilaç Sanayi-Turkey.

Animal groups: The experimental animals were divided into three groups, healthy control (HG) group aspirin (ASA) group and nimesulide+aspirin (NASA) group.

Experimental procedure: To conduct the experiment, nimesulide at a dose of 100 mg kg^{-1} was administered via oral gavage to the NASA group (n-6) animals. About 0.5 mL of distilled water as solvent was administered orally to the ASA (n-6) and HG (n-6) groups. Aspirin at a dose of 1000 mg kg^{-1} was administered via gavage to the NASA and ASA groups one hour after the administration of nimesulide and solvent. This procedure was repeated once a day for 7 days. Then, the animals were euthanized with a high dose of anaesthesia (50 mg kg^{-1} thiopental sodium) and their cochlea and cochlear nerve tissues were removed. The malondialdehyde

(MDA), total glutathione (tGSH), COX-1 and COX-2 levels were measured on the extracted nerve tissues. The tissues were also examined histopathologically. The findings from the NASA and HG groups were evaluated in comparison with the findings from the ASA group.

Biochemical analyses

Preparing the samples: During this phase of the study, 0.2 g were weighed from each removed tissue. For the MDA assignment, a 1.15% potassium chloride solution was completed to 2 mL in a phosphate buffer that was pH = 7.5 for the tGSH measurement and homogenized in an icy environment. Then, it was centrifuged for 15 min at +4°C, 10000 rpm. Supernatant part was further used for analysis.

Malondialdehyde (MDA) measurement: The MDA measurement was performed according to the method described by Ohkawa *et al.*[15] which was based on the principle of measurement of the optic density of the color at 532 nm, which is formed by the reaction of MDA with thiobarbituric acid in a hot acidic environment. About 25 µL sodium dodecyl sulfate (80 g L^{-1}) and 1 mixture (200 g L^{-1} acetic acid+1.5 mL 8 g L^{-1} 2-thiobarbituric acid) were added to 25 µL sample and the samples were heated at 95°C for 60 min. After being cooled, they were centrifuged at 4000 rpm for 10 min. The absorbance of the upper layer was measured at 532 nm. The MDA content of the sample was calculated from the calibration graph drawn using 1,1,3,3-tetraethoxypropane as standard.

Total glutathione (tGSH) measurement: The tGSH analysis was performed according to the method described by Sedlak *et al.*[16]. When disulfide sulfhydryl groups of DTNB (5,5 -dithiobis [2-nitrobenzoic acid]) that is a color compound are reduced, a yellow compound forms and it is measured at a wavelength of 412 nm. For the measurement, all the samples were treated with meta phosphoric acid at 1:1 for deproteinization and then centrifuged. About 150 µL mixture (5.85 mL 100 mM Na-phosphate buffer, 2.8 mL 1 mM DTNB 3.75 mL 1 mM NADPH and 80 µL 625 U L^{-1} Glutathione reductase) was added to 50 µL supernatant from the upper layer. The measurements were performed at 412 nm according to the standard graph prepared with GSSG.

Measurement of COX activity: Cyclooxygenase activity in rat liver tissue was measured using a COX activity assay kit (Cayman, AnnArbor, MI, USA). Tissue was collected free of ovarian membranes and washed thoroughly with ice-cold Tris buffer, pH 7.4, containing 0.16 mg mL^{-1} of heparin (to remove any red blood cells and clots) and stored at -80°C until assay. A sample of liver tissue was homogenized in 5 mL of cold buffer (0.1 mol Tris-HCl, pH 7.8, containing 1 mmol EDTA) per gram of tissue and centrifuged at 10000 g for 15 min at 4°C. The supernatant was removed for assay measurements and stored on ice. The protein concentration in the supernatant was measured using the Bradford method[17]. The COX kit measures the peroxidase activity of COX. The peroxidase activity is assayed colorimetrically by monitoring the appearance of oxidized N,N,N′,N′-tetramethyl-p-phenylenediamine at 590 nm[18]. COX-2 activity was measured using a potent COX-1-specific inhibitor, SC-560 (the reagent is ready to use as supplied). The results are given as unit per milligram of protein for COX-1 and COX-2 activity.

Histopathological examination: The tissues were fixed in a 10% formaldehyde solution for 72 hrs. After the fixation, the tissues were transferred to cassettes and flushed with running water for 24 hrs, then dehydrated by passing through a series of increasing alcohol concentrations (70, 80, 90 and 100%). The testicular tissues were made transparent in xylol and then embedded in paraffin blocks, from which 4-5micron thick sections were removed. The sections were stained with hematoxylin-eosin and evaluated and photographed using Olympus DP2-SAL firmware program (Olympus® Inc. Tokyo, Japan). The histopathological evaluation was performed by a histology specialist who was blind to the study groups. The severity of histopathological findings in each section was scored between 0-3. 0-normal tissue, 1-mild damage, 2-moderate damage and 3-severe damage.

Statistical analysis: The experimental results were expressed as "Mean±Standard Deviation" (SD). The significance of difference among the groups was determined using a one-way ANOVA test. Then, Fisher's post-hoc LSD (least significant differences) test was performed. All statistical analyses were performed with "SPSS for Windows, 18.0" statistic program and the $p<0.05$ were considered significant.

RESULTS

Biochemical findings

MDA and tGSH analysis results of the cochlea tissue: As it is understood from Fig. 1(a-b), an apparent increase was observed in the MDA content of the cochlea tissue of the animals to which aspirin was administered. The statistical analyses showed that aspirin significantly increased the MDA content compared to the healthy and nimesulide groups

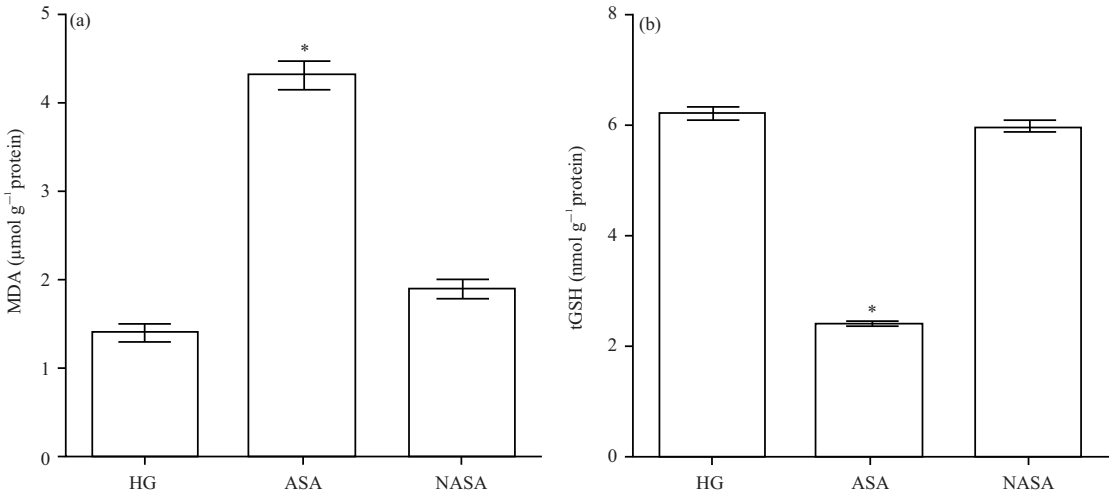

Fig. 1(a-b): Malondialdehyde (MDA) and total glutathione (tGSH) levels in the cochlea tissue of the rats, (a) MDA in the cochlea tissue and (b) tGSH levels in the cochlea tissue, all study groups compared with ASA
*p<0.0001, n = 6

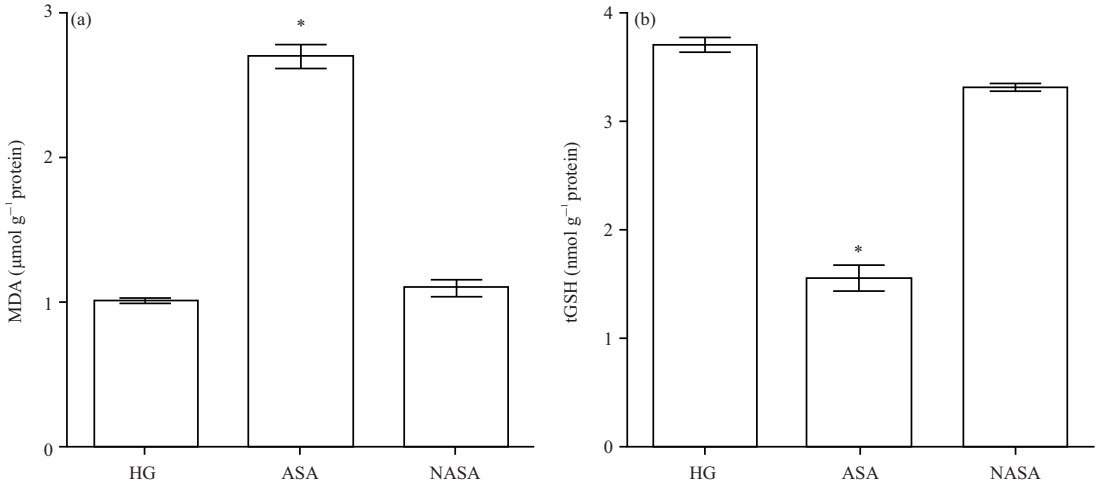

Fig. 2(a-b): Malondialdehyde (MDA) and total glutathione (tGSH) levels in the cochlear nerve tissue of the rats, (a) MDA and (b) tGSH levels in the cochlear nerve tissue, all study groups compared with ASA
*p<0.0001, n = 6

(p<0.0001). However, the difference in MDA content was found statistically insignificant between the nimesulide group and the healthy group (p>0.05). It was found that the tGSH content in the cochlea tissue was significantly lower in the aspirin group than the healthy and nimesulide groups (p<0.0001). However, when the tGSH content was compared between the nimesulide group and the healthy group, statistically insignificant results were obtained (p<0.05).

MDA and tGSH analysis results of the cochlear nerve tissue: The aspirin administration causes an increase in MDA content in the cochlear nerve tissue of the rats. Nimesulide significantly inhibited the aspirin-related increase in MDA (p<0.0001). The difference in MDA content between the nimesulide group and the healthy group was found to be statistically insignificant (p>0.05). A significant decrease was also found in the tGSH content of the cochlear nerve tissue in the aspirin group compared to the healthy and nimesulide groups (p<0.0001). It was seen that nimesulide inhibited the aspirin-related decrease in tGSH (p<0.0001). The difference in tGSH between the nimesulide group and the healthy group was considered insignificant as shown in Fig. 2(a-b).

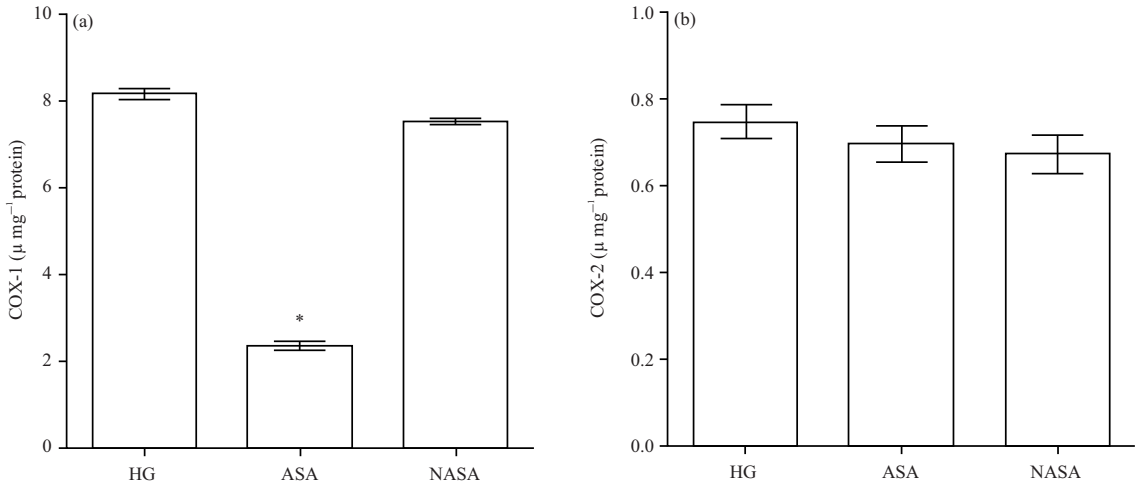

Fig. 3(a-b): Cyclooxygenase-1 (COX-1) Cyclooxygenase-2 (COX-2) levels in the cochlea tissue of the rats, (a) COX-1 levels in the cochlea tissue and (b) COX-2 levels in the cochlea tissue, all study groups compared with ASA
*p<0.0001, n = 6

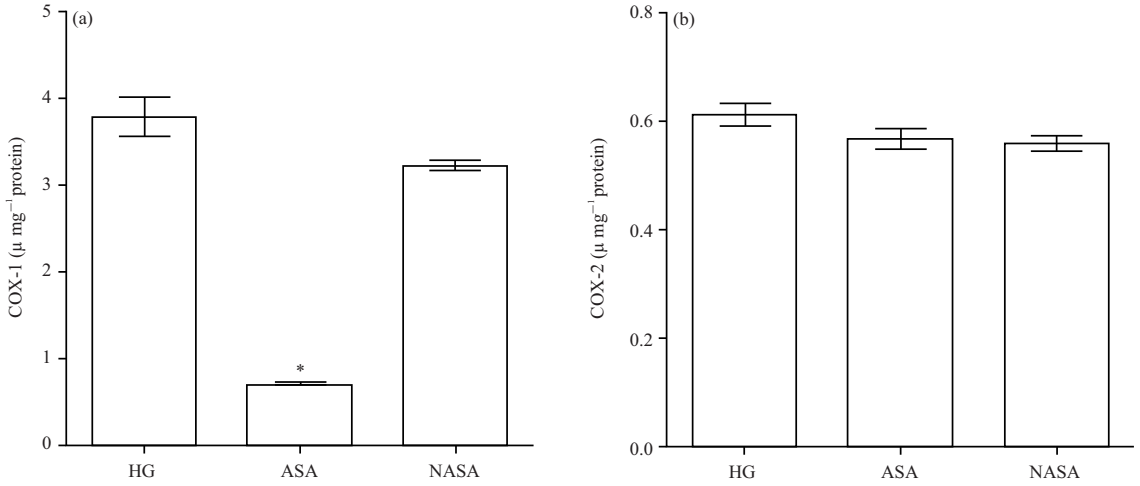

Fig. 4(a-b): Cyclooxygenase-1 (COX-1) Cyclooxygenase-2 (COX-2) levels in the cochlear nerve tissue of the rats (a) COX-1 levels in the cochlear nerve tissue and (b) COX-2 levels in the cochlear nerve tissue, all study groups compared with ASA
*p<0.0001, n = 6

COX-1 and COX-2 analysis results of the cochlea tissue: The COX-1 activity in cochlea tissue was found to be significantly reduced in the aspirin group than the healthy and nimesulide groups ($p<0.0001$). Nimesulide significantly inhibited the aspirin-related decrease in COX-1 activity ($p<0.0001$). The COX-1 activities were found similar between the nimesulide group and the healthy group, with a statistically insignificant difference($p>0.05$). However, the inhibitory effect of aspirin on COX-2 activity was found insignificant ($p>0.05$). The COX-2 activities were found similar between the aspirin group, the nimesulide group and the healthy group, with a statistically insignificant difference ($p>0.05$) (Fig. 3).

COX-1 and COX-2 analysis results of the cochlear nerve tissue: Aspirin significantly decreased the COX activity in cochlear nerve tissue, compared to the healthy group and the nimesulide group ($p<0.0001$). However, nimesulide prevented the aspirin-related decrease in COX-1 activity. No significant difference was found in COX-1 activity between the nimesulide group and the healthy group ($p>0.05$). Aspirin could not significantly decrease the COX-2 activity in the cochlear nerve tissue ($p>0.05$). The difference in COX-2 activity was found insignificant between the aspirin, nimesulide and healthy groups ($p>0.05$) (Fig. 4).

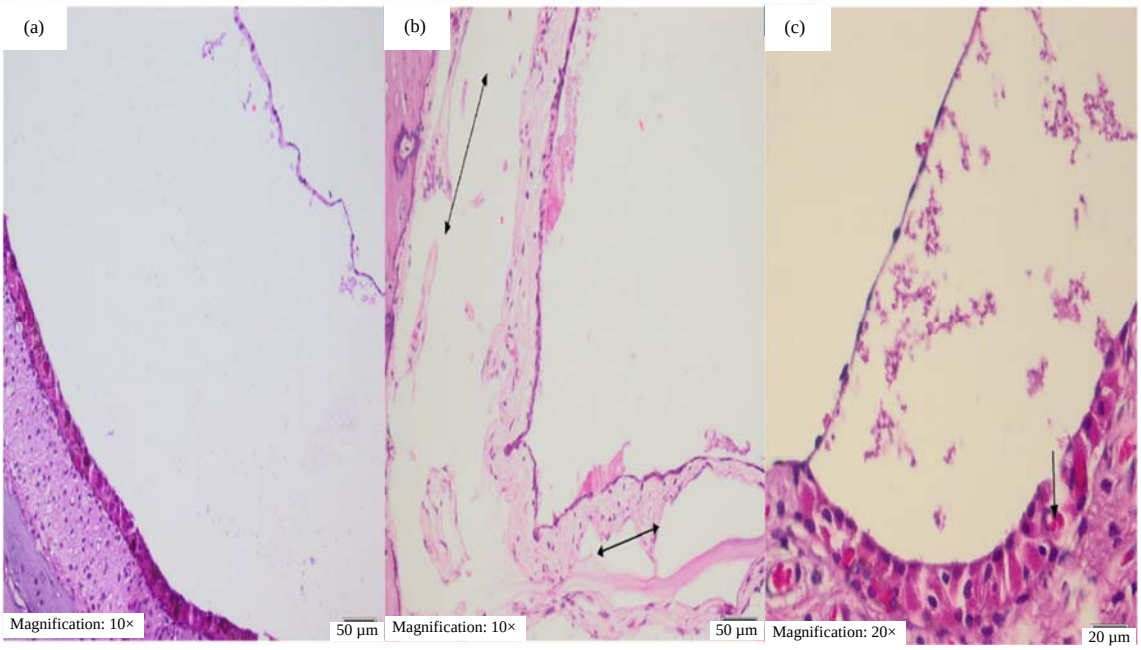

Fig. 5(a-c): Histopathological examination of Healthy (HG), Aspirin (ASA) and Nimesulide (NASA) groups in cochlea tissue, (a) Section showing healthy cochlea tissue (HE×200), (b) Section showing diffuse destruction and edema (double-sided arrows) in the cochlea tissue of the aspirin group (HE×200) and (c) Section showing a small number of mildly congested blood vessels (arrow) in the cochlea tissue of the nimesulide group (HE×400)

Table 1: Semiquantitative evaluation of histopathological changes

	Cochlear tissue damage		Cochlear nerve tissue	
Groups	Destruction	Edema	Myelinated nerve fibers	Schwann cell
SG	0	0	0	0
	p<0.001	p<0.001	p<0.001	p<0.001
ASA	2.83	3	2.83	3
	p<0.001	p<0.001	p<0.001	p<0.001
NASA	0.5	0	0.67	0.5
	p<0.002	p<0.001	p<0.003	p<0.001

p<0.05 were considered significant for all groups

Histopathological findings

Histopathological findings of the cochlea tissue: No pathological finding (grade-0) was found in the cochlea tissue of the healthy group as shown in Fig. 5(a). However, diffuse severe destruction and edema (grade-3) were observed in the cochlea tissue of the aspirin group (Fig. 5b). In the group treated with nimesulide, the injury was considered as grade-1. A small number of mildly congested blood vessels were also noted (Fig. 5c) (Table 1).

Histopathological findings of the cochlear nerve tissue: When the cochlear nerve tissue sections from the healthy group were evaluated histopathologically, it was seen that myelinated nerve fibers along with surrounding Schwann cell nuclei and supporting tissue had a normal histological structure (Fig. 6a). The histopathologic injury in the sections from the aspirin group was considered as grade3. It was found that myelinated nerve fibers were quite swollen and edematous and axons generally lost their central locations. When most nerve fibers were evaluated, it was seen that they lost their Schwann cell connections. The Schwann cells surrounding nerve fibers seemed to be hypertrophic and hyperplasic. Collagen deposits were noted in the tissue, while the blood capillaries were seen to be normal (Fig. 6b). In the group treated with nimesulide, the histopathological findings were mild (Grade 1) and had an overall appearance similar to the healthy group (Fig. 6c).

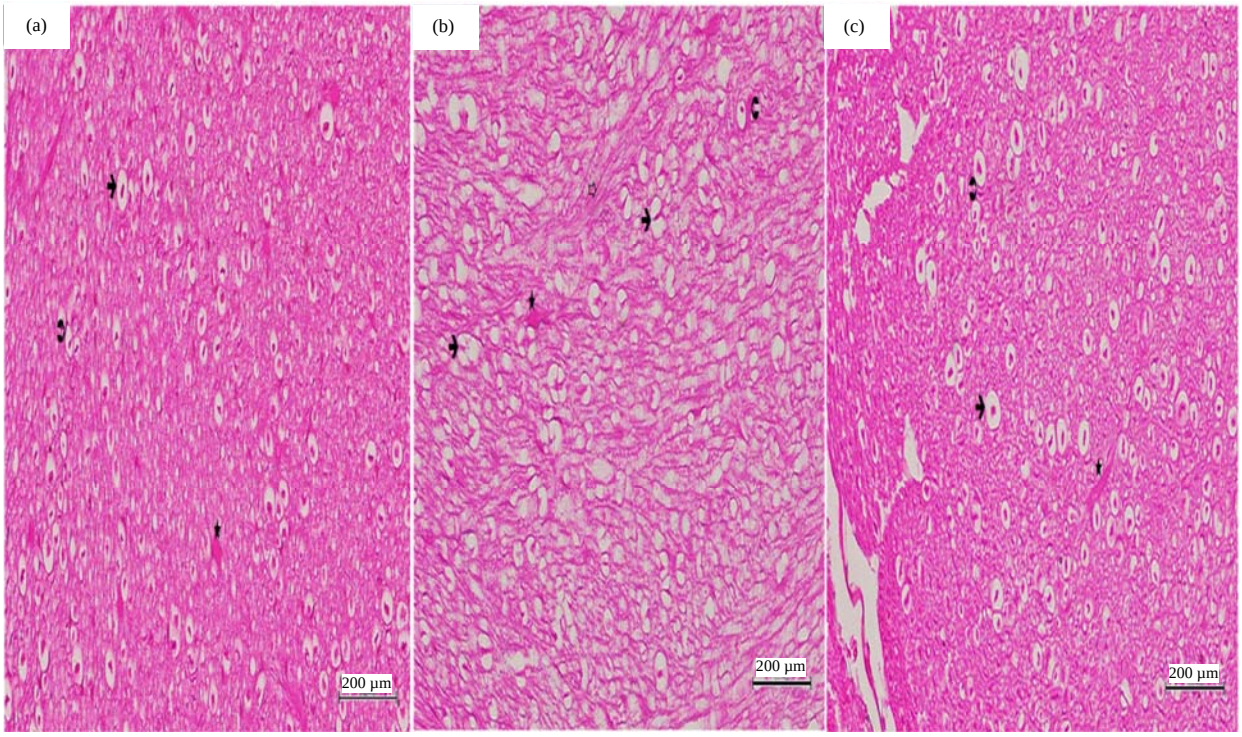

Fig. 6(a-c): Histopathological examination of Aspirin (ASA) and Nimesulide (NASA) groups in cochlear nerve tissue, (a-b) Blood vessels in the cochlear nerve tissue of the aspirin and (c) Nimesulide group (HE×400)

Section a showing ➔: Healthy ⊃: Myelinated axon and ★: Schwann cell. Section b showing ➔: Degenerated and edematous myelinated axon, ⊃: Hypertrophic and hyperplasic schwann cell and ➪: Collagen deposit. Section c showing ➔: Myelinated axon and ⊃: Schwann cell

DISCUSSION

Current study investigated whether aspirin caused an injury to the cochlea and cochlear nerve tissue in rats. Also, the protective effect of nimesulide against aspirin-induced injury of the cochlea and cochlear nerve tissue was examined biochemically and histopathologically. Our biochemical experiment results showed that malondialdehyde (MDA) content in both cochlea and cochlear nerve tissue was significantly increased in the aspirin group, compared to the healthy and nimesulide groups. It was also showed that the total glutathione (tGSH) content decreased. The MDA and tGSH levels in live organs and tissues are used to evaluate the oxidant/antioxidant balance[19]. As is known, MDA is the final product of lipid peroxidation (LPO) and a marker of oxidative tissue injury[20]. MDA, causes serious injury to cells, leading to cross-linking and polymerization of cell membrane components[21]. Living cells have several endogenous antioxidant defense mechanisms to inhibit the LPO reaction induced by excessively produced reactive oxygen species (ROS)[22]. One of these antioxidant mechanisms is GSH[23]. GSH, c-L-glutamyl-L-cysteinyl-glycine, is the most important low molecular weight antioxidant synthesized by cells. GSH reacts with hydrogen peroxide (H_2O_2) and organic peroxides, detoxifying H_2O_2 and protecting the cells from ROS injury[24-25]. A previous study showed biochemically and histopathologically that oxidative injury occurred in cochlea tissue with a high MDA level and a low tGSH level[26]. Bayram *et al.*[27] showed that in case of oxidative injury of the auditory nerve, the MDA content significantly increased and the tGSH decreased, compared to the healthy group. Salcan I *et al.*[28] showed that damage developed in the cochlear nerve tissue with high MDA and low tGSH histopathologically. This information obtained from our experimental results and literature indicated that aspirin disrupts the normal oxidant/antioxidant balance in the cochlea and cochlear nerve tissue in favor of oxidants.

This study also showed that aspirin significantly decreased the COX-1 activity in the cochlea and cochlear nerve tissue, compared to the healthy and nimesulide group. COX-1 is a structural enzyme and plays an important role in maintaining cell integrity[29]. It is suggested that aspirin-induced histopathological injury in the cochlea and cochlear nerve tissue might result from COX-1 inhibition. COX-1 is

responsible for the synthesis of cytoprotective prostaglandin in organs and tissues[5]. Aspirin administration did not cause a significant decrease in COX-2 activity in our study. However aspirin inhibits COX-1 and COX-2[5]. Although we could not show the reason why aspirin could not significantly decrease COX-2 activity in the cochlea and cochlear nerve tissue, there are indications to explain this phenomenon in the literature, COX-2 is an isoform which is induced by proinflammatory cytokines and is responsible for the production of pro-inflammatory prostaglandin E2 (PGE2)[29-30]. Fever or elevated body temperature completely arises from the production of PGE2[31]. Also, aspirin does not affect normal body temperature[32]. This shows that aspirin inhibits the increased COX-2 activity and the production of COX-2 products in pathological conditions, instead of healthy tissues. Also, the fact that aspirin decreased the elevated body temperature in pathological conditions but did not cause hypothermia in healthy individuals, supports our experiment results.

The previous studies have reported that salicylates have toxic effects on the cochlea and cochlear nerve tissue. However, in the literature, no clear evidence was found about the aspirin-related histopathological injury of the cochlea and cochlear nerve tissue. In our study, apparent destruction and edema were found in the cochlea tissue of the aspirin group as well as morphological deformations were noted in the Schwann cells, myelinated nerve fibers and axons in their cochlear nerve tissue. The MDA level was found low and the tGSH and COX-1 levels were found high in the group received nimesulide which significantly decreased the aspirin-related histopathological injury of the cochlea and cochlear nerve tissue. In the literature, no study was found regarding the effect of nimesulide on oxidant-antioxidant and COX levels in the cochlea and cochlear nerve tissue. However, it was reported that nimesulide prevented liver injury induced by ischemia-reperfusion, inhibiting the increase in MDA and COX-2 and the decrease in tGSH and COX-1 levels[6] Recent studies suggest that there is a link between increased MDA amount and increased COX-2 activity[33]. Furthermore, the fact that a high dose of celecoxib being a selective COX-2 inhibitor is not ototoxic in animals[34] supports our experiment results.

CONCLUSION

Consequently, no apparent histopathological injury was found in the cochlea and cochlear nerve tissue in animals in the aspirin group with a high MDA level and low COX-1 and tGSH level. It was seen that histopathological injury was minimal in the nimesulide group whose MDA, COX-1 and tGSH levels were found close to that of the healthy group. The experimental results of this study suggested that nimesulide inhibits the aspirin-related increase in MDA and decrease in tGSH and COX-1 levels, protecting the cochlea and cochlear nerve tissues from aspirin-induced injury. This information indicated that the co-administration of nimesulide and aspirin will have a potent analgesic, antipyretic and anti-inflammatory activity and reduce the ototoxicity of aspirin. COX-1 products in the cochlea and cochlear nerve tissues should be measured to explain the otoprotective action mechanism of nimesulide.

SIGNIFICANCE STATEMENT

This study discovers the nimesulide can be useful on aspirin ototoxicity. It has been noted that nimesulide decreased the COX-1 activity in the cochlea and cochlear nerve tissue. Thus, a new theory on nimesulide, the co-administration of nimesulide and aspirin will increase the therapeutic effect of aspirin and reduce its side-effects.

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