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维生素 E 对 CBA 小鼠年龄相关性听力损失的保护作用及其机制

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[摘　要]　**目的：**探讨维生素E(Vitamin E)能否改善年龄相关性CBA小鼠听力损失及其机制。**方法：**CBA小鼠分为对照组与Vitamin E干预组，其中Vitamin E干预组给予含Vitamin E的饲料，对照组给予正常饲料。通过检测小鼠听觉脑干反射阈值(auditory brainstem response，ABR)评估小鼠听力情况；用荧光素488标记的鬼笔环肽计算外毛细胞个数；扫描电镜检测外毛细胞静纤毛状态；检测耳蜗超氧化物歧化酶(superoxide dismutase，SOD)和谷胱甘肽过氧化物酶(glutathione peroxidase，GPx)活性水平；超氧阴离子荧光探针检测外毛细胞的超氧阴离子水平；免疫荧光检测外毛细胞cleaved caspase-3阳性细胞数。**结果：**CBA小鼠10月龄时，对照组有明显的听力损失。与对照组相比，Vitamin E干预组小鼠ABR阈值降低，外毛细胞数增加，静纤毛结构病变明显缓解，耳蜗SOD及GPx活性增高，超氧阴离子阳性细胞数减少，外毛细胞的cleaved caspase-3阳性细胞数降低。**结论：**Vitamin E能减轻与年龄有关的听力丧失，这种作用可能与抑制耳蜗氧化应激、外毛细胞丧失、静纤毛结构改变有关。

[关键词]　维生素E；CBA小鼠；年龄相关性听力损失

Protective effect and mechanism of vitamin E on age related hearing loss in CBA mice

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Abstract　**Objective:** To examine the effect on Vitamin E on age related hearing loss in CBA mice and its mechanism. **Methods:** CBA mice were treated with food contained Vitamin E as a Vitamin E intervention group and standard food as a control group. Hair cells were calculated by F-actin with Alexa Fluor-488 labeled phalloidin. Stereocilia morphology were evaluated by Scan electron microscope. The levels of superoxide dismutase (SOD) and glutathione peroxidase (GPx) activities were measured. The level of superoxide anion in outer hair cells by using a dihydroethidium probe. The number of cleaved caspase-3 positive outer hair cells was detected by immunofluorescence staining. **Results:** CBA mice aged 10 months had significant hearing loss in the control

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group. Compared with the control group, the auditory brainstem response (ABR) threshold was decreased, the number of outer hair cells was increased, the alteration of stereocilia was reduced, the levels of SOD and GPx activities in cochlea were increased, the number of superoxide anion-positive cells in outer hair cells was decreased, the number of cleaved caspase-3 positive cells in outer hair cells was decreased in mice from the Vitamin E intervention group. **Conclusion:** Vitamin E can relieve age-related hearing loss, which may be related to the inhibition of cochlear oxidative stress, loss of outer hair cells, and structural changes of stereocilia.

Keywords vitamin E; CBA mice; age related hear loss

年龄相关的听力损失(age related hearing loss，AHL)或老年性耳聋是常见的内耳退行性疾病，且是老年人最常见的慢性病[1]。在中国，60岁以上人群中患有不同程度的听力障碍患者已达到45%[2]。同时，AHL会导致患者孤立和沮丧，严重者会导致抑郁症[3]。随着当今人口老年化严重，AHL的机制阐明及治疗越来越紧迫。

年龄相关性听力损失主要是随着年龄增长，氧化应激、线粒体障碍及环境因素导致的[4-6]。氧化应激损伤是年龄相关性听力损失最常见的病理生理学改变。活性氧化因子(reactive oxygen species，ROS)是介导氧化应激的主要因子。ROS随着年龄增长在线粒体中积聚，因此线粒体氧化损伤是导致年龄相关性听力损失的主要机制[7]。研究[8]表明：随着年龄增大，细胞内抗氧化酶超氧化物歧化酶(superoxide dismutase，SOD)及谷胱甘肽过氧化物酶(glutathione peroxidase，GPx)缺乏会导致小鼠外毛细胞病理改变。CBA小鼠随着年龄增大，氧化应激蛋白积聚，在6月龄时ROS与RNS已开始增高，而到10月龄时已患有严重的AHL[9]。综上，氧化应激可导致内耳外毛细胞的退行性变，而抑制内耳外毛细胞的氧化应激过程能否缓解AHL症状还未见报道。因此，本研究利用CBA小鼠作为AHL模型，利用临床上常用的抗氧化药物维生素E(Vitamin E)来干预，观察Vitamin E能否能缓解听力损失以及具体机制。

1 材料与方法

1.1 材料

1.1.1 动物

40只CBA新生鼠购自华中科技大学动物实验中心[SCXK(鄂)2016—0020]，饲养于华中科技大学动物实验中心[SYXK(鄂)2010—0057]，相对湿度45%~55%，每天光照12 h。

1.1.2 实验仪器及试剂

SOD(货号：BC0170)和GPx(货号：BC1195)分析试剂盒购于北京Solarbio公司；Alexa Fluor-488-labeled phalloidin(货号：AT89)购于美国Thermofisher公司；抗cleaved caspase-3抗体(货号：ab32042)、抗myosin VIIa抗体(货号：ab3481)购于美国Abcam公司；超氧化物阴离子荧光探针(货号：S0063)购于江苏碧云天公司。FC型酶标仪购于美国Thermo公司；MB11型小动物听觉脑干反应(auditory brainstem response，ABR)测试系统购于德国MAICO公司；Tecnai F589型扫描电镜购于美国FEI公司；CSU-SD型激光共聚焦显微镜购于日本NIKON公司。

1.2 方法

1.2.1 小鼠分组

40只CBA鼠分为对照组和Vitamin E干预组，每组20只。Vitamin E干预组：出生后3周龄断奶时给予Vitamin E添加入饲料，质量比为7.76 g/kg(每公斤饲料)。对照组给予正常饮食，待10月龄时进行实验。

1.2.2 ABR测量

小鼠麻醉后置于恒温垫上维持体温为37 ℃，将电极插入头顶皮下、耳廓后方乳突皮下及尾部皮下，利用MB11型小动物ABR测试系统对小鼠进行click及4，8，16及32 Hz短纯音刺激ABR获取。在每个刺激水平，收集512个响应。

1.2.3 扫描电镜观察外毛细胞的静纤毛形态

从颞骨中分离耳蜗组织，4 ℃条件下2.5%戊二醛固定过夜，PBS冲洗后，用1%锇酸染色后干燥，喷涂铂层，利用Tecnai F589型扫描电镜观察毛细胞静纤毛形态。

1.2.4 毛细胞鬼笔环肽染色

分离耳蜗，经常规浓度蔗糖脱水和OCT包埋后，作纵向8 μm厚度冰冻切片，将耳蜗切片固定在玻片上，用Alex Fluor-488标记的鬼笔环肽(1:500)对切片染色1 h以显示毛细胞形态，DAPI染核后封片，CSU-SD型激光共聚焦显微镜观察外毛细胞形态并任选5个视野拍照。计算外毛细胞个数和有完整“V”字形的毛细胞。

1.2.5 SOD与GPx活性的检测

从颞骨中分离耳蜗匀浆后，利用1 000 r/min离心10 min收集上清液。上清液按照SOD活性分析试剂盒说明书步骤进行检测，利用FC型酶标仪检测560 nm处的吸光度，按照预先制作的标准曲线计算SOD活性。上清液按照GPx活性分析试剂盒说明书步骤检测，利用FC型酶标仪检测340 nm处的吸光度，按照预先制作的标准曲线计算GPx活性。SOD与GPx活性均以U/mL表示。

1.2.6 超氧化物阴离子水平的检测

耳蜗组织经常规浓度蔗糖脱水和OCT包埋后，制作冰冻切片。纵向切为8 μm厚度切片，将耳蜗切片固定在玻片上，利用超氧化物阴离子荧光探针说明书步骤，利用CSU-SD型激光共聚焦显微镜任选5个视野拍照。

1.2.7 Cleaved caspase-3免疫荧光

从颞骨中分离耳蜗经4%PFA固定、30%蔗糖脱水、石蜡包埋后5 μm切片，先用枸橼酸钠高温修复后，过氧化氢阻断内源性过氧化物酶，之后以1:200稀释比例孵育一抗(Cleaved caspase-3)，4 ℃过夜。次日，PBS洗涤后用孵育二抗1 h，利用CSU-SD型激光共聚焦显微镜任选5个视野拍照。

1.3 统计学处理

采用SPSS 17.0软件进行数据分析。数据均以均数±标准差($\bar{x}\pm s$)表示，两组计量资料比较采用双侧t检验。P<0.05为差异有统计学意义。

2 结果

2.1 Vitamin E可降低年龄相关性听力障碍ABR

在CBA小鼠听力损失模型中，Vitamin E处理可明显降低ABR阈值，且能缓解不同频率(4，8，16及32 Hz)刺激下的ABR阈值(图1)。

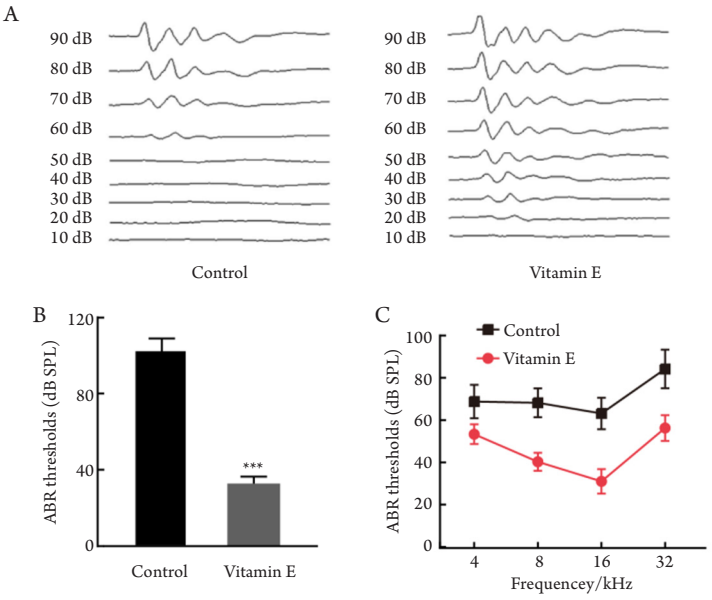

图1 Vitamin E可缓解CBA小鼠年龄相关性听力损失

Figure 1 Vitamin E alleviates the age-related hearing loss in CBA mice

(A)每组小鼠对点击刺激的ABR的原始痕迹；(B)每组小鼠对点击刺激的ABR阈值；(C)各组小鼠纯音刺激的ABR阈值(n=20)。与对照组相比，***P<0.001。

(A) Raw traces of ABR responses to click stimuli in each group mice; (B) ABR thresholds for click stimuli in each group mice; (C) ABR thresholds for pure tone stimuli in each group mice (n=20). ***P<0.001 vs Control group.

2.2 Vitamin E可缓解年龄相关性听力障碍的外毛细胞及静纤毛退行性病变

利用Alexa Fluor-488标记的鬼笔环肽对外毛细胞肌动蛋白微丝进行染色，从而计算外毛细胞数量，结果显示：Vitamin E处理组小鼠外毛细胞数较对照组多(图2A)。扫描电镜结果显示：未经Vitamin E处理的小鼠外毛细胞的静纤毛变的不规则且受到损伤，甚至缺失，毛细胞形态不规则、畸形、电子致密度浅及出现类似凋亡特征，而给予Vitamin E处理小鼠外毛细胞规则，纤毛完整(图2B)。

2.3 Vitamin E抑制年龄相关性听力损失CBA小鼠外毛细胞凋亡

对外毛细胞进行cleaved caspase-3检测，结果显示：Vitamin E可明显减少年龄相关性听力损失CBA小鼠外毛细胞cleaved caspase-3阳性细胞数目(图3)。

2.4 维生素可增高耳蜗SOD及GPx水平

检测耳蜗中对氧化应激有保护作用的两种酶SOD及GPx的活性，结果显示：经Vitamin E处理后，年龄相关性听力损失CBA小鼠SOD(图4A)及GPx(图4B)的活性均明显升高。

2.5 Vitamin E可降低超氧化阴离子水平

对超氧阴离子进行测定，结果显示：Vitamin E可明显降低年龄相关性听力损失CBA小鼠外毛细胞的边缘细胞、前庭膜细胞及螺旋器细胞的超氧阴离子阳性细胞数(图5)。

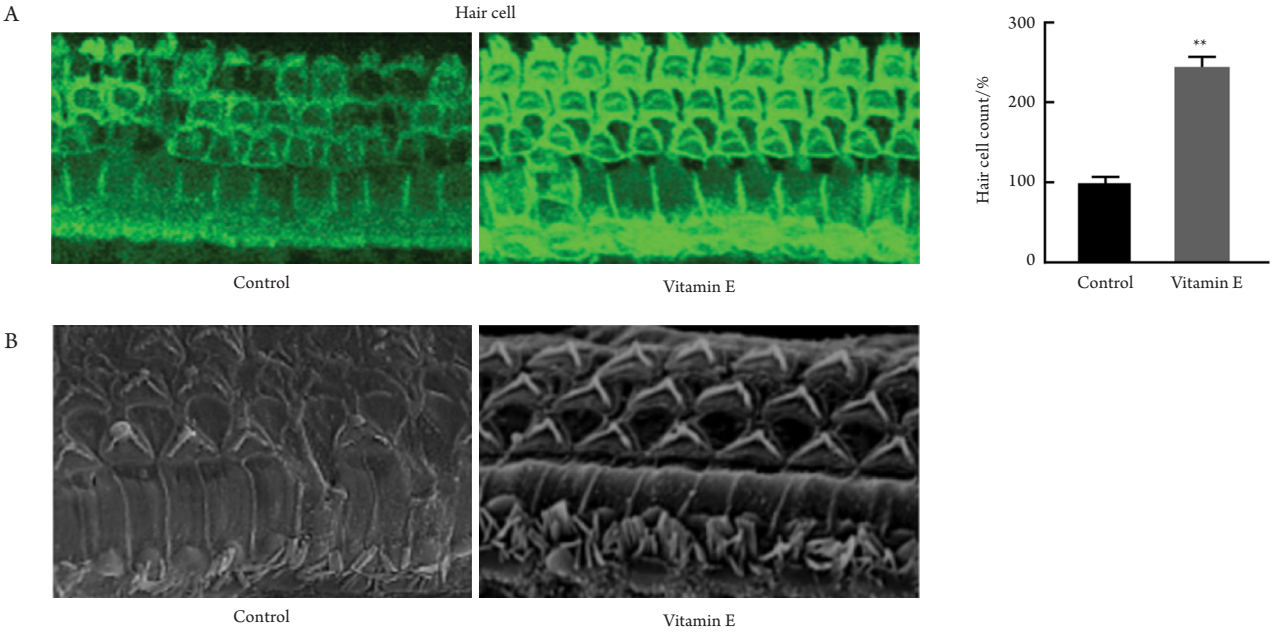

图2 Vitamin E可缓解年龄相关性听力障碍的CBA小鼠外毛细胞及静纤毛损伤

Figure 2 Vitamin E alleviates the degeneration effect of outer hair cells and stereocilia in CBA mice

(A)用Alexa fluor-488标记的鬼笔环肽对外毛细胞进行F-actin染色(×600)；(B)各组小鼠耳蜗基底部静纤毛的改变。Vitamin E组小鼠表现出正常的静纤毛外观，而CBA对照组小鼠的静纤毛缺失，外毛细胞形态结构呈畸形、不规则，并有趋于凋亡的特点(×4 000)。与对照组相比，$^{**}P<0.01$。

(A) Outer hair cells were stained for F-actin with Alexa Fluor-488-labeled phalloidin (×600); (B) Alterations of stereocilia in the basal turns of cochleae in each group mice. Vitamin E group mice showed normal appearance of stereocilia, however, stereocilia in the CBA Control group mice were missing, the morphological structural of outer hair cells was deformity, irregular and tend to apoptosis characteristics (×4 000). $^{**}P<0.01$ vs Control group.

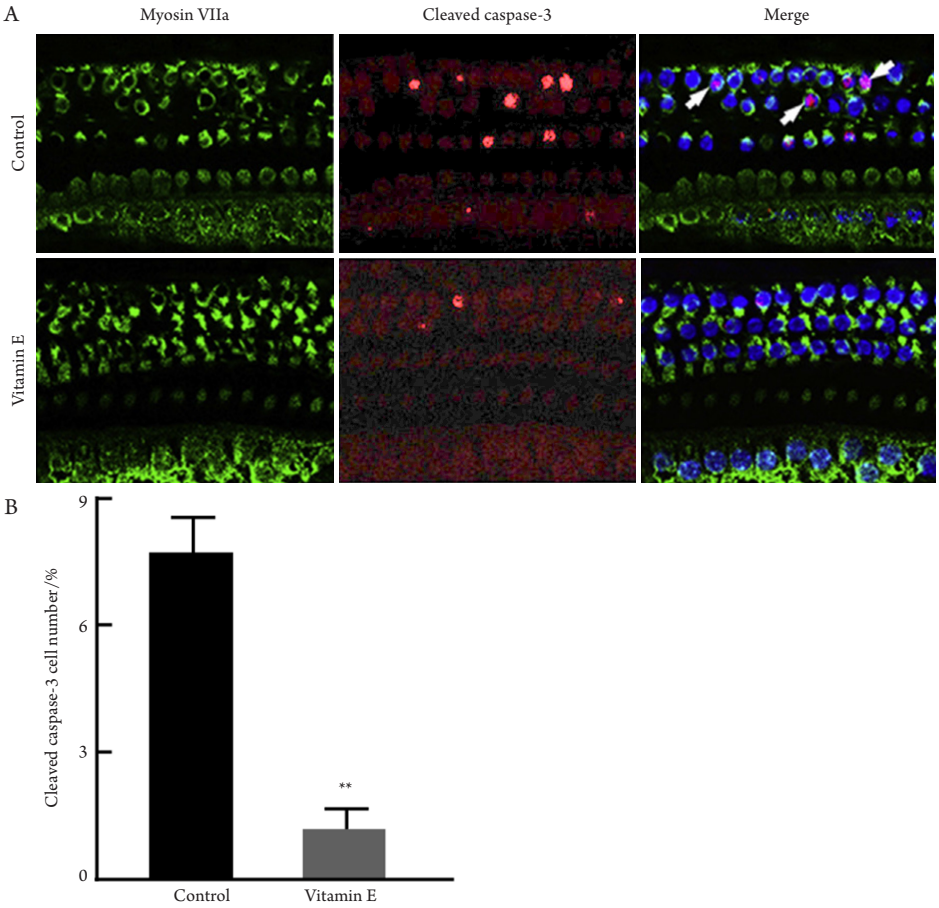

图3 Vitamin E对年龄相关性听力障碍CBA小鼠外毛细胞的抗凋亡作用

Figure 3 Anti-apoptosis effect of vitamin E on outer hair cells in CBA mice with age-related hearing impairment

(A)各组小鼠代表性的cleaved caspase-3(红色)、myosin VIIa(绿色)、DAPI(蓝色)染色图片。凋亡的外毛细胞(cleaved caspase-3和myosin VIIa双染色细胞，×200)。(B) 各组小鼠cleaved caspase-3细胞数(%)的统计分析(n=20)。与对照组相比，**P<0.01。

(A) Represent images showed staining of cleaved caspase-3 (red), myosin VIIa (green), and DAPI (blue) in each group mice. Outer hair cells undergoing apoptosis (cleaved caspase-3 and myosin VIIa double-stained cells). Arrows indicate positive reactions on hair cells (× 200). (B) Statistical analyses of cleaved caspase-3 cell number (%) in each group mice (n=20). **P<0.01 vs Control group.

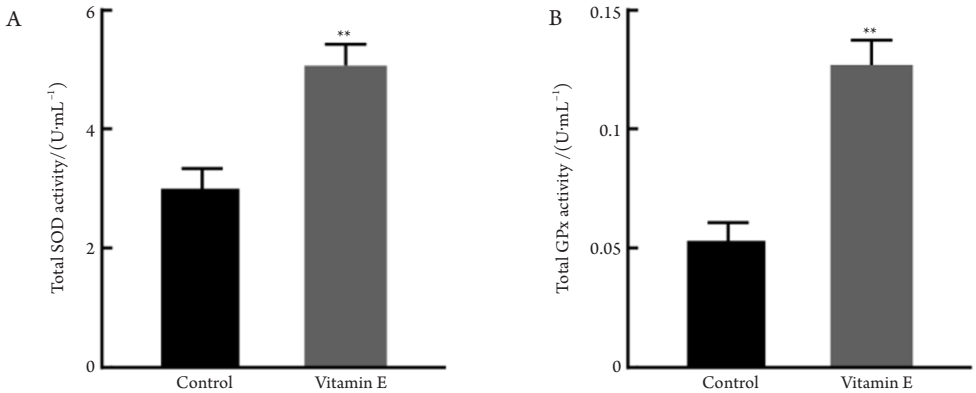

图4 Vitamin E可增高年龄相关性听力障碍CBA小鼠耳蜗SOD及GPx活性水平(n=20)

Figure 4 Vitamin E increases the levels of SOD and GPx activities in cochlea tissues of CBA mice with age-related hearing impairment (n=20)

各组小鼠耳蜗总SOD (A)和GPx (B)活性测定。与对照组相比，**P<0.01。

(A) Total SOD and (B) GPx activities were measured in whole mice cochlear in each group. **P<0.01 vs Control group.

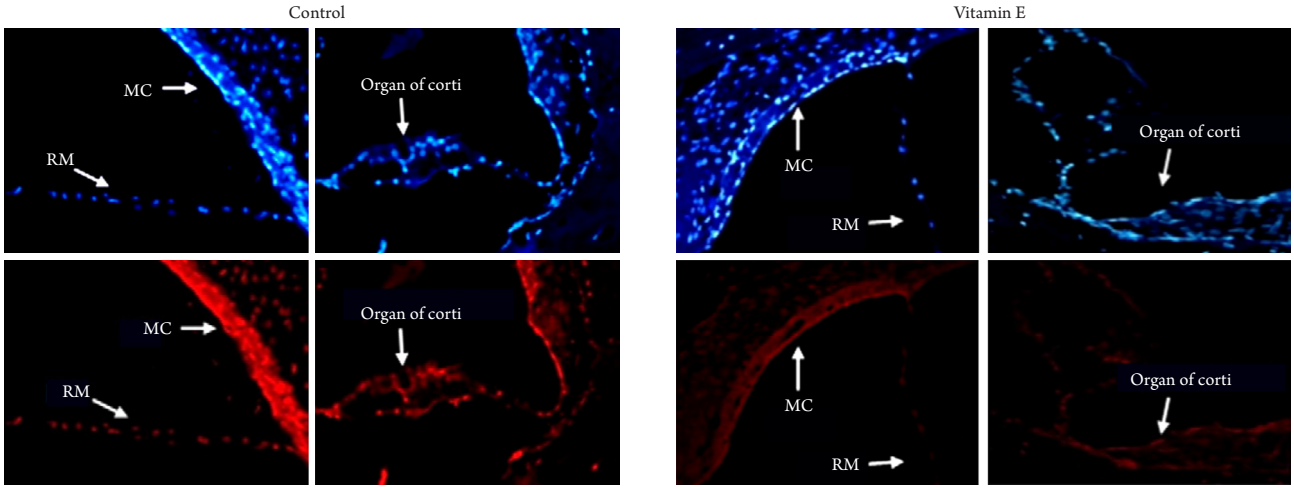

图5 Vitamin E降低年龄相关性听力障碍CBA鼠外毛细胞超氧阴离子水平

Figure 5 Vitamin E decreases the level of superoxide anion in outer hair cells of CBA mice with age-related hearing impairment

利用超氧阴离子荧光探针检测在未经治疗或维生素治疗的小鼠耳蜗组织的外毛细胞的超氧阴离子水平(× 100)。MC：耳蜗边缘细胞；RM：耳蜗前庭膜细胞；Organ of Corti：耳蜗螺旋器细胞。蓝色：DAPI；红色：超氧阴离子。

The level of superoxide anion was detected by using a dihydroethidium probe in outer hair cells of the cochlea tissues from mice either untreated or treated with vitamin (× 100). MC: marginal cells of stria vascularis; RM: Reissner's membrane cells of stria vascularis; Organ of Corti: Organ of Corti cells of stria vascularis. Blue: DAPI; Red: Superoxide anion.

3 讨论

衰老、应激、缺血、噪音和耳毒性因子可引起一系列病理生理变化对耳蜗边缘细胞造成不可逆转的损害，从而导致大量ROS生成，听觉损伤[4]。而有研究[10]表明：在听力障碍的小鼠模型中，给予维生素A、维生素C及其他微量元素处理能缓解听力损失，表明抑制内耳的氧化应激可缓解基因突变引起的听力损失。CBA鼠具有先天性遗传缺陷，出生后从6个月起耳蜗即开始退行性变，在10月龄时已有较严重的听力障碍[9]。因此，本研究利用10月龄CBA鼠作为年龄相关性听力损失模型。而Vitamin E同样具有抗氧化应激的作用，经Vitamin E干预后，CBA小鼠ABR阈值均较对照组下降，表明Vitamin E可缓解年龄相关性CBA小鼠的听力损失。

有研究[4]表明：年龄相关性听力损失与耳蜗氧化应激有直接联系。过氧化氢形成的过氧化物蛋白积累是导致氧化应激的重要机制，而GPx是分解过氧化物的主要蛋白[11]。SOD在线粒体中可使细胞内超氧化物自由基失活，从而发挥保护作用[12]。本研究中Vitamin E可使年龄相关性听力损失CBA小鼠耳蜗GPx及SOD水平升高。另有研究[13]表明：耳蜗的前庭膜及边缘细胞的ROS及超氧化物阴离子水平与听力损失有关，超氧阴离子阳性细胞数目增多可导致耳蜗功能的损害。本研究中Vitamin E可使年龄相关性听力损失CBA小鼠耳蜗边缘细胞、前庭膜细胞及螺旋器细胞的超氧阴离子水平的下降。以上数据表明：Vitamin E能通过抑制氧化应激来保护CBA小鼠听力损失。

过多ROS会导致细胞凋亡。有研究[9,14]发现：CBA小鼠随着年龄增大，ROS逐渐积聚，听觉感受器螺旋器最初表现为毛细胞的畸形及凋亡，外毛细胞的静纤毛病变及缺失，最终导致听神经的退行性病引发听力损失。本研究中在对照组中，纤毛的退行性变主要发生在外毛细胞层，而外毛细胞层在声音诱发的振动过程中有放大效应，因此，纤毛的退行性变可能直接导致了听力损失，在Vitamin E处理后，外毛细胞的凋亡被抑制，且外毛细胞及静纤毛的病理改变也减轻，证明了Vitamin E可通过抑制外毛细胞及纤毛退行性病变来改善听力。

综上，Vitamin E通过抑制耳蜗氧化应激，抑制内耳外毛细胞的凋亡及改善毛细胞纤毛损伤对年龄相关性CBA小鼠听力损失起缓解作用。

参考文献

1. Su P, Hsu CC, Lin HC, et al. Age-related hearing loss and dementia: A 10-year national population-based study[J]. Eur Arch

Otorhinolaryngol, 2017, 274(5): 2327-2334.
2. Liu XY, Han Y, Yang SM. A hearing self-reported survey in people over 80 years of age in China by hearing handicap inventory for the elderly-complete version vs screening version[J]. Acta Otolaryngol, 2016, 136(12): 1242-1247.
3. Brewster KK, Ciarleglio A, Brown PJ, et al. Age-related hearing loss and its association with depression in later life[J]. Am J Geriatr Psychiatry, 2018, 26(7): 788-796.
4. Tu NC, Friedman RA. Age-related hearing loss: Unraveling the pieces[J]. Laryngoscope Investig Otolaryngol, 2018, 3(2): 68-72.
5. Li Y, Zhao X, Hu Y, et al. Age-associated decline in Nrf2 signaling and associated mtDNA damage may be involved in the degeneration of the auditory cortex: Implications for central presbycusis[J]. Int J Mol Med, 2018, 42(6): 3371-3385.
6. Tavanai E, Mohammadkhani G. Role of antioxidants in prevention of age-related hearing loss: a review of literature[J]. Eur Arch Otorhinolaryngol, 2017, 274(4): 1821-1834.
7. Kamogashira T, Fujimoto C, Yamasoba T. Reactive oxygen species, apoptosis, and mitochondrial dysfunction in hearing loss[J]. Biomed Res Int, 2015, 2015: 617207.
8. Kinoshita M, Sakamoto T, Kashio A, et al. Age-related hearing loss in Mn-SOD heterozygous knockout mice[J]. Oxid Med Cell Longev, 2013, 2013: 325702.
9. Williamson TT, Zhu X, Walton JP, et al. Auditory brainstem gap responses start to decline in mice in middle age: A novel physiological biomarker for age-related hearing loss[J]. Cell Tissue Res, 2015, 361(1): 359-369.
10. Mukherjea D, Ghosh S, Bhatta P, et al. Early investigational drugs for hearing loss[J]. Expert Opin Investig Drugs, 2015, 24(2): 201-217.
11. Pacuła AJ, Kaczor KB, Wojtowicz A, et al. New glutathione peroxidase mimetics-Insights into antioxidant and cytotoxic activity [J]. Bioorg Med Chem, 2017, 25(1): 126-131.
12. Ishihara T, Shibui M, Hoshi T, et al. Scavenging of superoxide anions by lecithinized superoxide dismutase in HL-60 cells[J]. Mol Biosyst, 2016, 12(1): 274-282.
13. Fetoni AR, Rolesi R, Paciello F, et al. Styrene enhances the noise induced oxidative stress in the cochlea and affects differently mechanosensory and supporting cells[J]. Free Radic Biol Med, 2016, 101: 211-225.
14. Ding B, Walton JP, Zhu X, et al. Age-related changes in Na, K-ATPase expression, subunit isoform selection and assembly in the stria vascularis lateral wall of mouse cochlea[J]. Hear Res, 2018, 367: 59-73.

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