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ORIGINAL ARTICLE

Evaluation of Effect of Vitamin B12 on Noise-Induced Hearing Loss by Distortion Product Otoacoustic Emission (Dpoae) and Scanning Electron Microscopy

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Objective: Evaluation of protective effect of vitamin B12 on hearing loss following repeated noise by scanning electron microscopy (SEM) and Distortion Product Otoacoustic Emission (DPOAE).

Materials and Methods: In this study, eight adult albino guinea pigs were used as the study group (n = 4) and control group (n = 4). DPOAE measurements of both groups were performed before the procedure. 2 hours before administration of the noise, 0.9% NaCl solution was intraperitoneally injected to control group and vitamin B12 to study group once a day for 5 days. 105 dB SPL 4 KHz frequency-based narrow-band noise was administered to both groups 2 hours per day for 5 days. DPOAE measurements were performed again at 6th day and cochleae were dissected and examined by SEM at 7th day.

Results: Regarding results of DPOAE, noise induced hearing loss (NIHL) was observed in both groups at 6th day. Loss, flattening and fusion, which are findings of permanent hearing loss, were determined in stereocilias of inner and outer hair cells by SEM. These findings were evaluated as signs of permanent threshold shift. When DPOAE measurements and SEM results were evaluated in Vitamin B12 study group, no significant difference was observed in NIHL compared to control group ($p > 0.05$).

Conclusion: In our study, it was observed that simultaneously administration of vitamin B12 during noise had no protective effect on permanent threshold shift. However, new studies on noise and long-term use of vitamin B12 can be performed.

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Introduction

Noise-induced hearing loss (NIHL) is one of the most common occupational diseases, causing labor and economic losses. NIHL or acoustic trauma occurs in two ways. The first, temporary threshold shift (TTS) occurs with exposure to moderate intensity noise that recovers within minutes and days. If the noise causing TTS is continuous or frequent, a permanent threshold shift (PTS) develops in this case.[1,2] Cochlear hypoxia is the first pathological condition in NIHL. Oxidative stress has the major role in permanent hearing loss due to cochlear damage. Antioxidants, such as D-methionine, are effective on cortical body and NIHL by reducing the free oxygen radicals.[3]

Vitamin B12 is essential cofactor in methylation of myelin basic protein and cell membrane phospholipids and also plays a role in methionine synthesis from homocysteine. In case of Vitamin B12 deficiency, anemia, demyelination, axonal degeneration and finally neuronal loss occurs. Supplementary vitamin B12

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treatment is used for prevention of vascular diseases and neural tube defects and to enhance vascular endothelial function in coronary heart disease. Conditions such as thrombosis, embolism, hemorrhage, spasm, hypercoagulability at arterial system of the inner ear disturb inner ear perfusion. Homocysteine is a vascular and thrombotic risk factor and causes vascular injury by reducing the amount of nitric oxide (NO). Increased homocysteine concentrations cause vascular complications. Supplementary vitamin B12 and folate reduce homocysteine synthesis, and leads to vasodilatation as a result of an increase in the amount of NO. Thus, vitamin B12 causes an increase in vascular perfusion and cellular metabolism in the cochlea. Studies are currently underway examining the effect of vitamin B12 on NIHL.[4-9]

In this study, protective effect of vitamin B12 on NIHL, a widely observed major public health problem with no treatment accepted by everyone, was evaluated for the first time with SEM and Distortion Product Otoacoustic Emission (DPOAE) data.

Materials and Methods

This study was approved by the Committee for Ethics in Animal Experiments of the Current Marmara University (MUHDEK) with protocol number (44.2011.mar). Eight healthy mature male albino guinea pigs weighing between 440 and with normal Preyer's reflexes and normal tympanic membranes were used for these experiments. These animals were provided with free access to food and water. DPOAE were evaluated before the experiments all animals' both ears. The animals were assigned to one of two groups. In the experimental group (n=4) vitamin B12, (Dodex amp, Deva, Turkey, 300mg/kg) was administered intraperitoneally (i.p) 2 hours before noise exposure. In the control group (n=4), the same dosage of % 0,9 NaCl solution was administered i.p. All application was done 5 consecutive days. All groups were exposed to narrow band noise centered 4 kHz at 105 dB SPL (sound pressure level) for 2 hours every 5 consecutive days. The noise we used was designed PTS and to damage cochlear hair cells. All animals were awake when they were exposed to that noise. Noise presentation was performed by a high fidelity sound system (CD player Pioneer PD-306, amplifier Yamaha P4500, loudspeaker). The loudspeaker was centered over the animal's head at distance of . Sound intensity was monitored with a sound-level meter (Tromer, P.R.C.) positioned near the external auditory canal (Figure 1). One day after finished noise exposure DPOAE was measured again. 7th day after begining the experiment, the animals were deeply anesthetised with sodium thiopental (Pentothal , Abbott , U.S.A, 100 mg/kg, i.p.), and after killing them by decapitation, the cochleae were dissected by macroscopic (Figure 2). And then they were perfused with glutaraldehyde (3% in 0.1 mol cacodylat buffer pH 7.4).

Preparation of the cochleae

The cochleae were fixed with 2.5% glutaraldehyde in phosphate buffer (pH 7.4) for 4 hours and rinsed with phosphate buffer. After incubation in rapid decalcifier

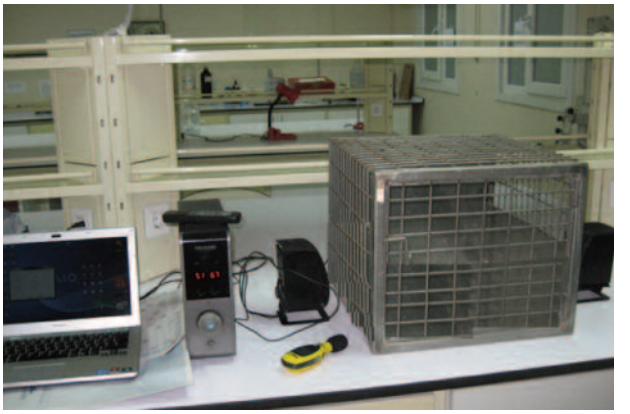


Figure 1. The equipments of experiment for generate noise.

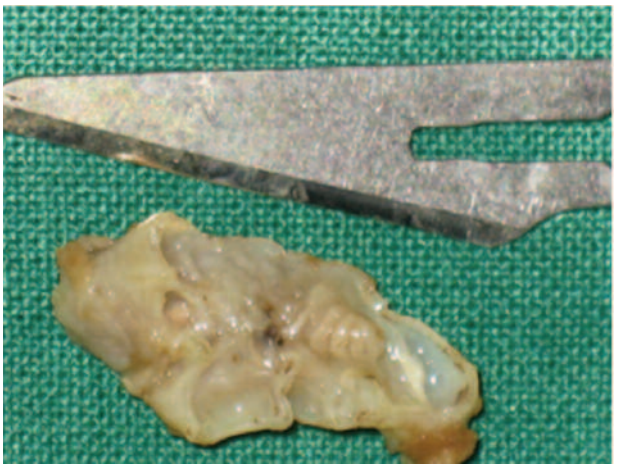


Figure 2. After incubation in decalcifier, the cochleae is seen before dissected to remove the bony capsule

overnight, the cochleae were further dissected in order to remove the bony capsule, the spiral ligament, the stria vascularis, and Reissner's and the tectorial membranes. The exposed organ of Corti was post-fixed in 1% osmium tetroxide in phosphate buffer for one hour and then rinsed with phosphate buffer. The tissues were dehydrated through an increasing series of ethanol, critical point dried with the critical point drying (Bio-Rad E 3000, Hemel Hempstead, Herts, UK) under liquid CO2 pressure. Finally they were sputter-coated with gold (Bio-Rad SC502, Hemel Hempstead, Herts, UK).The tissues were were viewed on a JEOL JSM-5200 SEM (Tokyo, Japan). Scores were done for each basal,middle and apical turn seperately. Damage scores were evaluated both inner (IHCs) and outer hair cells (OHCs) for sterocilia loss, fusion, length shortening. Scores were done like 0=undamaged, 1 =mild, 2 = moderate and 3= severe damage

DPOAE Test Application

Animals were sedated using an intraperitoneal solution of 50 mg/kg ketamin hidroklorur (Ketalar, Eczacibasi, Turkey) and 7.5 mg/kg xylazine. For the recording and subsequent analysis of DPOAE, a GSI Audera (Grason-stadler,California-USA) recorder was used. The acoustic probe was hand-held to the opening the external auditory meatus with gentle pressure. The measurements were carried out in a soundproof chamber. Both ears were measured at the frequencies f2= 1, 1.5, 2 3, 4, 6 and 8 kHz with one pair of stimulus tones (f2/f1=1.22, DP definition =2f1-f2; L1=65, L2=55,respectively) at approximately one measurement every 4s. Only DPOAE were included in the analysis that were at least 3 dB above background noise. All DPOAE levels with a signal to noise ratio were analyzed.

The data were analyzed using the Wilcoxon paired 2-sample test and Mann–Whitney U test variance analysis in SPSS (Statistical Package for the Social Sciences, version 15.0) for Windows. P value of less than or equal to 0.05 was considered significant.

Results

When DPOAE values before and after the noise were compared in 0.9% NaCl solution and Vitamin B12 given group, statistically significant decrease was found in post- noise values ($p \leq 0.05$) (Figure 3-4). Decrease in DPOAE values at 4, 6, 8 kHz frequencies were significantly higher in both groups ($p \leq 0.05$).

When study and control groups were compared in terms of hearing loss after noise, DPOAE values at 1, 1.5, 2, 4, 6, 8 kHz frequencies between the two groups showed no significant difference regarding hearing loss ($p > 0.05$).

SEM analysis of control and study group revealed findings of development of PTS such as hair loss, length shortening, fusion to each other, bending, irregular settlements, flattening of the IHCs and OHCs.

There was slight damage in basal and middle turn of IHCs and OHCs in both control and study groups (Figure 5a, 5b, 6a, 6b). In both groups, minimal damage to the IHCs and severe-to-moderate damage to OHCs was observed in the apical turn (Figure 7a, 7b). When IHC and OHC stereocilias of basal, middle and apical turns of cochlea were compared in terms of damage, there was no statistically significant difference between the control and study groups ($p > 0.05$) (Figure 8).

When DPOAE and SEM analysis were evaluated together, no significant difference was found between the right and left ears ($p > 0.05$). When DPOAE values and stereocilia injury rates were evaluated, similar rates were found regarding IHC and OHC cilia damage and decrease in DPOAE in both groups.

Discussion

It is reported that two major permanent morphological changes were observed in the noise-exposed cochleae: hair cell losses and stereocilia changes as for the histological data, However the acoustic trauma was characterized less by the lack of cells than by injured stereocilia.[10] First, at the ultrastructural level, it is likely that alterations in the stereocilia in the form of shortened or broken rootlets are involved in the initial pathologic processes that lead to TTS and, if such injuries are not repaired, then PTS.[11-13] More recent findings showed that hair bundles are capable of rebuilding their ultrastructure from top to bottom over a 48-hour period.[14] If damage is so severe that it overwhelms this self-repair mechanism as exposure continues, a discrete but direct mechanical disruption likely results in a toxic mixing of endolymph and perilymph through microbreaks in the structural

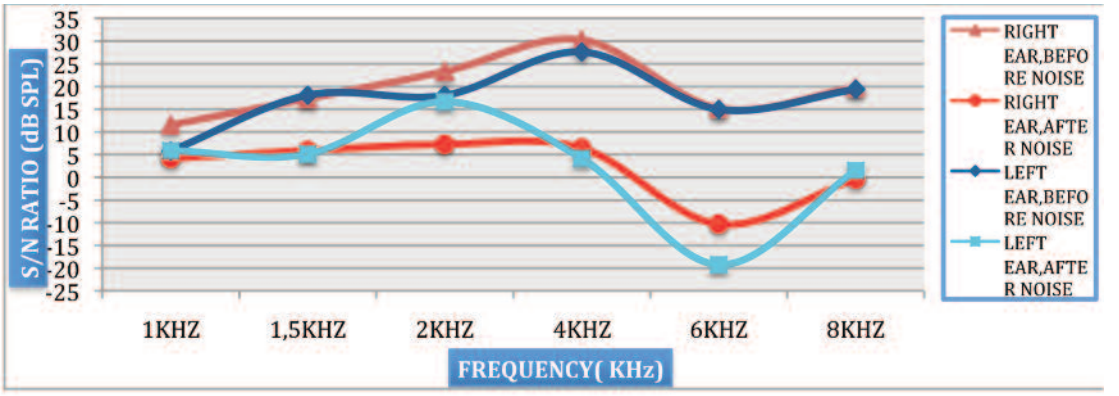


Figure 3. DPOAE's results, between of the ears in the experimental group.

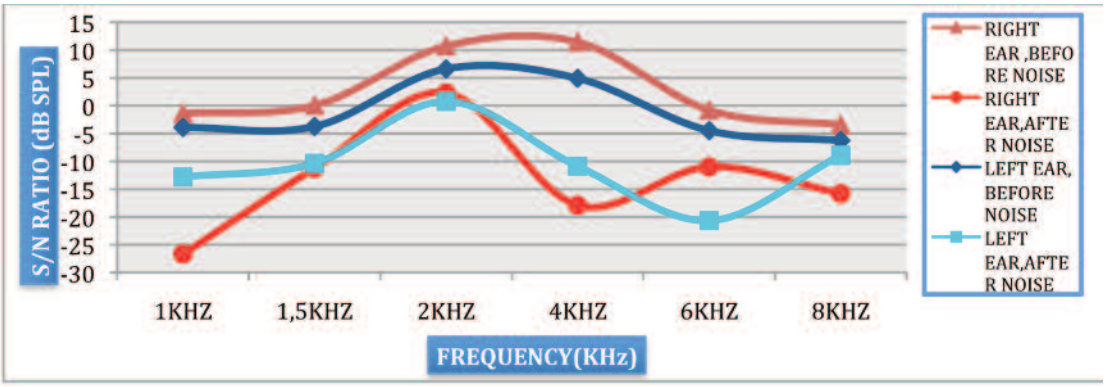


Figure 4. DPOAE's results between of the ears in the control group.

framework of the cochlear duct, which leads to secondary effects, including loss of hair cells and their corresponding nerve fibers.[15]

In NIHL, damage spreads to OHCs followed by IHCs as exposure to noise continues.[2] In our study; loss, length shortening, irregular settlement and flattening of stereocilia were detected in NIHL. This explains that PTS developed in both groups. In our study, we found that damage spreads to the IHCs. In study and control group, mild damage of IHCs and OHCs were detected in basal and middle turns of cochlea, and less damage rate were observed in IHCs of the apical turn than the other turns. The reason of this damage, which is thought to be related to 4000 Hz. centered narrow band noise, is because high frequencies affect basal turn more.[2] The amount of loss of stereocilia bundles was higher in OHCs in the apical turn than other turns. As a general consideration regarding hair cell loss in the apical cochlear turn, it should be mentioned that missing OHCs in the apical turn are not representative for damage by noise exposure. In the apical turn, many missing OHCs or incomplete OHC rows are observed in normal animals as well.[16] Zhou and Pickles concluded that early hair cell degeneration is a possible reason.[17] Missing hair cells in all other turns represent a damage following noise exposure or drug treatment etc.[18]

Former microscopic studies revealed a contradiction between reduced cochlear microphonics shortly after an impulse noise exposure, indicating an impairment of the OHC function, but OHCs that appeared morphologically unchanged. However, if post-mortum microscopy is applied to evaluate the OHC condition, it has to be taken into account that the degeneration of the stereocilia bundles is a temporal progressive process.[19] The complete degeneration of the OHCs and IHCs may last several weeks, and the degeneration of the ganglion spiral cochleae follows with a time delay of a few weeks or months. In line with previous experiments that

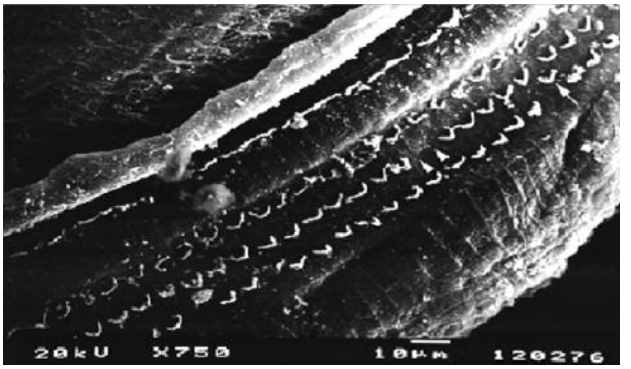


Figure 5a. Outer hair cells' stereocilia loss (arrowhead) and disorganized shape (arrow) are shown in the basal turn of the experimental group. SEM micrograph, x750.

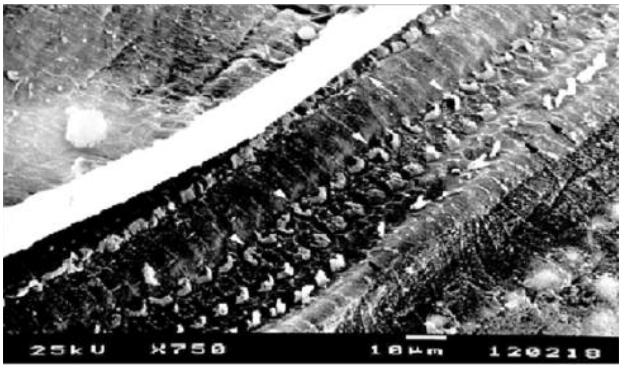


Figure 5b. Outer (white arrow) and inner (black arrow) hair cell' stereocilia loss were observed in the basal turn of the control group. Arrowhead; disorganized shape stereocilia in the outer hair cells. SEM micrograph, x750.

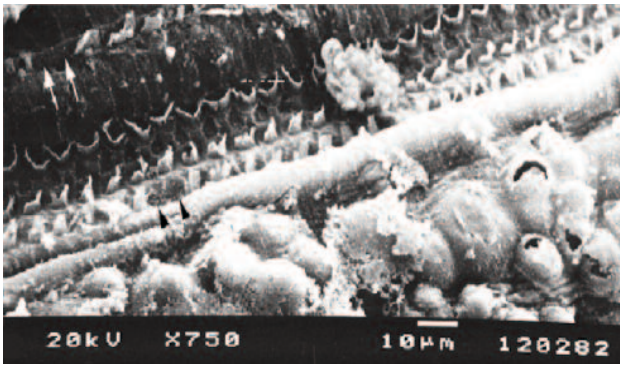
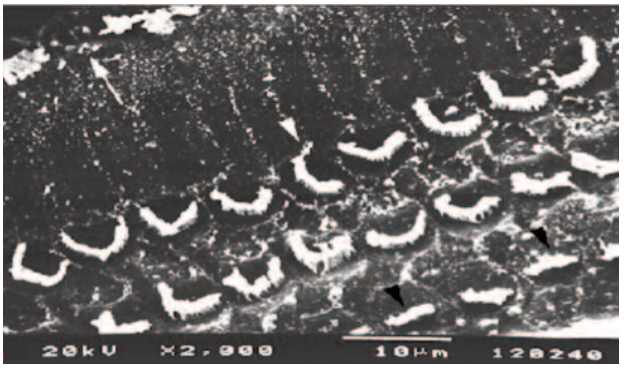


Figure 6a. In the middle turn of the experimental group, inner (white arrow) and outer (black arrowhead) hair cells' stereocilia loss. SEM micrograph, x750



Picture 6b. In the middle turn of the control group, inner hair cells' stereocilia loss (arrow) with outer hair cells' disorganized shape (white arrowhead) and splayed stereocilia (black arrowhead). SEM micrograph, x2000

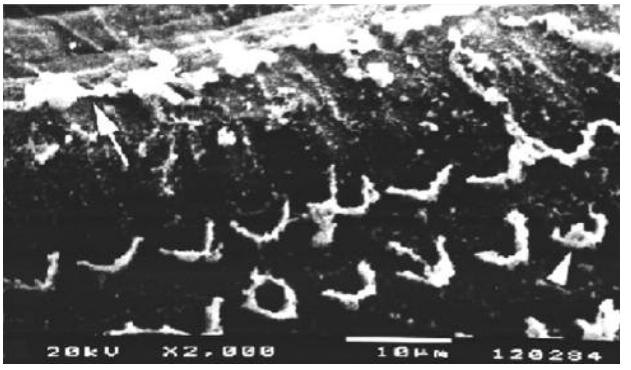


Figure 7a. In the apical turn of the experimental group, inner (arrow) and outer (arrowhead) cells' damaged stereocilia can be observed. SEM micrograph, x2000

Picture 7b. In the apical turn of the control group, inner (arrow) and outer (arrowhead) hair cells' stereocilia loss. fused shape and shortening are showed outer hair cells' sterocilia. SEM micrograph, x2000

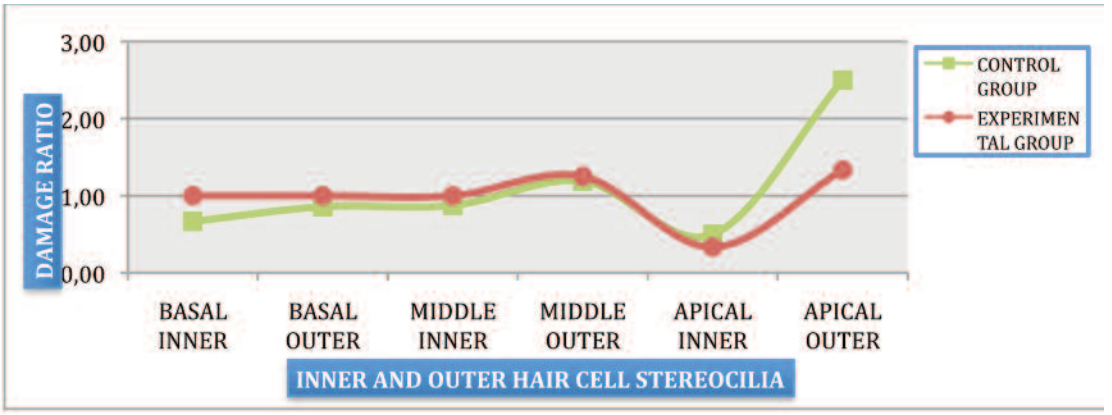


Figure 8. The damage results of the inner and outer hair cells' stereocilia in the scanning electrone microscopy.

showed long-lasting after effects of noise exposure, an observation time of 3–4 months after noise exposure was given before the morphological analysis was performed.[20] New studies can be done for long-term effects of vitamin B12 in NIHL.

Fused or disconnected stereocilia bundles with probably impaired tip-links were considered as "abnormal". It is important to note that, to a certain extent, fused stereocilia bundles are also found in normal animals. Geleoc et al. have measured the maximum transducer conductance of mouse OHCs and concluded that there is a direct correlation between the number of single transducer channels and the number of tip-links.[21] The localization of transduction channels at the tip-links has been concluded by Denk et al.[22] Meyer et al. supposed that tip-links are required for mechanoelectrical transduction.[23]

DPOAE shows frequency-specific cochlear functions. A closer relationship between the decline of amplitudes of DPOAE and the number of missing and changed OHCs (fused stereocilia bundles, missing tip links) could be established. The number of lost OHC does not reflect the decline in DPOAE in all cases.[22,23] In our study, both decreased hearing in DPOAE analysis and loss and damage in stereocilias were detected in morphological analysis at 7th day. When conventional audiometry (0.25-8 kHz) and extended high-frequency audiometry (9-20 kHz) were compared in people who were exposed to acoustic trauma, it was found that acoustic trauma exposure is mostly between 4-8 kHz and conventional audiometry was reported to be adequate for the evaluation of hearing.[24] Our study also showed more loss in the high frequencies, 4-8 kHz. Basal turn, excluding early degeneration of apical turn outer hair cells, was found to be more affected in SEM examination.

Preyer et al. have shown that the non-linearity of the mechanoelectrical transducer function of the OHCs as found in guinea pigs is essential for non-linear movement of the basilar membrane being considered as a source for the DPOAE generation.[25] Fused or otherwise changed stereocilia bundles have lost at least a part of their function, and this may result in a reduced DPOAE level as observed in previous experiments.[26] Our study also showed fusion in stereocilias. Lataye et al observed flattening in stereocilias at most damaging frequencies.[10] Our study also showed flattening of stereocilias in basal and middle turns.

There are several studies on the relationship between vitamin B12 and noise. Quaranta et al. observed decrease in TTS in adults who were administrated vitamin B12 for 1 week after the noise for creating TTS than the control group.[8] Shemes et al. found significantly higher vitamin B12 deficiency in soldiers with hearing loss and tinnitus and who were exposed to noise. They reported that dysfunction in hearing pathways may be associated with vitamin B12 deficiency.[27] Campbell et al. reported that administration of D- methionine after noise does a significant decrease in ABR thresholds at 2-4 kHz and outer hair cell loss.[28] In another study, hepatic glycogen levels were found to decrease in rats exposed to 95-110 dB noise and vitamin B12, vitamin C and vitamin E

administration were found to suppress the reduction in the levels of this hepatic glycogen.[29] Gök et al. examined the amount of serum B12, folic acid, and homocysteine in NIHL. In patients with hearing loss, serum homocysteine values were found to be high and folic acid and vitamin B12 values were significantly lower.[30] They reported that people who work in a noisy place should be looked for vitamin B12 and folic acid values routinely.[31] In studies on the role of free oxygen radicals in the pathophysiology of acoustic trauma, although those who have low levels of serum vitamin B12 and folic acid are more prone to hearing loss caused by acoustic trauma is shown, there are also studies in which such a relationship can not be demonstrated.[3,32]

Unlike the noise applied for the creation of acoustic trauma or TTS in previous studies, repetitive noise was performed to establish permanent hearing loss. Although in previous studies results indicating preventive effect of vitamin B12 on TTS of NIHL were found, regarding SEM and DPOAE findings of our study, no statistically significant difference was observed when compared with the control group and concluded that it has no preventive effect on permanent hearing loss. Except this study, there are no other experimental studies evaluating the protective effect of vitamin B12 in NIHL. However, new wide-ranging studies are needed to compare the long-term effects.

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