Estrogen inhibits apoptosis of pericytes in the cochlea stria vascularis through downregulating TMEM16A

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Abstract

Background: Cochlear cell apoptosis is an important factor influencing presbycusis. Estrogen has a protective effect on hearing, but the specific mechanism of its protective effect on presbycusis is not clear. We studied whether estrogen affects apoptosis by regulating TMEM16A, thus preventing presbycusis.

Methods: In an animal model, the hearing threshold was detected by auditory brainstem response (ABR). HE and transmission electron microscopy was used to observe morphological changes. qRT-PCR was used to analyze mRNA level changes. In experiments with cells, primary culture and identification of PCs in the cochlear stria vascularis (SV) were performed. The expression of TMEM16A was detected by immunofluorescence, and the apoptosis rate was detected by flow cytometry. **Results:** The results showed that estrogen intervention could improve presbycusis, cochlear SV atrophy and PCs degeneration in aged mice, and it could reduce expression of TMEM16A and apoptosis. In addition, experiments with cells showed that TMEM16A expression and apoptosis were increased in the aging cell group, but these effects were reversed by estrogen or T16Ainh-A01 intervention.

Conclusions: These results show that estrogen might reduce the apoptosis of PCs by downregulating TMEM16A, which would then protect against hearing loss via presbycusis. This study may provide a novel potential treatment and prevention method for presbycusis.

* Xuerui Li and Yang Zhang contributed equally to this work.

Introduction

With the aging of the population, organs age and change in function, and the number of patients with age-related hearing loss (ARHL) is increasing (Ciorba et al. 2015). ARHL is also known as presbycusis, and moderate or more severe presbycusis can affect communication and contribute to depression and dementia (Jafari et al. 2019). Until now, the specific pathogenesis of presbycusis has not been clear, and there is still a lack of effective prevention and treatment measures.

Age-related deterioration of the stria vascularis (SV) is one of the main causes of presbycusis (Jafari et al. 2019). A large number of studies have found that SV capillary loss is the main pathological change of presbycusis (Bielefeld et al. 2010). PCs are an important component of the SV capillary and play an important role in maintaining SV homeostasis (Shi et al. 2008). Some studies have shown that the cochlear vascular capillaries of aged animals are damaged and degenerated, and the density of PCs on the cochlear SV is significantly decreased (Neng et al. 2015). Previous studies have found that abnormal PCs in the cochlea can cause capillary leakage and lead to hearing loss (Shi 2009). Although the mechanism of these phenomena is still unclear, the data suggest that PCs may be a new target for the prevention and treatment of presbycusis.

There are gender differences in the damage of presbycusis. Women exhibit presbycusis after menopause, while men exhibit presbycusis earlier than women(Davis et al. 1995;Helzner et al. 2005), and estrogen replacement therapy can delay presbycusis(Hederstierna et al. 2007;Williamson et al. 2020). Some studies have shown that estrogen is related to apoptosis. Estrogen not only can reduce apoptosis of vascular smooth muscle cells(Ling et al. 2004) but also can reduce the apoptosis of cochlear outer hair cells induced by gentamicin ototoxicity, which it achieves by inhibiting the JNK pathway(Nakamagoe et al. 2010). Can estrogen inhibit the apoptosis of PCs and further protect against presbycusis?

Transmembrane member 16A (TMEM16A), as the main component of CaCCs(Betto et al. 2014), was found to be expressed in cochlear spiral ganglion cells(Zhang et al. 2015), cochlear vascular striatal basal cells(Jeon et al. 2011) and cochlear Sertoli cells(Wang et al. 2015), and previous research by our group found that TMEM16A exhibits age-related changes in cochlear SV PCs(Zhou et al. 2019). The coding sequence of TMEM16A is located within the 11q13 region, which contains a series of proteins related to apoptosis(Wilkerson and Reis-Filho 2013). Recent studies have shown that TMEM16A can promote apoptosis of endothelial cells(Allawzi et al. 2018) and vascular smooth muscle cells(Zeng et al. 2018). It has been confirmed that autophagy and apoptosis increase in mice with presbycusis(Menardo et al. 2012). However, the effect of TMEM16A on PCs apoptosis in presbycusis has not yet been reported.

At present, the specific mechanism by which estrogen acts in presbycusis is not clear. Based on the protective effect of E2 on cochlear cell apoptosis, this study established an ovariectomized C57BL/6J mouse model to investigate the effect of estrogen on the expression of TMEM16A in PCs of aged C57BL/6 mice and to study whether estrogen affects the apoptosis of PCs through TMEM16A. Our research results have laid an experimental foundation for elucidating the potential mechanism of estrogen regulating auditory function and have provided new strategies for the prevention and treatment of presbycusis.

Materials And Methods

Experimental grouping and treatment

Animals were provided by the Animal Experiment Center of Beijing Vital River Laboratory Animal Technology Co (animal use license batch no. SCXK Beijing 2016-0006). The use of animals in the present study was approved by the Committee of Animal Experimental Ethics of The First Affiliated Hospital of Medical College, Shihezi University (permit no. A2018-160-01). A total of 40 C57BL/6J female mice were divided into the following four groups (10/group): 3-month-old (3 m) group, 12-month-old (12 m) group, 12-month-old ovariectomized (12 m OVX) group (OVX at the age of 9 months) and 12 m OVX group treated with E2 (OVX at the age of 9 months and after 1 month treat with E2 [0.10 mg/kg/d] until the age of 12 months(Wu et al. 2016)).

Cell-based experiments were divided into a group of 5th generation (P5) PCs, a group of 13th generation (P13) PCs, a group of P13 PCs given DMSO, a group of P13 PCs given E2 (treated with 10^{-8} mmol/L E2

for 24 h), a group of P13 PCs given T16Ainh-A01 (treated with 30 $\mu\text{mol/L}$ T16Ainh-A01 for 24 h), and a group of P13 PCs given T16Ainh-A01 and E2 (treated with 30 $\mu\text{mol/L}$ T16Ainh-A01 for 24 h and then treated with 10^{-8} mmol/L E2 for 24 h).

Auditory Brainstem Response (abr) Detection

C57BL/6J mice were anesthetized by intraperitoneal injection and were placed in a soundproof screening room. Three platinum needle electrodes were placed subdermally at the vertex (positive electrode), the left mastoid (negative electrode) and right hind leg (ground electrode). ABR waves were recorded in response to a series of click stimuli. The intensity of acoustic stimulation was 30 ~ 100 dB SPL, the scanning time was 10 sec, and the number of superimposed stimuli was 1,024. The stimulation protocol included stimulus intervals of 11.10 beats/sec. The intensity of each stimulus decreased every 20 dB SPL. When approaching the threshold, the decrease was every 5 dB SPL. The amplitude and threshold changes of ABR III waves in each group were observed.

Hematoxylin-eosin Staining

Mice were euthanized by cervical dislocation, and the bilateral temporal bone was quickly collected. Samples were immersed in 4% paraformaldehyde solution at 4 °C for 24 h. The cochlea in all groups were decalcified in 10% EDTA (25 °C) for 7 days. Then, the cochlea was embedded in paraffin so that its orientation was parallel to the cochlea axis. Thereafter, they used a microtome to section the sections with a thickness of 3 μm . Finally, the sections were stained with hematoxylin-eosin (HE), analyzed by light microscopy and then photographed (automatic biochemical analyzer, Olympus).

Transmission Electron Microscopy

The cochleae from different groups of mice were isolated. Segments of the cochleae were fixed overnight in phosphate-buffered 3% glutaraldehyde, and then the cochlear tissue was cut into 1 mm $\times$ 1 mm $\times$ 1 mm pieces and was postfixed in 1% osmium. Then, the osmium was flushed from the tissue, and it was dehydrated, saturated and embedded in Epon 812 epoxy resin. An LKB-2188 ultrathin slicer was used to generate sections, and uranium dioxide acetate and lead citrate double electron staining was performed. Observation and photography were performed using a transmission electron microscope (JEM-1230, JEOL, JPN).

Reverse Transcription-quantitative Pcr

The cochlear SV of each group of mice was removed under aseptic conditions. An RNeasy Mini Kit was used to extract the total RNA, and the RNA was pre-denatured at 70 °C for 5 min, annealed at 42 °C for 60 min, and extended at 4 °C for 5 min to generate cDNA through reverse transcription. Then, the target

gene and internal reference were amplified. The following primers were used for qPCR amplification: β -actin, forward 5'-ACTGCTCTGGCTCCTAGCAC-3', reverse 5'-ACATCTGCTGGAAGGTGGAC-3'; TMEM16A, forward 5'-CACTCTTCGCCCTGCTAAAC-3', reverse 5'-ACCAGATGCCGATGTCTTTG-3'; Bax, forward 5'-TGAAGACAGGGGCCTTTTTG-3', reverse 5'-AATTCGCCGGAGACACTCG-3'; Bcl-2, forward 5'-ACGGTGGTGGAGGAACTCTTCAG-3', reverse 5'-GGTGTGCAGATGCCGGTTCAG-3'; and Caspase-3, forward 5'-TGGTGATGAAGGGGTCAATTTATG-3', reverse 5'-TTCGGCTTTCCAGTCAGACTC-3'. The thermocycling conditions consisted of 2 min of hot-start enzyme activation at 95 °C, which was followed by 40 cycles of PCR at 95 °C for 5 sec (denaturation), 60 °C for 40 sec (annealing) and 72 °C for 30 sec (elongation). Amplification was confirmed by the presence of a single peak in the melting temperature analysis and linear amplification throughout the PCR cycles. Relative mRNA expression of target genes was analyzed using β -actin as the internal control.

Primary Culture Of Pcs

Primary cell culture was performed as described above, with some modifications(Zhang et al. 2017). Using C57BL/6J mice aged 8–11 days ($n = 3$), cochlear tissues were removed under sterile conditions and were soaked in 4 °C D-Hanks solution. The SV was gently separated under a dissecting microscope and transferred to 0.7 mL of cell culture medium. Next, the SV was minced into 0.15–0.20 mm³ pieces, and the pieces were evenly arranged on the bottom surface of the culture dish using sterile ophthalmology. Tissues were incubated at 37 °C in an atmosphere containing 5% CO₂. After 24 h, 1 mL of cell culture solution was carefully added. On day 3, the unattached fragments were washed with phosphate-buffered saline, and new cell culture solution was added. Every day, the color, appearance, tissue mass, and cell growth were observed under an inverted microscope. After 2 ~ 3 washes per week, the PCs were purified by digestion passage.

Immunofluorescence

Immunofluorescence was used to identify the primary cultured PCs. The cells were evenly plated in a 6-well plate with glass slides, washed with 37 °C PBS after 3 days, and then fixed with 4% paraformaldehyde for 15 min. Subsequently, the cells were incubated in 0.3% Triton-100 (100 μ l) for 30 min and blocked in 5% bovine serum albumin (BSA; cat. no. B2064; Sigma-Aldrich; Merck KGaA) at room temperature for 1 h. Then, the cells were incubated with a mouse anti-desmin (1:100; cat. no. ab8470; Abcam) and an α -SM-actin monoclonal antibody (1:100; cat. no. ab7817; Abcam) at 4 °C overnight. The next day, slides were incubated with a FITC-conjugated goat anti-mouse secondary antibody (1:100, ZF-0312; OriGene Technologies, Inc.) at 37 °C for 60 min and then were washed with PBS. Nuclear staining by DAPI (Solarbio Science and Technology Co.) at 37 °C for 15 min. Finally, the glycerin buffer was sealed and photographed by laser scanning confocal microscopy (Zeiss LSM 510 META, Carl Zeiss AG).

Immunofluorescence detection of TMEM16A expression was performed as follows: first, the initial steps are the same as the above PCs immunofluorescence steps. The sealing fluid was shaken off and then the slides were incubated with a rabbit anti-TMEM16A antibody (1:100; cat. no. ab64085; Abcam) at 4 °C overnight. The next day, slides were incubated with a FITC-conjugated goat anti-rabbit (1:100, ZF-0311; OriGene Technologies, Inc.) at 37 °C for 1 h and then washed with PBS. Sections were stained with DAPI at 37 °C for 15 min. Finally, pictures were taken with a laser scanning confocal microscope.

Cell Aging Model

The cells of the 5th, 9th and 13th generations were placed in six-well plates and were cultured under standard conditions for 24 h. A β -galactosidase staining kit (cat. no. C0602; Beyotime, Haimen, China) was then used according to the manufacturer's instructions, and 1 mL of β -galactosidase fixed solution was added to each well. After being fixed at room temperature for 15 min, 1 mL of working dye solution was added to each well. After the six-well plate was sealed with plastic wrap and incubated overnight in a 37°C incubator, the results were finally observed and photographed under an ordinary optical microscope.

Flow Cytometry

Cells were plated in groups in six-well plates. Trypsin digestion was used to collect the cells. Cells were centrifuged at 1000 rpm for 6 min and then were washed twice with precooled PBS. Each tube contained 500 μ L of binding buffer, in which the cells were resuspended. Then, 5 μ L of Annexin V-FITC and 10 μ L of PI staining solution were added. After mixing, the cells were incubated in the dark for 30 min. The resuspended liquid was transferred to a flow cytometry sample tube for machine detection.

Statistical analysis

Statistical analysis was performed using SPSS 19.0 software, and GraphPad Prism was used to draw graphs. All the results were expressed by $(\bar{x} \pm s)$. Every data point was analyzed by one-way ANOVA. Results with P values of less than 0.05 were considered statistically significant.

Results

ABR was detected in different groups of mice

The change in the ABR III wave threshold was observed in each group (Fig. 1). The ABR threshold of the 12 m group was higher than that of the 3 m group (**P < 0.01). However, the ABR threshold of the 12 m OVX group was higher than that of the 12 m group (#P < 0.05). When the 12 m OVX group was treated

with E2, the ABR III wave threshold was decreased ($P < 0.05$). These findings suggest that E2 has a protective effect on presbycusis in C57BL/6J mice.

Morphological observation of cochlear stria vascularis and pericytes

Morphological observation of the cochlear SV and HE staining of the mouse cochlear SV varied between the different groups (Fig. 2A). In the 3 m group, the SV was normal in shape and nuclear boundary. There was a large degree of vacuolar degeneration that appeared in the cochlear SV of the 12 m group, and the structure was disordered. In the 12 m OVX group, the cochlear SV was atrophic, the nucleus shrank, and the cytoplasm decreased. In the 12 m OVX group treated with E2, the cytoplasm increased significantly, vacuole degeneration was decreased, cell morphology was changed, and the cochlear SV increased in width.

The studies revealed PCs morphology under a transmission electron microscope (Fig. 2B). In the 3 m group, PCs were flat and slender with good shape, and the cytoplasmic and nuclear components of the PCs were abundant. In the 12 m group, PCs chromatin organelles were lost, and cytoplasmic swelling was observed. In the 12 m OVX group, the irregularly shaped PCs had sparse caveolae, the nuclear boundaries were not clear, and the chromatin was compact. In the 12 m OVX group treated with E2, PCs chromatin content was dense. It is suggested that E2 has a protective effect on the degeneration of SV and PCs in aging mouse cochlea.

mRNA content of TMEM16A, Bax, Bcl-2 and Caspase-3 in the cochlear SV

The mRNA expression of TMEM16A, Bax, Bcl-2 and Caspase-3 was analyzed in cochlear SV cells from the different groups (Fig. 3). Compared with the 3 m group, the TMEM16A, Bax and Caspase-3 mRNA levels were increased in the 12 m group ($n = 3$, $^*P < 0.05$, $^{***}P < 0.001$), and Bcl-2 decreased significantly ($n = 3$, $^{***}P < 0.001$). The levels of TMEM16A, Bax and Caspase-3 mRNA in the 12 m OVX group were higher than those in the 12 m group ($n = 3$, $^{\#}P < 0.05$, $^{\#\#}P < 0.01$), and Bcl-2 levels significantly decreased ($n = 3$, $^{\#\#\#}P < 0.001$). In the 12 m OVX group treated with E2, the expression of TMEM16A, Bax and Caspase-3 decreased ($n = 3$, $^{\&}P < 0.05$, $^{\&\&}P < 0.01$), and Bcl-2 increased significantly ($n = 3$, $^{\&\&\&}P < 0.001$). These results showed that with increasing age, the expression of TMEM16A and apoptosis in cochlear SV were increased, while estrogen downregulated the expression of TMEM16A and reduced apoptosis.

Primary culture and identification of PCs determined the cell aging model

In the in vitro cell experiments, we performed primary culture of isolated cochlear SV PCs and established an aging cell model. The studies revealed the growth state of PCs under an ordinary optical microscope (Fig. 4A). On the second day, we found adherence of the cochlear stria vascular tissue fragments. On the 7th day, the PCs had begun to grow out from the cochlear stria vascular tissue fragments and showed typical long spindle or few polygonal cells. The cells proliferated rapidly throughout the culture dish by day 12, reaching 90% density and passaging. Primary cultured PCs were observed (Fig. 4B) using fluorescence inverted microscope ($\times 400$). In the primary culture of PCs, the expression of Desmin and α -

SM-actin was positive (green), and there was no observed expression of vWF (a specific marker of endothelial cells). Thus, we generated pure primary cultures of PCs. After continuous passage to the 5th, 9th and 13th generation, cells were evenly spread in 6-well plates and stained with β -galactosidase (Fig. 4C). In the P13 group, the cultured cells were obviously aged, and the number of positive cells was significantly higher than that of the P5 group ($n = 3$, $^{***}P < 0.001$). Therefore, we selected the P13 PCs as the cell aging group.

Immunofluorescence detection of TMEM16A expression

We used immunofluorescence to observe the expression of TMEM16A in PCs. As shown in Fig. 5, TMEM16A was labeled with green fluorescence, and DAPI was used to stain the nuclei. Compared with the P5 group, the results showed that the expression of TMEM16A was increased in the P13 group ($n = 6$, $^{**}P < 0.01$). When the P13 PCs were treated with E2, the expression of TMEM16A was decreased ($n = 6$, $^{##}P < 0.01$). These results indicated that estrogen could reduce the expression of TMEM16A in the aging PCs group.

Apoptosis was detected by flow cytometry

To further determine whether estrogen has an effect on PCs apoptosis through TMEM16A, we detected the apoptosis rate of PCs in each group by flow cytometry (Fig. 6). Compared with the P5 group, the P13 group of the apoptosis rate were significantly increased ($n = 3$, $^{***}P < 0.001$). When the P13 PCs were treated with E2 or T16Ainh-A01, the apoptosis rate was decreased ($n = 3$, $^{##}P < 0.01$, and $^{###}P < 0.001$), and after intervention with both T16Ainh-A01 and E2 the rate was also decreased ($n = 3$, $^{###}P < 0.001$). The results show that T16Ainh-A01 and E2 can reduce the apoptosis of PCs, and because estrogen can reduce the expression of TMEM16A, it is suggested that estrogen may inhibit the apoptosis of PCs through TMEM16A.

Discussion

We produced meaningful experimental results, and the data showed that the morphology in the SV of the cochlea changed in 12-month-old C57BL/6J mice, and TMEM16A expression and apoptosis increased in PCs. Estrogen can downregulate the expression of TMEM16A and decrease apoptosis in cochlear SV PCs of aged mice. In addition, cell-level experiments showed that T16Ainh-A01, a specific blocker of TMEM16A, could reduce the apoptosis of PCs. Estrogen downregulated the expression of TMEM16A and inhibited the apoptosis of PCs in cochlear SV. These results suggest that TMEM16A is closely related to the apoptosis of aging cochlear SV PCs and that estrogen can inhibit the apoptosis of PCs in the cochlea SV by downregulating TMEM16A to protect against presbycusis.

C57BL/6J mice are commonly used in the study of presbycusis, and there is significant hearing loss at the age of 12 months. In this study, C57BL/6J mice were selected as the model, and mice that were 3 months old were used as the young group and those that were 12 months old were used as the old group.

The ABR threshold was significantly increased in 12-month-old mice, which was consistent with the process of presbycusis. Some studies have shown that estrogen has a protective effect on hearing(Hederstierna et al. 2007). The ABR threshold of auditory brainstem response in postmenopausal women is significantly higher than it is in young women and men(Jerger and Johnson 1988;Wharton and Church 1990), suggesting that this difference may be related to the level of estrogen. Animal experiments have shown that the hearing threshold in ovariectomized rats is significantly higher than it is in normal rats(Coleman et al. 1994). We found that the ABR threshold of ovariectomized 12-month-old C57BL/6J mice increased. After estrogen intervention, the ABR threshold of ovariectomized 12-month-old C57BL/6J mice decreased, indicating that estrogen has a protective effect on presbycusis.

The normal metabolism and physiological function of the cochlea SV is the basis for maintaining normal hearing(Spicer and Schulte 2005). Cochlea SV atrophy is the key factor leading to presbycusis. Early studies have reported cochlear SV contraction in gerbils, and the contraction rate increases with age(Gratton and Schulte 1995). When the atrophy of the cochlear SV reaches a certain extent, the capillaries and PCs are reduced or lost(Ocho et al. 2000), the PCs cell structure is abnormal, the cytoplasm and chromatin are lost, and the cell vacuoles are abundant(Neng et al. 2015). The results of our experiment showed more cochlear SV atrophy in the 12-month-old mouse group than in the 3-month group. Transmission electron microscopy showed that the nuclei of PCs in the cochlear SV of 12-month-old mice had unclear boundaries, chromatin was compact, and cell vacuoles appeared. It was verified that with increasing age, the morphological structure changes of cochlear SV PC, which may lead to hearing loss. In addition, after estrogen intervention in ovariectomized mice, we found that the atrophy of the cochlear SV was improved, the cytoplasm of PCs increased, the number of vacuoles decreased, and the cell morphology improved. It is suggested that estrogen may affect the function of PCs and protect against presbycusis by improving SV atrophy and the morphological structure of PCs in the aging cochlea.

TMEM16A is an important constituent protein of CaCCs(Forge and Wright 2002). In the study of hearing and TMEM16A, it was found that TMEM16A is closely related to many cells in the cochlea(Jeon et al. 2011;Wang et al. 2015;Zhang et al. 2015). Early studies in our laboratory found that there were also age-related TMEM16A changes in the PCs of guinea pig cochlear SV(Jeon et al. 2011;Wang et al. 2015;Zhang et al. 2015). In our study, the expression of TMEM16A mRNA in the cochlear SV of aging C57BL/6J mice was increased. Cell level studies have found that TMEM16A was also expressed in primary cultured PCs and that its expression increased in the aging cell group, which corresponded to the animal experiment. The results showed that the change in TMEM16A expression was related to presbycusis. The expression and function of TMEM16A vary with cell type. Some studies have shown that TMEM16A can aggravate the disease by inhibiting apoptosis in cancer and tumors, suggesting that TMEM16A may be a potential biomarker of some aggressive diseases(Deng et al. 2016;Song et al. 2018;Frobom et al. 2019). For normal cells of the body, such as podocytes(Lian et al. 2017) and smooth muscle cells(Lian et al. 2017), overexpression of TMEM16A can promote apoptosis of these cells and further lead to the occurrence of disease. As many studies on presbycusis have found that it can cause cochlear cell degeneration, autophagy and apoptosis were increased(Huang et al. 2017;Sanchez-Rodriguez et al. 2018;Yuan et al.

2018). However, it has not been reported whether TMEM16A can affect presbycusis by regulating cochlear cell apoptosis. In our study, animal experiments have shown that the expression of Caspase-3 and Bax mRNA increased and Bcl-2 mRNA decreased in 12-month-old mice, indicating that apoptosis occurred in the cochlear SV of 12-month-old mice. Experiments in cells showed that the apoptosis rate increased significantly in the aging PC group. After administration of T16Ainh-A01, a specific blocker of TMEM16A, the apoptosis rate was decreased significantly in the aging PC group. These results suggested that TMEM16A might be closely related to the apoptosis of cochlear SV PCs and that TMEM16A may further affect the occurrence and development of presbycusis by regulating the apoptosis of PCs.

There have been multiple theories about how estrogen directly or indirectly protects auditory function. Currently, estrogen has been proven to be an anti-apoptotic drug. Estrogen can reduce the apoptosis of cochlea outer hair cells against gentamicin ototoxicity through inhibition of the JNK pathway (Nakamagoe et al. 2010). Based on the protective effect of estrogen on cochlea cell apoptosis, our results proved that apoptosis of PCs in cochlear SV of ovariectomized C57BL/6J mice was increased. After estrogen intervention, the expression of Caspase-3 and Bax mRNA decreased, and Bcl-2 mRNA increased, indicating that cochlear SV apoptosis was improved. At the cellular level, the apoptosis of PCs in the aging group decreased after estrogen intervention. These results suggest that estrogen can reduce apoptosis of PCs. In addition, we studied the relationship between estrogen and TMEM16A. We found that after ovariectomized mice were treated with estrogen, the expression of TMEM16A mRNA on the cochlear SV PCs was decreased. Cell experiments showed that the expression of TMEM16A decreased after estrogen intervention in the aging PC group. These results suggest that estrogen is related to TMEM16A, and estrogen may play a protective role in presbycusis by downregulating TMEM16A to inhibit the apoptosis of cochlear SV PCs.

However, this study only reveals that estrogen may only partially affect presbycusis by regulating the effect of TMEM16A on apoptosis. However, how estrogen specifically regulates the expression or function of TMEM16A and through what molecular mechanism are the issues that we need to focus on in the future. Nevertheless, the present findings provide a clearer understanding of the effects of estrogen on the internal microcirculation and auditory function of the cochlea, and they provide a theoretical basis for the use of estrogen as a potential drug in the prevention and treatment for presbycusis.

Conclusions

In summary, due to the increased expression of TMEM16A, which can lead to an increase in the apoptosis of PCs, estrogen can downregulate the expression of TMEM16A and can also reduce the apoptosis of PCs. These data suggest that estrogen may reduce apoptosis in PCs by downregulating the expression of TMEM16A, which provides a protective effect on presbycusis.

Abbreviations

ABR	Auditory brainstem response
BM	Basement membrane
CaCCs	Calcium-activated chloride channels
DAPI	4',6-diamidino-2-phenylindole
E2	Estrogen
HE	Hematoxylin and eosin
PBS	Phosphate buffer saline
PCs	Pericytes
SV	Stria vascularis
TMEM16A	Transmembrane member 16A

Declarations

Ethics approval and consent to participate

The use of animals in the present study was approved by the Committee of Animal Experimental Ethics of The First Affiliated Hospital of Medical College, Shihezi University (permit no. A2018-160-01).

Consent for publication

Not applicable.

Availability of data and materials

The analysed data sets generated during the study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

LL, KM and JS designed the experiments. XL, YZ, LC and ZF conducted the experiments. XL, YZ, LC and ZF acquired the data. XL, YZ, KM, LL and JS analyzed the data. XL and LL wrote the manuscript. All authors read and approved the final manuscript.

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Figures

A

Figure 1

ABR threshold results of each group The results are presented as the mean $\pm$ SD, n = 10. **P < 0.01 vs. the 3 m group. #P < 0.05 vs. 12 m group. &P < 0.05 vs. the 12 m OVX group.

Figure 2

Fig. 2A HE staining of the cochlear SV in each group Fig. 2A1 to D1 inverted microscope ($\times 100$) and Fig. 3A2 to D2 inverted microscope ($\times 400$) show the HE staining images of the changes in the cochlear SV.

Fig. 2B Morphological structure of cochlear SV PCs under transmission electron microscopy The following are indicated in the figure: Vascular basement membrane (BM) and pericytes (PCs). PCs were closely attached to the lateral wall of the BM. The shape of the PCs is shown by the arrow in the picture.

Figure 3

mRNA content in the cochlear SV A: The mRNA expression of TMEM16A in the cochlear SV cells between the different groups. B: The mRNA expression of Caspase-3 in cochlear SV cells. C: The mRNA expression of Bax in cochlear SV cells. D: The mRNA expression of Bcl-2 in cochlear SV cells. *P < 0.05, ***P < 0.001 vs. the 3 m group. #P < 0.05, ##P < 0.01, ###P < 0.001 vs. 12 m group. &P < 0.05, &&&P < 0.001 vs. 12 m OVX group.

Figure 4

Primary culture of PCs and analysis of the cell aging group A: Primary culture of PCs growth processes under an inverted microscope (×200). B: Identification of primary cultured PCs. Fluorescence inverted microscope (×400). The expression of Desmin and α-SM-actin was positive (green). The EC-specific marker vWF was negative. C: β-galactosidase staining of PCs. In Figs. a1, b1 and c1 are 10× microscope observations. In Figs. a2, b2 and c2 are 40× microscope observations. D: Counting of positive cells stained with β-galactosidase. *P < 0.05, ***P < 0.001 vs. the P5 group.

Figure 5

Expression of TMEM16A in primary cultured PCs Fluorescence inverted microscope (×400) A: TMEM16A is labeled with green fluorescence, and DAPI staining labeled the nucleus. B: Expression changes of TMEM16A in PCs. **P < 0.01 vs. the P5 group. ##P < 0.01 vs. P13 group.

Figure 6

The apoptosis rate of PCs A: Apoptosis was detected by flow cytometry. B: Apoptosis rates. *** $P < 0.001$ vs. the P5 group. ## $P < 0.01$ and ### $P < 0.001$ vs. P13 group.