Synergistic Effect of combined antioxidants on Noise-Induced Acoustic Trauma in Adult Guinea Pigs. Audiological and Histological Study

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Abstract: Introduction: Cochlear noise injury is considered one of the most debilitating diseases worldwide. Numerous drug trials have been made for complete protection from this acoustic trauma, unfortunately with little success. Recently, drug combination has showed promising effects in treating this trauma; however, this has to be further documented by in-depth researches. Aim of the work: To estimate the effect of combination of the antioxidants; vitamins A, C and E, plus magnesium (A, C, E+ Mg) in either protection or treatment of noise-induced cochlear injury in adult guinea pigs. Materials and methods: Twenty five guinea pigs were used in this study and were divided equally into five groups. Group I served as a control group. Group II administered the drug combination for 5 successive days. Group III exposed once to 120 dB SPL octave band noise for 5 successive hours. Group IV pre-treated with vitamins A, C, E+ Mg for 5 successive days prior to noise exposure. Group V first exposed to same noise injury, and then same drug combination was administered for 5 successive days, starting one day after noise exposure. Results: Noise exposure resulted in profound cochlear damage. Prophylactic administration of the drug combination showed partial protection of the cochlea as detected audiologically and histologically. In contrast, significant improvement of both function and structure of the cochlea was revealed with post-treatment 1 day after noise-induced cochlear damage. Conclusion: Delayed treatment by this combination of drugs (vitamins A, C, E+ Mg) proved to be effective even if started one day after noise exposure. However, drug combination used as prophylactic treatment was not as effective.

Key words: noise- cochlea- antioxidants- magnesium- guinea pigs.

1. Introduction:

With rapid industrialization in modern society, noise pollution is an ever-increasing problem. The resulting noise induced hearing loss (NIHL) is considered one of the most common causes of hearing disabilities. Millions of workers in many industrial environments are exposed to noise induced acoustic trauma despite their usage of mechanical noise protection (1). Others, as orchestra musicians (2) and military personnel (3) are also exposed to similar trauma. Furthermore, NIHL definitely has a negative effect on the quality of life (4) so that finding an effective protection or therapy for the noise induced cochlear damage would be extremely beneficial.

Modern researches had provided new insights for the possible mechanisms of NIHL. Oxidative stress has been widely implicated in neuronal cell degeneration (5) and has been well characterized after noise-induced cochlear trauma (6). Therefore, antioxidant drug administration, as vitamins, has been widely proposed as a potential therapeutic intervention in acoustic trauma (7). Additionally, magnesium supplements (Mg) have also been used as a potential treatment for noise trauma owing to its positive action on noise-induced micro-circularity impairment (8).

Unfortunately, any single protective agent to be effective, it must be provided for long periods of time prior to noise exposure. High-dose vitamin C, as an example, did not completely prevent NIHL even with 35 days pre-treatment. Its serum level has been found to be stabilized in humans after a minimum of 3 weeks of daily intake (9). Vitamin E serum levels have also been found to stabilize after over a month of daily intake in human subjects (10). In addition, authors recommended using Mg with other agents to improve its therapeutic efficiency (8). Emerging from the previous point, several studies showed that using combination of several agents had fast promising effects and were more effective than single agent (6, 11, 12, 13). This was accompanied by confirmation of the safety of combined use of antioxidants in several studies in humans or experimental animals (5, 11, 12).

Timing of intervention obviously has a key role in success of either protection or therapeutic regimen. A previous study reported that initiation of combined treatment as shortly as one hour before noise exposure failed to prevent hair cell death (11). On the other hand, delayed treatment initiated 5 days post-
noise exposure has not met any mentionable success (13).

So, the aim of the present study was to verify and provide further insight into the potential efficiency of combined use of vitamins A, C, E + magnesium as otoprotectants as well as a delayed therapy for noise-induced cochlear damage.

2. Materials and Methods
Twenty five adult male guinea pigs were used in this study. They were purchased and housed at the Medical Research Center, Faculty of Medicine, Ain Shams University. The animals were put in wired mesh cages with food and water *ad libitum*. They were divided equally into 5 groups as follows:

**Group I**: comprised 5 animals that served as a control group.

**Group II**: comprised 5 animals that received combined vitamin A, C, E and magnesium (A, C, E + Mg) once daily for 5 days. Details of doses and routes of administration are described later in the drug regimen.

**Group III**: comprised 5 animals that were exposed to noise trauma once for 5 continuous hours.

**Group IV**: comprised 5 animals that received vitamin A, C, E + Mg in same routes and doses as group II once daily, beginning 5 days before the day of noise exposure.

**Group V**: comprised 5 animals that received vitamin A, C, E + Mg in same routes and doses as group II, once daily for 5 successive days, starting one day after noise exposure.

**Drug regimen**: (11):
- Vitamin A was given in a dose of 2.1 mg/kg/day orally by an intragastric tube (β carotene forte®, 15 mg capsules, equivalent natural vitamin A 25000 IU, Medizen Pharmaceutical Industries for Arab Co. for Pharm. & medicinal plants “Mepaco-Medifood”, Enshas, Sharkeya, Egypt).
- Vitamin C was given in a dose of 71.4 mg/kg/day intraperitoneally. (Cevarol® 1000 mg ampoules, Memphis Co. for Pharmaceutical & Chemical Industries, Cairo, Egypt).
- Vitamin E was given in a dose of 26 mg/kg/day orally by an intragastric tube (Vitamin E- antioxidant 400 mg capsules, Pharco Pharmaceuticals, Alexandria, Egypt).
- Magnesium (Mg) was given in a dose of 343 mg/kg/day intramuscularly (10% magnesium sulphate, Egypt Otsuka Pharm. Co).

**Audiological study:**

**A) Auditory Brainstem Response (ABR):**

This was used to measure hearing threshold in all guinea pigs before administration of drugs or noise exposure. All recording was conducted under anesthesia by ketamine hydrochloride (Ketalar®, Sigma), 40 mg/kg (14) in a soundproof chamber.

**Stimulus parameters:**
The ABRs were generated in response to 100 µs alternated clicks at a range of 2-4 KHz. The stimulus was presented at a rate of 21 pulses / second. Monaural thresholds were obtained via headphone at 10 dB steps between 100 dBSPL down to threshold.

**Recording parameters:**
The ABRs were recorded by means of three platinum-iridium needle electrodes, placed subdermally over the vertex (positive), the mastoid (negative) and the contra-lateral mastoid (ground). The recording window included a 10-millisecond post-stimulus times. ABRs were amplified 20000-fold and filtered from 30 Hz to 3000 Hz. At least two repeatable traces with approximately 1000 response sweeps for each trace were collected for each subject. The test session including electrode application and evoked response recording for each subject lasted for about 30 minutes.

**Response analysis:**
The ABRs was defined by three positive peaks (I, III, V) at supra-threshold intensity (100 dB SPL). Three recording parameters were analyzed. Absolute and inter-peak latencies for wave I, III and V measured. Threshold was defined as the lowest intensity capable of producing a visually detectable, reproducible wave V.

**B) Noise exposure:**
All animals were housed in wired mesh cages anaesthetized, and unrestrained with free access to food and water. They were exposed to noise simultaneously at the same session to ensure the same testing environment and the same level of noise exposure. They were exposed to 120 dBSPL, continuous octave band noise (centered at high frequency of 4 KHZ) in a ventilated sound treated chamber via loud speakers. Sound was delivered from Single channeled audiometer (Maico). Noise was calibrated prior to testing and at the end of the experiment using sound level meter to ensure uniformity of the stimulus through the entire test that lasted for 5 continuous hours.

On day 14 after noise exposure, ABR in all guinea pigs in groups II, III, IV and V were re-measured by the same procedure. Hearing loss induced by single noise exposure in guinea pig has been found to be stabilized by 10-14 days (10, 15).
Histological study:
After the final ABR measurements on day 14, all animals were anesthetized with ketamine hydrochloride, 40 mg/kg (14). Transcardial perfusion was done by cold 2.0% paraformaldehyde /2.0% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) (16). The animals were then sacrificed by decapitation. The temporal bone was taken, and the cochleae were dissected carefully. The right cochleae of all groups were processed for light microscopic examination (LM), and the left cochleae of all groups were processed for transmission electron microscopic examination (TEM). Complete infiltration of the cochlea by the proper fixative was ensured by making a tiny hole at the apex of the cochlear capsule by a curved stapes pick, and gently forcing the fixative using a fine needle fitted onto a tuberculin syringe.

A) Light microscopic study (LM):
The right cochleae were further fixed in 10% formalin for 2 days. Decalcification was then done for 4 weeks using Di-sodium EDTA. Specimens were processed to form paraffin blocks and five µm-thick serial mid-modiolar longitudinal sections were cut and subjected to Haematoxylin and Eosin (H&E) staining (17).

B) Transmission electron microscopic study (TEM):
The left cochleae were further fixed in Phosphate buffered gluteraldehyde and processed to form capsules. Semi-thin sections of 1 µm were cut and stained by toluidine blue. Ultra-thin sections (50-60nm in thickness) were then cut using ultra-microtome. Sections were then mounted on copper grids and stained with saturated solution of uranyl acetate followed by lead citrate (17). Stained ultra-thin sections were examined and photographed by JEOL-1010 JEM transmission electron microscope in The Regional Center for Mycology and Biotechnology, Al Azhar University.

Morphometric and Statistical study:
1- Auditory brainstem response (ABR) thresholds and threshold shifts were measured.
2- Measurements of the spiral ligament thickness. The central parts of the ligament were examined in serial H&E-stained sections from all animals. Histological measurements were done in five high power fields /section. The measurements were performed using Image Analyzer (Olympus Image J, NIH, 1.41b, USA) in the Oral Pathology Department, Faculty of Dentistry, Ain Shams University. The standard error of means (SEM) of the audiological and histological data was calculated and statistical analysis was carried out using SPSS statistical program version 17; IBM Corporation, NY 10589. One-way analysis of variance test (ANOVA) was used to evaluate the data. Post hoc least significant difference (LSD) was used for comparison of measurements between all groups. All data were expressed as (mean±SEM). The P value considered significant when less than 0.05.

3. Results
I) Audiological results:
Twenty five adult guinea pigs were enrolled in the present study (groups I, II, III, IV and V). Prior testing, all animals showed normal mobile tympanic membranes together with normal ABR morphology and thresholds (Table 1).

Table (1): Showing the Mean ± SEM of different ABR parameters and comparison between all groups: (Before noise exposure or administration of drugs)

<table>
<thead>
<tr>
<th></th>
<th>Group I</th>
<th>Group II</th>
<th>Group III</th>
<th>Group IV</th>
<th>Group V</th>
</tr>
</thead>
<tbody>
<tr>
<td>Threshold</td>
<td>10.00 ± 0.00</td>
<td>10.00 ± 0.00</td>
<td>10.00 ± 0.00</td>
<td>10.00 ± 0.00</td>
<td>10.00 ± 0.00</td>
</tr>
<tr>
<td>I Lat.</td>
<td>1.07 ± 0.03</td>
<td>1.18 ± 0.09</td>
<td>1.19 ± 0.05</td>
<td>1.13 ± 0.06</td>
<td>1.17 ± 0.04</td>
</tr>
<tr>
<td>III Lat.</td>
<td>2.41 ± 0.13</td>
<td>2.56 ± 0.05</td>
<td>2.47 ± 0.07</td>
<td>2.38 ± 0.12</td>
<td>2.42 ± 0.11</td>
</tr>
<tr>
<td>V Lat.</td>
<td>3.69 ± 0.05</td>
<td>3.75 ± 0.03</td>
<td>3.63 ± 0.06</td>
<td>3.68 ± 0.04</td>
<td>3.67 ± 0.03</td>
</tr>
<tr>
<td>V Lat Ths</td>
<td>4.35 ± 0.10</td>
<td>4.51 ± 0.08</td>
<td>4.35 ± 0.15</td>
<td>4.41 ± 0.11</td>
<td>4.37 ± 0.12</td>
</tr>
<tr>
<td>I-III</td>
<td>1.34 ± 0.14</td>
<td>1.38 ± 0.05</td>
<td>1.28 ± 0.04</td>
<td>1.25 ± 0.09</td>
<td>1.31 ± 0.06</td>
</tr>
<tr>
<td>III-V</td>
<td>1.28 ± 0.10</td>
<td>1.28 ± 0.06</td>
<td>1.09 ± 0.06</td>
<td>1.25 ± 0.06</td>
<td>1.27 ± 0.09</td>
</tr>
<tr>
<td>I-V</td>
<td>2.62 ± 0.05</td>
<td>2.62 ± 0.08</td>
<td>2.56 ± 0.05</td>
<td>2.51 ± 0.07</td>
<td>2.59 ± 0.06</td>
</tr>
</tbody>
</table>

-Values are mean ± SEM. - Number in parenthesis indicates the number of guinea pigs.
As shown in table (1), all Animals of all groups showed normal mean hearing thresholds with normal mean absolute latencies of waves I, III and V & normal inter-peak latencies (I-III, III-V and I-V). There was non-significant statistical difference (p>0.05) in all groups of the study prior to noise exposure and prior to the administration of any drug. This emphasized that all animals were normal hearers before any intervention.

Table (2): Showing the Mean ± SEM of different ABR parameters and comparison between all groups: (After noise exposure and administration of drugs).

<table>
<thead>
<tr>
<th></th>
<th>Group I (Control)</th>
<th>Group II (drug combination)</th>
<th>Group III (Noise)</th>
<th>Group IV (pre-noise treated)</th>
<th>Group V (post-noise treated)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Threshold</td>
<td>10.00 ± 0.00</td>
<td>10.00 ± 0.00</td>
<td>63.33 ± 3.33 *</td>
<td>50.00 ± 3.16 **</td>
<td>18.00 ± 3.74 **</td>
</tr>
<tr>
<td>I Lat.</td>
<td>1.07 ± 0.03</td>
<td>1.13 ± 0.06</td>
<td>1.09 ± 0.16</td>
<td>1.24 ± 0.12</td>
<td>1.14 ± 0.08</td>
</tr>
<tr>
<td>III Lat.</td>
<td>2.41 ± 0.13</td>
<td>2.44 ± 0.07</td>
<td>2.45 ± 0.09</td>
<td>2.70 ± 0.10</td>
<td>2.65 ± 0.17</td>
</tr>
<tr>
<td>V Lat.</td>
<td>3.69 ± 0.05</td>
<td>3.79 ± 0.07</td>
<td>3.88 ± 0.08</td>
<td>3.80 ± 0.08</td>
<td>3.68 ± 0.04</td>
</tr>
<tr>
<td>V Lat Ths</td>
<td>4.35 ± 0.10</td>
<td>4.49 ± 0.08</td>
<td>4.43 ± 0.20</td>
<td>4.43 ± 0.08</td>
<td>4.35 ± 0.05</td>
</tr>
<tr>
<td>I-III</td>
<td>1.34 ± 0.14</td>
<td>1.39 ± 0.08</td>
<td>1.48 ± 0.06</td>
<td>1.36 ± 0.07</td>
<td>1.34 ± 0.09</td>
</tr>
<tr>
<td>III-V</td>
<td>1.28 ± 0.10</td>
<td>1.29 ± 0.07</td>
<td>1.49 ± 0.11</td>
<td>1.31 ± 0.08</td>
<td>1.30 ± 0.08</td>
</tr>
<tr>
<td>I-V</td>
<td>2.62 ± 0.05</td>
<td>2.64 ± 0.05</td>
<td>2.78 ± 0.06</td>
<td>2.70 ± 0.09</td>
<td>2.63 ± 0.03</td>
</tr>
</tbody>
</table>

-Values are mean ± SEM. - Number in parenthesis indicates the number of guinea pigs.
- a: significance of difference by LSD from Group I (Control) at least P<0.05.
- b: significance of differences by LSD from Group III (Noise) at least P <0.05.
- c: significance of differences by LSD from Group IV (pre-treated ) at least P <0.05.

As shown in table (2) and diagram (1), guinea pigs of group II (drug combination), showed non-significant statistical difference (P>0.05) in mean threshold and all other parameters means of ABR testing compared with group I (control). This documented that this drug combination is not ototoxic.

Noise exposure in group III resulted in “No Response” in 2 animals in ABR parameters, at highest threshold (70 dB). The remaining 3 guinea pigs of group III (noise-exposed) were included in the statistical different ABR parameters. They showed significant statistical difference in mean threshold parameter only (P<0.05) with non-significant statistical difference as regards the other parameters means of ABR testing (P>0.05) compared with group I (control). This documented the ototoxic effect of noise in both ears.

In-addition, guinea pigs of group IV (pre-noise treated group) showed significant statistical difference in mean threshold parameter only (P<0.05) with non-significant statistical difference as regards the other parameters means of ABR testing (P>0.05) compared with group I (control) and group III (noise-exposed). This documented the limited protective effect of combined drugs when administered prior to noise exposure on ototoxicity of noise in both ears.

On the other hand, guinea pigs of group V (post-noise treated group) showed significant statistical difference in mean threshold parameter only (P <0.05) with non-significant statistical difference as regards the other parameters means of ABR testing (P>0.05) compared with group II (noise-exposed) and group IV (pre-noise treated group). However, group V (post-noise treated group) showed non-significant statistical difference (P>0.05) in mean threshold and all other parameters means of ABR testing compared with group I (control). This documented the marked anti-ototoxic effect of combined drugs when administered one day after noise exposure on ototoxicity of noise in both ears.
II-Histological results:

**Group I** (Control animals):

Examination of H&E-stained sections of this group showed the wedge-shaped cochlear duct roofed by Reissner’s membrane separating it from the scala vestibuli, while the basilar membrane made its floor separating it from the scala tympani. The basilar membrane extended from the spiral lamina medially to the spiral ligament on the lateral wall, supporting the organ of Corti (Figure 1). Neuroepithelial cells of the organ of Corti were seen as three outer hair cells (OHCs) with acidophilic cytoplasm and basal rounded vesicular nuclei and one inner hair cell (IHC) with central rounded vesicular nucleus. Outer phalyngeal cells supported the OHCs and the inner phalyngeal cell was seen supporting the IHC. Outer and inner pillars surrounded the tunnel of Corti. Other supporting cells as Hensen, Claudius, Böttcher cells laterally and border cells medially could be recognized. The tectorial membrane was seen hanging as homogenous acidophilic structure (Figure 2). Stria vascularis covered the spiral ligament at the lateral wall of the cochlear duct. The three layers of strial epithelium were seen; marginal, intermediate and basal layers. The fibrous connective tissue meshwork of the spiral ligament was seen underneath (Figure 3). Mean thickness of the spiral ligament measured 23.64 ± 0.52 (mean± SEM) (Table 3 and Histogram 1).

Toluidine blue-stained sections showed the spiral ganglion neurons studded with Nissl’s granules (Figure 4).

Ultrastructural examination by TEM showed the OHCs containing multiple mitochondria with apparent cristae and their nuclei showed regular chromatin pattern. Stereocilia were seen projecting from the apical surface of the cells (Figure 5). Nerve fibers of the spiral ganglion neurons showed regular myelin wrapping (Figure 6).

**Group II** (animals that received drug combination):

No structural and statistical differences were found in the animals’ cochleae of this group compared with the control group.

**Group III** (animals exposed to noise):

Examined H&E-stained sections showed that the OHCs were the most affected after noise exposure especially in the basal turn. Loss of OHCs was noticed in many sections mostly in the third row, with degeneration of the remaining OHCs (Figure 7). Other sections showed OHCs with cytoplasmic vacuolization and karyolytic nuclei (Figures 7, 8). The IHCs were seen slightly affected showing few cytoplasmic vacuolization (Figures 7, 8). Supporting cells were seen degenerated, vacuolated. Disrupted pillar cells were frequently encountered (Figures 7, 8). Vacuolated cytoplasm of strial cells was noticed with apparent increase in melanin pigment as compared to the control. Congested capillaries could be easily noticed. The fibrocytes of the spiral ligament were seen highly vacuolated, degenerated with pyknotic nuclei (Figure 9). Statistically significant decrease in mean thickness of the spiral ligament was obvious (P< 0.05) as compared with the control sections, with mean thickness measuring 12.60 ± 0.70 (mean± SEM) (Table 3 and Histogram 1).

Toluidine blue-stained sections showed apparent decrease in Nissl’s granules content of the spiral
ganglion neurons. The neurons were widely spaced, showed paler cytoplasm and apparent decrease in size compared with control sections (Figure 10).

Examination by TEM showed irregular, disrupted OHCs with degenerated mitochondria. Disarranged, bent or lost OHCs’ stereocilia, together with shrunken electron dense nuclei were also observed (Figure 11). The IHC exhibited only few cytoplasmic vacuolization (Figure 12). Supporting cells with vacuolated cytoplasm and irregular electron dense nuclei. The heterochromatin was seen accumulated under the nuclear envelope (Figure 13). Few nerve fibers showed swelling with very thin and disrupted myelin sheath compared with the control sections (Figure 14).

**Group IV (pre-noise treated animals):**

Examined H&E-stained sections of the cochleae revealed that treatment by the drug combination five days before the day of noise exposure had partial protective effect. Degenerated OHCs with pyknotic nuclei were seen in some sections (Figure 15). Other sections showed OHCs with vacuolated cytoplasm and loss of some OHCs’ nuclei particularly in the third row (Figure 16). The IHC was seen apparently normal. Some supporting cells were extremely thin and degenerated with pyknotic nuclei (Figure 15), while others showed vacuolated cytoplasm (Figure 16). Cytoplasmic vacuolization could be noticed in the strial epithelial cells. The melanin content of the stria was apparently increased compared with the control. The spiral ligament showed vacuolated cytoplasm of many fibrocytes (Figure 17). Its mean thickness measured $22.98 \pm 0.35$ (mean±SEM) showing statistically non-significant difference ($P>0.05$) compared with the control group, and with the pre-noise treated group. However, it was significantly increased ($P<0.05$) as compared with the noise-exposed group (Table 3 and Histogram 1).

Toluidine blue-stained sections showed the structure and Nissl’s granules content in the spiral ganglion neurons apparently similar to the control (Figure 21). Examination by TEM showed that the OHCs comparable to the control however, few supporting cells appeared vacuolated (Figure 24). Myelin sheaths of the nerve fibers showed similar appearance as the control sections (Figure 25).

**Group V (post-noise treated animals):**

Examination of the H&E-stained sections of the cochleae showed that treatment of animals by drug combination after one day of noise exposure efficiently improved the OHCs’ structure. The OHCs were seen comparable to the control appearing organized, with homogenous acidophilic cytoplasm and vesicular nuclei. The supporting cells’ cytoplasm was also comparable to the control sections (Figure 21). The structure and melanin content of the stria vascularis as well as structure of the spiral ligament were apparently similar to the control (Figure 22). The mean thickness of the spiral ligament measured $22.47 \pm 0.53$ (mean±SEM). It showed statistically non-significant difference ($P>0.05$) compared with the control group, and with the pre-noise treated group. However, it was significantly increased ($P<0.05$) as compared with the noise-exposed group (Table 3 and Histogram 1).

Toluidine blue-stained sections showed the structure and Nissl’s granules content in the spiral ganglion neurons apparently similar to the control (Figure 23).

Examination by TEM showed that the OHCs comparable to the control however, few supporting cells appeared vacuolated (Figure 24). Myelin sheaths of the nerve fibers showed similar appearance as the control sections (Figure 25).

**Fig. 1:** Showing the wedge-shaped cochlear duct (CD) lying between the scala vestibuli (SV) above and scala tympani (ST) below it. Basilar membrane (↑) is seen extending between spiral ligament (▲) and the spiral limbus (△). Reissner’s membrane is seen making the roof of the cochlear duct as 2 layers of simple squamous cells (↑↑). [Group I (control): H&E × 250]
Fig. 2: Showing the organ of Corti resting on the basilar membrane (↑). OHCs (O), IHC (I), pillar cells (P) can be seen. Other supporting cells as outer phalangeal (OP), inner phalangeal (IP), border cells (B), Hensen cells (H) can be noticed. Notice the homogenous acidophilic tectorial membrane hanging over the hair cells (↑↑). [Group I (control): H&E × 640]

Fig. 3: Showing the marginal cells (M), intermediate cells (I) and basal cells (B) of stria vascularis epithelium. Notice the strial melanin granules (Δ) and fibrocytes of the spiral ligament (↑). [Group I (control): H&E × 640]

Fig. 4: Showing the spiral ganglion neurons and their Nissl’s granules content (↑). [Group I (control): Toluidine blue × 640]

Fig. 5: Showing OHCs (O) with multiple mitochondria and apical projecting stereocilia (Δ). The IHC (I) can be noticed with its stereocilia (↑). [Group I (control): TEM × 3000]

Fig. 6: Showing regular myelin wrapping of the nerve fibers. [Group I (control): TEM × 10000]

Fig. 7: Showing lost OHC in the third row (Δ) and degeneration of the other OHCs (↑). Few vacuolization of IHC can be observed (♦). Vacuolated border cells (♦) and disrupted outer pillar cell (▲) can be noticed. [Group III (noise exposed): H&E × 640]
Fig. 8: Showing vacuolated OHCs (Δ) and karyolytic nucleus of one of them (↑). Notice the slightly vacuolated IHC (♦) and the degenerated supporting cells (*). [Group III (noise exposed): H&E × 640]

Fig. 9: Showing vacuolated strial cells (V), with apparently increased melanin content compared with the control (†). Congested blood vessel can be seen (Δ). Apparently decreased thickness of the spiral ligament compared with control can be observed. Notice highly vacuolated (*) and degenerated fibrocytes (▲). [Group III (noise exposed): H&E × 640]

Fig. 10: Showing apparently decreased Nissl’s granules content of the spiral ganglion neurons compared with the control. Notice the widely separated neurons and the apparent decrease in size of some neurons (†). [Group III (noise exposed): Toluidine blue × 640]

Fig. 11: Showing irregular, disrupted OHCs with degenerated mitochondria (↑) and a shrunken electron dense nucleus (▲). Disarranged, bent or lost OHCs’ stereocilia can be observed. [Group III (noise exposed): TEM × 4000]

Fig. 12: Showing the IHC with few cytoplasmic vacuolization (V). [Group III (noise exposed): TEM × 8000]

Fig. 13: Showing a supporting cell with an electron dense irregular nucleus (†). The heterochromatin is seen accumulating under the nuclear envelope. Notice the vacuolated cytoplasm (*). [Group III (noise exposed): TEM × 12000]
Fig. 14: Showing very thin disrupted myelin sheath (↑) wrapping the apparently swollen nerve fibers. [Group III (noise exposed): TEM × 10000]

Fig. 15: Showing degenerated OHCs (↑) with pyknotic nuclei. Degenerated outer phalangeal (Δ), Hensen’s (▲) and border cells (*) can be noticed. [Group IV (pre-noise treatment): H&E × 640]

Fig. 16: Showing vacuolated cytoplasm of the OHCs (↑) with loss of OHC in the third row (*). Degeneration of supporting cells can be seen. [Group IV (pre-noise treatment): H&E × 640]

Fig. 17: Showing cytoplasmic vacuolization in the strial epithelial cells (V). The melanin content of the stria is apparently increased compared with the control. Many fibrocytes are noticed with vacuolated cytoplasm (↑). Thickness of the spiral ligament is seen comparable to the control. [Group IV (pre-noise treatment): H&E × 640]

Fig. 18: Showing widely spaced spiral ganglion neuron. Notice the apparently decreased Nissl’s granules content and decreased size of some neurons (↑) compared with the control. [Group IV (pre-noise treatment): Toluidine blue × 640]

Fig. 19: Showing OHCs with slightly vacuolated cytoplasm (Δ), shrunken nuclei (▲) and degenerated mitochondria (↑). Notice the supporting cell with degenerated electron dense and cytoplasmic vacuolization (S). [Group IV (pre-noise treatment): TEM × 4000]
Fig. 20: Showing apparently normal myelin sheath appearance.  
[Group IV (pre-noise treatment): TEM × 6000]

Fig. 21: Showing the hair and supporting cells comparable to the control sections.  
[Group V (post-noise treatment): H&E × 640]

Fig. 22: Showing the stria vascularis and the spiral ligament apparently similar to the control.  
[Group V (post-noise treatment): H&E × 640]

Fig. 23: Showing structure and Nissl’s granules’ content in the spiral ganglion neurons apparently similar to the control.  
[Group V (post-noise treatment): Toluidine blue × 640]

Fig. 24: Showing OHCs ($) comparable to the control.  
[Group V (post-noise treatment): TEM × 6000]

Fig. 25: Showing myelin sheath comparable to the control.  
[Group V (post-noise treatment): TEM × 10000]
Table (3): Showing the Mean ± SEM of the thickness of the central part of the spiral ligament in µm:

<table>
<thead>
<tr>
<th>Group</th>
<th>Thickness of</th>
<th>Values</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group I (control)</td>
<td>spiral ligament</td>
<td>23.64 ± 0.52 (5)</td>
</tr>
<tr>
<td>Group II (drug combination)</td>
<td></td>
<td>23.36 ± 0.53 (5)</td>
</tr>
<tr>
<td>Group III (Noise)</td>
<td></td>
<td>12.60 ± 0.70 * (5)</td>
</tr>
<tr>
<td>Group IV (pre-noise treated)</td>
<td></td>
<td>22.47 ± 0.53 * (5)</td>
</tr>
<tr>
<td>Group V (post-noise treated)</td>
<td></td>
<td>22.98 ± 0.35 * (5)</td>
</tr>
</tbody>
</table>

Values are mean ± SEM. - Number in parenthesis indicates the number of guinea pigs. - a: significance of difference by LSD from Group I (Control) at least p<0.05. - b: significance of differences by LSD from Group III (Noise) at least p<0.05.

4. Discussion:
In the present study, combined drug administration had no negative effect on the cochlear function or structure. This coincides with previous studies that confirmed that this drug combination was not ototoxic (4, 11).

Acoustic trauma caused by noise exposure in the present study exhibited profound cochlear damage as documented audiologically and histologically. Exposure to high intense noise led to hearing loss in guinea pigs (Table 2). These findings extend the results of previous studies reporting elevated ABR thresholds and threshold shifts after noise exposure (1). In agreement, other researchers reported missing OHCs (18, 19) and vacuolization of the sensory and supporting cells of the organ of Corti (20) after noise exposure. Minimal affection of the IHC was noticed in the present study coinciding with other researches reporting no loss of IHC’s stereocilia with non significant loss of IHCs (18, 21).

Degradation of the filamentous actin (F-actin) - a cytoskeleton protein of OHCs- has been reported after noise exposure in murine cochleae (19). This F-actin is primarily distributed in the stereocilia of the hair cells and found to be involved in their motility and maintenance of cell shape (22). Therefore degradation of F-actin might pave the way to the disarrangement of hair cells’ stereocilia noticed in the present study. Moreover, lethal alteration of cytoskeletal organization has been found to be triggered by increased Ca++ influx (23). This might also explain the irregularly disrupted shapes of OHCs seen in the present study by TEM.

Noise-induced lateral wall histopathology found in the present study in the form of atrophic spiral ligament with vacuolated strial cells. This was in agreement with what described in previous studies which confirmed its significant contribution to noise-induced hearing loss (16, 24). Vacuolization of strial basal cells and type II fibrocytes was reported in a recent study. They added that degeneration of type I fibrocytes was also observed 2 weeks after noise exposure. It was suggested that noise exposure might lead to interruption of the cochlear ion homeostasis at multiple points along normal route of ion transfer (16).

Melanocytes have been known for their action in maintaining ion homeostasis (25) and for their antioxidant properties by inhibition of reactive oxygen species (ROS) formation (26). Other studies reported increased strial pigmentation after acoustic trauma (27, 28). An earlier study stated that melanin
migrated from the intermediate to the marginal layer possibly to be involved in mechanisms underlying prevention of acoustic trauma (29). Therefore, increased strial pigmentation as noticed in the present study in group III could be considered a protective way against noise exposure.

Myelin sheath degradation in apparently swollen nerve fibers was observed after noise exposure in the present study. These were in accordance with other studies reporting degeneration and swelling of afferent nerves (30, 31). Some authors suggested that after degeneration of hair cells, neurons lack either stimulation or neurotrophic growth factors normally provided by these cells (32). Other investigators stated that noise-induced excessive release of glutamate from the damaged hair cells might be responsible for these effects (33). Moreover, decreased Nissl’s granules in some neurons of the spiral ganglia were also detected in semi-thin toluidine blue-stained sections in the present study. Some authors suggested that this might be a reflection of cell stress (34). Moreover, it has been found to be associated with increased Ca++ in noise-induced damage of hippocampal cells of rats (35). Other authors attributed it to decreased neuronal function by noise-induced excitotoxicity (36).

Noise-induced hearing loss (NIHL) was assumed to be primarily caused by mechanical destruction of organ of Corti (37). Later on, intense metabolic activity has been claimed to be the major cause of this cochlear damage. In turn, this would increase mitochondrial activity with subsequent increase in free radical formation (38). Both oxidative stress and mitochondrial destruction have been found to increase intracellular Ca++ that activates apoptosis (39, 40). This might occur through an apoptotic inducer known as BCL-2 associated death promoter (BAD) that works via the Ca++-dependant calcineurin. Next, translocation of BAD into the mitochondria might lead to the release of apoptosis-inducing factors initiating DNA fragmentation and apoptosis (41). Additionally, this Ca++ overload could be higher in the basally-located OHCs, thereby might have intrinsically higher susceptibility to the reactive oxygen species (ROS) that was found to be increased following noise exposure (42). This might explain that the findings of the present study were seen mostly in the basal turn. Oxidative stress has also been implicated in lipid peroxidation (43) that has been involved in the damage of proteins embedded in the cell membranes with subsequent cell death (9).

Decreased blood flow of the inner ear was also implicated in NIHL (7, 37). Some researchers found that noise decreased red blood cell velocity with blood stagnation in strial capillaries (44-46). This coincides with the finding of congested strial capillaries observed in noise-exposed group in the present study. In addition, noise has been found to decrease blood vessel diameter due to formation of 8-isoprostaglandin F₂α, a vasoactive byproduct of free radicals (7) or due to Noise-induced hypoxia (47).

Most of the recent studies have focused on antioxidants in treatment of NIHL. The antioxidants properties of each of vitamins A, E and C have been well known and documented by many studies (48, 49). B-carotene -which is metabolized into vitamin A in vivo- has been found to scavenge singlet oxygen radicals. These free oxygen radicals are known to react with lipid to form lipid hydroperoxides, thus scavenging them by vitamin A prevents lipid peroxidation. Vitamin E as well is a well known fat-soluble antioxidant that reacts with and decrease peroxyl radicals within the cell membrane inhibiting the propagation of lipid peroxidation cycle (49). In contrast to vitamins A and E, vitamin C is water-soluble antioxidant detoxifying free radicals by reducing and scavenging them in the aqueous phase (48). Furthermore, the two lipid soluble vitamins E and A are complementary to each other since the antioxidant properties of vitamin E is effective at high oxygen concentrations, however that of vitamin A is effective at low oxygen concentrations. Moreover, it was reported that vitamin C could reduce vitamin E radical, thus restore back vitamin E after its possible oxidation into vitamin E radical in the oxidative attack (50). In agreement the present study showed that these vitamins acted in synergism since they significantly inhibited the noise-induced acoustic trauma both audiologically and histologically particularly when administered one day post noise.

Beneficial effect of magnesium (Mg) on noise trauma have been well documented, however, the specific molecular mechanisms underlying this effect have not yet been explained. It seems to be a complex process, including protection against impairment of cochlear microcirculation and oxygenation (8). Previous studies reported decrease in noise-induced vasoconstriction increasing blood flow to the cochlea (51, 52). In addition, Mg has a calcium antagonistic action by modulating Ca++ channels permeability, decreasing Ca++ influx into the cochlear cells, reducing NIHL (52). Magnesium also may have an otoneuroprotective effect via N-methyl-D-aspartate receptor antagonism. Hence, in this way Mg reduces intracellular glutamate release that is responsible for noise-induced neuronal damage (53). Furthermore, Mg may also act as an antioxidant protecting the cochlea against free radicals which
have been shown to be partly responsible for noise-induced hearing loss (54).

The mechanisms of noise injury include parallel pathways that are not limited to oxidative stress. Thus, therapies combining multiple antioxidants may have advantages over single-agent approaches. Several trials using combination therapies have demonstrated clear additive and beneficial interactions against different damaging mechanisms in NIHL (4, 6, 11).

In the present study, the combination of drugs taken one day after noise exposure were more effective than pre-treatment 5 days pre-noise. These findings were matched audiologically and histologically. In agreement, previous studies reported that administration of drugs shortly before noise exposure failed to prevent hair cell death in mice (4, 11). However, some authors reported that pre-treatment with drug combination decreased the loss of fibrocytes in the lateral wall of the cochlea (5). This coincides with the results of the present study reporting nearly similar thickness and structure of the spiral ligament in the pre-noise treated group as compared to the control sections. Better hearing outcomes were reported with treatment by drug combination initiated 1 and 3 days post-noise. This was evidenced by significant functional recovery and reduction of acute temporary threshold shifts in guinea pigs (12). Previous investigations documented the presence of late-forming free radicals in NIHL. Several studies reported that noise-induced oxidative stress begins early and became substantial with time (15, 55, 56). This would potentially explain observations of hair cell death that accelerates with time up to 14 days after noise exposure. Using 4-hydroxynonenal, it was confirmed that peak ROS production in cells in the organ of Corti occurs 7–10 days post-noise, and that noise-induced hair cell death is similarly delayed (15). It could be assumed that pre-treatment with a variety of scavengers reduced the early formation of free radicals that has been well characterized by previous investigators (38, 56). However, it would be more effective if these drugs continued for 5 more days after noise exposure. This also could justify why post-noise treatment was more effective than pre-noise administration of drugs in the present work.

Conclusion:

The results of the present study provide a window of opportunity for rescue from noise cochlear trauma. It also provides morphological and functional evidences that delayed treatment one day after noise exposure may have beneficial clinical outcomes especially after unexpected noise exposure.

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