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Original Article

Fetal bovine serum inhibits neomycin-induced apoptosis of hair cell-like HEI-OC-1 cells by maintaining mitochondrial function

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Abstract: Aging and exposure to noise or ototoxic drugs are major causes of hair cell death leading to human hearing loss, and many agents have been developed to protect hair cells from apoptosis. Fetal bovine serum (FBS) is a fundamental ingredient in the culture medium of hair cell-like House Ear Institute Organ of Corti 1 (HEI-OC-1) cells, but there have been no reports about the function of FBS in HEI-OC-1 cell apoptosis. In this study, we found that FBS deprivation alone significantly increased HEI-OC-1 cell apoptosis in the absence of neomycin exposure and that the presence of FBS significantly inhibited HEI-OC-1 cell apoptosis after neomycin exposure compared to FBS-deprived cells. Further, we found that the protective effect of FBS was dose dependent and more effective than the growth factors B27, N2, EGF, bFGF, IGF-1, and heparan sulfate. We also found that FBS deprivation significantly disrupted the expression level of mitochondrial proteins, increased pro-apoptotic gene expression, decreased the mitochondrial membrane potential, and increased reactive oxygen species accumulation in HEI-OC-1 cells after neomycin exposure. These findings indicate that FBS is involved in maintaining the level of mitochondrial proteins, maintaining the balance of oxidant gene expression, and preventing the accumulation of ROS, and thus FBS maintains normal mitochondrial function and inhibits apoptosis in HEI-OC-1 cells after neomycin exposure.

Keywords: Fetal bovine serum, HEI-OC-1 cells, neomycin, apoptosis, protection

Introduction

Aging and exposure to noise or ototoxic drugs are major causes of hair cell (HC) damage leading to human hearing loss. Aminoglycosides are highly potent antibiotics used for treating severe gram-negative bacterial infections [1], but in addition to their therapeutic effects, aminoglycosides are also well-known ototoxic drugs that can induce the loss of HCs [2, 3]. The formation of reactive oxygen species (ROS), the loss of the mitochondrial membrane potential (MMP), and the activation of caspases are well-known mechanisms that lead to the apoptosis of HCs caused by aminoglycoside exposure [2, 4]. Recent studies demonstrated that calcium influx and activation of the c-Jun N-terminal kinase pathway are involved in aminoglycoside ototoxicity [5, 6], and our previous studies showed that autophagy and the apoptosis repressor with caspase recruitment domain play an important roles in aminoglycoside ototoxicity [7, 8].

Many agents have shown protective effects in HCs in various models of damage both in vivo and in vitro [9-11]. Fetal bovine serum (FBS) is a fundamental ingredient in the culture medium of House Ear Institute Organ of Corti 1 (HEI-OC-1) cells, which are HC-like cells expressing Myosin 7a, Myosin 6, Math1, etc., and are commonly used to investigate the protective role or ototoxicity of different compounds [12-14]. Previous studies have reported that FBS supports the viability of pulmonary artery smooth muscle cells (PASMCs) and that FBS deprivation increases PASMC apoptosis through the accumulation of ROS, mitochondrial dysfunction, and increased caspase activity [15, 16]. These studies all indicate that FBS might also play a major role in regulating apoptosis in HEI-OC-1 cells.

To test this hypothesis, we investigated the effect of FBS on the viability of HEI-OC-1 cells after neomycin exposure. Our results indicate that FBS enhances HEI-OC-1 cell survival after neomycin exposure by maintaining the balance of oxidant factors, inhibiting ROS accumulation, maintaining normal mitochondrial function, and inhibiting the expression of pro-apoptotic factors.

Materials and methods

Cell culture and viability assay

HEI-OC-1 cells were cultured in DMEM medium supplemented with 10% FBS (Gibco, cat #10099141) and ampicillin (50 μg/ml, Sigma) at 37°C with 5% CO_2. In groups cultured without FBS, the cells were completely washed of FBS and cultured in DMEM medium and ampicillin only. In cells cultured with growth factors, the cells were completely washed of FBS and cultured in DMEM medium supplemented with B27 (1:50 dilution, Invitrogen), N2 (1:100 dilution, Invitrogen), epidermal growth factor (EGF, 20 ng/ml, Life Technologies), basic fibroblast growth factor (bFGF, 10 ng/ml, Peprotech), insulin-like growth factor-1 (IGF-1, 50 ng/ml, Sigma), heparan sulfate (50 ng/ml, Sigma), and ampicillin (50 μg/ml). HEI-OC-1 cells were then treated with neomycin for 24 h with increasing doses from 1 mM to 20 mM in 6-well plates and then immediately imaged on an inverted contrast-phase microscope. The survival rate was assessed as the percentage of plastic-adherent cells compared to the total cells. The survival rate was further confirmed by using the Cell Counting Kit (CCK-8; Protein Biotechnology). Briefly, HEI-OC-1 cells were exposed to neomycin with an increasing dose (1 mM to 20 mM) and for increasing time (1 h to 24 h) in 96-well plates, then incubated with 10 μl CCK-8 solution in each well for 0.5 h at 37°C. A microtiter plate reader (BIO-RAD) was used to measure the optical densities at 450 nm.

Immunofluorescence

Briefly, cells were washed with PBS, fixed in 4% paraformaldehyde, and permeabilized in 0.3% Triton X-100. After nonspecific binding sites were blocked with 5% bovine serum albumin (BSA) for 1 h, samples were incubated with polyclonal anti-Myosin 7a (1:1,000 dilution, Proteus Biosciences) overnight at 4°C, washed with PBS, and stained with the secondary antibodies conjugated with Cy5 (Abcam). DAPI (Sigma-Aldrich) was used to counterstain the nuclei. Images were taken using a confocal fluorescence microscope (Leica SP5).

Western blot

HEI-OC-1 cells were lysed in RIPA buffer (Protein Biotechnology), and protein concentrations were assessed with the BCA Protein Assay Kit (Protein Biotechnology). Proteins were separated on an SDS-PAGE gel and then transferred to a nitrocellulose membrane and blocked with 5% BSA. The membrane was incubated with rabbit polyclonal anti-Myosin 7a (1:2,000 dilution, Proteus Biosciences), rabbit monoclonal anti-Mfn2 (1:3,000 dilution, Cell Signaling Technology), mouse monoclonal anti-HSP60 (1:3,000 dilution, Proteintech), rabbit polyclonal anti-TOM20 (1:4,000 dilution, Proteintech), rabbit polyclonal anti-COXIV (1:5,000 dilution, Proteintech), rabbit monoclonal anti-Tubulin (1:5,000 dilution, Cell Signaling Technology), and mouse monoclonal anti-GAPDH (1:5,000 dilution, Abcam). Peroxidase-conjugated immunoglobulin G (1:2,000 dilution, Abcam) was added as the secondary antibody. A SuperSignal West Dura chemiluminescent substrate kit (Thermo Scientific) was used to detect the proteins according to the manufacturer's instructions.

Reverse-transcription PCR and quantitative PCR

RNA was prepared using TRIzol reagent (Protein Biotechnology), and cDNA was synthesized with the RevertAid First Strand cDNA synthesis kit

Table 1. PCR sequences used in the experiments

Gene	Forward sequence	Reverse sequence
β-actin	ACGGCCAGGTCATCACTATTG	AGGGGCCGGACTCATCGTA
GADPH	GCAAGAGAGAGGCCCTCAG	TGTGAGGGAGATGCTCAGTG
Myosin 7a	CTTTAACAAGCGTGGTGCCATC	GATTGCTGCGTTGATCTTCTCC
Myosin 6	TCAGAAGACATCAGGGAGAAGC	TGTTCTTCAGATTGCAGCCACC
Casp3	AATCATGCCATTTGCCCAGC	CTCAAGTGTGTAGGGGGAGG
Apaf-1	AGGGTGTGAGAGGAGTGTGT	ATCACCTCGATGGACTTGCC
Casp9	CTGTCCCGTGAAGCAAGGAT	CAGGGCACACATGACAATGC
Fadd	ACAATGTGGGGAGAGACTGG	CCCTTACCCGATCACTCAGG
Gsr	TGCACTTCCCGGTAGGAAAC	GATCGCAACTGGGGTGAGAA
Sod1	GGAGCAAGGTCGCTTACAGA	AGTGACAGCGTCCAAGCAAT
Nqo1	TCCGAAGCATTTCAGGGTCG	GGGCCAATACAATCAGGGCT
Alox15	TCGGGACTCGGAAGCAGAAT	CCCATCGGTAACAGGGGAAC

(Thermo Fisher Scientific). The primer sequences are shown in **Table 1**. For reverse transcription-PCR (RT-PCR), the reaction conditions were as follows: 94°C for 3 min followed by 36 cycles of denaturation at 94°C for 30 s, annealing at 58°C for 60 s, and extension at 72°C for 40 s followed by a final extension at 72°C for 7 min. The PCR samples were separated on a 1% agarose gel and observed using ethidium bromide staining. For quantitative PCR (qPCR), SYBR Green (Roche) was used on a Real-Time PCR apparatus (CFX96, BIO-RAD). The reaction conditions were 20 s at 95°C followed by 40 cycles of denaturation at 95°C for 15 s and annealing at 62°C for 1 min followed by a final extension of 20 s at 72°C. *GADPH* was used as the reference endogenous gene.

TUNEL staining

Apoptotic cells were detected using the TUNEL Kit (Roche). In brief, HEI-OC-1 cells were fixed in 4% paraformaldehyde for 30 min, permeabilized with 0.3% Triton X-100, and blocked with 5% BSA for 1 h at room temperature. The HEI-OC-1 cells were then stained with the TUNEL reaction mix for 1 h at 37°C and counterstained with DAPI (Sigma-Aldrich). TUNEL-positive nuclei and DAPI-positive nuclei were counted separately in five separate fields (200 × magnification) using a confocal fluorescence microscope, and the percentage of TUNEL-positive nuclei out of all DAPI-positive nuclei was calculated.

Detection of MMP and the determination of ROS levels

TMRE (Sigma-Aldrich) and Mito-SOX Red (Life Technologies) were applied separately to measure the MMP and ROS levels, respectively. The cells were incubated with TMRE or Mito-SOX Red for 10 min at 37°C then washed with prewarmed PBS and imaged using a confocal fluorescence microscope.

Flow cytometry

Annexin V-FITC and propidium iodide (BD) were used to detect the apoptotic cells. In brief, the cells were collected, washed with cold PBS, and then resuspended in 1 × binding buffer at a concentration of 1 × 10^6 cells/ml. A total volume of 5 μl Annexin V-FITC and 5 μl propidium iodide were added and gently mixed with 100 μl cells, incubated for 15 min at room temperature in the dark, and mixed with 400 μl 1 × binding buffer. For determining MMP and analyzing ROS production, HEI-OC-1 cells were collected and incubated with TMRE or Mito-SOX Red for 10 min at 37°C, washed twice with PBS, and analyzed by flow cytometry (FACSCanto, BD) within 1 h.

Statistical analyses

Data are presented as means ± S.D. Statistical analyses were conducted using SPSS version 13.0 and GraphPad Prism6 software. Two-way ANOVA followed by the Student-Newman-Keuls (SNK) post hoc test was used for the cell viability analysis in **Figures 2** and **3**, and one-way ANOVA followed by the SNK post hoc test was performed in other experiments. A p-value < 0.05 was considered statistically significant.

Results

HEI-OC-1 cells expressed the HC markers Myosin 7a and Myosin 6

To confirm that HEI-OC-1 cells still expressed HC markers and could be used as an HC-like cell line, RT-PCR, western blot, and immunohistochemistry were used, and the results showed that this cell line expressed Myosin 7a and Myosin 6 (**Figure 1**).

FBS significantly increased the viability of HEI-OC-1 cells after neomycin exposure

To determine the function of FBS in HEI-OC-1 cell apoptosis induced by neomycin exposure, we treated HEI-OC-1 cells with an increasing

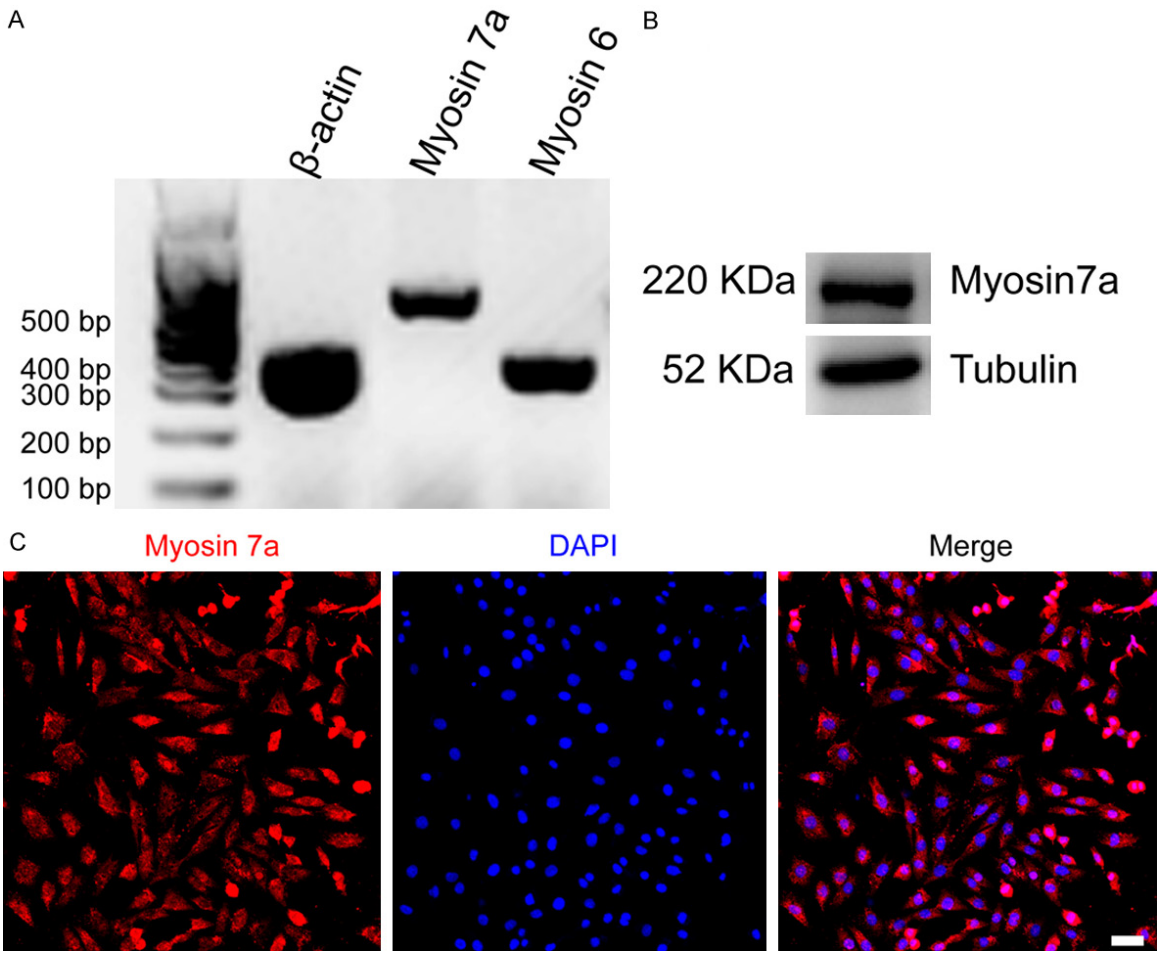

Figure 1. HEI-OC-1 cells expressed the HC markers Myosin 7a and Myosin 6. A. RT-PCR showed that HC markers Myosin 7a and Myosin 6 were expressed in HEI-OC-1 cells. B. Western blot showed that HEI-OC-1 cells expressed Myosin 7a. C. Immunofluorescence demonstrated that HEI-OC-1 cells expressed Myosin 7a. Scale bar = 50 μm.

dose of neomycin (0 mM to 20 mM) for 24 h. We found that the survival rate decreased significantly as the dose of neomycin increased both in cells cultured with and cells cultured without FBS. Interestingly, after neomycin exposure the FBS cultures had significantly higher survival rates compared to the cells cultured without FBS (**Figure 2A** and **2B**). We also used the CCK-8 Kit to measure the survival rate of HEI-OC-1 cells. The CCK-8 results demonstrated that the survival rate of HEI-OC-1 cells cultured with or without FBS decreased significantly with the increasing neomycin dose or increasing exposure time (**Figure 2C** and **2D**), and the results confirmed that the survival rate of HEI-OC-1 cells was significantly greater in the cells cultured with FBS compared to those cultured without FBS after neomycin exposure. Besides, we also found that FBS deprivation had an interaction with the neomycin-induced cytotoxicity that the viability of HEI-OC-1 cells affected more by FBS deprivation while the neomycin exposure dose and time were 10 mM and 24 h (**Figure 2C** and **2D**).

The protective effect of FBS was dose-dependent and more effective than the growth factors B27, N2, EGF, bFGF, IGF-1, and heparan sulfate

To determine whether the protective effect of FBS is dose dependent and to determine the major component in FBS that protects HEI-OC-1 cells from neomycin damage, we treated HEI-OC-1 cells with 10% FBS, 5% FBS, growth factors, or DMEM medium only. We found that the 10% FBS cultures had significantly greater survival rates compared with the 5% FBS cultures after exposure to 10 mM neomycin for 24 h, and both the 10% and 5% FBS cultures had

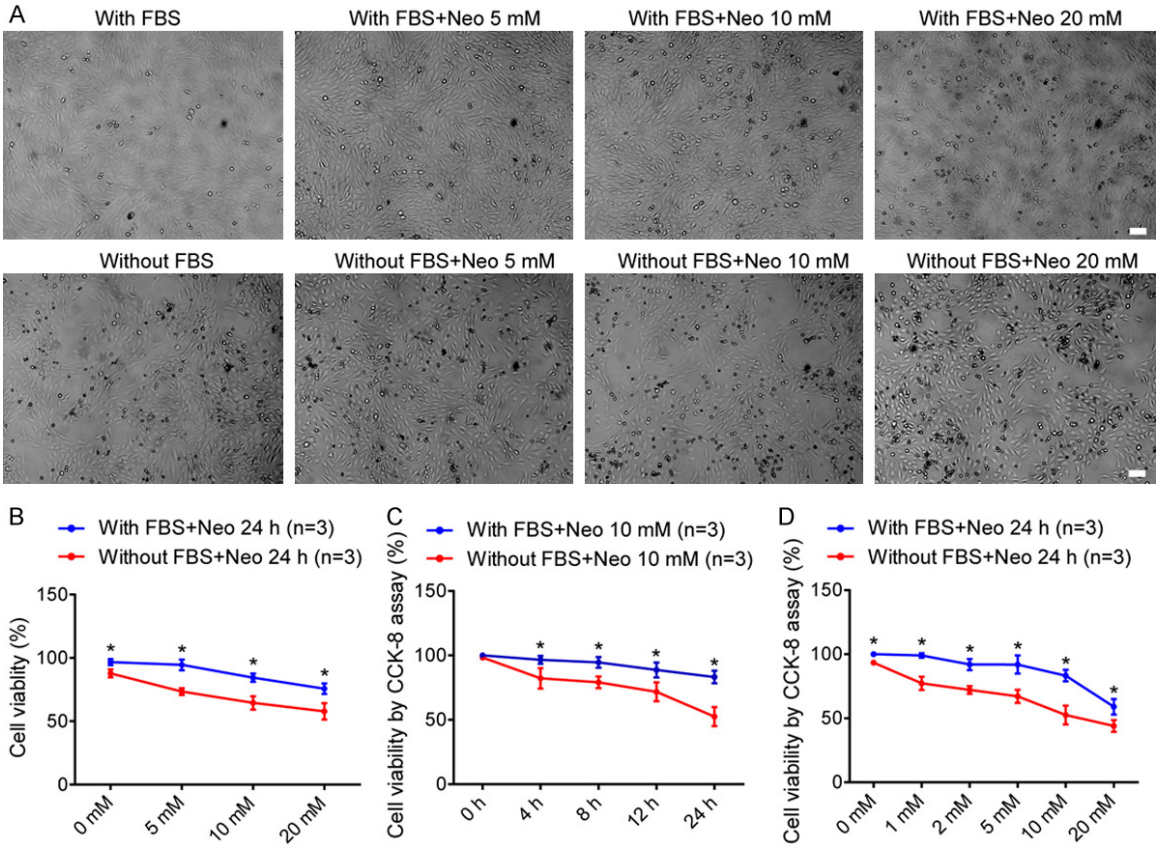

Figure 2. FBS significantly enhanced the viability of HEI-OC-1 cells after neomycin exposure. A and B. In groups cultured with FBS, HEI-OC-1 cells were cultured in DMEM medium supplemented with 10% FBS and ampicillin at 37°C with 5% CO_2. In groups cultured without FBS, the cells were completely washed of FBS and cultured in DMEM medium and ampicillin only. HEI-OC-1 cells were then treated with neomycin for 24 h with increasing doses from 1 mM to 20 mM in 6-well plates and then immediately imaged on an inverted contrast-phase microscope. The survival rate was assessed as the percentage of plastic-adherent cells compared to the total cells. The survival rate of HEI-OC-1 cells was 87.8 ± 3.1%, 73.5 ± 2.6%, 64.5 ± 5.3%, and 57.8 ± 6.5% in cultures without FBS and exposed to 0 mM, 5 mM, 10 mM, and 20 mM neomycin for 24 h, respectively, compared to 96.7 ± 2.3%, 94.5 ± 4.2%, 84.4 ± 3.2%, and 75.7 ± 4.1% in the FBS cultures. Two-way ANOVA analysis showed that there were significant differences in cell viability between with FBS and without FBS, and there were significant differences in cell viability with increasing neomycin-treated dose. Further, SNK test showed that the survival rate decreased significantly as the dose of neomycin increased in cells cultured with or without FBS, and the survival rate of HEI-OC-1 cells was significantly greater in the cells cultured with FBS compared to cells cultured without FBS after neomycin exposure. C and D. HEI-OC-1 cells were exposed to neomycin with an increasing dose (1 mM to 20 mM) and for increasing time (1 h to 24 h) in 96-well plates, then incubated with 10 μl CCK-8 solution in each well for 0.5 h at 37°C. A microtiter plate reader (BIO-RAD) was used to measure the optical densities at 450 nm. The CCK-8 results demonstrated that the survival rate of HEI-OC-1 cells was 98.5 ± 0.5%, 82.3 ± 7.9%, 79.2 ± 4.5%, 71.9 ± 7.3%, and 52.3 ± 7.2% in cultures without FBS after 10 mM neomycin exposure for 1 h to 24 h, compared to 99.2 ± 0.7%, 96.6 ± 3.0%, 94.6 ± 4.1%, 88.8 ± 5.8%, and 83.3 ± 4.9% in the FBS cultures. And the survival rate of HEI-OC-1 cells was 93.5 ± 0.6%, 77.2 ± 5.1%, 72.2 ± 3.0%, 67.2 ± 5.1%, 52.3 ± 7.2%, and 44.1 ± 4.5% in cultures without FBS and exposed to 0 mM, 5 mM, 10 mM, and 20 mM neomycin for 24 h, respectively, compared to 99.2 ± 0.7%, 99.0 ± 1.7%, 92.0 ± 4.3%, 92.0 ± 7.1%, 83.3 ± 4.9%, and 59.0 ± 6.0% in the FBS cultures. Two-way ANOVA analysis showed that the cell viability were significantly different between cells cultured with FBS and cells cultured without FBS, and there were interaction between neomycin cytotoxity and FBS deprivation that the viability of HEI-OC-1 cells affected more by FBS deprivation while the neomycin exposure dose and time were 10 mM and 24 h, respectively, and the cell viability of HEI-OC-1 cells cultured with increasing neomycin-treated time and with increasing neomycin-treated dose were significantly different. Further, SNK test showed that that the survival rate of HEI-OC-1 cells was significantly greater in the cells cultured with FBS compared to those cultured without FBS after neomycin exposure, and the survival rate of HEI-OC-1 cells cultured with or without FBS decreased significantly with increasing neomycin exposure time and increasing neomycin-treated dose. *$P < 0.05$. Scale bars = 100 μm.

significantly higher survival rates compared with the growth factor cultures after neomycin exposure (**Figure 3A** and **3B**). Further, we measured the survival rate of HEI-OC-1 cells with the CCK-8 Kit. The CCK-8 results confirmed that the survival rate of HEI-OC-1 cells in the 10% FBS cultures was significantly greater compared to the 5% FBS cultures after exposure to 10 mM or 20 mM neomycin for 24 h (**Figure 3C** and **3D**). The CCK-8 results also demonstrated that the 10% FBS cultures had significantly greater survival rates compared with the growth factor cultures after neomycin damage, but there was no significant difference between the growth factor cultures and the cultures without FBS after neomycin damage (**Figure 3E** and **3F**). These results suggest that the protective effect of FBS is dose dependent, that 10% FBS is more effective than 5% FBS in protecting HEI-OC-1 cells from apoptosis, and that the protective effect of FBS (both the 10% and 5% concentrations) against neomycin injury is greater than the growth factors used in this study.

FBS significantly decreased the expression of apoptotic factors and the percentage of apoptotic HEI-OC-1 cells after neomycin exposure

To determine the effect of FBS on HEI-OC-1 cell apoptosis induced by neomycin, we first tested whether FBS deprivation alone would increase apoptosis in HEI-OC-1 cells under neomycin-free conditions using flow cytometry. The results demonstrated that FBS deprivation alone increased apoptosis in HEI-OC-1 cells (**Figure 4A** and **4B**). Next we determined the effect of FBS on HEI-OC-1 cell apoptosis after neomycin exposure. The results demonstrated that the cells cultured with FBS after neomycin exposure had significantly lower rates of apoptosis compared to cells cultured without FBS (**Figure 4A** and **4B**). Besides, there was no significant difference in the cell viability between the cells cultured without FBS and the cells cultured with 10 mM neomycin exposure for 24 h, and the cell viability decreased more significantly in the cells cultured without FBS and with 10 mM neomycin exposure for 24 h compared to the cells cultured without FBS or with 10 mM neomycin exposure for 24 h alone (**Figure 4A** and **4B**). We further used TUNEL staining to measure the amount of apoptosis in HEI-OC-1 cells. TUNEL staining showed that FBS deprivation increased HEI-OC-1 cell apoptosis under neomycin-free conditions (**Figure 5A** and **5B**), and the percentages of TUNEL-positive cells in the cells cultured with FBS were significantly lower compared to the cells cultured without FBS after neomycin exposure (**Figure 5A** and **5B**). These results indicated that FBS inhibits the HEI-OC-1 cell apoptosis that results from neomycin exposure. qPCR results showed that the expression levels of pro-apoptotic factors like Casp3, Casp9, and Apaf-1 were significantly decreased in cells cultured with FBS compared to cells cultured without FBS both with and without neomycin exposure (**Figure 5C** and **5D**). Together, our results suggest that FBS inhibits HEI-OC-1 cell apoptosis by suppressing the expression of pro-apoptotic factors after neomycin exposure.

FBS significantly increased the MMP of HEI-OC-1 cells after neomycin exposure

To further investigate the mechanism behind the role of FBS in HEI-OC-1 apoptosis induced by neomycin exposure, the TMRE kit was used to measure changes in the MMP in HEI-OC-1 cells using immunofluorescence staining and flow cytometry analysis. The results demonstrated that FBS deprivation alone increased TMRE intensity under neomycin-free conditions (**Figure 6A-C**), and the cells cultured with FBS had significantly greater TMRE intensity compared to cells cultured without FBS after neomycin exposure (**Figure 6A-C**). Besides, there was no significant difference in the MMP between the cells cultured without FBS and the cells cultured with 10 mM neomycin exposure for 24 h, and the MMP decreased more significantly in the cells cultured without FBS and with 10 mM neomycin exposure for 24 h compared to the cells cultured without FBS or with 10 mM neomycin exposure for 24 h alone (**Figure 6A-C**). These results suggest that FBS protects HEI-OC-1 cells from apoptosis by inhibiting the disruption of MMP.

FBS significantly increased antioxidant gene levels and decreased the ROS level in HEI-OC-1 cells after neomycin exposure

Mito-SOX Red has been reported to selectively detect mitochondrial superoxide, and it was used in this study to measure mitochondrial ROS levels in HEI-OC-1 cells using immunofluorescence staining and flow cytometry analysis. The results demonstrated that FBS deprivation

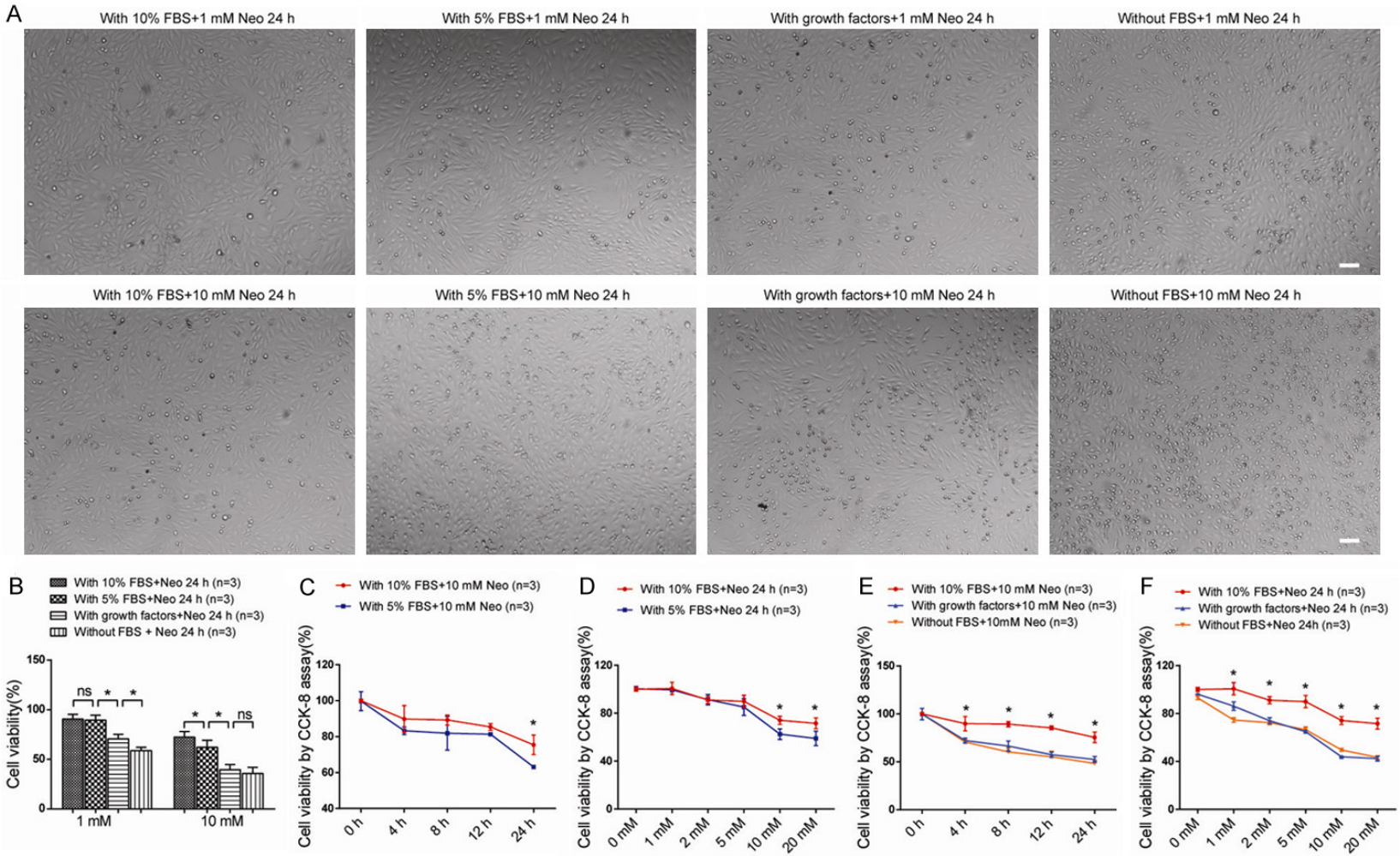

Figure 3. The protective effect of FBS was dose dependent and was more effective than the growth factors B27, N2, EGF, bFGF, IGF-1, and heparan sulfate. A and B. In groups cultured with FBS, HEI-OC-1 cells were cultured in DMEM medium supplemented with 10% FBS or 5% FBS at 37 °C with 5% CO_2. In groups cultured without FBS, the cells were completely washed of FBS and cultured in DMEM medium only. In cells cultured with growth factors, the cells were completely washed of FBS and cultured in DMEM medium supplemented with B27 (1:50 dilution), N2 (1:100 dilution), EGF (20 ng/ml), bFGF (10 ng/ml), IGF-1 (50 ng/ml), heparan sulfate (50 ng/ml), and ampicillin (50 μg/ml). HEI-OC-1 cells were then treated with neomycin for 24 h with increasing doses from 1 mM to 20 mM in 6-well plates and then immediately imaged on an inverted contrast-phase microscope. The survival rate was assessed as the percentage of plastic-adherent cells compared to the total cells. Cell counting under the inverted contrast-phase microscope showed that the survival rate was 90.0 ± 4.8%, 89.0 ± 5.0%, 70.7 ± 4.6%, and 58.8 ± 3.3% in groups cultured with 10% FBS, with 5% FBS, with the growth factors, and without FBS, respectively, after 1 mM neomycin exposure for 24 h. And the survival rate was 72.3 ± 5.8 %, 62.0 ± 7.2 %, 39.5 ± 5.3%, and 37.5 ± 6.2 in groups cultured with 10% FBS, with 5% FBS, with the growth factors, and without FBS, respectively,

after 10 mM neomycin exposure for 24 h. Two-way ANOVA followed by SNK test showed that the survival rate in the 10% FBS culture was significantly greater compared to the 5% FBS culture after 10 mM neomycin exposure for 24 h, and the FBS cultures (both the 10% and 5% concentrations) had significantly higher survival rates compared with the growth factor cultures B27, N2, EGF, bFGF, IGF-1, and heparan sulfate after neomycin exposure. C and D. For the CCK-8 assay, HEI-OC-1 cells were exposed to neomycin with an increasing dose (1 mM to 20 mM) and for increasing time (1 h to 24 h) in 96-well plates, then incubated with 10 μl CCK-8 solution in each well for 0.5 h at 37°C. A microtiter plate reader (BIO-RAD) was used to measure the optical densities at 450 nm. The CCK-8 results demonstrated that the survival rate was 74.1 ± 3.3% and 71.6 ± 4.5% in groups cultured with 10% FBS compared to 62.5 ± 4.4% and 60.0 ± 6.0% in the 5% FBS cultures after 10 mM or 20 mM neomycin exposure, respectively, for 24 h. Two-way ANOVA showed that there were significant differences in cell viability between with 10% FBS culture and with 5% FBS culture after neomycin exposure, and the cell viability of HEI-OC-1 cells cultured with increasing neomycin-treated time and with increasing neomycin-treated dose were significantly different. Further, SNK test showed that the survival rate of HEI-OC-1 cells in the 10% FBS culture was significantly higher compared with the 5% FBS culture after exposure to 10 mM or 20 mM neomycin for 24 h. E and F. The CCK-8 results demonstrated that the survival rate was 89.8 ± 7.5%, 89.3 ± 2.8%, 85.4 ± 1.8%, and 75.5 ± 5.4% in groups cultured with 10% FBS compared to 72.2 ± 2.7%, 66.4 ± 5.3%, 57.4 ± 2.8%, and 52.5 ± 3.1% in groups cultured with the growth factors after neomycin exposure for 4 h, 8 h, 12 h, and 24 h, respectively. The survival rate was 100.0 ± 5.1%, 91.1 ± 3.0%, 89.9 ± 5.1%, 74.1 ± 3.3%, and 71.6 ± 4.5% in groups cultured with 10% FBS compared to 86.2 ± 3.5%, 74.0 ± 2.4%, 64. 9 ± 1.1%, 43.8 ± 0.6%, and 42.3 ± 2.0% in groups cultured with the growth factors after 1 mM, 2 mM, 5 mM, 10 mM, and 20 mM neomycin exposure for 24 h, respectively. Two-way ANOVA analysis showed that there were significant differences in cell viability between with 10% FBS cultures, with growth factors cultures and without FBS cultures after neomycin exposure, and there were significant differences in cell viability with increasing neomycin exposure time and doses. Further, SNK test showed that the 10% FBS cultures had significantly higher survival rates compared with the growth factor cultures after neomycin exposure, but there was no significant difference between the growth factors cultures and the without FBS cultures. *$P < 0.05$. Scale bars = 100 μm.

alone significantly increased the mitochondrial ROS level in HEI-OC-1 cells under neomycin-free conditions (**Figure 7A-C**) and that the cells cultured with FBS had significantly lower mitochondrial ROS levels compared with the cells cultured without FBS after neomycin exposure (**Figure 7A-C**). Besides, there was no significant difference in the mitochondrial ROS levels between the cells cultured without FBS and the cells cultured with 10 mM neomycin exposure for 24 h, and the mitochondrial ROS levels decreased more significantly in the cells cultured without FBS and with 10 mM neomycin exposure for 24 h compared to the cells cultured without FBS or with 10 mM neomycin exposure for 24 h alone (**Figure 7A-C**). The expression levels of four oxidant genes were further measured using qPCR. The results showed that FBS deprivation alone significantly decreased the expression level of the antioxidant factor *Gsr* under neomycin-free conditions (**Figure 7D**). After neomycin exposure, the cells cultured with FBS had significantly greater levels of the antioxidant genes *Gsr*, *Nqo1* and *Sod1* compared to those cultured without FBS (**Figure 7D**). Together, our data demonstrate that FBS increased antioxidant gene expression and prevented the accumulation of mitochondrial ROS and thus prevented HEI-OC-1 cells from undergoing apoptosis after neomycin exposure.

FBS deprivation significantly disrupted mitochondrial protein levels in HEI-OC-1 cells after neomycin exposure

To further determine whether FBS mediates its protective effects by maintaining mitochondrial protein levels, we measured levels of mitofusin 2 (Mfn2), heat shock protein 60 (HSP60), translocase in the outer mitochondrial membrane 20 (TOM20), and cytochrome c oxidase subunit IV (COX IV) by western blot. The results demonstrated that FBS deprivation alone did not affect the levels of these proteins under neomycin-free conditions (**Figure 8A-E**). The results also demonstrated that the cells cultured without FBS had significantly higher levels of the mitochondrial protein TOM20 compared to the cells cultured with FBS after neomycin exposure (**Figure 8A** and **8D**), and the cells cultured without FBS had significantly lower levels of the mitochondrial protein COX IV compared to the cells cultured with FBS after neomycin exposure (**Figure 8A** and **8E**). We also found that the level of mitochondrial proteins TOM20 increased and the level of COX IV decreased more significantly in the cells cultured without FBS and with 10 mM neomycin exposure for 24 h compared to the cells cultured without FBS or with 10 mM neomycin exposure for 24 h alone (**Figure 8D** and **8E**). These results suggest that FBS deprivation disrupts mitochondrial protein

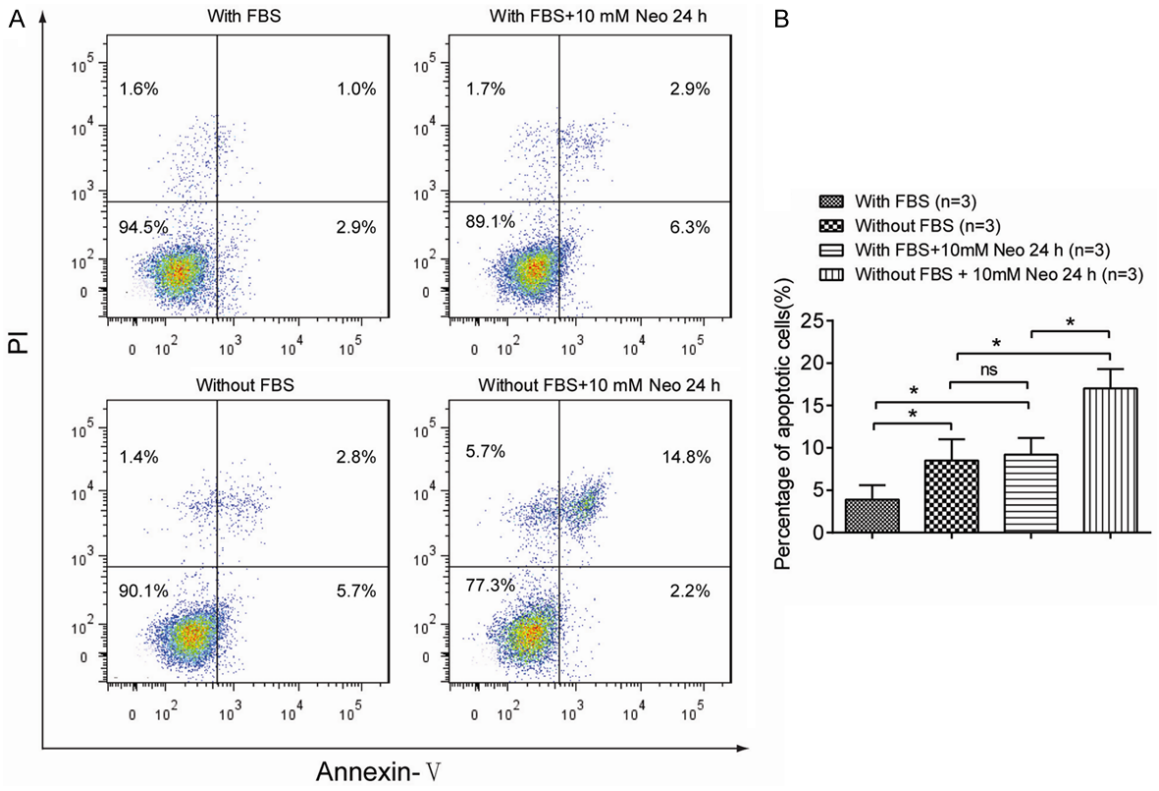

Figure 4. Flow cytometry showed that FBS significantly decreased the percentage of apoptotic HEI-OC-1 cells after neomycin exposure. A and B. Annexin V-FITC and propidium iodide were used to detect the apoptotic cells. In brief, the cells were collected, washed with cold PBS, and then resuspended in 1 × binding buffer at a concentration of 1 × 10^6 cells/ml. Total volumes of 5 μl Annexin V-FITC and 5 μl propidium iodide were added and gently mixed with 100 μl cells, incubated for 15 min at room temperature in the dark, and mixed with 400 μl 1 × binding buffer. Flow cytometry showed that the percentage of apoptotic HEI-OC-1 cells was 3.9 ± 1.7%, 8.5 ± 2.5%, 9.2 ± 1.9%, and 17.0 ± 2.3% in cultures with FBS, without FBS, with FBS and with 10 mM neomycin exposure for 24 h, and without FBS and with 10 mM neomycin exposure for 24 h, respectively. Flow cytometry showed that the apoptosis was significantly increased after neomycin exposure, and the cells cultured without FBS had significantly greater rates of apoptosis compared to the cells cultured with FBS under neomycin-free conditions. After neomycin exposure, the cells cultured with FBS had significantly lower rates of apoptosis compared to the cells cultured without FBS, and the cell viability decreased more significantly in the cells cultured without FBS and with 10 mM neomycin exposure for 24 h compared to the cells cultured without FBS or with 10 mM neomycin exposure for 24 h alone, but there was no significant difference between the cells cultured without FBS and the cells cultured with 10 mM neomycin exposure for 24 h. *$P < 0.05$ by one-way ANOVA and the SNK test.

levels, and thus affects mitochondrial function and induces apoptosis in HEI-OC-1 cells.

Discussion

Previous studies have reported that FBS plays a critical role in apoptosis of PASMCs [15-18], but to our knowledge there have been no reports about the function of FBS in HEI-OC-1 cell apoptosis. In this study, we first investigated the effect of FBS in maintaining the viability of HEI-OC-1 cells without neomycin exposure, and our results showed that FBS deprivation alone significantly increased apoptosis in HEI-OC-1 cells under neomycin-free conditions (**Figures 1**-**4**). We next determined the function of FBS in HEI-OC-1 cell apoptosis after neomycin exposure, and we found that FBS significantly decreased the apoptosis of HEI-OC-1 cells after neomycin exposure (**Figures 1**-**4**). Both of these results suggest that FBS has an important effect in maintaining the viability of HEI-OC-1 cells and that it has a protective function against HEI-OC-1 cell death induced by neomycin.

We also found that the protective effect of FBS is dose dependent and that 10% FBS is a more effective concentration than 5% FBS in protecting HEI-OC-1 cells from apoptosis (**Figure 3A-D**). Further, we investigated the major component in FBS that protects HEI-OC-1 cells from neo-

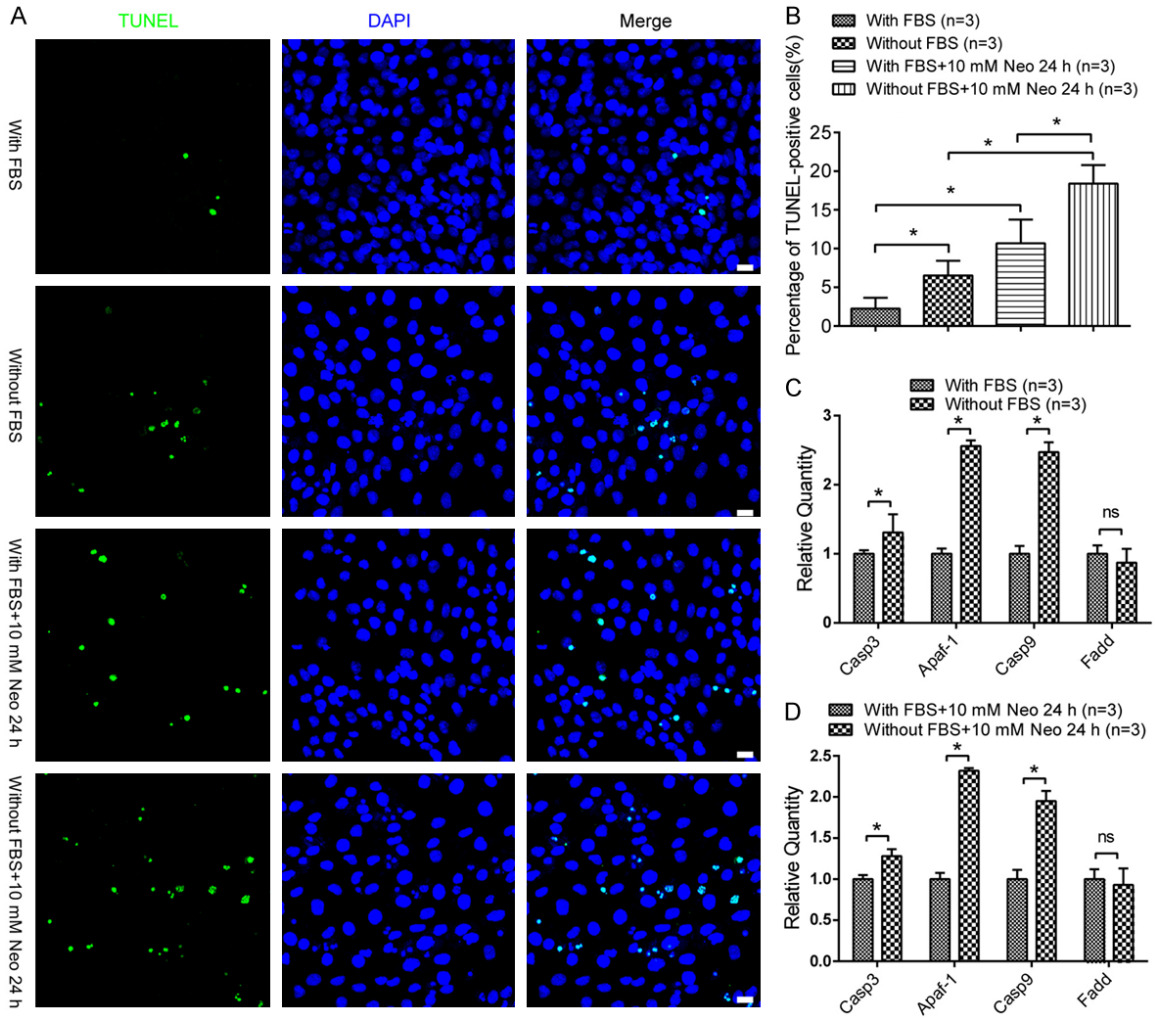

Figure 5. TUNEL staining showed that FBS significantly reduced the percentages of apoptotic HEI-OC-1 cells after neomycin exposure. A and B. The HEI-OC-1 cells were stained with the TUNEL reaction mix for 1 h at 37 °C and counterstained with DAPI. TUNEL-positive nuclei and DAPI-positive nuclei were counted separately in five separate fields (200 × magnification) using a confocal fluorescence microscope, and the percentage of TUNEL-positive nuclei out of all DAPI-positive nuclei was calculated. The percentage of TUNEL-positive HEI-OC-1 cells was 2.3 ± 0.8%, 6.5 ± 1.1%, 10.7 ± 1.8%, and 18.4 ± 1.4% in cultures with FBS, without FBS, with FBS and with 10 mM neomycin exposure for 24 h, and without FBS and with 10 mM neomycin exposure for 24 h, respectively. The results showed that the percentage of TUNEL-positive cells increased significantly after neomycin exposure compared to the controls, and the percentage of TUNEL-positive apoptotic cells increased significantly in cells cultured without FBS compared to cells cultured with FBS under neomycin-free conditions. After neomycin exposure, the percentage of TUNEL-positive cells was significantly lower in the cells cultured with FBS compared to the cells cultured without FBS. C and D. qPCR results showed that the expression of pro-apoptotic factors like Casp3, Casp9, and Apaf-1 was significantly decreased in cells cultured with FBS compared to cells cultured without FBS both with and without neomycin exposure. *P < 0.05 by one-way ANOVA and the SNK test. Scale bars = 20 μm.

mycin damage, and we found that FBS (both the 10% and 5% concentrations) is more effective than the growth factors B27, N2, EGF, bFGF, IGF-1, and heparan sulfate (**Figure 3A-F**). These results indicate that the major component in FBS that protects HEI-OC-1 cells from neomycin damage might be complex and thus needs further study.

Apoptosis is mainly controlled by the activation of both intrinsic and extrinsic pathways in which Casp3, Casp9, Apaf-1, and Fadd are the main pro-apoptotic factors [19-22]. Here we demonstrated that FBS deprivation significantly increased the expression level of pro-apoptotic genes under neomycin-free conditions (**Figure 5D**), and conversely that FBS decreased

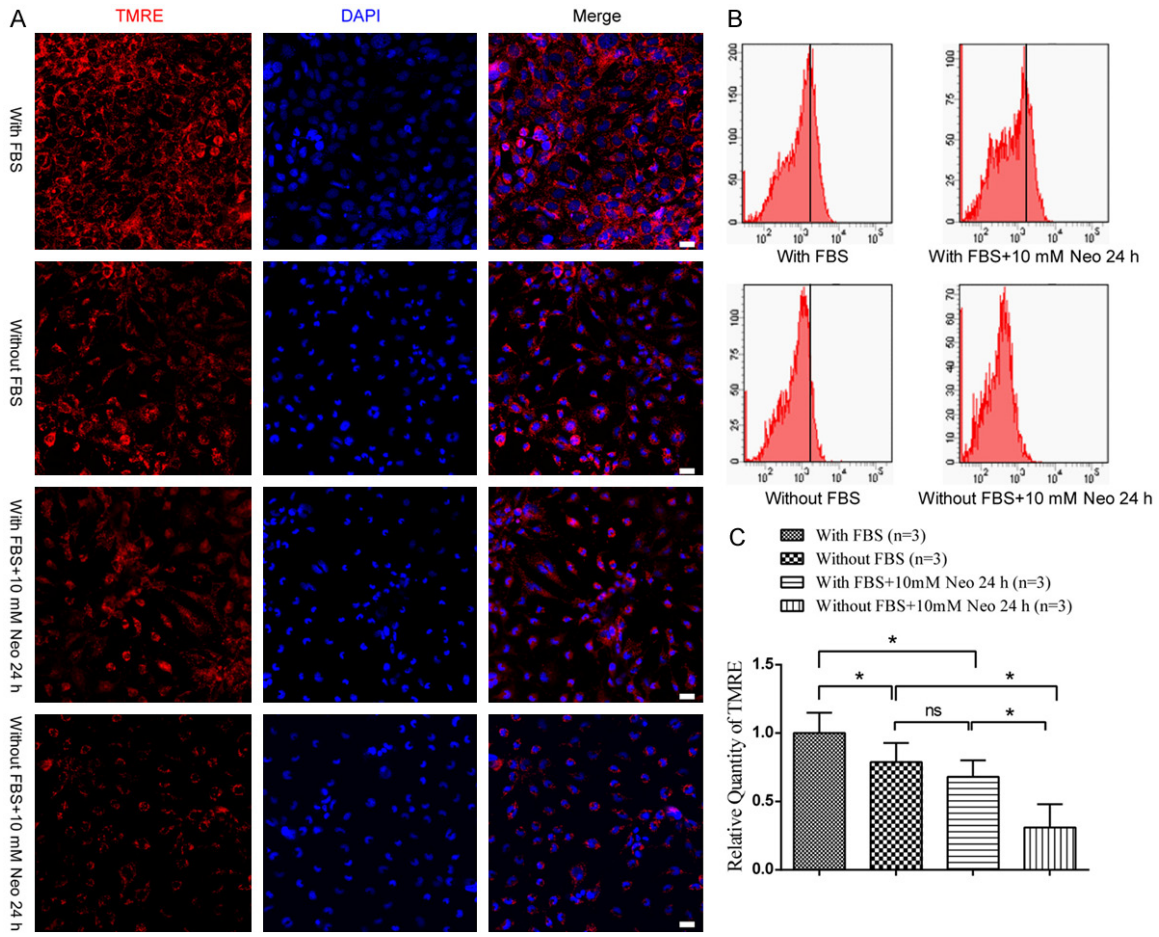

Figure 6. FBS significantly increased the MMP of HEI-OC-1 cells after neomycin exposure. A-C. For determining MMP, HEI-OC-1 cells were collected and incubated with TMRE for 10 min at 37°C, washed twice with PBS, and analyzed by flow cytometry within 1 h. The immunofluorescence and flow cytometry results showed that TMRE intensity was reduced after neomycin exposure compared with the controls, and the TMRE intensity in the HEI-OC-1 cells cultured without FBS was significantly decreased compared to the cells cultured with FBS under neomycin-free conditions. After neomycin exposure, the cells cultured with FBS had significantly greater TMRE intensity compared to the cells cultured without FBS, and the MMP decreased more significantly in the cells cultured without FBS and with 10 mM neomycin exposure for 24 h compared to the cells cultured without FBS or with 10 mM neomycin exposure for 24 h alone, but there was no significant difference between the cells cultured without FBS and the cells cultured with 10 mM neomycin exposure for 24 h. The flow cytometry results showed that the relative quantity of TMRE was 1.0 ± 0.2, 0.8 ± 0.1, 0.7 ± 0.1, and 0.3 ± 0.2 in cultures with FBS, without FBS, with FBS and with 10 mM neomycin exposure for 24 h, and without FBS and with 10 mM neomycin exposure for 24 h, respectively. *$P < 0.05$ by one-way ANOVA and the SNK test. Scale bars = 20 μm.

the expression of pro-apoptotic genes after neomycin exposure (**Figure 5E**). Together, these results suggest that FBS might regulate HEI-OC-1 cell apoptosis by suppressing pro-apoptotic gene expression.

Mitochondrial dysfunction and ROS accumulation have been reported to be involved in HC apoptosis [23-28]. ROS accumulation triggers mitochondrial depolarization, which results in the loss of MMP and the release of apoptotic factors from the mitochondria [29-32]. Our current study found that FBS deprivation alone significantly increased the mitochondrial ROS level and decreased the MMP of HEI-OC-1 cells under neomycin-free conditions (**Figures 6** and **7**) and that FBS significantly increased the MMP and decreased the ROS level in HEI-OC-1 cell mitochondria after neomycin exposure (**Figures 6** and **7**). These results suggest that FBS plays a critical role in regulating mitochondrial function after neomycin exposure.

Under physiological conditions, ROS production and scavenging keep the ROS level within a certain range, and this is regulated by numer-

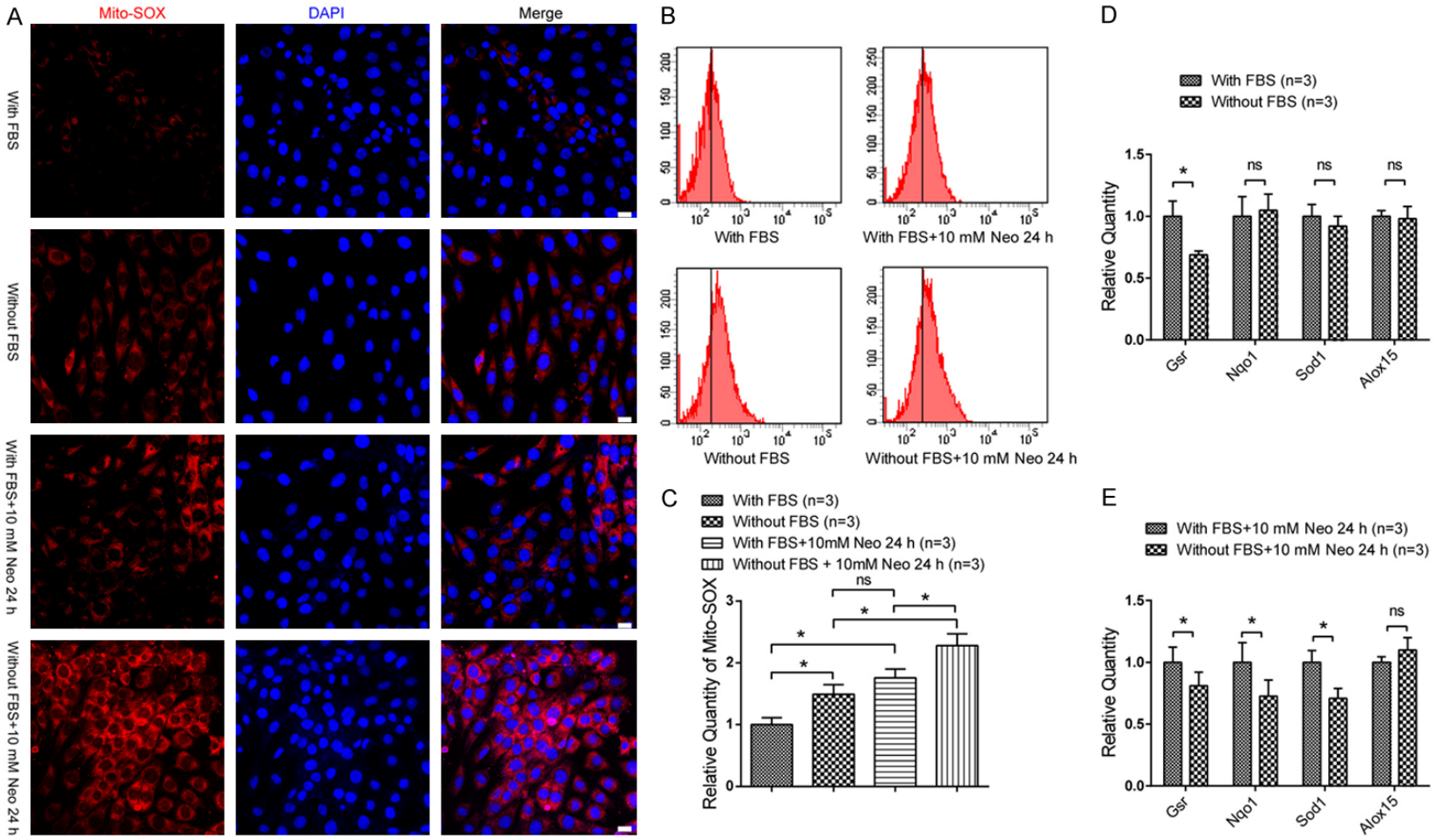

Figure 7. FBS significantly increased the antioxidant gene expression and decreased the ROS level in HEI-OC-1 cells after neomycin exposure. A-C. For analyzing ROS production, HEI-OC-1 cells were collected and incubated with Mito-SOX Red for 10 min at 37 °C, washed twice with PBS, and analyzed by flow cytometry within 1 h. Mito-SOX intensity was reduced after neomycin exposure compared with the controls, and Mito-SOX intensity in the HEI-OC-1 cells cultured without FBS was significantly increased compared to the cells cultured with FBS under neomycin-free conditions. After neomycin exposure, the cells cultured with FBS had significantly lower mitochondrial ROS levels compared with the cells cultured without FBS, and the mitochondrial ROS levels decreased more significantly in the cells cultured without FBS and with 10 mM neomycin exposure for 24 h compared to the cells cultured without FBS or with 10 mM neomycin exposure for 24 h alone, but there was no significant difference between the cells cultured without FBS and the cells cultured with 10 mM neomycin exposure for 24 h. The flow cytometry results showed that the relative quantity of Mito-SOX was 1.0 ± 0.1, 1.5 ± 0.2, 1.8 ± 0.1, and 2.3 ± 0.2 in cultures with FBS, without FBS, with FBS and with 10 mM neomycin exposure for 24 h, and without FBS and with 10 mM neomycin exposure for 24 h, respectively. D. qPCR showed that cells cultured without FBS had significantly decreased expression of the antioxidant factor *Gsr* under neomycin-free conditions. E. qPCR showed that the cells cultured with FBS had significantly higher levels of the antioxidant genes *Gsr*, *Nqo1*, and *Sod1* compared to cells cultured without FBS after neomycin exposure. **P* < 0.05 by one-way ANOVA and the SNK test. Scale bars = 20 μm.

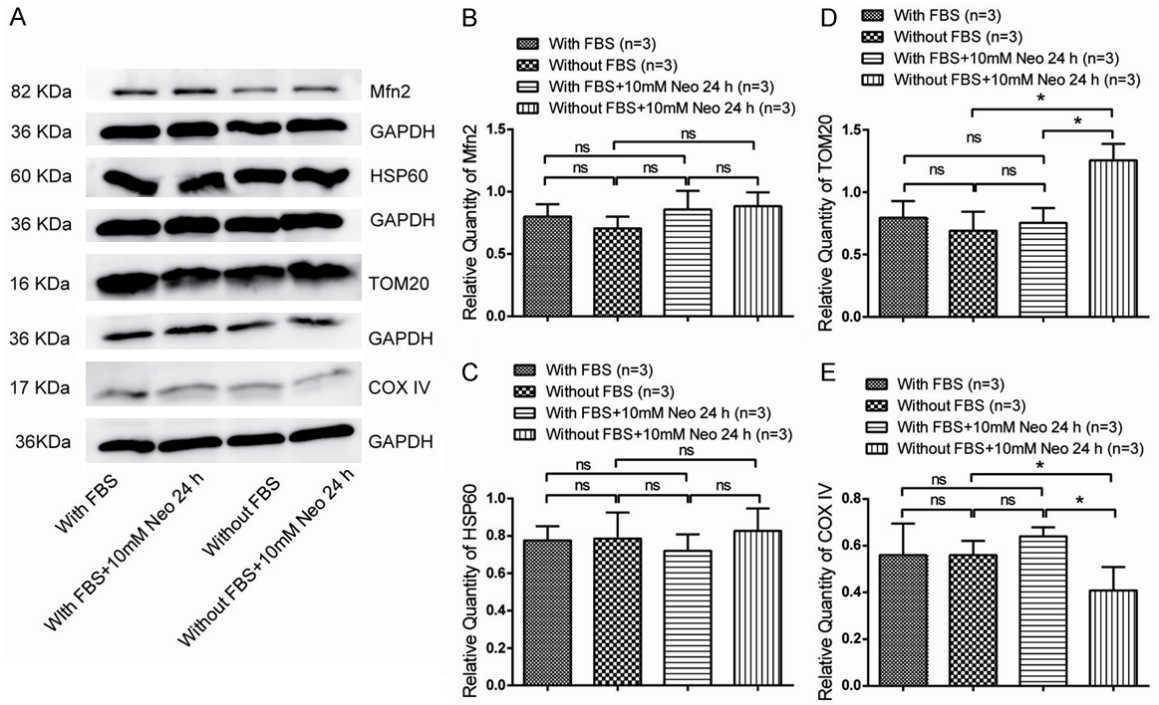

Figure 8. FBS deprivation significantly disrupted mitochondrial protein levels in HEI-OC-1 cells after neomycin exposure. A-C. Western blot showed that there was no significant difference in the Mfn2 and HSP60 expression between cells cultured with FBS, without FBS, with FBS and with 10 mM neomycin exposure for 24 h, and without FBS and with 10 mM neomycin exposure for 24 h. A and D. Western blot showed that the cells cultured without FBS had significantly higher levels of the mitochondrial protein TOM20 compared to cells cultured with FBS after neomycin exposure, and that the cells cultured with 10 mM neomycin exposure for 24 h had significantly higher levels of the mitochondrial protein TOM20 compared to cells cultured without FBS under neomycin-free conditions. The results also showed that there was no significant difference in the TOM20 expression between cells cultured with FBS and without FBS, between cells cultured without FBS and cells cultured with FBS and with 10 mM neomycin exposure for 24 h, and between cells cultured with FBS and cells cultured with FBS and with 10 mM neomycin exposure for 24 h. A and E. Western blot showed that the cells cultured without FBS had significantly lower levels of the mitochondrial protein COX IV compared to cells cultured with FBS after neomycin exposure, and that the cells cultured with 10 mM neomycin exposure for 24 h had significantly lower levels of the mitochondrial protein COX IV compared to cells cultured without FBS under neomycin-free conditions. The results also showed that there was no significant difference in the COX IV expression between cells cultured with FBS and without FBS, between cells cultured without FBS and cells cultured with FBS and with 10 mM neomycin exposure for 24 h, and between cells cultured with FBS and cells cultured with FBS and with 10 mM neomycin exposure for 24 h. *$P < 0.05$ by one-way ANOVA and the SNK test.

ous oxidant genes [7, 20, 25, 33]. In this study, we have shown that FBS deprivation alone significantly decreased the expression of the antioxidant gene *Gsr* under neomycin-free conditions (**Figure 7D**). Conversely, FBS significantly increased the expression of the antioxidant genes *Gsr*, *Sod1* and *Nqo1* after neomycin exposure (**Figure 7E**).

Mitochondrial proteins are critical for the maintenance of mitochondrial function and cell viability; for example, Mfn2 is the key regulator for mitochondrial outer membrane fusion and fission [34-36], HSP60 plays important roles in folding key proteins after import into the mitochondria and in stress-induced apoptosis [37, 38], TOM20 is essential for protein translocation into the mitochondrion [39, 40], and COX IV is the terminal electron transport protein in the oxidative respiratory chain in the mitochondria [41]. Further, previous studies have shown that the expression of mitochondrial proteins like Mfn2, HSP60, TOM20 and COX IV are disrupted after exposure to some toxic reagents, which impairs mitochondrial function, renders cells vulnerable to cytotoxic-effects, and induces apoptosis [36, 38, 41-49]. Our current study showed that FBS mediates its protective effects by maintaining normal mitochondrial protein levels, and we found that FBS deprivation significantly increased the level of TOM20 and decreased the level of COX IV after neomycin

exposure (**Figure 8**), suggesting that FBS is essential for maintaining the expression level of mitochondrial proteins in order to maintain mitochondrial homeostasis and inhibit cell apoptosis.

Besides, we also found that FBS deprivation had an interaction with the neomycin-induced cytotoxicity that the viability of HEI-OC-1 cells affected more by FBS deprivation while the neomycin exposure dose and time were 10 mM and 24 h, respectively (**Figure 2**), and that the combined insults with FBS deprivation and neomycin exposure were more serious compared to FBS deprivation or neomycin exposure alone (**Figure 4**). Further, we investigated the mitochondrial changes under FBS deprivation and 10 mM neomycin exposure for 24 h. Our results found that FBS deprivation or neomycin exposure alone significantly increased the mitochondrial ROS level and decreased the MMP of HEI-OC-1 cells, but each of them did not disrupted the level of mitochondrial proteins like Mfn2, HSP60, TOM20 and COX IV (**Figures 6**-**8**). And we also found that the level of MMP, ROS and mitochondrial proteins were not significantly different between cells cultured with FBS deprivation alone and cells with 10 mM neomycin exposure for 24 h alone (**Figures 6**-**8**). But the combined insults with FBS deprivation and neomycin exposure were more serious than FBS deprivation alone or neomycin exposure alone, we also found that the level of mitochondrial ROS increased and the level of MMP decreased more significantly in the cells cultured without FBS and with 10 mM neomycin exposure for 24 h compared to the cells cultured without FBS or with 10 mM neomycin exposure for 24 h alone. Further, we found that the level of mitochondrial proteins TOM20 increased and the level of COX IV decreased more significantly in the cells cultured without FBS and with 10 mM neomycin exposure for 24 h compared to the cells cultured without FBS or with 10 mM neomycin exposure for 24 h alone (**Figures 6**-**8**). Together, our finding indicate that FBS deprivation had cytotoxic effect like neomycin insults, and FBS deprivation had an interaction with the neomycin-induced cytotoxicity.

Conclusion

Together, our findings indicate that FBS is involved in maintaining the level of mitochondrial proteins, maintaining the balance of oxidant gene expression and preventing the accumulation of ROS, and thus FBS maintains normal mitochondrial function and inhibits the apoptosis of HEI-OC-1 cells after neomycin exposure. We also found that the protective effect of FBS was dose dependent and was more effective than the growth factors B27, N2, EGF, bFGF, IGF-1, and heparan sulfate.

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Disclosure of conflict of interest

None.

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