

# Up-regulation thioredoxin contributes to inhibit diabetic hearing impairment

**Xiang Ren**

Dalian Medical University

**Jin Juan Lv**

Dalian Medical University

**Yu Zhen Fu**

Dalian Medical University

**Na Zhang**

The Second Hospital of Dalian Medical University

**Cheng hong Zhang**

Dalian Medical University

**Zheng hao Dong**

Dalian Medical University

**Maryam Chudhary**

Dalian Medical University

**Si wen Zhong**

Dalian Medical University

**Li Kong**

Dalian Medical University

**Hui Kong** (✉ [konghui@dmu.edu.cn](mailto:konghui@dmu.edu.cn))

---

## Research

**Keywords:** Thioredoxin, Diabetes, Hearing impairment, mitochondria, endoplasmic reticulum stress, autophagy

**Posted Date:** April 12th, 2021

**DOI:** <https://doi.org/10.21203/rs.3.rs-392135/v1>

**License:**   This work is licensed under a Creative Commons Attribution 4.0 International License.

[Read Full License](#)

---

# Abstract

## Background

Diabetes mellitus (DM) is a metabolic disorder characterized by chronic hyperglycemia. An association between DM and hearing impairment has been widely discussed. It's essential to find effective prevention or diagnosis of diabetic hearing loss. Thioredoxin (Trx) is a small molecule protein (12kDa) and plays biological functions such as anti-apoptotic, transcriptional regulation. In this study, our aim is to clarify the protective effect of Trx on diabetic hearing loss to find the early potential therapeutic target of diabetic hearing impairment in clinic in the future.

## Methods

Trx transgenic(Tg) mice was used to induce diabetic model by intraperitoneal injected Streptozotocin (STZ) and with/without SF or PX12 treatment. Succinate Dehydrogenase (SDH) Staining was used to evaluate the loss of hair cells. The relative expression of related proteins and genes was detected by Western blot and qRT-PCR.

## Results

In diabetic mice, the outer hair cells were lost significantly. However, the loss of hair cells was delayed over-expression Trx. Moreover, the expression of PGC-1 $\alpha$ , bcl-2 and LC3 was increased in Tg(+)-DM mice compared with Tg(-)-DM mice. The expression of ASK1, Txnip, GRP78, CHOP and p62 was decreased in Tg(+)-DM mice compared with Tg(-)-DM mice.

## Conclusion

Taken together, up-regulation Trx can protect cochlear hair cell from damage in diabetes. The underlying mechanism may be related to regulate ER stress through ASK1 and mitochondria pathway or autophagy via Txnip.

## Introduction

Diabetes mellitus (DM) is a metabolic disorder characterized by chronic hyperglycemia. The number of patients with DM is increasing each year and there will be a variety of complications such as diabetic retinopathy (DR)[1], nervous system damage[2], kidney damage[3] and cardiovascular disease[4] with the progression of diabetes. Besides, an association between DM and hearing impairment has been widely discussed. The pathogenesis of diabetic hearing loss remains unclear[5,6] and affected the life of diabetes patients. Thus, it is strongly necessary to understand the mechanisms and find some ways to

inhibite diabetic heraing impairment. There are many factors participated in this process such as oxidative stress[7], endoplasmic reticulum stress [8] (ERS), and autophagy[9]etc.

Endoplasmic reticulum is involved in many important physiological functions in eukaryotic cells such as post-translational modification, folding, assembly, lipid synthesis and intracellular calcium storage[10]. Exposure to high glucose for a long time may lead to protein synthesis disorders or accumulation of misfolded proteins in endoplasmic reticulum lumen, which may lead to ERS[11]. Glucose-regulated protein 78 (GRP78) plays an important role in regulating ERS[12] .

Autophagy is a highly conservative catabolic process in eukaryotic cells, which plays a crucial role in maintaining normal life activities. It has been reported that autophagy induction can be against inflammation[13], improve senescence[14], and prevent neurodegeneration[15]. As a protective mechanism of cells in an adverse environment, with the development of diabetes, autophagy disorders lead to autophagic apoptosis[16].

Thioredoxin (Trx) is a small redox protein existing in tissues which contains a dithiol-disulfide active site: -Cys-Gly-Pro-Cys-[17]. It can remove intracellular ROS and protect cell from oxidative damage effectively[18]. Trx is a vital regulator of apoptotic signal-regulating kinase 1(ASK1) functions. When Trx is oxidized, ASK1 is dissociated from Trx and interacts with TRAF2/6 to form ASK1-TRAF2-IRE1 $\alpha$  (ERS marker) complex which ultimately promote cell apoptosis and death[19,20]. Moreover, we also found that Trx can regulate autophagy through thioredoxin-interacting protein (Txnip)-mTOR pathway[21]. Our former researches indicated that Sulforaphane (SF) which is widely found in cruciferous plants, including cabbage, broccoli and radish also can up-regulate Trx expression[22] to delay photoreceptor degeneration in diabetes [23].

Basing on these evidences, in this study we will up-regulate Trx expression through SF treatment or transgenetic method to explore the protective effect of Trx on diabetes-indueced cochlear hair cell degeneration and related mechanism aiming to provide some evidence for diabetic hairing impairment prevention or treatment in clinic in the future.

## Materials And Methods

### Patients

A total of 45 patients with diabetes in the Endocrinology Department of the Second Affiliated Hospital of Dalian Medical University. According to the average value of pure tone audiometry from 0.5kHz-4kHz, the patients were divided into two groups: Diabetics with normal hearing (DM1, 20 cases, 40 ears) and diabetics with abnormal hearing (DM2, 25 cases, 50 ears). People with healthy examination were selected as the normal control group (NC, 25 cases, 50 ears). Signed consent was obtained from all patients.

### Otoacoustic emissions

OAEs are sounds caused by the motion of the cochlea's sensory hair cells as they energetically respond to auditory stimulation. The method of OAEs recording used in this study were the distortion product otoacoustic emissions(DPOAEs) and transient evoked otoacoustic emissions (TEOAEs). The instruments were GSI-61 dual channel diagnostic audiometer. Automatic tympanometer of GSI-39version and Cochlear Emission Analyzer of Capella Denmark GN Audiometrics A/S company. The frequencies analyzed in this study were 0.5 k, 1 k, 2k, 4 k and 8 kHz.

## **Animal Care**

Male Trx transgenic (Tg) mice (C57BL/6J background, six-week-old, 20±2g) were obtained from Dalian Medical University Laboratory Animal Center. All experiments involving animals in this study were executed in keeping with institutional guidelines for the care and use of laboratory animals, the experimental program was approved by the institutional Animal Care and Use committees of Dalian Medical University Laboratory Animal Center. All experimental animals were fed adaptively (immobile temperature 22±2°C, sufficient water and food, 12 hours light / dark: average illuminance of 80 lx). Genomic DNA from mouse tail was amplified by PCR using the following sets of primers: HTXN-380F 5'-CAGTACATCAATGGGCGTGG-3', HTXN-956R 5'-CGCGGTACCGTCGACTTAGA-3'. The conditions of PCR amplification for synthesized Trx were as follows: 95°C for 30 seconds, 95°C for 10 seconds, 65°C for 30 seconds, 72°C for 1 minutes and for 35 cycles, 72°C for 10 minutes, 4°C for endless. The PCR products were running on a 1% agarose gel at 80 V for 40 minutes and stained with ethidium bromide (EB). The PCR product of Tg-Trx is 577 bp. The data was analyzed with a GDS-8000 Bio imaging system (UVP, Upland, CA).

## **Diabetes model establishment**

After adaptive feeding, the diabetic model was induced by intraperitoneal injected streptozotocin(50mg/kg) continued for 5 days and control group was given intraperitoneal injection of citrate buffer. Diabetes was defined as follow: random blood-glucose  $\geq 16.7$  mmol/L [24].

## **Drug treatment**

The diabetic mice were intraperitoneal injection with 1.0 mg/kg SF (S8046, LKT Laboratories Inc., St. Paul, MN, USA) and/or 1.0 mg/kg PX12 (Trx inhibitor) for 2 weeks. The control mice were injected with saline solution. Animals were sacrificed by CO<sub>2</sub> asphyxiation after treatment. .

## **Hair cell counting**

Animals were sacrificed with overdose CO<sub>2</sub> and the cochleae were quickly removed from the skull and put them into the NBT (Solarbio<sup>®</sup>, N8140 ) working fluid (1:1:2, 0.2 M sodium succinate, 0.2 M PBS pH 7.6, 0.1% tetranitro-blue tetrazolium). The round and oval windows were removed and the apex of the cochlea was opened to facilitate perfusion. NBT working fluid is slowly injected into the cochlea along the circular window. The working fluid flowed from the hole at the top of the cochlea, the perfusion was successful.



The cochlea was placed in NBT working fluid, incubated at 37°C for 1 hour, and fixed with 10% formalin for 2 days. 7% EDTA decalcified solution at room temperature until cochlear osteomalacia. The hair cells were counted from the top to the bottom of the cochlea at the same distance (200 µm) under a light microscope. The hair cells numbers were compared to the normal numbers obtained in the group and cell losses were expressed as percentage. Cell losses were plotted as a function of cochlear length (cochleogram).

### **Quantitative real-time PCR**

Total RNA of cochlea was isolated using a TRIZOL (Takara) reagent according to the manufacturer's instructions. The cDNA was synthesized from 500ng of RNA in a 10µl reaction with RNA PCR Kit (AMV) Ver.3.0 (Takara). GAPDH was used as reference for PCR amplification. The conditions of RNA PCR amplification for synthesized cDNA were as follows: 37°C for 15 minutes, 85°C for 5 seconds and 4°C for endless. The primers were used as following:

GAPDH forward 5'-TGTGATGGGTGTGAACACGAGAA-3', reverse 5'-GAGCCCTTCCACAATGCCAAAGTT-3'; Txnip forward 5'-ACTCCTCAAGATGGGTGGCAATC-3', reverse 5'-ACATCCACCCAGCAAACACTCCT-3'; Trx forward 5'-CAAATGCATGCCGACCTTCCAGTT-3', reverse 5'-3'; Tfam forward 5'-TGGCAGTCCATAGGCACCGTATT-3', reverse 5'-ACAGACAAGACTGATAGACGAGGG-3'; ASK1 forward 5'-TTTGTTCGTGAGACTGCGTACC-3', reverse 5'-AGACACTTGGGCACACTACACA-3'. The conditions of PCR amplification were as follows: 94°C 30 seconds, 94°C 5 seconds, 55°C 15 seconds, 72°C 10 seconds for 40 cycles and the data were analyzed by  $\Delta\Delta C_t$  method.

### **Immunofluorescence staining**

The mice were euthanized with excessive CO<sub>2</sub> and their cochlear were removed. The cochlear were fixed in 10% formaldehyde solution for 2 days after removing the auditory vesicles. 7% EDTA decalcified solution at room temperature until cochlear osteomalacia. After dehydration in alcohol, the cochlear were cut into 5-µm paraffin sections that parallel to the axis of the cochlea. Primary antibodies were anti-Trx (Proteintech®, 14999-1-AP, 1:200), anti-ASK1 (Santa Cruz, sc-5294, 1:200). Antigens were visualized after incubation with Alexa Fluor® 488 or 649-conjugated goat IgGs (Molecular Probes). Fluorescent images were captured using microscope (Leica DM6000B fluorescence microscope, Germany).

### **Western Blot**

The cochlea were lysed in a buffer (50mM Tris-HCl pH 8.0, 150mM NaCl, 1% Nonidet P-40, 0.5% deoxycholate, 0.1% SDS, 1mM PMSF and 150 units/ml aprotinin) and ultrasound pyrolysis on ice. Tissues were centrifuged (12000g, 4°C, 15min) and extracted the liquid supernatant for another tubes. The solution was disposed by SDS-PAGE and electroblotted onto polyvinylidenedifluoride (PVDF) membranes (Millipore, Billerica, MA). The membranes, with 5% blocking milk for 1h at room temperature, were incubated by anti-GAPDH (Proteintech®, 10494-1-AP, 1:4000), anti-Trx (Cell Signaling Technology, 2429 1:1000), anti-Txnip (Proteintech®, 18243-1-AP, 1:1000), anti-ASK1 (Santa Cruz, sc-5294, 1:1000),

anti-Bax (Proteintech<sup>®</sup>, 50599-2-Ig, 1:1000), anti-Bcl-2 (abcam, ab185002, 1:1000), anti-PGC-1 $\alpha$  (Abcam, ab54481, 1:1000), anti-LC3-II (Proteintech, 12135-1-AP, 1:1000), anti-p62 (ABclonal, A7758, 1:1000), anti-Beclin 1 (ABclonal, A7353, 1:1000), anti-GRP78 (Proteintech<sup>®</sup>, 66574-1-Ig, 1:1000), anti-CHOP (Proteintech<sup>®</sup>, 15204-1-AP, 1:1000) that diluted in 5% skim milk. All of membranes were incubated overnight. Subsequently, the membranes were washed three times with 1 $\times$ TTBS for 15min. Then every membrane was incubated with goat anti-mouse IgG (Abclonal, AS003, 1:2000) and goat anti-rabbit IgG (Abclonal, AS014, 1:2000) for 1.5h and washed three times with 1 $\times$ TTBS for 15min. The membranes were then exposed to X-ray film using an enhanced chemiluminescence system. The intensities of the bands were measured using LabWorks 4.5.

## Statistical analysis

The data were presented using mean $\pm$ SD and the differences among groups were determined via one-way analysis of variance and t test. All performs were made by GraphPad Prism software (Ver.5.0). A p value < 0.05 was considered significant.

# Results

## Diabetes induced hearing impairment in pateints.

As show in Fig. 1 A, the average PTA value of DM1 and the normal group are within the normal range, there is no significant difference between the PTA value of DM1 and normal group in 0.25kHz and 0.5kHz, while it's significantly higher in DM1 than control group in 1k-8kHz. And the PTA value of DM2 in frequency 0.5k-8kHz is significantly increased compared with DM. The OAE results which including DPOAE level, S/N-ratio and TEOAE correlaton in DM1 and DM2 are all significantly increased compared with control group. Simultaneously, they are significantly decreased in DM2 compared with DM1(Fig. 1B-D). The auditory testing results indicate the diabetic cochlea have been damaged before the hearing change, the early hearing loss in diabetics are mainly in high frequencies and involve full frequency gradually.

## Diabetes induced cochlear hair cell degeneration*in vivo*.

As shown in Fig.2A&B, as the progression of diabetes, the FBG and homeostasis model assessment-insulin resistance index (HOMA-IR) were increased significantly in diabetic group compared with control group. The cochlea from diabetic mice were isolated for SDH staining to observe the degeneration of the outer hair cell (OHC) and inner hair cell (IHC). Fig.2 C-E present OHC loss (D) and IHC loss (E) in diabetic mice as a function of cochlear length (cochleogram). The degeneration started from the basal turn and expanded progressively with time towards the apical turn. At 10 day, both OHC loss and IHC loss occurred. The percentage of loss was increasing with the diabetes process. However, IHC degeneration appeared to develop more slowly than the OHC. There was no hair cell loss observed in control group.

We also used western blot to detect the expression of Trx. The results showed that the relative expression of Trx was decreased gradually and was positively correlated with the loss of cochlear hair cells in diabetic mice (Fig.1 F&G).

#### **Up-regulated Trx expression by SF inhibited diabetic induced cochlear hair cell injury.**

As show in Fig.3 A&B, Trx expression was significantly up-regulated after treated with SF. The total loss ratio of OHC/IHC at 31.8%/16.3% in diabetic group compared with WT group. However, the loss ratio of OHC/IHC(26.5%/13.8%) was inhibited in diabetic mice after SF treatment compared with diabetic mice. The process could be inhibited by PX12 (Fig.3 C&D&E). we also detect the expression of Trx and Txnip. The results showed that the expression of Trx and Txnip was decreased or increased significantly in diabetic group compared with WT group, however, the expression of Trx and Txnip was up/down-regulated after SF treatment in diabetic group which can be reversed by PX-12 (Fig.3 F&G).

#### **Up-regulated Trx expression by SF inhibited diabetic induced cochlear hair cell injury through down-regulating ASK1 and Txnip.**

As shown in Fig.4 A, Trx (red) and ASK1 (green) were mainly located at the OHC/IHC of cochlea, the red fluorescence of Trx in the diabetic group was significantly decreased, while the green fluorescence of ASK1 was significantly increased compared with the control group, which suggesting that diabetes can down-regulate Trx expression and up-regulate ASK1 expression in cochlea. After SF treatment, the red fluorescence was increased and the green fluorescence was significantly decreased compared with the diabetic group. However, the green fluorescence (ASK1) was significantly increased and the red fluorescence (Trx) was significantly decreased after SF and PX12 treatment. We also used western blot to detect the expression of Trx and ASK1(Fig.4 B-D).

#### **Over-expressed Trx can delay diabetes induced cochlear hair cell degeneration *in vivo*.**

As shown in Fig.5 A, over-expressed Trx transgenic mice were identified and randomly divided into Tg(-)-DM group and Tg(+)-DM group. As show in Fig.5 B, FBG in DM group was up to the standard of diabetes. Subsequently, we counted the hair cell under microscope with SDH staining to observe the loss of cochlear hair cells. The results demonstrated that OHC/IHC loss was significantly delayed in Tg(+)-DM group compared with Tg(-)-DM group (Fig.5 C&D). Moreover, we used qRT-PCR to measure gene expression of Tfam, ASK1 and Txnip. As shown in Fig.5 E-G, the expression of Tfam was increased and the expression of ASK1 and Txnip was decreased in Tg(+)-DM group compared with Tg(-)-DM group.

#### **Over-expressed Trx can delay diabetes induced cochlear hair cell degeneration through regulating mitochondrial pathway/ endoplasmic reticulum stress (ERS) / autophagy *in vivo*.**

We used western blot to detect protein expression for mitochondrial pathway, ERS and autophagy. As shown in Fig.6 A-D, the expression of PGC1 $\alpha$  was decreased in the Tg(-)-DM group compared with Tg(-) group, while it was increased in the Tg(+)-DM group(Fig. 6 B). The expression of pro-apoptotic protein Bax in Tg(+)-DM group was significantly decreased compared with Tg(-)-DM group(Fig. 6 C). Moreover, the

expression of bcl-2 expression in Tg(-)-DM group was significantly decreased compared with Tg(-) group, however, it was increased in Tg(+) group compared with Tg(-) group (Fig. 6 D). As shown in Fig.6 E-H, the expression of ASK1, GRP78 and CHOP in Tg(-)-DM group was significantly increased compared with Tg(-) group, while the expression of ASK1, GRP78 and CHOP was significantly decreased in the Tg(+)-DM group. As shown in Fig.6 I-L, the expression of Txnip significantly increased in Tg(-)-DM group, which it was reversed by over-expression Trx. The expression of LC3-II was decreased in the Tg(-)-DM group, while the expression in the Tg(+)-DM group was increased than Tg(-)-DM group(Fig.6 K). Besides, the expression of p62 was increased in Tg(-)-DM group, while it was decreased in Tg(+) group compared with the Tg(-) group (Fig. 6 L). These results was summarized in Fig.7.

## Discussion

Diabetes induced hearing impairment is one of the serious complications of DM. Because it's high incidence, it was attracted more and more people's attention. The onset of diabetic hearing impairment is hidden and the course of the disease is slow, which seriously affects the life quality of patients. Most patients have irreversible hearing impairment[25]. In our research, we found 84% of diabetics in DM2 had bilateral hearing loss. We also found cochlear impairment in diabetes patients with normal PTA by otoacoustic emission (OAE). Apply easily, possible without cooperation, and not taking a long time are main features of the test. The sensitivity of TEOAE is higher in detection, while DPOAE has been used more frequently in clinic, because it enables the detection of frequencies in wider intervals in the early detection of cochlear disorders[26]. The frequencies analyzed in this study were 0.5k, 1k, 2k, 4k and 8kHz. The value of DPOAE level records was lower with the frequencies increasing in diabetes pateints compared with the healthy people.

With the development of diabetes, permanent hearing loss or imbalance is caused by hair cell loss, internal auditory arteriosclerosis and delayed auditory brainstem response (ABR) delayed wave V[27]. Therefore, illuminating the relationship between diabetes and hearing impairment and its pathogenesis is of great significance for early diagnosis, prevention and treatment. Mitochondrial damage, endoplasmic reticulum stress and autophagy disorder caused by persistent hyperglycemia in diabetes plays an important role in cochlear hair cell damage and hearing loss[28,29,30].

Along with glutathione, thioredoxin (Trx), a small 12kDa protein widely found in organism, is one of the two main antioxidants in mammals[31]. Moreover, Trx plays a pivotal role in maintaining the intracellular environment and regulating proliferation and transcriptional [32]. Our previous study also found that the crucial biological functions of Trx on anti-oxidation and anti-apoptosis in cells, which is important for maintaining cell metabolism[33,34].

In this study, up-regulating Trx by SF and transgenic mice (Trx) were used to explore the role of Trx in the process of diabetes-induced cochlear hair cell damage and hearing loss in mice. We demonstrated that there was a significant correlation between Trx expression decreasing and cochlear hair cell degeneration in diabetic mice. Moreover, we also found that up-regulating Trx by SF could protect the hair cells loss

induced by diabetes. Furthermore, the expression of ASK1 can be inhibited after Trx expression increasing which was detected in the cochlear hair cells (Fig.4). Besides, in our former research reported that Grape Seed Proanthocyanidin Extract (GSPE) can prevent retinal degeneration in diabetic mice by mediating up-regulation Trx and reducing ER stress [35]. These results indicated up-regulating Trx would be a potential way to delay neurodegeneration induced by diabetes. After over-expression Trx in mice, we further explored the protective effect and mechanism of Trx on the diabetes-induced cochlear hair cells. We found that the outer hair cells were arranged loosely and irregularly in Tg(-)-DM, while the volume of hair cells was increased and arranged regularly in Tg(+)-DM group compared with Tg(-)-DM group.

In our research, we found that mitochondrial pathway, ERS and autophagy involved in process of cochlear hair cell damage. In Tg(+)-DM group the expression of PGC1 $\alpha$  and Bcl-2 was increased and the expression of Bax was decreased compared with Tg(-)-DM. It suggested that Trx can effectively improve the loss of cochlear hair cells in diabetes through regulating mitochondrial pathway. Scholars also have proved that Trx over-expression extends antioxidant protection, attenuates mitochondrial damage, and activates mitochondrial turnover such as mitophagy and biogenesis [36].

Endoplasmic reticulum (ER) is a dynamic intracellular organelle with multiple functions. It is essential for cell homeostasis, development and stress response [10]. And it can be activated by many unfavorable factors. Studies have shown that high glucose stimulation can lead to the occurrence of ER stress response [37]. When ER stress is activated, several apoptotic signaling pathways can be activated to induce cell apoptosis. Trx can alleviate the neurodegeneration of the disease by inhibiting the endoplasmic reticulum stress in MPP<sup>+</sup> environment [17]. Our work also confirmed that the expression of ER stress marker protein GRP78 in DM group was significantly increased compared with non-diabetic group. In transgenic Trx diabetic mice with down-regulating the expression of ASK1 leading to the expression of GRP78 and CHOP was decreased in cochlear compared with Tg(-)-DM mice. It indicates that diabetes can significantly activate ER stress. Trx can suppress ER stress via ASK1 to protect hair cells from diabetes.

Autophagy is also a highly conserved lysosome degradation pathway in eukaryotes, responsible for providing metabolic energy, circulating intracellular components, and eliminating harmful molecules, organelles and abnormal accumulation of substances [38]. Studies have shown that autophagy is essential for the development and maturation of the inner ear of vertebrates, which is the basic intracellular pathway [30]. Autophagy is essential in neurons and the postnatal onset of hearing and the concomitant increase in neuronal activity is correlated with the induction of autophagy in the cochlea [39,30]. After over-expression of Trx with down-regulating Txnip expression, the expression of LC3-II, an autophagy marker protein, was up-regulated in Tg(+)-DM, suggesting that Trx can activate autophagy and increase the encapsulation of products to be degraded in cochlear cells. Trx can reduce the expression of autophagic lysosome degradation protein p62 and promote autophagic lysosome degradation which accelerated the autophagy flux.

In summary, up-regulate Trx by SF and over-expression of Trx in mice can protect the cochlea hair cell damage induced by diabetes. The underlying mechanism may be related to regulate ER stress through ASK1 and autophagy or mitochondria pathway through Txnip.

## Conclusion

Up-regulating the expression of Trx can delay diabetes-induced cochlear hair cells degeneration and the underlying mechanism may be related to regulate ER stress through ASK1 and autophagy or mitochondria pathway through Txnip.

## Declarations

### Ethics approval and consent to participate

All animal experiments followed the institutional Animal Care and Use committees of Dalian Medical University Laboratory Animal Center.

### Consent to publication

All authors read and approve of the final manuscript for publication.

### Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

### Competing interests

The authors declare that they have no competing interests.

### Funding

This project was supported by Grant No. 31371218 from the National Natural Science Foundation of China, National and Local Joint Engineering Research Center for Mongolian Medicine Research and Development (MDK2019075) and the Natural Science Foundation of Liaoning Province Grant No. 2020-BS-189. The Liaoning Provincial Program also supported this work for the Top Discipline of Basic Medical Sciences.

### Author's contribution

Xiang Ren, Jinjuan Lv, Yuzhen Fu and Na Zhang designed, performed the experiments, and evaluated the data. Yuzhen Fu, Na Zhang, Chenghong Zhang, Zhenghao Dong, Maryam Chudhary and Shiwen Zhong contributed to reagents, materials and analytical tools and provided records and interpretation of the data. Xiang Ren, Jinjuan Lv and Na Zhang contributed to manuscript preparation. Li Kong and Hui Kong

were the guarantor for this work and had full access to all the data in the work and takes responsibility for the integrity of the data and the accuracy of the data analysis.

**Acknowledgements**

We thank professor Kong for insightful comments. We also thank our colleagues in the Li Kong's lab and Hui Kong's lab for assistance, critical comments, and suggestions. We specially thank professor Hui Kong for their assistance.

**Abbreviations**

DM	Diabetes mellitus
DR	Diabetic retinopathy
Trx	Thioredoxin
SF	Sulforaphane
PX-12	Trx inhibitor
ERS	Endoplasmic reticulum stress
GRP78	Glucose-regulated protein 78
ASK1	Apoptotic signal-regulating kinase 1
DPOAEs	Distortion product otoacoustic emissions
TPOAEs	Transient evoked otoacoustic emissions
STZ	Streptozotocin
SDH	Succinate Dehydrogenase

**References**

1: Ma J, Wang J, Liu Y, Wang C, Duan D, Lu N, Wang K, Zhang L, Gu K, Chen S, Zhang T, You D, Han L. Comparisons of serum miRNA expression profiles in patients with diabetic retinopathy and type 2 diabetes mellitus. Clinics (Sao Paulo). 2017 Feb 1;72(2):111-115.

2: Ahmed AS, Elgharabawy RM, Al-Najjar AH. Ameliorating effect of anti-Alzheimer's drugs on the bidirectional association between type 2 diabetes mellitus and Alzheimer's disease. Exp Biol Med (Maywood). 2017 Jul;242(13):1335-1344.

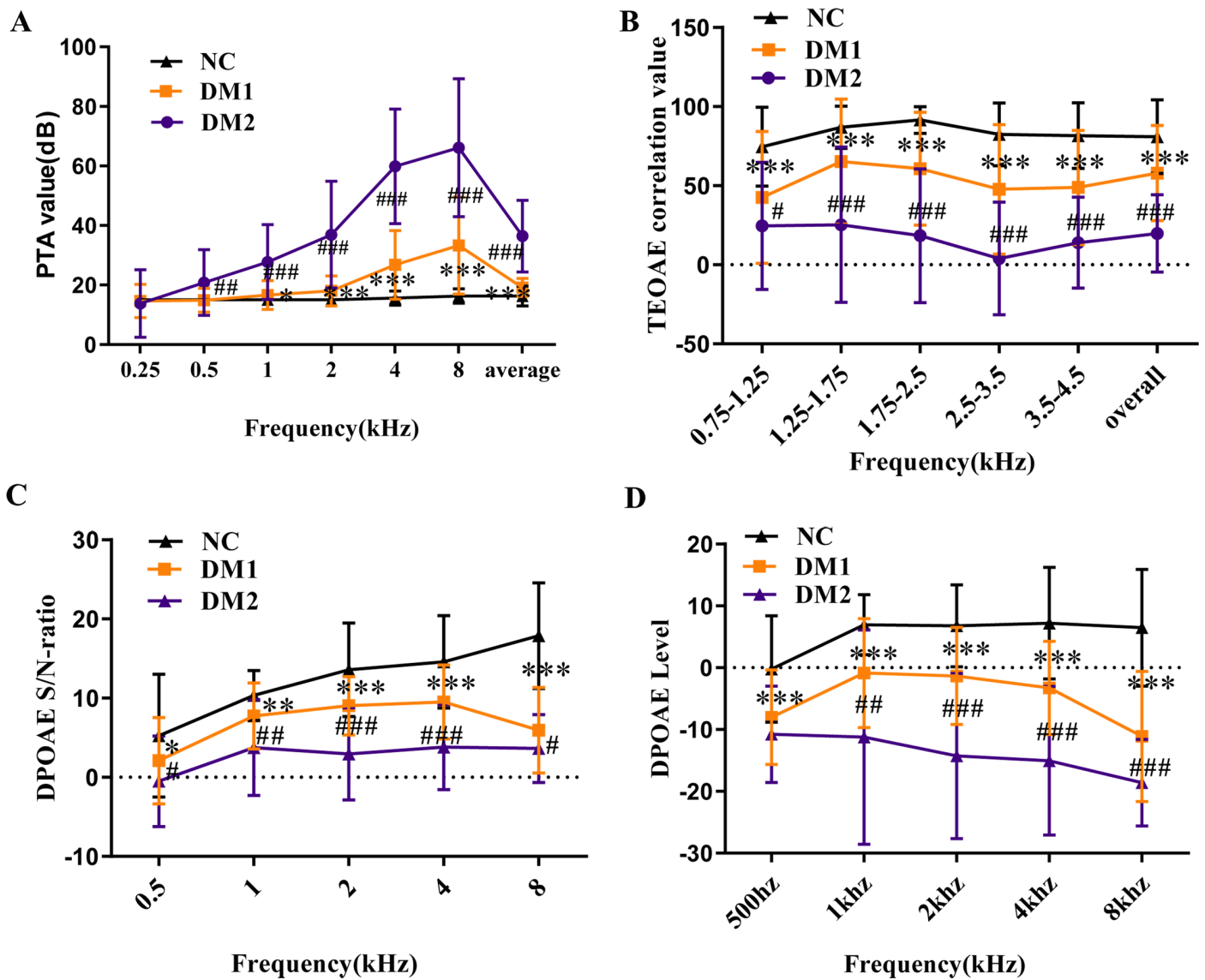
- 3: Lambooy SPH, Bidadkosh A, Nakladal D, van Buiten A, Girgis RAT, van der Graaf AC, Wiedenmann TJ, Koster RA, Vogelaar P, Buikema H, Henning RH, Deelman LE. The Novel Compound Sul-121 Preserves Endothelial Function and Inhibits Progression of Kidney Damage in Type 2 Diabetes Mellitus in Mice. *Sci Rep*. 2017 Sep;11(7):11165.
- 4: Szuszkiewicz-Garcia MM, Davidson JA. Cardiovascular disease in diabetes mellitus: risk factors and medical therapy. *Endocrinol Metab Clin North Am*. 2014 Mar;43(1):25-40.
- 5: Felício JS, de Souza d'Albuquerque Silva L, Martins CLELP, et al. Cochlear dysfunction and microvascular complications in patients with type 1 diabetes mellitus. *Diabetol Metab Syndr*. 2018; 10:81. Published 2018 Nov 9.
- 6: Meyer zum Gottesberge AM, Massing T, Sasse A, Palma S, Hansen S. Zucker diabetic fatty rats, a model for type 2 diabetes, develop an inner ear dysfunction that can be attenuated by losartan treatment. *Cell Tissue Res*. 2015;362(2):307-315.
- 7: Helzner EP, Contrera KJ. Type 2 Diabetes and Hearing Impairment. *Curr Diab Rep*. 2016 Jan;16(1):3.
- 8: Tsuchihashi NA, Hayashi K, Dan K, Goto F, Nomura Y, Fujioka M, Kanzaki S, Komune S, Ogawa K. Autophagy through 4EBP1 and AMPK regulates oxidative stress-induced premature senescence in auditory cells. *Oncotarget*. 2015 Feb 28;6(6):3644-55.
- 9: Zong S, Liu T, Wan F, Chen P, Luo P, Xiao H. Endoplasmic Reticulum Stress Is Involved in Cochlear Cell Apoptosis in a Cisplatin-Induced Ototoxicity Rat Model. *Audiol Neurotol*. 2017;22(3):160-168.
- 10: Bravo R, Parra V, Gatica D, Rodriguez AE, Torrealba N, Paredes F, Wang ZV, Zorzano A, Hill JA, Jaimovich E, Quest AF, Lavandero S. Endoplasmic reticulum and the unfolded protein response: dynamics and metabolic integration [J]. *Int Rev Cell Mol Biol*, 2013, 301: 215-290.
- 11: Manish Mishra, R.A.K., Retinal Mitochondrial DNA Mismatch Repair in the Development of Diabetic Retinopathy, and Its Continued Progression After Termination of Hyperglycemia [J]. *investigative ophthalmology & visual science*, 2014. 55(10): p. 8.
- 12: Gething M. Role and regulation of the ER chaperone BiP [J]. *Semin Cell Dev Biol*, 1999, 10(5): 465-472.
- 13: Menardo J, Tang Y, Ladrech S, Lenoir M, Casas F, Michel C, Bourien J, Ruel J, Rebillard G, Maurice T, Puel JL, Wang J. Oxidative stress, inflammation, and autophagic stress as the key mechanisms of premature age-related hearing loss in SAMP8 mouse Cochlea. *Antioxid Redox Signal*. 2012 Feb 1;16(3):263-74.
- 14: Nixon RA. The role of autophagy in neurodegenerative disease. *Nat Med*. 2013 Aug;19(8):983-97.
- 15: Rubinstein AD, Kimchi A. Life in the balance - a mechanistic view of the crosstalk between autophagy and apoptosis. *J Cell Sci*. 2012 Nov 15;125(Pt22):5259-68.



- 16: Piano I, Novelli E, Della Santina L, Strettoi E, Cervetto L, Gargini C. Involvement of Autophagic Pathway in the Progression of Retinal Degeneration in a Mouse Model of Diabetes. *Front Cell Neurosci*. 2016 Feb 19; 10:42.
- 17: Zeng XS, Jia JJ, Kwon Y, Wang SD, Bai J: The role of thioredoxin-1 in suppression of endoplasmic reticulum stress in Parkinson disease. *Free Radic Biol Med* 2014; 67:10-18.
- 18: Behl, T., I. Kaur, and A. Kotwani, Implication of oxidative stress in progression of diabetic retinopathy[J]. *Surv Ophthalmol*, 2016. 61(2): p. 187-96.
- 19: eng T, Peng L, Chao H, et al. IRE1 $\alpha$ -TRAF2-ASK1 complex-mediated endoplasmic reticulum stress and mitochondrial dysfunction contribute to CXC195-induced apoptosis in human bladder carcinoma T24 cells. *Biochem Biophys Res Commun*. 2015;460(3):530-536.
- 20: Chen H, Yang H, Pan L, et al. The molecular mechanisms of XBP-1 gene silencing on IRE1 $\alpha$ -TRAF2-ASK1-JNK pathways in oral squamous cell carcinoma under endoplasmic reticulum stress. *Biomed Pharmacother*. 2016; 77:108-113.
- 21: Ren X, Wang NN, Qi H, et al. Up-Regulation Thioredoxin Inhibits Advanced Glycation End Products-Induced Neurodegeneration. *Cell Physiol Biochem*. 2018;50(5):1673-1686.
- 22: Tanito M, Masutani H, Kim YC, Nishikawa M, Ohira A, Yodoi J. Sulforaphane induces thioredoxin through the antioxidant-responsive element and attenuates retinal light damage in mice. *Invest Ophthalmol Vis Sci*. 2005;46(3):979-987.
- 23: Lv J, Bao S, Liu T, et al. Sulforaphane delays diabetes-induced retinal photoreceptor cell degeneration. *Cell Tissue Res*. 2020;382(3):477-486.
- 24: Tan S, Song L, Wang M, Zhao W, Yang Y. ARF influences diabetes through promoting the proliferation and malignant development of  $\beta$  cells. *Artif Cells Nanomed Biotechnol*. 2018;46(sup3): S702-S706.
- 25: Horikawa C, Kodama S, Tanaka S, et al. Diabetes and risk of hearing impairment in adults—a meta-analysis[J]. *J Clin Endocrinol Metab*. 2013; 98(1): 51-58.
- 26: Kemp DT. Otoacoustic emissions, their origin in cochlear function, and use. *Br Med Bull*. 2002; 63:223-241.
- 27: Fukushima H, Cureoglu S, Schachern PA, Paparella MM, Harada T, Oktay MF. Effects of type 2 diabetes mellitus on cochlear structure in humans. *Arch Otolaryngol Head Neck Surg*. 2006 Sep;132(9):934-8.
- 28: Du Z, Yang Y, Hu Y, et al. A long-term high-fat diet increases oxidative stress, mitochondrial damage and apoptosis in the inner ear of D-galactose-induced aging rats. *Hear Res*. 2012;287(1-2):15-24.

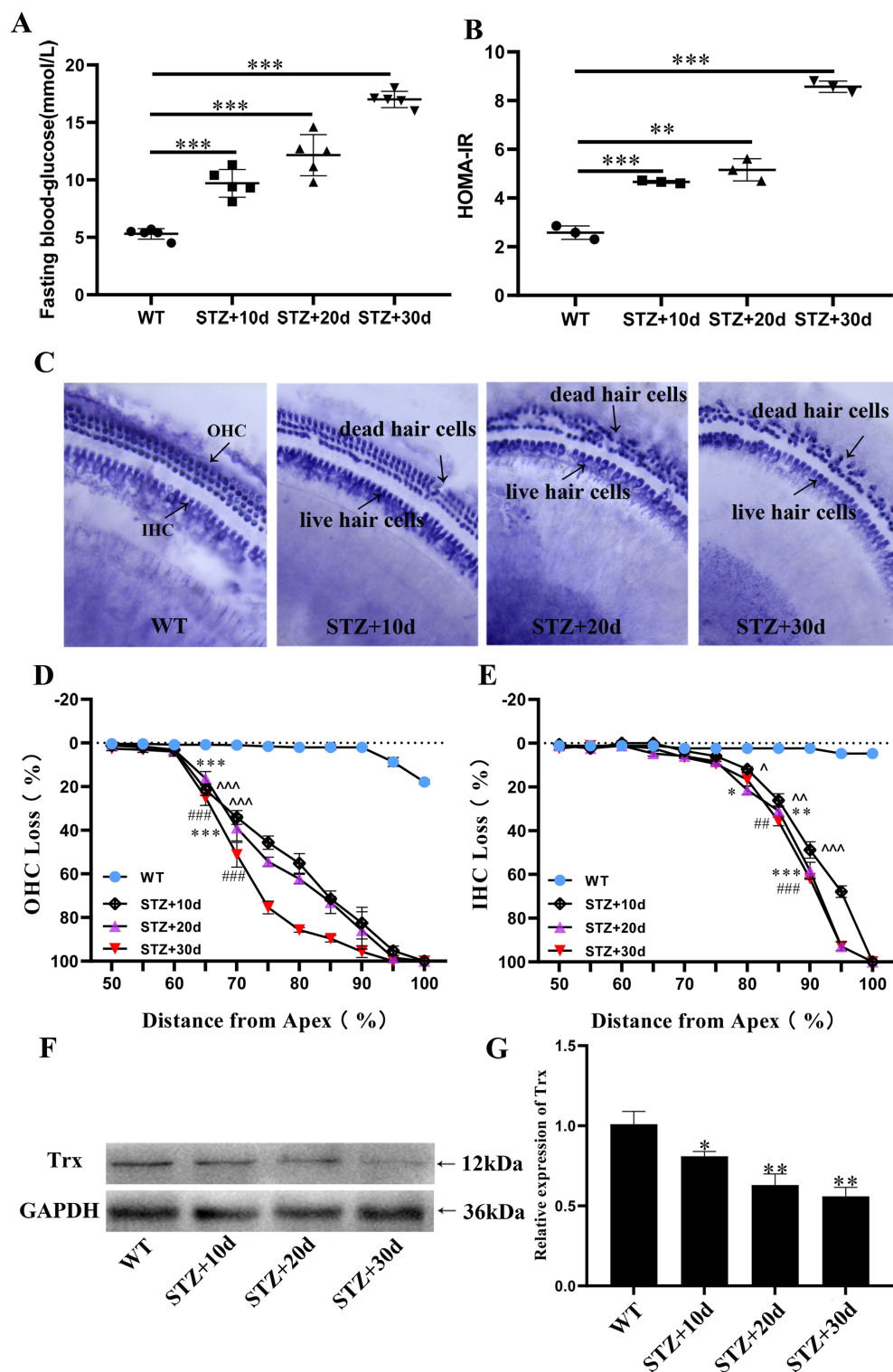
- 29: Liu Z, Fei B, Du X, Dai Y, She W. Differential Levels of Endoplasmic Reticulum Stress in Peripheral Blood Mononuclear Cells from Patients with Sudden Sensorineural Hearing Loss. *Med Sci Monit.* 2020; 26:e927328. Published 2020 Nov 10.
- 30: Magariños M, Pulido S, Aburto MR, de Iriarte Rodríguez R, Varela-Nieto I. Autophagy in the Vertebrate Inner Ear. *Front Cell Dev Biol.* 2017 May 26; 5:56.
- 31: Lu J, Holmgren A. The thioredoxin antioxidant system. *Free Radic Biol Med.* 2014 Jan; 66:75-87.
- 32: Zhang J, Li X, Han X, Liu R, Fang J. Targeting the Thioredoxin System for Cancer Therapy. *Trends Pharmacol Sci.* 2017 Sep;38(9):794-808.
- 33: Ren X, Li C, Liu J, Zhang C, Fu Y, Wang N, Ma H, Lu H, Kong H, Kong L. Thioredoxin plays a key role in retinal neuropathy prior to endothelial damage in diabetic mice. *Oncotarget.* 2017 May 24;8(37):61350-61364.
- 34: Luo M, Shang L, Brooks MD, Jiagge E, Zhu Y, Buschhaus JM, Conley S, Fath MA, Davis A, Gheordunescu E, Wang Y, Harouaka R, Lozier A, Triner D, McDermott S, Merajver SD, Luker GD, Spitz DR, Wicha MS. Targeting Breast Cancer Stem Cell State Equilibrium through Modulation of Redox Signaling. *Cell Metab.* 2018 Jul 3;28(1):69-86.e6.
- 35: Ren X, Lu H, Wang N, Zhang C, Ji Y, Cui S, Dong Y, Yang K, Du M, Diao F, Kong L. Thioredoxin is implicated in the anti-apoptotic effects of grape seed proanthocyanidin extract during hyperglycemia. *Mol Med Rep.* 2017 Nov;16(5):7731-7737.
- 36: Sánchez-Villamil JP, D'Annunzio V, Finocchietto P, et al. Cardiac-specific overexpression of thioredoxin 1 attenuates mitochondrial and myocardial dysfunction in septic mice. *Int J Biochem Cell Biol.* 2016;81(Pt B):323–334.
- 37: Huang Y, Leng TD, Inoue K, et al. TRPM7 channels play a role in high glucose-induced endoplasmic reticulum stress and neuronal cell apoptosis. *J Biol Chem.* 2018;293(37):14393–14406.
- 38: de Iriarte Rodríguez R, Pulido S, Rodríguez-de la Rosa L, Magariños M, Varela-Nieto I. Age-regulated function of autophagy in the mouse inner ear. *Hear Res.* 2015 Dec;330(Pt A):39-50.
- 39: Son JH, Shim JH, Kim KH, Ha JY, Han JY. Neuronal autophagy and neurodegenerative diseases. *Exp Mol Med.* 2012;44(2):89-98.

## Figures



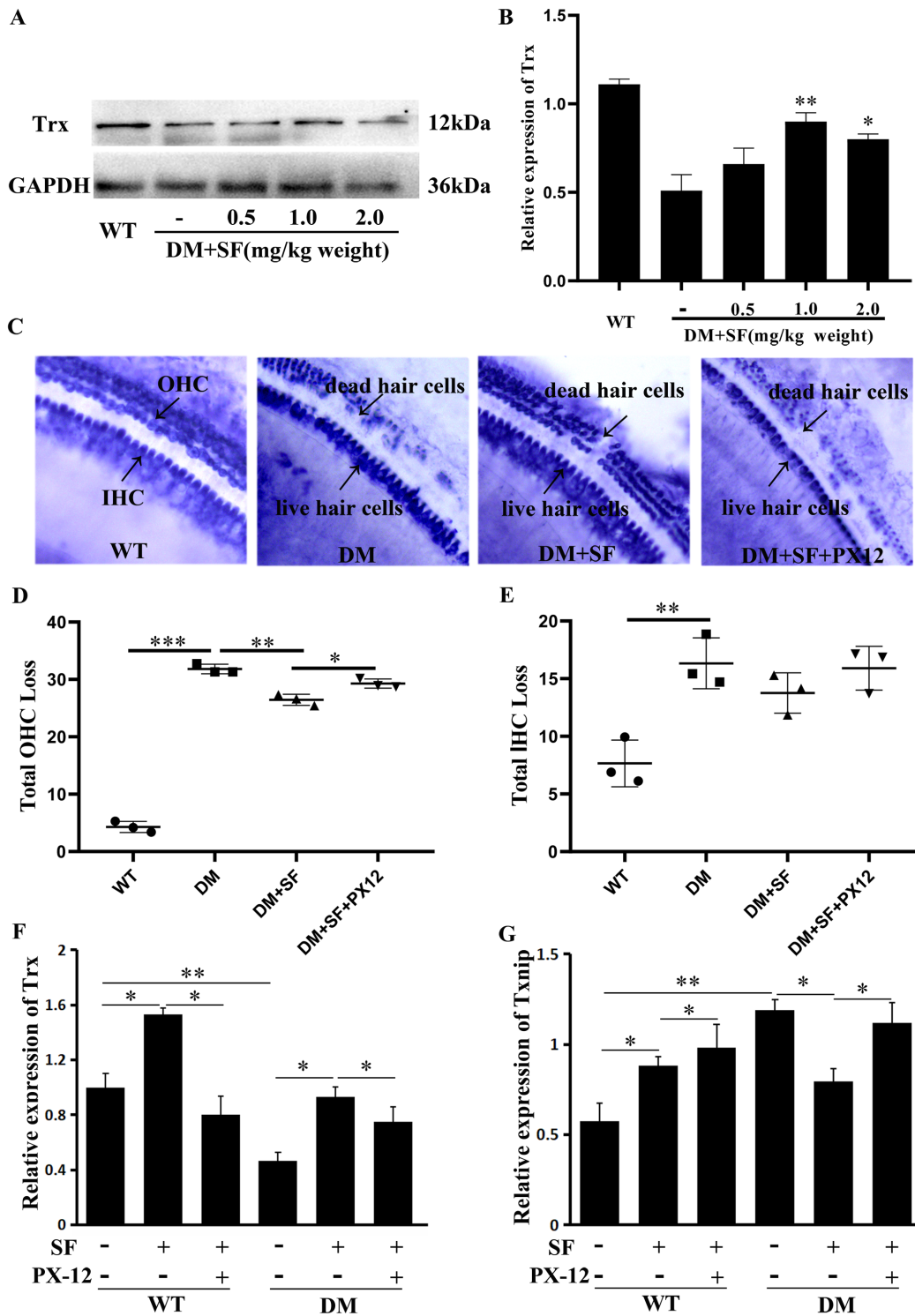
**Figure 1**

Pure tone audiometry was performed in diabetic patients. (A) Pure tone Listening Test was used to detect changes in hearing. The function of cochlear hair cells was detected by OAE results which including TEOAE correlaton(B), S/N-ratio(C) and DPOAE level(D).



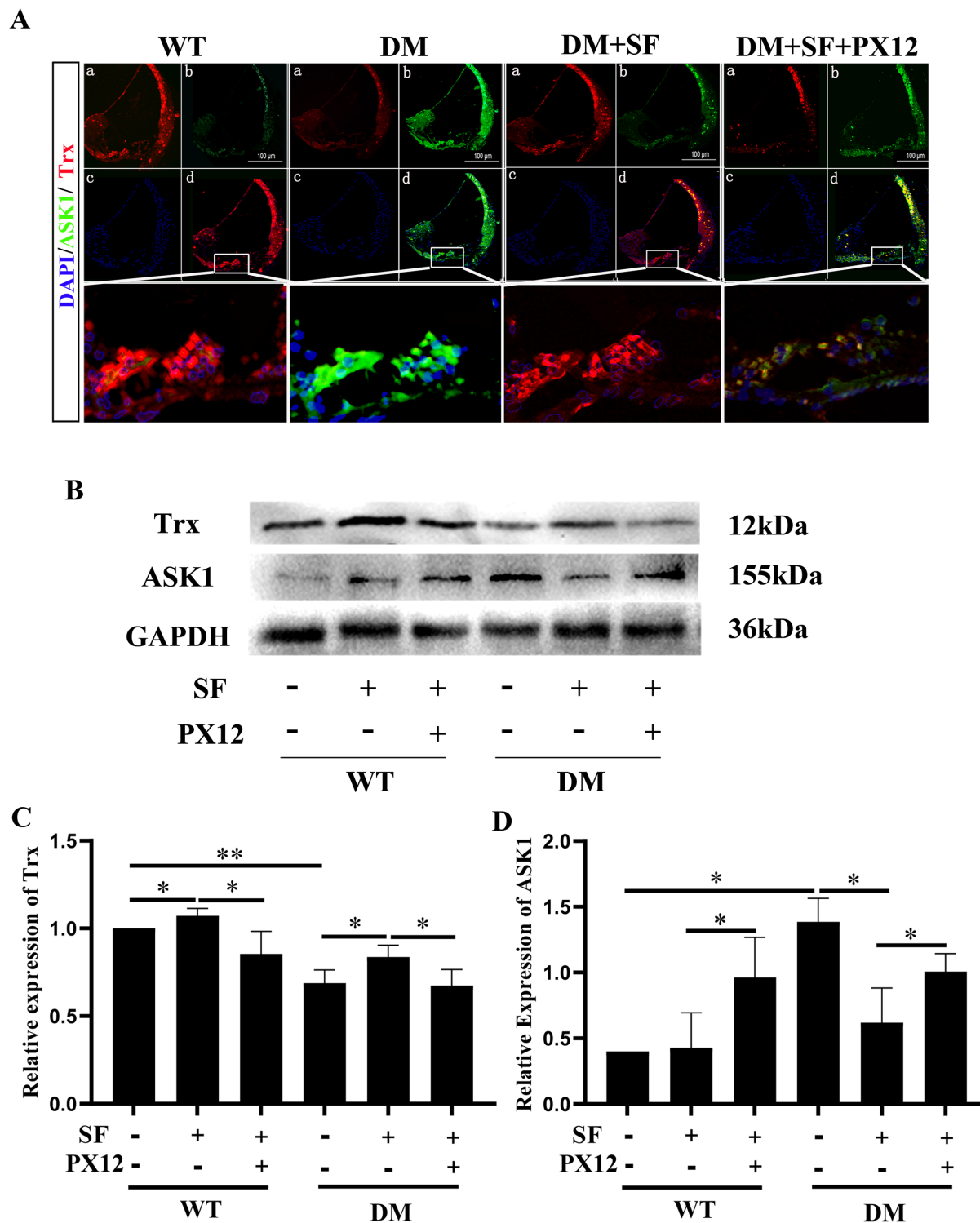
**Figure 2**

The damage of cochlea hair cell in diabetic mice. (A)&(B) The diabetic model was determined by FBG and HOMA-IR. The cochlea hair cell loss was observed by SDH staining (C) and the count of OHC/IHC (D)&(E). (F)&(G) The expression of Trx was analyzed by western blot in diabetes mice. The data are expressed as the mean  $\pm$  SD ( $n \geq 3$  for each group). \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ .



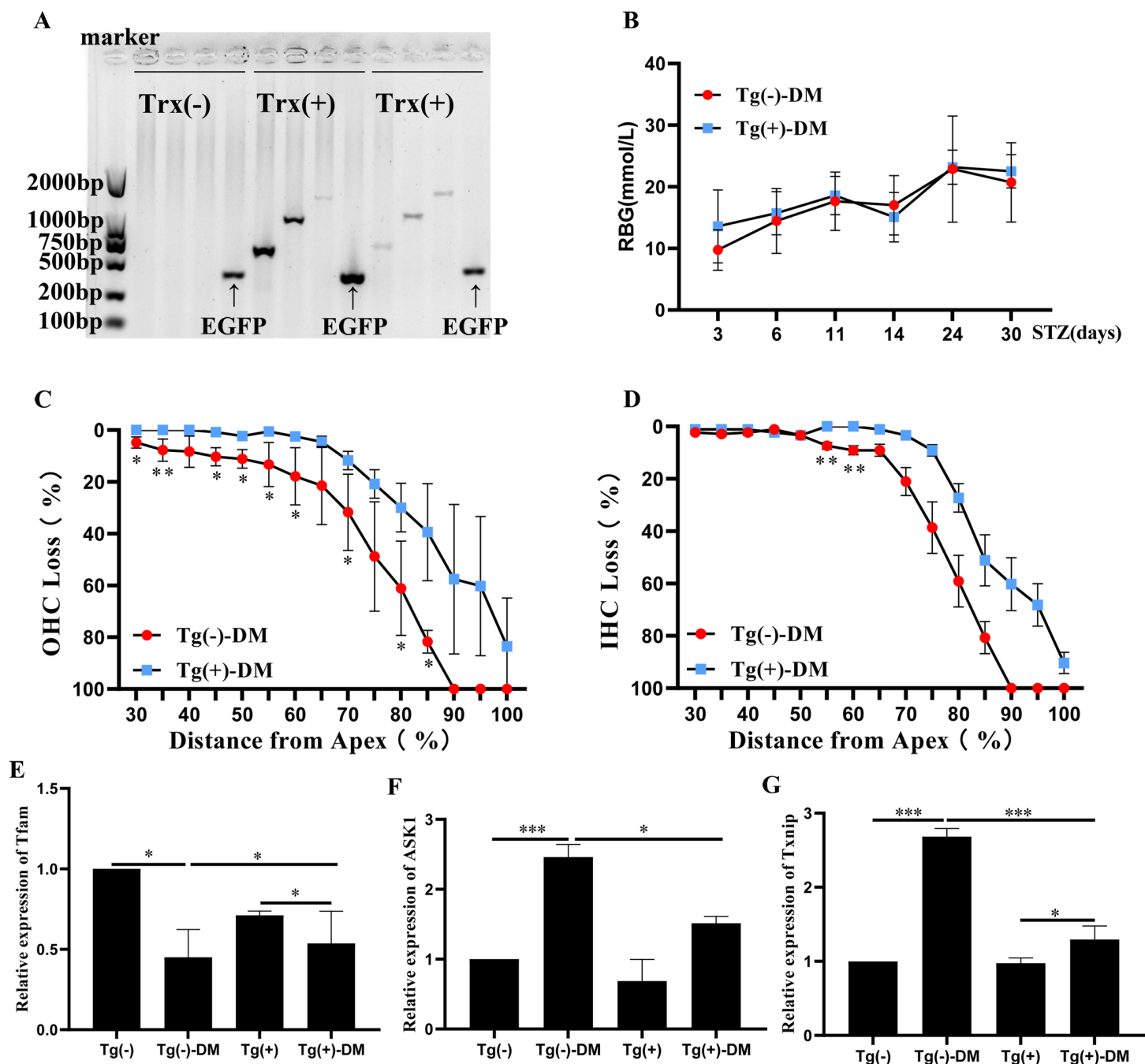
**Figure 3**

The protective effect of up-regulating Trx via SF on cochlea hair cell in diabetic mice. (A)&(B) SF up-regulated Trx expression was detected by western blot. Cochlea hair cells loss was analyzed by SDH staining in diabetic mice (C) and OHC/IHC were counted (D)&(E). The related expression of Trx and Txnip were analyzed by qRT-PCR (F&G). The data are expressed as the mean  $\pm$  SD (n=3 for each group). \* $p$ <0.05, \*\* $p$ <0.01, \*\*\* $p$ <0.01.



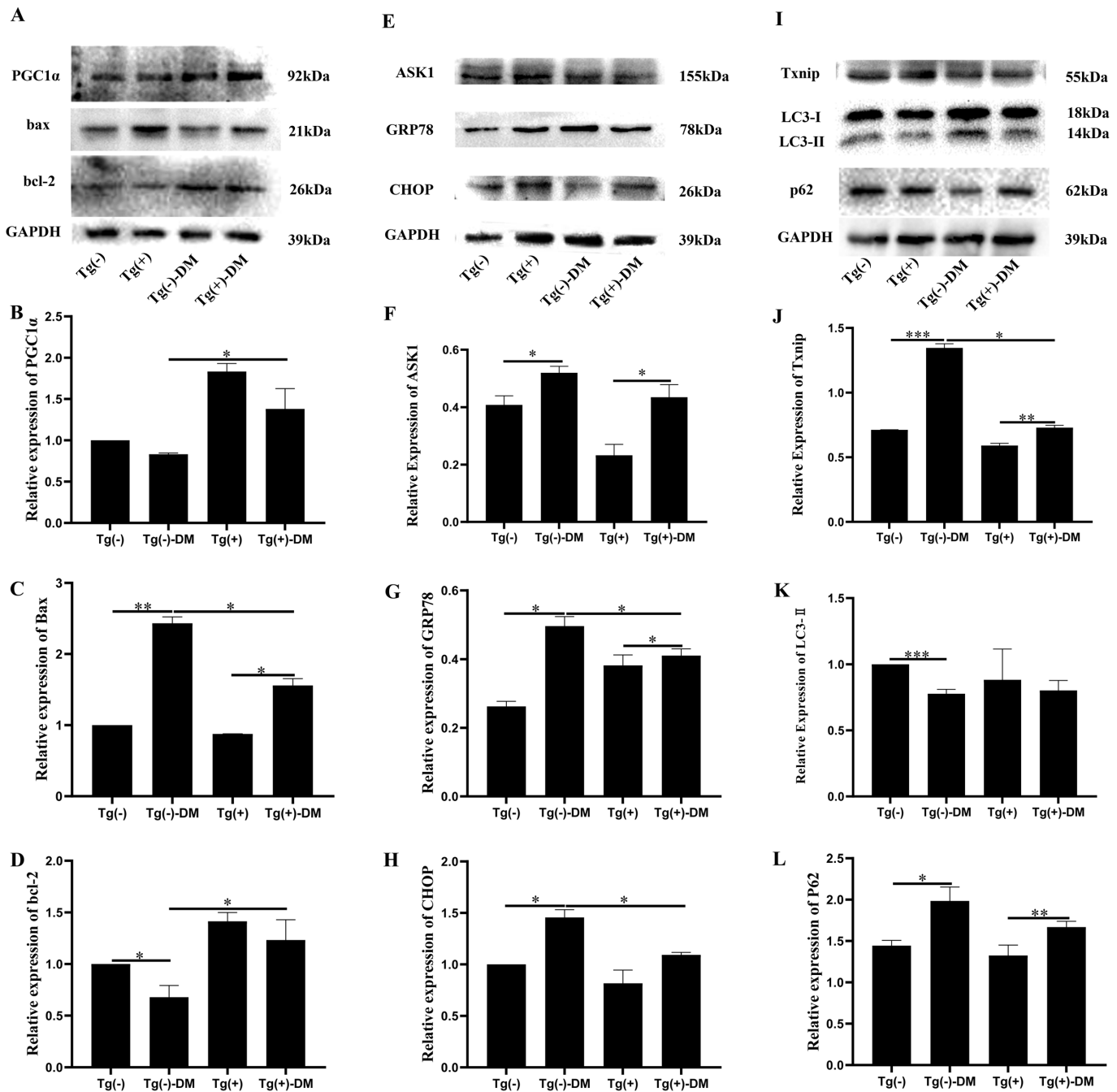
**Figure 4**

The expression of Trx/ASK1 in diabetic mice cochlea after up-regulation Trx via SF. (A) The expression of Trx/ASK1 in diabetic mice cochlea by Immunofluorescence. Western blot (B)-(D) were used to evaluate the expression of Trx and ASK1. The data are expressed as the mean  $\pm$  SD ( $n=3$  for each group). \* $p<0.05$ , \*\* $p<0.01$ .



**Figure 5**

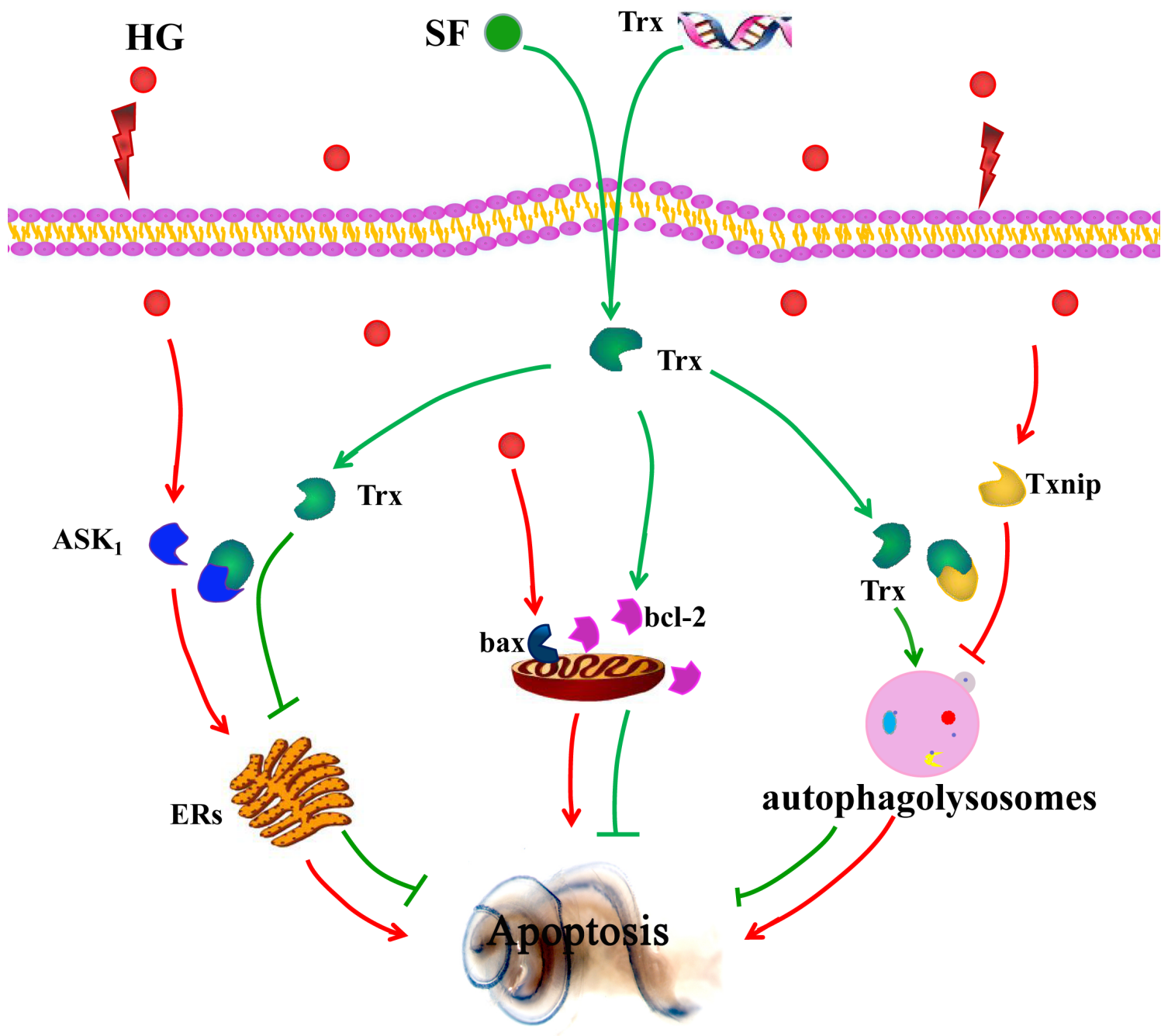
The protective effect of Trx on cochlear hair cell in diabetic mice. Trx over-expression mice were identified(A) and RBG was used to evaluate diabetic model(B). Counting the outer cochlear hair cells(C) and inner cochlear hair cells(D) in diabetic mice. Q-PCR were used to evaluate the expression of Tfam (E), ASK1 (F) and Txnip (G) in diabetic mice. The data are expressed as the mean  $\pm$  SD (n=3 for each group). \*p<0.05, \*\*p<0.01, \*\*\*p<0.01.



**Figure 6**

The underlying protective mechanism of Trx on cochlear hair cells in diabetes. Western blot was used to detected the experssion of mitochondrial related proteins(A), ERS related proteins(E) and autophagy related proteins(I) of cochlear hair cells in diabtict mice. Densitometric analysis showed that the elevation of PGC1α(B), Bax(C), bcl-2 (D), ASK1(F), GRP78 (G), CHOP (H), Txnip (J), LC3-II (K) and p62 (L). (n=3 for each group). \*p<0.05, \*\*p<0.01, \*\*\*p<0.001.





**Figure 7**

Summarize the protective effect and related mechanism of Trx on diabetes-induced cochlea hair cell damage.