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Experimental Study / Deneysel Çalışma

Prenatally exposure to exogenous glucocorticoids and stress may affect the inner ear

Prenatal dönemde ekzojen glukokortikoid ve stres maruziyeti iç kulağı etkileyebilir

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Objectives: This study aims to investigate the effect of exogenous glucocorticoid exposure in the prenatal period on hearing and to evaluate the effectiveness of caffeic acid phenethyl ester (CAPE), an antioxidant, on the prevention of the inner ear injury.

Materials and Methods: Dexamethasone was given to half of twelve Sprague-Dawley pregnant rats and the distilled water was given to the remaining half. The real subjects were obtained by born of the offsprings. When the all subjects were two months of age, they were exposed to 110 dB noise during four hours as a stressor effect. These subjects were divided into three groups. Group 1: subjects to whose mothers were given distilled water; Group 2: subjects to whose mothers were given dexamethasone; Group 3: subjects to whose mothers were given dexamethasone and CAPE.

Results: While there was no statistical significance in hearing thresholds which exposed and not exposed to exogenous dexamethasone before noise exposure ($p>0.05$) between the groups, the elevation of hearing thresholds of subjects which exposed to exogenous dexamethasone was statistically significant after noise exposure ($p<0.05$).

Conclusion: Prenatally exposure to exogenous glucocorticoids may cause the inner ear susceptible to the effect of noise, and CAPE is effective to prevent the possible damage.

Key Words: Caffeic acid phenethyl ester; delayed effect; glucocorticoid; hearing; prenatal exposure.

Amaç: Bu çalışmada prenatal dönemde ekzojen glukokortikoidlere maruz kalınmasının işitme üzerine etkisi incelendi ve bir antioksidan olan kafeik asit fenetil esterin (KAFE) iç kulakta oluşabilecek olası hasarı önlemede etkili olup olmadığı değerlendirildi.

Gereç ve Yöntem: On iki hamile Sprague-Dawley cinsi sıçanın, yarısına deksametazon ve diğer yarısına da distile su enjekte edildi. Esas denekler, bu sıçanların yavrulamasıyla elde edildi. Esas denekler iki aylık olduklarında stres etkisi oluşturmak için dört saat süresince 110 dB gürültüye maruz bırakıldı. Denekler üç gruba ayrıldı. Grup 1: annelerine distile su enjekte edilen denekler; Grup 2: annelerine deksametazon verilen denekler; Grup 3: annelerine deksametazon ve KAFE verilen denekler.

Bulgular: Gürültüye maruziyet öncesi dönemde, ekzojen deksametazon verilen ve verilmeyen gruplar arasında işitme eşikleri bakımından istatistiksel anlamlı farklılık saptanmazken ($p>0.05$), gürültü maruziyetinden sonraki dönemde, ekzojen deksametazon verilen deneklerde işitme eşikleri istatistiksel olarak anlamlı derecede yüksekti ($p<0.05$).

Sonuç: Prenatal dönemde ekzojen glukokortikoidlere maruz kalınması iç kulağı gürültünün etkilerine karşı hassas hale getirebilir ve KAFE olası bu hasarı önlemede etkilidir.

Anahtar Sözcükler: Kafeik asit fenetil ester; gecikmiş etki; glukokortikoid; işitme; perinatal maruziyet.

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Substantial evidence from several epidemiological studies indicates that some diseases of adult life may arise from early events occurring in the prenatal period.[1] The intrauterine environment programs the metabolic and endocrine balance of the individual, inducing responses which may lead to dysfunction later in life. Results of experiments in animals and clinical investigations in newborn children with risk factors relating to hearing function demonstrate that the developing auditory system is more susceptible to acoustic trauma than the mature system.[2]

Prenatal stress has been associated with a variety of alterations in offspring. Prenatal stress may increase the organism's vulnerability to aversive life events.[3] Exposure of pregnant animals to exogenous or endogenous glucocorticoid hormones not only reduces birth weight but also alters the development of several organs including the cardiovascular system and kidney, leading to hypertension in adult life.[4] Recent studies have implied that children of mothers under stress during pregnancy may be at risk for developing sensorineural hearing loss, and prenatal exposure to the synthetic glucocorticoid (dexamethasone) increased hearing loss after noise exposure in adult rats offsprings.[5,6] Administration of corticosteroids to pregnant women at risk of preterm labor is an established intervention with proven reductions in the rates of neonatal respiratory disease, intraventricular hemorrhage and death.[7,8] It has been known that cortisol may inhibit growth, and reductions in birth weight after corticosteroids have been shown in several species.[9]

Noise trauma is known to cause oxidative stress and formation of reactive oxygen species in the inner ear, leading to cell damage and hearing loss as does prenatal exposure to glucocorticoid hormones.[10] Complications resulting from noise exposure are well-described in both the epidemiological and experimental literature. Researches indicate that noise-induced hearing loss can be attenuated by exogenous or endogenous reactive oxygen species scavengers such as superoxide dismutase, allopurinol, lazaroids and melatonin.[11-13] Caffeic acid phenethyl ester (CAPE) is an active component of propolis from honey hives. It has antiviral, antiinflammatory and immunomodulatory properties, and has been shown to inhibit the growth of different types of transformed cells. It also has very important antioxidant effects.[14] For example, it has been demonstrated that CAPE has anti-proliferative and radiosensitizing effects on medullablastoma cells.[15]

In this study, we investigated the effect of prenatal exposure to glucocorticoid hormones on the auditory system and evaluated the effect of CAPE as an antioxidant agent on the auditory system by BERA (brainstem evoked response audiometry) and immunohistochemistry techniques.

MATERIALS AND METHODS

Subjects

This study was performed in the experimental surgical laboratory with approval of Ethical Committee of the Institute of Experimental Medicine of Fırat University Elazığ, Turkey and guided for the care and use of laboratory animals. Twelve pregnant Sprague-Dawley rats were distributed reasonably in white plastic cages (25x45x20 cm) with corn cob bedding. Environmental conditions were automatically controlled with a 12-hour light-dark cycle. Food and tap water were available. The cleaning of cages and new bedding were provided twice weekly.

Six Sprague-Dawley pregnant rats were injected intraperitoneally (IP) with dexamethasone (0.1 mg/kg) once daily from day 14 of pregnancy (first day of third trimester) until parturition. Six other pregnant rats were injected IP with distilled water (0.1 mg/kg) once daily from day 14 of pregnancy until parturition. Thus, during the prenatal term, half of the subjects were exposed to the exogenous glucocorticoid and half were not. The experiments were subsequently performed on the offspring rats following parturition of the pregnant rats.

A total of 24 offspring rats were used as test subjects in this study. The subjects were two months of age at the onset of experiments. They were grouped as follows:

Group 1: The subjects whose mothers were injected with 0.1 mg/kg/day distilled water and exposed to 110 dB SPL (sound pressure level) noise for four hours (n=8).

Group 2: The subjects whose mothers were injected with 0.1 mg/kg/day dexamethasone and exposed to 110 dB SPL noise for four hours (n=8).

Group 3: The subjects whose mothers were injected with 0.1 mg/kg/day dexamethasone, and exposed to 110 dB SPL noise for four hours and injected with 10 μmol/kg CAPE (n=8).

Noise exposure of subjects

The broad band noise (800-20.000 Hz) was generated by a Wavetek signal generator (Wavetek Corporation; San Diego CA USA. Model 187), amplified by audio amplifiers (NAD 216 THX). One week after measurements of their hearing thresholds by BERA, all subjects were exposed for four hours to a broad band noise at an intensity of 110 dB SPL delivered by the loudspeakers (Vifa Loudspeakers, D26TG-05-06) situated 50 cm above the floor of a sound isolated cage (1x0.5x0.5 meter). Calibration of the sound conditioner in open field was performed with a condenser microphone (Bruel and Kjaer model type 2213). The measurements were performed at four points within the cage and standardized at 110±2 dB SPL and applied for four hours.

Antioxidant agent (CAPE) regimen

An antioxidant agent (CAPE) was injected IP to group 3 subjects at a concentration of 10 μmol/kg. The antioxidant regimen consisted of a total of five injections given 30 minutes before noise exposure, immediately after noise exposure (which lasted 4 hours) and once daily for three days.

Measurement of subjects' hearing thresholds by BERA

The subjects were anesthetized with a combination of 2% xylazine hydrocloride (Rompun, Bayer, Turkey) 5 mg/kg and ketamine hydrocloride (Ketalar, Eczacıbaşı, Turkey) 60 mg/kg by intramuscular (IM) route. The hearing thresholds of the subjects were determined by Homoth BERA-System 4000 (Homoth Medizinelektronik GmbH & Co KG-Germany) in the sound isolated room at 26-28 ºC. BERA tests were performed subcutaneously with stainless steel electrodes as the potential difference between an electrode on the vertex and an electrode on the mastoid. The stimuli consisted of full-cycle sine waves at 32, 16, 8, 4, 2, 1 and 0.5 kHz. The hearing thresholds of all subjects were measured one week before, 48 hours after and two months after noise exposure.

Sacrifice of the subjects

When the subjects were four months of age at the end of the measurement of hearing thresholds by BERA, they were decapitated by guillotine system after being anesthetized with high dose ketamine hydrocloride and xylazine hydrocloride. The skull bones of the subjects were cut in the midline, tympanic bullas were revealed and their cochleas were dissected and removed en-bloc.

The cochleas of each subject were kept in 2% phosphate buffered glutaraldehyde. These cochleas were used for immunohistochemical evaluation. Caspase immunoreactivity that indicates to apoptosis was evaluated by light microscopy.

Tissue preparation for light microscopy

The dissected cochleas were decalcified for three weeks in 10% EDTA solution. These cochleas were then fixed for 24 hours in 10% formaldehyde solution. After the fixation process, the cochleas were washed with tap water for 24 hours, and dehydrated by reaction with graded alcohol series. The cochleas were sliced and blocked after infiltration with paraffin. Five micro meter sections of paraffinized blocks were mounted on microscope slides.

Immunohistochemistry technique

The cochlear sections were deparaffinized and washed after being reacted with alcohol series, and after incubation with 0.1-1% H_2O_2, washed with PBS (phosphate buffered saline) and dyed with avidin-biotine. The sections were inoculated with 10% normal bovine serum. Then caspase-3 goat polyclonal primary immunoglobulin G (IgG) antibodies were diluted with 1:400 normal bovine serum. The sections were incubated with caspase overnight. Phosphate buffered saline was dripped onto negative control sections. Next day, the sections were washed with PBS and incubated first with secondary antibody (biotin bovine antigoat) then HRP (horse radish peroxidase), and after washing with PBS, conducted with DAB (diaminobenzidine) chromogen. The sections washed with distilled water were stained with hemotoxylin eosine. Then, the sections were washed with distilled water until blueness disappeared, and closed after reacted with alcohol and xylol. The sections were evaluated according to size of caspase immunoreactivity as follows: low (+), mild (++) and intense (+++).

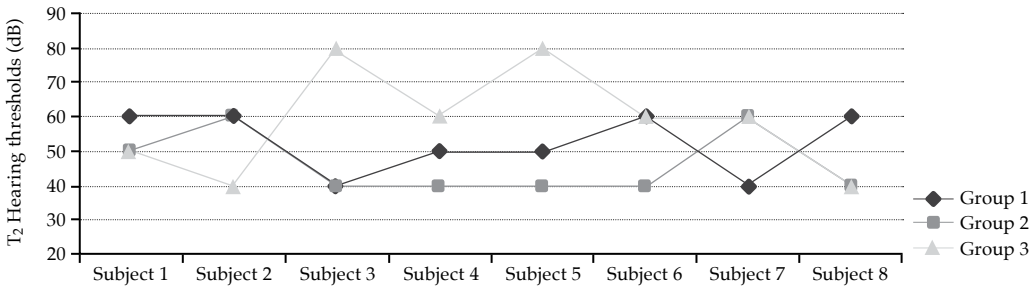

Figure 1. *T_2 is the hearing thresholds (dB) at 16.384 Hz measured 48 hours after noise exposure.*

Statistical analysis

The statistical comparison of the hearing thresholds was calculated with double direct variance analysis. Tukey's t-statistical analysis was used for Post-hoc calculation. Differences were considered statistically significant when $p<0.05$.

RESULTS

BERA tests

Hearing thresholds before noise exposure

When BERA tests were performed one week before the noise exposure, V. waveform was obtained at 40 dB SPL as hearing thresholds of all subjects of all groups (whose mothers were injected with dexamethasone or not) and there was no statistically significant difference between the groups.

Changes in group 1

The mean of hearing thresholds of group 1 subjects measured before noise exposure was 13 dB better than that measured 48 hours after noise exposure, and the difference was statistically significant ($p<0.05$). The mean of hearing thresholds measured 48 hours after noise exposure was 10 dB worse than measured two months after noise exposure, but this difference was not statistically significant ($p>0.05$) (Figure 1).

Changes in group 2

The mean of hearing thresholds of group 2 subjects measured before noise exposure was 12 dB better than measured 48 hours after noise exposure, and the difference was statistically significant ($p<0.05$). There was no statistically significant difference between the means of hearing thresholds measured 48 hours after noise exposure and measured two months after noise exposure ($p>0.05$) (Figure 2).

Changes in group 3

The mean of hearing thresholds of group 3 subjects measured before noise exposure was 22 dB better than measured 48 hours after noise exposure, and the difference was statistically significant ($p<0.05$). There was no statistically significant difference between the means of hearing thresholds measured 48 hours after and measured two months after noise exposure ($p>0.05$) (Figure 3).

Immunohistochemistry investigations

Group 1: (++) caspase immunoreactivity in the spiral ganglion cells (Figure 4) and stria vascularis (Figure 5) was observed in group 1 subjects.

Group 2: Either cytoplasmic or nuclear caspase immunoreactivity (+) was observed in the organ of corti (Figure 6, 7) and caspase immunoreactivity

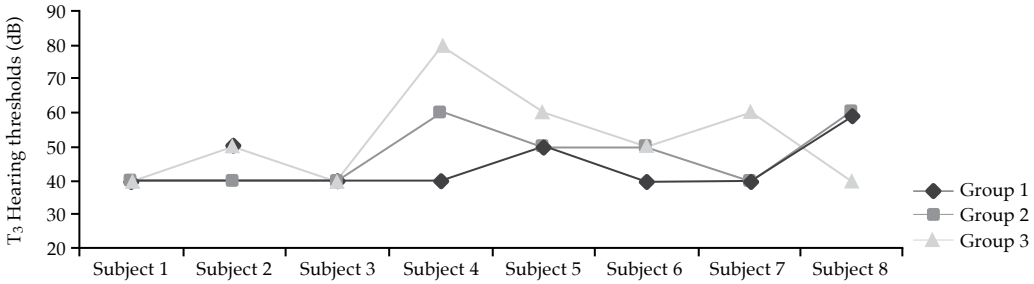

Figure 2. *T_3 is the hearing thresholds (dB) at 16.384 Hz measured two months after noise exposure.*

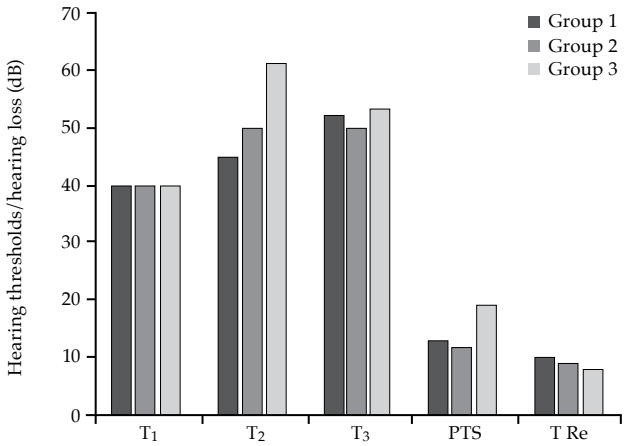

Figure 3. *Hearing threholds at 16.384 Hz measured before noise exposure (T_1), 48 hours after noise exposure (T_2) and 2 months after noise exposure (T_3). Permanent thresholds shift (PTS) is the difference between T_1 and T_2, and threshold recovery (T Re) is the difference between T_2 and T_3.*

(+++) was observed in the stria vascularis and spiral ganglion cells (Figure 7, 8).

Group 3: Caspase expression was not observed in the organ of corti of group 3 subjects (injected with CAPE) against the susceptible effects of the prenatal stress and dexamethasone. But (+) caspase immunoreactivity was observed in spiral ganglion cells and stria vascularis (Figure 9).

DISCUSSION

Any change in fetal environment (prenatal stress, nutrition status of the mother, exposure to exogenous drugs) can affect the future life of the developing organism. But there are some conditions which need external interference for the fetus to continue living. Treatment with exogenous glucocorticoids can diminish the risk of neonatal respiratory disease, intraventricular bleeding and death.[8] Nonetheless, prenatal treatment with exogenous glucocorticoids may pose some drawbacks for the future life of the fetus. It has been shown that prenatal administration of steroids can negatively affect body size and brain development, and play a role the origin of future disease.[16]

Ahlbom et al.[17] injected third trimester pregnant rats with 0.1 mg/kg/day dexamethasone and added hydrogen peroxide (H_2O_2) and methylmercury (MeHg) to cell cultures obtained from the offspring rats. Consequently, they determined the number of apoptotic cells of granular cells of rats given dexamethasone, H_2O_2 and MeHg were more than controls. In this study, we observed that the caspase expression as an apoptotic marker in the organ of corti, stria vascularis and spiral ganglion cells in group 2 was more than in group 1 which was not exposed to exogenous dexamethasone.

Hougaard et al.[18] concluded that neither prenatal exposure to mild stress nor to dexamethasone is detrimental to the hearing organ per se, and this study does not support the previous reports. Rybalko and Syka[19] determined that the hearing thresholds of rats 3-6 months of age before exposure to noise were similar to thresholds of adult rats. Exposure to noise caused similar threshold shifts in all rats, and hearing thresholds began to improve two weeks after noise exposure. In our study, the hearing thresholds of group 3 subjects began to improve two months after noise exposure,

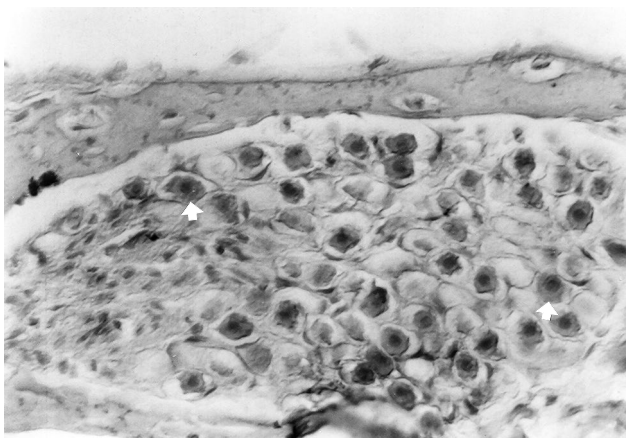

Figure 4. *Immunoreactivity in spiral ganglion cells (arrows) (H-E x 20).*

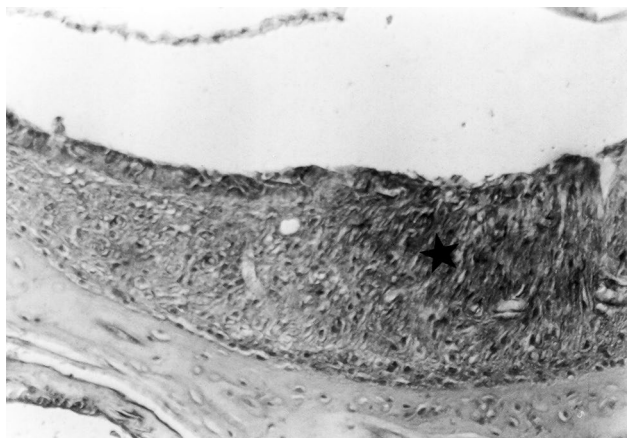

Figure 5. *(++) Caspase expression in stria vascularis (*) (H-E x 10).*

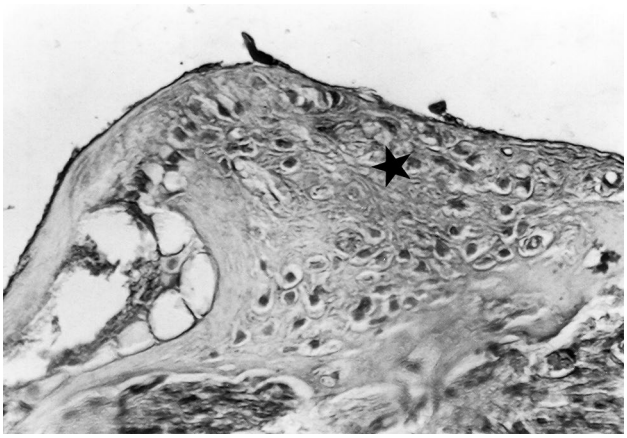

Figure 6. *(+) caspase immunoreactivity in the organ of corti (*) (H-E x 20).*

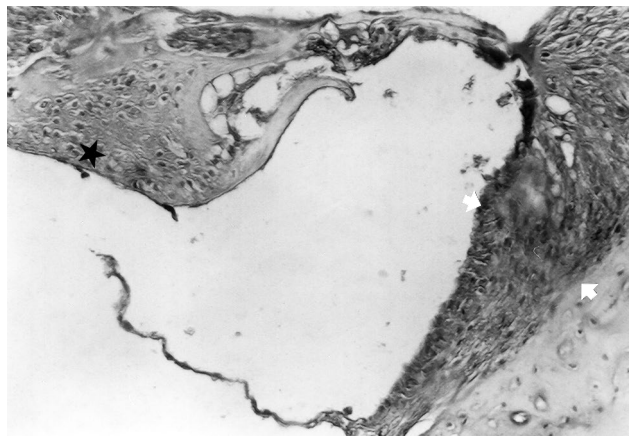

Figure 7. *Caspase expression (+) in the organ of Corti (*) and (+++) in stria vascularis (arrow) (H-E x 10).*

and this improvement can be related to positive effects of CAPE.

Stenqvist[20] treated rats with *Pseudomonas aeroginosa* exotoxin. After instillation, they found that threshold elevation occurred at all frequencies. They concluded that aging-related hearing threshold changes of Sprague-Dawley albino rats started at different ages. Kadner et al.[5] observed that prenatal stress caused hearing loss at low frequencies, possibly due to increased vulnerability to noise induced hearing loss. Canlon et al.[6] showed that the loss of cochlear hair cells in rats given dexamethasone was within normal limits, unless noise which induced oxidative stress was applied. Some changes occur in the stereocilia of hair cells after expose to noise. For example, stereocilia become soft and drooping, and the connections between stereocilia break off.[21] Gao et al.[22] proposed that exposure to intermediate and high intensity noises caused pathologies in the stereocilia of hair cells which might have been caused the threshold shifts. Sullivan and Conolly[23] exposed rats to 110, 100, 95 or 85 dB SPL for six hours/day, five days/week over four weeks. They observed outer cell losses in the organ of Corti in rats exposed to 95, 100 and 110 dB SPL.

Superoxide radical (SOR) blockers and membrane stabilizers which are effective in cochlear ischemia and reperfusion have been used to prevent cochlear damage. Seidman et al.[11] exposed rats to 90 dB SPL continuous noise over 60 hours in order to create cochlear damage, and investigated the effects of allopurinol and superoxide dismutase on the cochlear damage. They observed that allopurinol and

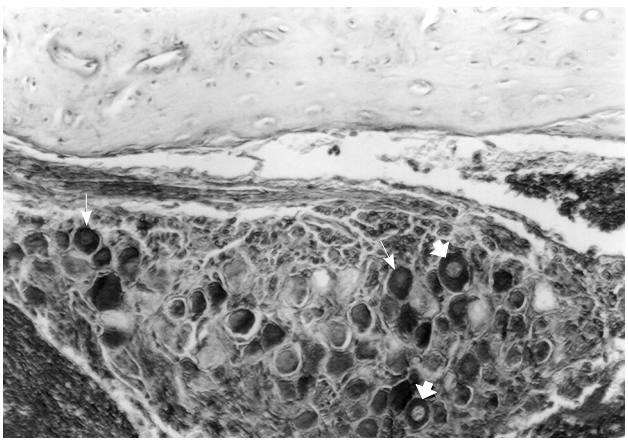

Figure 8. *Either cytoplasmic (thick arrow) or nuclear (slim arrow) (+++) caspase immunoreactivity in spiral ganglion cells (H-E x 10).*

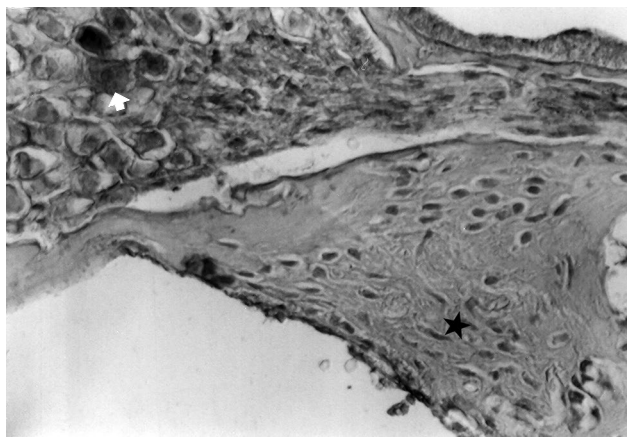

Figure 9. *No caspase immunoreactivity in the organ of Corti (*) and (+) caspase expression in spiral ganglion cells (arrow) (H-E x 20).*

superoxide dismutase were partially effective and concluded that SOR was effective to create cochlear damage and this damage might be partially prevented by antioxidant agents. In our study, the hearing thresholds of rats in group 3 (exposed to noise and injected with CAPE) improved partially.

It has been shown that ischemia/reperfusion (I/R), renal lipid oxidation and tissue damage can be suppressed by acute application of CAPE.[24] Irmak et al.[25] produced brain ischemia by occluding the carotid artery for 20 minutes in rats, and produced reperfusion by releasing the occlusion. They gave 10 μmol/L CAPE and 25 μmol/L alpha-tocopherol by IP before reperfusion, and observed that acute application of either CAPE or alpha-tocopherol suppressed lipid peroxidation induced by I/R, but CAPE had better results than alpha-tocopherol. Uzar et al.[26] investigated the role of CAPE on cerebellar oxidative stress induced by methotrexate in rats. They found that the increased activities of superoxide dismutase and catalase were significantly reduced by CAPE treatment and concluded that CAPE might have protected from oxidative damage caused by methotrexate treatment in rat cerebellum. Ek et al.[27] concluded that CAPE might have a positive effect on inflammatory bowel disease treatment due to its antiinflammatory and antioxidant activities. It has been shown that the anti-proliferative and radiosensitizing effects of 30 μmol/L CAPE on medulloblastoma cells might have been achieved through depleting glutathione, increased reactive oxygen species activity, and inhibiting nuclear factor-kappaB activity.[15] In this study, we used CAPE at a dose of 10 μmol/kg and observed no apoptotis in the organ of corti in rats injected with CAPE. But in an another study about SOR blockers, Bergmann[28] exposed guinea pigs to 120 dB SPL for one hour using allopurinol and dipyridamole as therapeutic agents, and observed that these agents had no preventive effect.

It is known that cisplatin causes high frequency sensorineural hearing loss due to the role of reactive oxygen species.[29] Kelly et al.[30] investigated whether nitric oxide played a role in ototoxicity caused by cisplatin, by application of aminoguanidine which inhibits inducible nitric oxide synthase (iNOS). They performed BERA three days before and just after application of cisplatin, and determined the levels of malondialdehyde which is the marker for nitric oxide and lipid peroxidation in cochlear tissues. They observed threshold shifts in rats treated with cisplatin. In our study, we observed that improvement in the hearing thresholds of group 3 subjects was statistically significant ($p<0.05$).

According to electrophysiological (BERA) and immunohistochemical evaluation, prenatal exposure to exogenous dexamethasone can decrease cochlear susceptibility to stress like noise trauma, and CAPE may be effective in preventing inner ear damage.

Declaration of conflicting interests

The authors declared no conflicts of interest with respect to the authorship and/or publication of this article.

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