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PARP-1-modulated AIF translocation is involved in streptomycin-induced cochlear hair cell death

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Abstract

Conclusion—SM-induced dose- and location-dependent cochlear hair cell death *in vitro*. AIF might be translocated from mitochondria to nucleus and cytoplasm within SM-treated hair cells. The translocation of AIF might be modulated by PARP-1.

Objective—Streptomycin (SM), one of the widely used aminoglycoside nowadays, is still causing significant permanent sensorineural hearing loss owing to sensory hair cell death. This study was designed to investigate the role of apoptosis-inducing factor (AIF), an important mitochondrial cell death regulator, in SM ototoxicity within neonatal rat cochleae and HEI-OC1 cells.

Methods—The viability of HEI-OC1 cells was quantified by MTT assay. AIF, PARP-1, and myosin VIIa distributions were achieved by immunofluorescence. mRNA and protein expression of AIF and PARP-1 were examined by q-PCR and Western-blot.

Results—The hair cell loss was concomitant with the SM concentration variation, and aggravated from apical to basal turn. AIF was detected in nuclear region and AIF mRNA was up-regulated after SM incubation. Besides, AIF protein expression in mitochondria was decreased, whereas in cytosol it was increased. PARP-1 mRNA and protein were also up-regulated. 3-AB could attenuate the cell death and reverse the changes of AIF distribution by blocking PARP-1.

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Keywords

Streptomycin ototoxicity; programmed cell death; cochleae organotypic culture; HEI-OC1 cell

Introduction

Aminoglycosides (AGs) have been used as a successful class of antibiotics because of its potent antibacterial activities and low cost. However, the permanent ototoxicity of AGs has set a great obstacle to its wider clinical application [1]. SM, the first isolated AG, owing to its unparalleled antibiotic property, still plays an essential role in the therapy of tuberculosis [2]. As tuberculosis is becoming an increasingly serious problem worldwide, the sensorineural deafness resulting from SM is drawing more and more attention. Although the previous studies have made observations concerning the morphological changes induced by SM, the specific mechanisms underlying the ototoxicity are still largely unknown.

Researchers previously classified the AGs-induced hair cell loss into apoptosis and placed caspases (a family of cys proteases), as a central role in carrying out cell death [3,4]. However, the limited protective effect by inhibiting caspases impelled the seeking of a new caspase-independent pathway [5]. Recent evidence suggested AIF as an essential mediator in caspase-independent cell death [6]. As a highly conserved mitochondrial intermembrane flavoprotein, the mature AIF (62 kD) performs an indispensable protective redox function when anchored to the inner mitochondrial membrane [7]. Under the stress of several pro-apoptotic signals, the mature AIF is cleaved into a soluble 57 kD protein (truncated AIF, tAIF), and exerts a special activity in inducing programmed cell death. Once released from mitochondria, AIF relocates into the nucleus and induces caspase-independent chromatinolysis, leading to cell death finally [8]. However, this process and its related mechanisms are still controversial. Reports suggested that poly (ADP-ribose) polymerase-1 (PARP-1), a nuclear enzyme responsible for DNA damage repairing, is an essential up-regulator of AIF translocation [9]. The over-expression of PARP-1 triggered by serious impairment could induce AIF translocation, while the blocking of PARP-1 could depress the translocation [10]. However, no study of AIF concerning SM ototoxicity has been reported yet.

Our previous study has implied that AIF might participate in gentamicin-induced hair cell death of vestibule [11]. In this study, we continued to investigate the potential role of AIF in two *in vitro* models of SM cochleotoxicity, so as to decipher the mechanisms of SM ototoxicity and to find a better target for the intervention of SM-induced deafness.

Materials and methods

Animals and cell line

Post-natal Wistar rats (3-day-aged) were purchased from the Animal Center of Shandong University (Jinan, China). The care and use of the animals were approved by the Animal Care and Use Committee of Shandong University. The HEI-OC1 cell line was a widely used

progenitor hair cell line derived from cochlea of the Immortomouse, expressing molecular markers of cochlear cells [12].

Culture of cochlear explants and HEI-OC1 cells

The anesthetic rats were quickly decapitated, and the whole inner ears were harvested. After opening bulla and removing the cochlear shell, the basilar membrane was quickly dissected out and incubated in DMEM/F12 medium (Gibco, Carlsbad, CA) intermingled with 10% fetal bovine serum (FBS, Gibco) and ampicillin (50 μg/ml). The explants were cultured at 37 °C in 5% CO_2. Following the assessment of destroying effect of SM on the cochlea, the length of each explant was measured and three regions were chosen representatively as the apical, middle, and basal turns, at 5–14%, 40–49%, and 75–84% of the total length of basilar membrane away from the apex, respectively.

HEI-OC1 cells were cultured in DMEM basic medium (Gibco) with 10% FBS under permissive conditions (33 °C, 10% CO_2). No antibiotics were added into the medium except the SM. All operations concerning this cell line were conducted at the logarithmic phase.

Administration of drugs

The streptomycin sulfate (Solarbio, Beijing, China; potency: 650–750 mcg/mg, the concentration of 1 mg/ml equaled 0.44–0.51 mM) was freshly diluted in non-serum culture medium to a concentration of 100 mg/ml before being used.

3-AB (Sigma, St. Louis, MO) was diluted in dimethyl sulfoxide (DMSO, Solarbio) to a stock concentration of 1 M. The cells were pre-incubated for 30 min with 1 mM 3-AB before SM treatment. The controls were treated with the same volume of solvent. MTT assay has confirmed that 1 mM 3-AB showed no obvious impact on HEI-OC1 cell viability (data not shown).

MTT assay for HEI-OC1 cell viability assessment

HEI-OC1 cells (6000/well) were seeded in 96-well flat-bottom plates (Corning Glass Works, Corning, NY) and incubated overnight under permissive conditions. After 24 h incubation of SM or 3-AB in 100 μl culture medium, 10 μl MTT (5 mg/ml) was added for another 4 h. After aspirating the supernatants, 150 μl DMSO was added to dissolve the precipitate. The optical density (OD) values were measured at 570 nm by an ELISA reader (Multi-skan MK3, Shanghai Bio-excellent, Shanghai, China). The positive control underwent the same operations, but without cell-seeding, whereas the negative control was just treated without drugs. Viabilities were calculated as follows, relative viability=$(OD_{experiment}-OD_{positive})/(OD_{negative}-OD_{positive})\times 100\%$.

Immunofluorescence staining

After being fixed in 4% paraformaldehyde, samples were permeabilized with Triton X-100 (cochlea for 1% and HEI-OC1 cell for 0.2%, Sigma) in PBS for 30 min. Subsequently, specimens were blocked in 10% donkey serum albumin in PBS for 1 h. After 20 h incubation with AIF antibody (1:200, goat polyclonal, Santa Cruz, CA), PARP-1 antibody (1:200, rabbit monoclonal, Abcam, Cambridge, MA) or myosin VIIa antibody (1:800, rabbit

polyclonal, CST, US or 1:800, mouse polyclonal, DSHB, Iowa City, IA) at 4 °C, samples were treated with donkey anti-goat, anti-mouse, or anti-rabbit secondary antibodies (1:1000, Life Technologies, Carlsbad, CA) and diamidino-phenyl-indole (DAPI, 1:1000) for 1 h. Then the samples were visualized with an inverted DMI 400CS confocal microscope (Leica, Wetzlar, Germany). Negative controls without primary antibodies had been performed to test the specificity.

mRNA extraction and quantitative real-time PCR (q-PCR)

Total RNA was extracted from HEI-OC1 cells using Trizol Reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. With the presence of random hexamer primer, 1 mg total mRNA was reverse-transcripted into complementary-DNA using the Revert Aid kit (Fermentas, Burlington, Canada). q-PCR was performed with the Light Cycler Fast Start DNA SYBR Green kit and run in triplicate on Master cycler ep realplex (Eppendorf AG, Hamburg, Germany). The sequence-specific primers of AIF, PARP-1, and β-Actin were as follows: AIF forward: 5′-GAGTGATTTGGGTCCTGATGTG-3′; AIF reverse: 5′-GCAGATTTTGGGTTGTCTTGTG-3′; PARP-1 forward: 5′GCCGCCTACTCTATCCTCAGG-3′; PARP-1 reverse: 5′-GGCTTCTTCATTCCAAAGTCAT-3′; β-Actin forward: 5′-AGGGCTATGCTCTCCCTCAC-3′; β-Actin reverse: 5′-CTCTCAGCTGTGGTGGTGAA-3′. The expression levels of AIF and PARP-1 were normalized by β-Actin.

Protein extraction and Western-blot analysis

Total protein of HEI-OC1 cells was extracted using radio-immune precipitation buffer protein lysis buffer. The isolation of mitochondrial and cytosol proteins were achieved according to the manufacturer's protocol (Beyotime, Shanghai, China); 20 μg protein were denatured at 95 °C, and separated by pre-cast sodium dodecyl-polyacrylamide gels electrophoresis. Then the separated protein was transferred onto polyvinylidene difluoride membrane and blocked in 5% non-fat dry milk for 1 h at room temperature. Next, the membranes were incubated in 3% non-fat dry milk with the primary antibodies of AIF (1:1000 for mitochondrial and 1:500 for cytosol, goat polyclonal, Santa Cruz, CA), PARP-1 (1:2000, rabbit monoclonal, Abcam), COX-4 (1:500, goat polyclonal, Santa Cruz), and β-actin (1:1000, mouse monoclonal, Santa Cruz) overnight at 4 °C. The membranes were incubated with the secondary donkey anti-goat, anti-rabbit, or anti-mouse IgG antibodies (1:5000, Santa Cruz) for 1 h at room temperature. Finally, the immunoblots were detected with an ECL kit (Santa Cruz) and visualized after exposure to X-ray films. The relative optical density ratio was calculated with the Image J software by comparison with COX-4 or β-actin.

Statistical analysis

Statistical analyses were performed by SPSS 17.0 software package. Student's *t*-test and one-way ANOVA were applied and data were expressed as the mean±SEM. $p<0.05$ was regarded as statistically significant.

Results

Effects of SM on hair cells

The hair cells of rat cochleae in the control group exhibited normal cell bodies and ordered arrangement, while occasional loss in the outer or inner hair cells occurred. In the SM group, hair cells showed a disordered arrangement and swollen or atrophic morphology. The loss of cochlear hair cells in the SM group was positively proportional to the SM concentration and progressed from the basal turn to the apex eventually (Figure 1A).

MTT assay revealed a dose-dependent cell loss manner in HEI-OC1cells treated with SM (Figure 1B). Survival ratios were significantly reduced with increase of SM concentration ($p<0.05$).

AIF translocation in SM-inducing cell death

AIF was mainly detected in the cuticular plate region in normal hair cells (not shown), while a weak distribution outside the nucleus and no distribution within the nucleus were exhibited in control (Figure 2A). After SM treatment, a strong fluorescence of AIF was detected around and within the nuclear region (arrows). In most SM-treated HEI-OC1 cells, a strong fluorescence of AIF was also detected, and AIF aggregated in the nuclear region (Figure 2B). These costainings revealed the translocation of AIF from mitochondria to the nucleus in both cochleae and HEI-OC1 cells. Staining was absent from negative control material tested without the primary antibody (not shown).

Within the HEI-OC1 cell line, protein from the SM group significantly decreased in mitochondria, but increased in cytoplasm, indicating the releasing of AIF from the mitochondria into cytoplasm ($p<0.05$) (Figure 3).

Inhibitory effect of PARP-1 blocking on AIF translocation

Immunofluorescences implied that PARP-1 expressions in both cultured cochleae and HEI-OC1 cell were strengthened and mainly aggregated in the nuclear region in the SM group compared with the controls (Figures 4A and B). q-PCR analysis showed up-regulation of PARP-1 mRNA in the SM group, while Western-blot also revealed up-regulation of PARP-1 ($p<0.05$) (Figures 4C and D). MTT assay showed that the viability of cells treated with 3-AB and SM was higher than that treated with SM only, but lower than the control group ($p<0.05$). After 3-AB pre-incubation, no difference was detected in the two groups with 3-AB compared with control in the mitochondria ($p>0.05$) (Figure 5A). When it came to the cytosol, the increase of AIF triggered by SM also disappeared (Figure 5B). These results indicated that PARP-1 blocking could attenuate hair cell death by inhibited AIF translocation.

Discussion

As SM preferably damages the vestibular organs within the inner ear, few works have been reported about how this antibiotic undermines the cochleae. In the present investigation, within two *in vitro* models of SM ototoxicity, we found that SM undermined cochlear hair cells markedly. Both immunofluorescence of myosin VIIa within the organ of Corti and

MTT assay in the HEI-OC1 cell line evidenced that SM reduced hair cell viability with the increasing concentration. In the organ of Corti, SM-induced hair cell loss varied with the location, which started from the basal turn and progressed to the apex eventually. The cochlear base-to-apex gradient injury observed above was also reported in other AGs-induced ototoxicity models [13]. However, unlike gentamicin and kanamycin that are prone to accumulate in the OHC much more than IHC, SM seemed to undermine OHC or IHC at a similar degree. Therefore, it could be ascertained that the impairment patterns caused by SM have several similar aspects, but also are particular in some aspects compared with other AGs.

It has been reported that, once entering hair cells, excessive AGs promote the production of the reactive oxygen species or free radicals and initiate the pathways leading to cell death [14]. However, the subsequent regulating pathways remain unclear, the conventional views are that caspase-mediated apoptosis is the predominant sub-routine during the process, whereas increasing dissenting voices suggest that caspase-independent pathways might play a critical role in sensory hair cell death. Jiang et al. [5] reported both apoptotic- and necrotic-like appearances were presented in hair cells treated with kanamycin *in vivo*, but markers for classic apoptotic pathways were absent. In our work, different appearances of hair cell death were also detected, implying that SM could cause multiple forms of `regulated cell death', inclusive of necroptosis (programmed necroptosis), parthanatos (PARP-1 dependent cell death), and the other modes, which could not be simply contributed into classic apoptosis according to the latest recommendation of the Nomenclature Committee on Cell Death [15,16].

To address this issue, we focused on AIF, an essential mediator in necroptosis and parthanatos [6]. In this study, mRNA expression of AIF was upregulated in the SM group and immunofluorescence showed a co-localization of AIF and nucleus of most SM-treated cells, which implied a possible involvement of AIF in hair cell death. The difference of translocated-AIF staining between cochleae and HEI-OC1 cells might be as a result of different mitochondria distribution patterns, whereas AIF intended to act in a similar mode within both cochleae and the cell line. Next we detected the protein expression of AIF, which showed a decrease in mitochondria but an increase in cytosol after SM exposure. These results certified that AIF was translocated from mitochondria to cytosol and nucleus of hair cell after SM incubation. After being released from mitochondria, AIF arrives in the nucleus and exerts a particular effect on interacting with DNA and/or RNA to cause caspase-independent chromatinolysis [17]. The changes in mitochondrial membrane permeability are required for AIF being translocated and executing its pro-apoptotic ability, and PARP-1 is reported as a potent inducer of mitochondria dysfunction and AIF release [18]. Pathological conditions like glutamate excitotoxicity or oxidative stress could trigger the activation of PARP-1 and the following generation of PAR polymer, which mediates the release of tAIF. In this work, after incubated with SM, both expressions of PARP-1 mRNA and protein increased significantly in HEI-OC1 cells, moreover, the immunostaining of PARP-1 was strengthened and aggregated in the nuclear area after being treated with SM, suggesting that PARP-1 has been activated both in HEI-OC1 cells and cochlear hair cell. The attenuation of cell death induced by 3-AB implied that the inhibition of PARP-1 could exert a protective effect on SM-induced hair cell death. Furthermore, when PARP-1 was blocked by 3-AB, the

expression alterations of AIF in mitochondria and cytosol of hair cells was depressed. These results suggested that translocation of AIF was blocked by inhibiting PARP-1, implying that PARP-1 plays an important role in promoting AIF translocation under SM exposure. However, the specific mechanism of AIF translocation remains to be further investigated. Researchers reported that disruption of intracellular Ca^{2+}homeostasis was essential for AGs ototoxicity, and calpain, a Ca^{2+}-regulated neutral cysteine protease, was also responsible for AIF translocation [19,20]. More efforts would be made for the interpretation of SM ototoxicity in our future work.

Conclusively, this work demonstrated that SM could induce a dose- and location-dependent cochlear sensory hair cell death. AIF was involved in this process by translocation from mitochondria to cytosol and nucleus. PARP-1 might also be involved by regulating AIF translocation. The investigation of AIF and its affiliates might offer a new chance of targeted therapeutics in SM-inducing deafness.

Acknowledgments

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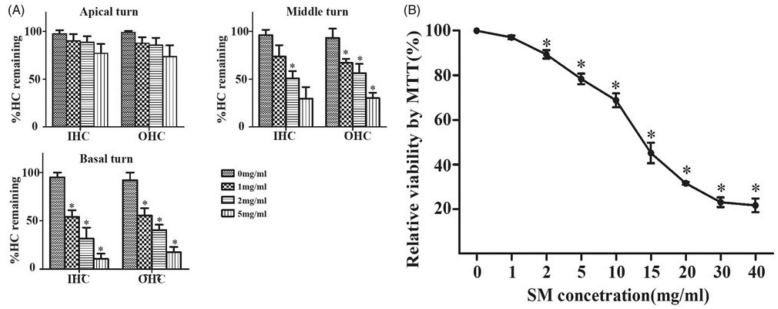

Figure 1.

The effects of SM on hair cells of cultured rat cochleae and HEI-OC1 cell line. (A) Statistics of hair cell loss of rat cochleae treated with SM. (B) The viabilities alterations of HEI-OC1 cells exposed to SM by MTT assay. SM could significantly trigger cell loss in a dose- and location-dependent manner compared with control (*p<0.05). SM, streptomycin.

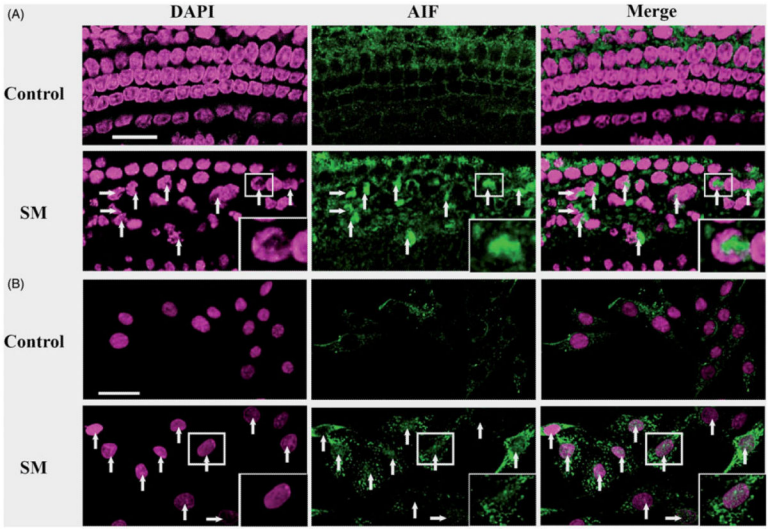

Figure 2.

AIF nuclear translocation after SM exposure. (A) Images from the middle turn of the cochlea treated with or without SM (2 mg/ml) using a confocal microscope at 630×. (B) Images of HEI-OC1 cell treated with or without SM (5 mg/ml) at 400×. Dual-stainings (arrows) of DAPI (rose-pink) and AIF (green) revealed that AIF was translocated into the nucleus after SM incubation. Pictures in the lower right of the SM group were magnifications of representative AIF translocation images. AIF, Apoptosis-inducing factor; DAPI, diamidino-phenyl-indole; SM, streptomycin. Scale bar represents 25 μm in (A) and 50 μm in (B).

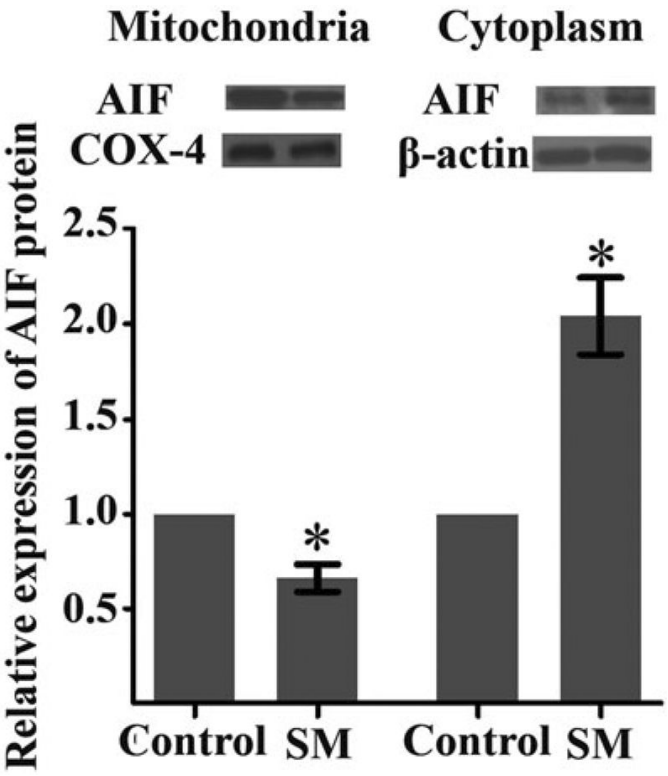

Figure 3.

Expressions of AIF protein of HEI-OC1 cells after SM (5 mg/ml) exposure by Western blot. Significant differences of AIF protein expression were detected in the SM group compared to the control (*p<0.05). AIF, Apoptosis-inducing factor; SM, streptomycin; COX-4, cytochrome c oxidase-4.

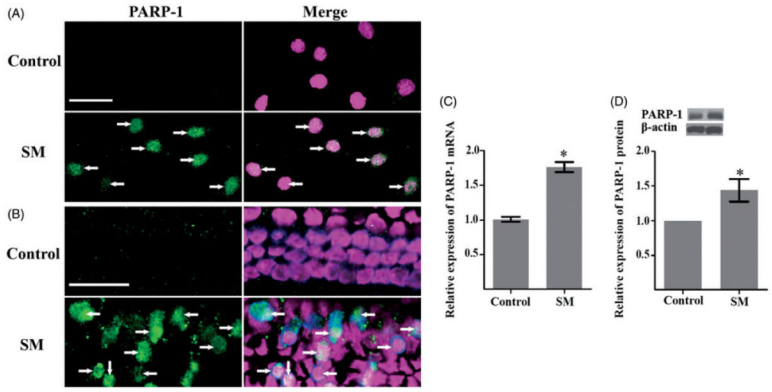

Figure 4.

Expressions of PARP-1 of cochlear explants and HEI-OC1 cells under SM incubation. (A) Images of PARP-1 immunofluorescences from the middle-basal turn of the cochlea treated with or without SM (2 mg/ml) using a confocal microscope at 630×. (B) Images of HEI-OC1 cells treated with or without SM (5 mg/ml) at 400×. (C) mRNA expression by q-PCR of HEI-OC1 cells. (D) Protein expression by Western blot of HEI-OC1 cells. Nuclei were marked with DAPI (rose pink) and cochlear hair cells were marked with myosin VIIa (blue). Arrows showed the strengthened stainings and accumulation in nuclei of PARP-1 in SM-treated cells. Significant differences of PARP-1 mRNA and protein expression were detected in the SM group (*p<0.05). Scale bar represents 25 μm in (A) and 50 μm in (B). PARP-1, poly (ADP-ribose) polymerase-1; SM, streptomycin; DAPI, diamidinophenyl- indole.

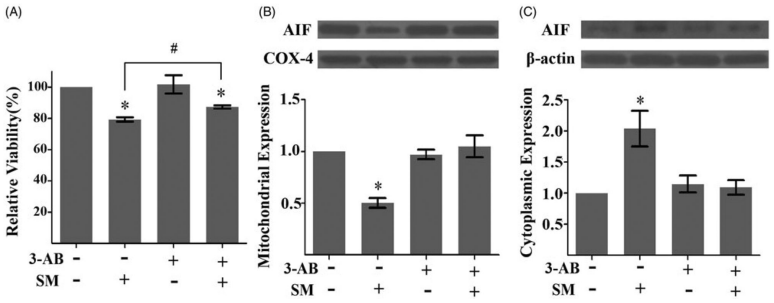

Figure 5.

Effects of 3-AB (PARP-1 inhibitor) on HEI-OC1 cells under SM. (A) The viabilities alterations of HEI-OC1 cells exposed to SM (5 mg/ml) or 3-AB (1 mM) by MTT assay. (B) Mitochondrial AIF protein expression after SM or 3-AB treatment by Western-blot. (C) Cytoplasmic AIF protein expression. Significant differences of viabilities were detected in groups treated with SM compared with control (*p<0.05); moreover, viability in the group treated with SM and 3-AB was higher than the group treated with SM only (#p<0.05). AIF protein in groups treated with SM only showed significant differences in the mitochondria and cytosol (*p<0.05), while the other two groups treated with 3-AB showed no difference (p>0.05). AIF, apoptosis-inducing factor; PARP-1, poly (ADP-ribose) polymerase-1; SM, streptomycin; COX-4, cytochrome c oxidase-4; 3-AB, 3-Aminobenzamide.