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Original Article

Estrogen Reduces Caspase-3 Expression in the Inner Ear of Guinea Pigs Exposed to Simulated Microgravity and Inboard Noises of Spaceship

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OBJECTIVE: In different tissues, oestrogen plays different roles in cell apoptosis. In this study, we investigated the effect of oestrogen on cell apoptosis in inner ear cells of guinea pigs exposed to simulated microgravity and inboard noises of a spaceship.

MATERIALS and METHODS: Three groups of guinea pigs received no oestrogen, a preventive dose of oestrogen, and treatment dose of oestrogen, respectively. Caspase-3 expression in the inner ears of different groups was evaluated using immunohistochemical staining. The hearing levels were tested by auditory brainstem response.

RESULTS: On the first day of exposure to the stimulus, the auditory brainstem response thresholds were lower in the treatment group than in the control and prevention groups. After 3-day recovery, the prevention group showed a greater auditory brainstem response threshold shift than the other two groups. The expression of caspase-3 in hair cells and stria vascularis cells was weaker in the treatment group on the first day of the exposure. However, after 3-day recovery, caspase-3 expression was weaker in the prevention group.

CONCLUSION: Estrogen had a protective effect on the hearing impairment caused by the combined factors of simulated inboard noises and microgravity of a spaceship, reflected by both the auditory brainstem response score and morphological characteristics. Different doses and periods of the administration of oestrogen showed different protective effects. Use of oestrogen for treatment resulted in a better protective effect right after the stimulus, whereas preventive use of oestrogen led to a better effect in the recovery period.

KEY WORDS: Sex hormone, apoptosis, hearing, auditory brainstem responses (ABR)

INTRODUCTION

Astronauts, when in the space, suffer weightlessness and are exposed to inboard noises, radiation, and a polluted environment [1]. It is reported that obvious hearing threshold shift, either temporary or persistent, may occur in astronauts back from the space [2, 3]. For some instances, this kind of hearing impairment is inevitable. However, there is currently no effective way to prevent it.

The pathology of acquired hearing loss is very complex, but essentially, cellular death plays a key role. Cellular death occurs by two major routes: apoptosis and necrosis. Apoptosis is an active, energy-requiring process that is initiated by specific pathways in the cell [4], while necrosis is a passive one requiring no energy and results in rupture of the cell body. Recent studies indicate that apoptosis is probably the more important cell death route in sensory hair cells in response to noise exposure [5]. This is further strengthened by the observation that several biochemical apoptotic markers are activated in outer hair cells of noise-insulted cochleae [6], such as the caspase cascade [7, 8], a key mediator of apoptosis. Among these apoptotic markers, caspase-3, a common effector activated by both extrinsic an intrinsic pathways, is of essential importance [9].

Oestrogens are a class of sex steroid hormones that are synthesised from cholesterol and are secreted primarily by the ovaries, with secondary contributions from the placenta, adipose tissue, testes, and adrenal glands [10]. It has been reported that oestrogen protects pancreatic beta [11], retina ganglion [12], neuronal [13], bone [14], and heart [15] cells via inhibition of apoptosis. However, oestrogen also plays a role in increasing apoptosis in other cell types, such as osteoclasts [16], breast cancer cells [17], and leukaemia cells [18]. For human hearing, oestrogen seems to have protective effects [19]. Our previous studies also found that oestrogen showed a hearing protective effect for guinea pigs exposed to wind tunnel noises, and inhibited the expression of caspase-3 [20, 21]. However, the underlying mechanism of this phenomenon still remains to be investigated, and the effect of oestrogen on hearing when suffering microgravity combined with inboard noises, a more realistic simulated space environment, still needs to be evaluated. Thus, we detected the expression of caspase-3 in the inner ears of guinea pigs exposed to this simulated space environment. Oestrogen was administered at different doses and for different periods, in order to evaluate the relationship between oestrogen and apoptosis of inner ear cells.

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MATERIALS and METHODS

Animals and Grouping

Thirty-six healthy adult red-eyed white guinea pigs, all male and weighing 300-350 g, were obtained from Xinlong Animal Center in Haidian District in Beijing. Only animals that demonstrated a classical Preyer's reflex, indicating normal hearing were included in the study. All the procedures were approved by the Animal Care and Use Committee of the 306th Hospital of People's Liberation Army. They were randomly divided into exposure group (WN), oestradiol treatment group (ET), and oestradiol prevention group (EP), n=10 each, and a normal control group (N, n=6). Group N was kept in normal conditions without exposure to microgravity and noise. Group WN was exposed to both noise and microgravity. Group ET was exposed to both noise and microgravity, with oestrogen administrated simultaneously and 3 days afterward. Group EP was exposed to both noise and microgravity, with oestrogen administrated 3 days before the exposure. Oestrogen administration ended when exposure started.

Oestradiol Administration

Oestradiol benzoate (Jinyao, China) was intramuscularly (i.m.) injected into the hind limbs at 0.08 mg/kg every day, with the first dose doubled. Animals in the ET group received the oestradiol injection from the first day of exposure to the third day after the exposure ended. Animals in the EP group received the oestradiol injection in the 3 days before the exposure.

Microgravity Simulation

In WN, EP, and ET groups, animals were constrained on special frames that were tilted at an angle of -30°. The animal was fixed on the frame by the hind limbs so as to let it breathe, eat, and excrete freely, but it could not move at will. This stage lasted 5 days.

Noise Exposure

Noise exposure was composed of two phases: white noise and intense noise. In the first phase, guinea pigs were exposed to white noise within wire cages suspended in an experimental shelf. The cage was positioned so that the sound pressure within the cage varied by less than 2 dB sound press level (SPL) at 72 dB SPL. The exposure stimulus was generated by a custom-made white noise source (UZ-3, Beijing, China), filtered by an equaliser (MEQ, Beijing, China), amplified (PA-1000, Beijing, China), and delivered to a loudspeaker placed in front of the cages. Sound exposure levels were measured at the same point within the cage before each exposure, using a BK2250 hand-held analyser to monitor the sound intensity of the noise. This stage continued for 5 days. In the second phase, guinea pigs were exposed to the impulse noise unanaesthetised, within a wire cage placed in front of the impulse noise source. The exposure stimulus was generated by an impulse noise source (BK1027, Beijing, China) at 160 dB SPL, with a pulse width of 30 ms and repetition rate of 1/min.

Recording of Auditory Brainstem Responses (ABRs)

The ABRs were recorded before the exposure, the day after the exposure, and after 3-day recovery in WN, EP, and ET groups. After the animals were deeply anaesthetised with ketamine (Gutian, 60 mg/kg, i.m.) and xylazine hydrochloride (Huamu, 4 mg/kg, i.m.), the ABRs evoked by a click were measured by an ABR recorder (Medsen) in a sound-isolated electrostatic-screening room. Stainless steel wires were used as electrodes: the active electrode was placed at the vertex area, the reference electrode was inserted beneath the skin of the mastoid process region, and the grounded electrode at the apex nasi. The measurement started from a high intensity of sound, which was gradually reduced from 20 to 5 dB SPL. The intensity that could evoke a discernible β wave was regarded as the ABR threshold. It was the lowest sound intensity that could evoke a wave and could repeat at least once. During the whole procedure, the body temperature of the animals was kept at about 38°C.

Immunohistochemistry

At each examination point, the animals were anaesthetised with ketamine (Gutian, 60 mg/kg, i.m.) and xylazine hydrochloride (Huamu, 4 mg/kg, i.m.). Then the acoustic capsules were taken out and subjected to intra-cochlea infiltration with 4% paraformaldehyde for 24 h. They were decalcified with ethylene diamine tetraacetic acid for 14 days, rehydrated in graded ethanol, lucidified with dimethyl benzene, and then embedded in paraffin. Slices of 3 μm thickness were cut perpendicular to the longitudinal axis the cochlea. The slices were washed consecutively in double distilled water and 0.01 M phosphate-buffered saline (PBS, pH 7.4). After washing, the slices were transferred to plastic jars containing 0.01 M citrate buffer (pH 6.0) and boiled for 10 min at 700 W. Samples were allowed to cool for 20 min before washing in PBS. Non-specific blocking was performed for 10 min with 1.5% normal calf serum. Excess liquid was removed and slides were incubated with the respective primary antibody.

The rabbit polyclonal caspase-3 antibody (Lorenzo Life, NY, USA), now known to recognise caspase-3, was diluted 1:100 in PBS and added to the slides overnight at 4°C. The primary antibodies were substituted with a normal rabbit immunoglobulin G (Xingbosheng, Shanghai, China) to obtain negative controls. After washing in PBS, sections were incubated for 60 min at room temperature. After an additional wash in PBS, the sections were incubated for 30 min at room temperature with avidin and biotinylated horseradish peroxidase macromolecular complex (Xingbosheng, Shanghai, China). After further washing in PBS, the sections were developed with 3,3P-diaminobenzidine (DAB; Xingbosheng, Shanghai, China) for 30 s, then washed with tap water for 5 min and counterstained with haematoxylin. The time in DAB (30 s) was set at the beginning of the experiment according to the time the nuclei of the positive control needed to stain brown in DAB. The sections were then rewashed in water and dehydrated by graded alcohol.

A microscope connected via a video camera to a computer using Olympus software (Olympus, Japan) was used to assess immunostaining images. Three different researchers indifferently classified all the specimens using the light microscope. In the cochlea areas used for comparison were ganglion cells (Types I and II), hair cells (inner and outer), and stria vascularis.

Statistical Analysis

ABRs are expressed as means±standard deviations. SPSS 17.0 was used to perform paired t-test and one-way analysis of variance. Differences between means were considered significant at $p<0.05$.

RESULTS

Results of ABRs in Each Group

In Group N, the threshold of the ABR was 11.25±3.77dB SPL. For the ABR thresholds of the other groups, see Table 1. As shown in Table 1, ABR thresholds showed no difference between the groups before the exposure (F=1.103, p=0.339) and a significant difference between the groups on the first day of the exposure (F=7.124, p=0.02). Inter-group comparisons by SNK showed that at this time point, a significant difference existed between ET and NW, and between ET and EP, with no significant difference between NW and EP (p>0.05). ABR thresholds showed no difference between the groups 3 days after the end of the exposure (p>0.05). In this study, we further evaluated the thresholds of the ABR in the different groups before and after the exposure and before and after recovery (see Table 2).

Immunohistochemistry of Caspase-3

The normal group demonstrated negative staining in hair cells, stria vascularis cells, and ganglion cells (Figure 1). Caspase-3-positive staining was found in hair cells on the first day of the exposure, in WN, ET, and EP. The staining in EP was the strongest. Three days after recovery, this group still showed positive staining (Figure 2).

Table 1. ABR thresholds of different Groups at each time point $\bar{X}$±s, dB SPL) (n=the number of ears)

Groups	Before the exposure	The first day of the exposure	3 days after the end of exposure
WN	9.25±6.13 (n=20)	64.50±16.30^ (n=20)	43.50±6.83 (n=10)
ET	7.50±3.04 (n=20)	50.00±11.24^ (n=20)	36.00±8.10 (n=10)
EP	8.75±4.83 (n=20)	59.00±7.71^ (n=20)	38.50±6.69 (n=10)

WN: exposure group; ET: oestradiol treatment group; EP: oestradiol prevention group

Table 2. ABR threshold shifts in different Groups before and after the exposure; before and after the recovery ($\bar{X}$±s, dB SPL) (n=the number of ears)

Groups	Before and after the exposure	Before and after the recovery
WN	55.25±14.73 (n=20)	9.00±9.18 (n=10)
ET	42.50±11.41 (n=20)	16.00±6.99 (n=10)
EP	50.25±7.69 (n=20)	23.50±6.69 (n=10)

WN: exposure group; ET: oestradiol treatment group; EP: oestradiol prevention group

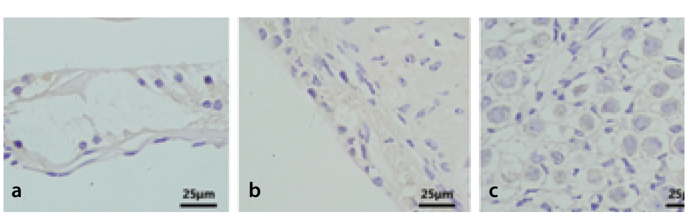

Figure 1. a-c. In normal group, immunohistochemistry slices of hair cells (a), stria vascularis cells (b) and ganglion cells (c), which demonstrate negative staining for Caspase-3 (×40 magnification)

In stria vascularis cells, on the first day of the exposure, caspase-3-positive staining was found in WN, ET, and EP. EP had the strongest staining, followed by WN and ET. Three days after recovery, the three groups still showed positive staining (Figure 3). The staining in WN and EP was weaker than that on the first day of the exposure, while the staining in ET was stronger than that on the first day of the exposure (Figure 3).

In ganglion cells, on the first day of the exposure, caspase-3-positive staining was found in WN, ET, and EP. Three days after recovery, ganglion cells still showed positive staining, with no obvious change compared with that on the first day of the exposure (Figure 4).

From the results mentioned above, we can summarise that:

1. On the first day of the exposure to the stimulus, the ABR threshold in the treatment group was lower than that in the control and prevention groups.
2. After 3-day recovery, the prevention group showed a larger ABR threshold shift than the other two groups.
3. Caspase-3 expression was weaker in the treatment group compared with the other groups on the first day of the exposure, in hair cells and stria vascularis cells. However, after 3-day recovery, it was weaker in the prevention group, in hair cells, stria vascularis cells, and ganglion cells.

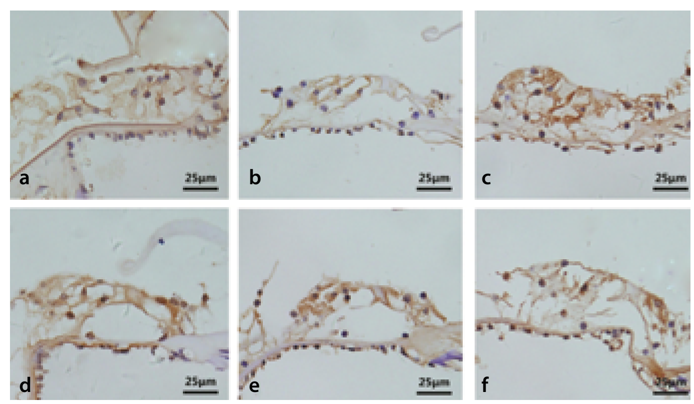

Figure 2. a-f. Caspase-3 starning in hair cells. WN, ET and EP on the first day of exposure (a-c) WN, ET and EP 3 days after recovery (d-f). Strongest staining (×40 magnification) (f)

WN: exposure group; ET: oestradiol treatment group; EP:oestradiol prevention group

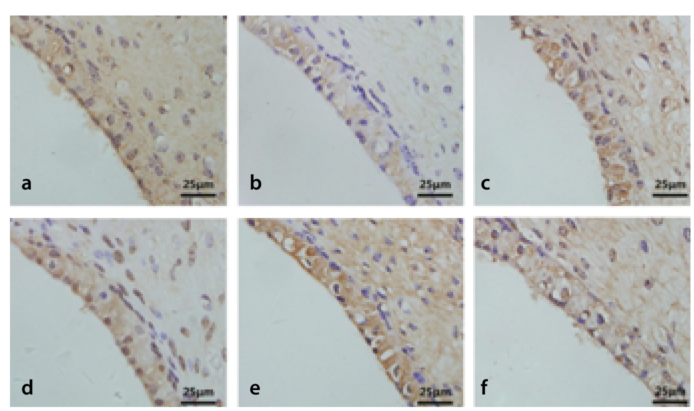

Figure 3. a-f. Caspase-3 staining in stria vascularis cells. WN, ET and EP on the first day of exposure (a-c). WN, ET and EP 3 days after recovery. c>a>b, d<a, e>b, f<c (×40 magnification) (d-f)

WN: exposure group; ET: oestradiol treatment group; EP:oestradiol prevention group

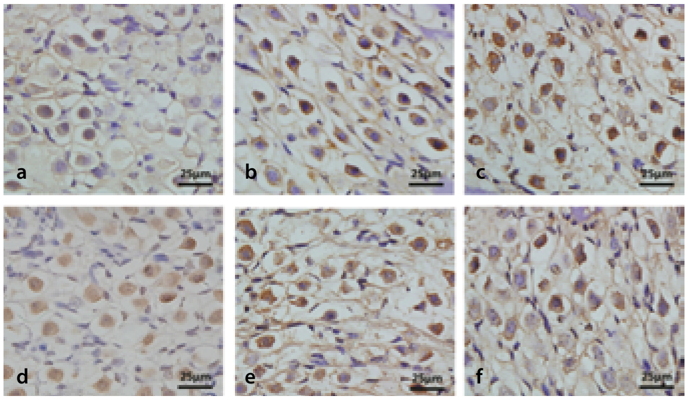

Figure 4. Caspase-3 staining in ganglion cells. WN, ET and EP on the first day of exposure (a-c). WN, ET and EP 3 days after recovery (×40 magnification) (d-f)
WN:exposure group; ET: oestradiol treatment group; EP:oestradiol prevention group

DISCUSSION

Both simulated inboard noises and microgravity can lead to hearing impairment in guinea pigs, as demonstrated by the threshold shift of ABRs. Furthermore, compared with exposure to the noises only, the combination of noises and microgravity can delay the process of recovery. One of the morphological characteristics of the impairment is increased expression of caspase-3, an important maker of apoptosis, in hair cells, spiral ganglion cells, and stria vascularis cells [22]. The same results were obtained in this study. It is concluded that noises lead to mechanical impairment and ischaemia, which cause necrosis of the functional cells; apoptosis of the cells also contribute greatly to this situation [9]. The combination of noises and microgravity, which may cause comparatively low blood infiltration in the cochlea, can synergistically worsen the hearing outcome [22].

Apoptosis can be induced through at least two pathways [23]. The extrinsic pathway is activated through death receptors that reside on the plasma membrane. Binding of a death ligand to its receptor causes activation of caspase-8 that is then able to activate effector caspases such as caspase-3 and caspase-7 [24]. The intrinsic pathway, on the other hand, is activated from the inside of the cell, with a close relationship to the mitochondria. The integrity of the mitochondrial membrane is controlled primarily by a balance between the antagonistic actions of the proapoptotic and antiapoptotic members of the Bcl-2 family [25]. Stimuli can cause mitochondrial damage, resulting in permeability changes of the outer mitochondrial membrane. These changes cause the release of several proapoptotic factors into the cytosol that also activate effector caspases (caspase-3 and caspase-7) and finally start apoptosis. Therefore, caspase-3/7 activity is universally increased during apoptosis. Caspase-3 activation is a critical determinant of apoptosis [26, 27]. This is why we chose caspase-3 as the target indicator for apoptosis.

In spite of the paradoxical role of oestrogen, regarding its effect on apoptosis of various cells [28, 29], oestrogen has shown its beneficial effects for the hearing function of humans and animals [19]. Oestrogens work through two intracellular receptors, oestrogen receptor alpha and beta. They have been shown to be present in the inner ear of rodents, humans, and fish [30-33]. Cells containing the two kinds of receptors in the inner ear of mice and rats were found to show a unique distribution pattern, both in the auditory pathways and in the water/ion-regulating areas [30]. This kind of distribution is in accordance with the potential protective effect of oestrogens on hearing function.

The results of our study indicate that right after the exposure, compared with the control group, the treatment group showed better hearing results, with a lower ABR threshold. Accordingly, the expression of capase-3 was lower. These findings further strengthen the theory that oestrogen can protect inner ear cells from impairing stimulus, through inhibiting apoptosis of the cells. It has been proved that both etoposide and TNFα stimulate caspase-3/7 activity in human osteoblastic cells and this activation is significantly inhibited by pretreatment of cells with oestradiol. Etoposide inhibits cellular topoisomerase and activates the intrinsic pathway of apoptosis, whereas TNFα binds to cell surface receptors that activate the extrinsic pathway of apoptosis. It is of interest that oestradiol inhibited caspase-3/7 activation by both agents [34], which indicates that oestrogen inhibits the two major pathways of cell apoptosis.

Another interesting finding in our study is that in the prevention group, in which oestradiol was administrated ahead of the exposure, right after the exposure caspase-3 showed a more intensive expression in the inner ears, followed by a lower expression 8 days later (3 days after the exposure). Also during this period, the ABR threshold showed a recuperative change and the threshold shift was larger than that in the other two groups. It reveals the complexity in the relationship between oestrogen and apoptosis. In this group, right after the exposure, the concentration of oestradiol did not reach the top level, unlike in the treatment group, but outer hair cells immediately start dying during the acoustic insult and continue to do so until at least 30 days thereafter [35, 36]. Thus, at this time point, the inhibitory effects of oestrogen in this group were lower than those in the treatment group. However, cell apoptosis is essentially controlled by a series of genes and the role of oestrogen involves gene regulation of proteins that influence apoptosis [37]. This may explain the long period of apoptosis, even 30 days after the stimulation. Monroe [38] has demonstrated that oestrogen has the potential to oppose apoptosis by regulating caspase activity through both transcriptional and posttranscriptional mechanisms in reproductive tissues. These data raise the possibility that oestrogen opposes apoptosis, at least in part, by regulating initiator caspase gene transcription, and may explain the result of caspase expression at 3 days after the end of the stimulus in this study.

In conclusion, the results of our study indicate that oestrogen has a protective effect on the hearing impairment caused by the combined factors of simulated inboard noises and microgravity of a spaceship, reflected by both the ABR score and morphological characteristics. Different doses and periods of the administration of oestrogen led to different results. The use of oestrogen as treatment demonstrated a better protective effect right after the stimulus, whereas the preventive use of oestrogen demonstrated better results in the recovery period. The exact intracellular mechanisms by which oestrogen opposes apoptosis still need to be revealed.

Ethics Committe Approval: Ethics committee approval was received for this study from the ethics committee of the Animal Care and Use Committee of the 306th Hospital of People's Liberation Army.

Peer-review: Externally peer-reviewed.

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