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Protective Effect of Retinoic acid preconditioning on H_2O_2-induced Apoptosis in Hair Cell-like Cells

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Abstract

Background: Several researches have proven that high levels of noise can induce oxidative stress and increase free radical's formation, particularly hydrogen peroxide (H_2O_2) and nitric oxide (NO) production. Retinoids are polyisoprenoid lipids derived from vitamin A or retinol. These molecules are essential regulatory elements in cell processes. All-trans retinoic acid (ATRA) plays a vital function in cellular growth, apoptosis, reproduction, cell differentiation, and immune feature by binding to its nuclear receptors.

Aim: In this study, we studied the protective effect of ATRA on H_2O_2-induced damage to bone marrow mesenchymal stem cell (BMSCs)-derived hair cells in culture.

Methods: Expression of MATH1 and SOX2 genes were assayed by immunocytochemistry (ICC). In order to evaluate the tolerance of ATRA-treated cells, after incubation of hair cells-like cells with ATRA, it was exposed to H_2O_2 as an oxidative stress model. Then, the apoptosis percentage of cultivated hair cell-like cells was evaluated by acridin-orange staining method.

Results: Our findings revealed that apoptotic cells were markedly diminished in the ATRA +H_2O_2 co-treated cells in comparison with the H2O2 only–treated group.

Conclusion: ATRA has the protective effect against oxidative stress damage in the cultivated hair cells- like cells by reducing the apoptosis.

Conflicts of Interest: The Authors declare no conflicts of interest.
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Introduction

Hearing loss is a common disorder that affects almost 10% of the population. Recent studies have investigated the reactive oxygen species (ROS) role such as free radicals and nitric oxide in the peripheral auditory system (1, 2). Many recent studies have shown that drug-induced hearing loss, noise pollution and ischemic reperfusion are associated with free radicals' formation. Over the last decade, oxidants play an important role in many illnesses. Several studies have shown that high-pitched sounds can lead to loss or cellular changes in various parts of the adult inner ear (3, 4). The mechanism of these changes is not exactly known, although previous studies have shown that intense sounds can produce oxidative stress

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and free radicals, especially hydrogen peroxide (4, 5), ischemia (6) or nitric oxide (7). ROS agents such as superoxide and hydroxyl radicals play an important role in brain injury (8). It seems that repair of nerve tissue after injury can be improved by reducing the formation of free radicals. Therefore, factors that can reduce free radicals may improve repair of damaged areas of the brain. Retinoic acid (RA) has been shown to have free radical scavenging activity, anti-apoptotic and neuroprotective properties (9, 10). Retinoids are polyisoprenoid lipids obtained from vitamin A or retinol. These molecules are important regulators of change in cellular processes. Retinoids control cellular proliferation, differentiation, and apoptosis (11). During mammalian development, RA elicits an intercellular response at specific locations required for differentiation and development**.** When RA binds to the RA receptor nuclear transcription factor (RAR), it can activate transcription of a wide range of target genes (12). Retinoid ability in regulating cell proliferation and increased cell differentiation has been demonstrated in many cell cultures and animal models (11, 13). RA was reported to play a regulatory role in increasing survival of B cells by inhibition of apoptosis in vitro (14). Although the RA requirement in cell proliferation and differentiation is well documented in cell culture, the protective action against oxidative stress-induced apoptosis in cultivated hair cell still not well understood.

Methods

Animals

Male Wistar rats weighing about 140-150 g were purchased from the Laboratory Animal Center of Shahid Beheshti University of Medical Sciences. Animals were kept in environmentally controlled rooms (under constant temperature (22 ± 2°C) and 12:12-h light: dark cycle) (15). The Ethics Committee of Shahid Beheshti University of Medical Sciences approved this study based on National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publication No. 85-23, revised 1985).

Isolation of bone marrow stem cells

Ketamine (90 mg/kg) and xylazine (10 mg/kg) combination (intraperitoneal injection) were used for anesthesia of rats. Bone mesenchymal stromal cells (BMSCs) were extracted from femur and tibia bone and washed with Phosphate Buffered Saline (PBS). Then cells were cultured in medium with DMEM/F12 (Gibco, USA) supplemented with 10% Fetal Bovine Serum (FBS, Gibco). Cells were cultivated at 37 °C in a humidified 5% CO_2. Cell morphology were observed by phase-contrast microscopy at passage 4.

Hair cell-like cells differentiation

BMSCs were initially differentiated into the neural stem cells (NSCs) in the presence of B27. Next, NSCs were differentiated into hair cell -like cells in the presence of growth factors including 20 ng/ml of epidermal growth factor (EGF), 20 ng/ml of basic fibroblast growth factor (bFGF) and 50 ng/ml of Insulin-like growth factor 1 (IGF-1) (R&D Systems, USA) for two weeks (16).

Immunocytochemistry

Cultured hair cells were fixed by incubating in 4% paraformaldehyde (PFA) in phosphate-buffered saline (PBS) for 15 minutes and permeabilized with Triton X-100 in PBS for 10–15 min. Anti-Math1 (1:100) and anti-SOX2 (1:200) were used. Cells were incubated with mentioned primary antibodies overnight at 4 °C, then rinsed with PBS and next incubated with secondary antibodies (1:2000) (Abcam, Cambridge, UK) for 1 h at room temperature in dark. Subsequently, cells were counterstained with DAPI. Following washing with PBS, cultured cells were observed under a fluorescent microscope.

H_2O_2 treatments

To evaluate the protective effects of ATRA against hair cell-like cells injury induced by H_2O_2 exposure, ATRA pretreated cells were

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rinsed with buffer of PBS and incubated with medium including 500 µM H_2O_2 for 4 h.

MTT assay

MTT assay measure cell viability using a colorimetric plate reader. Hair cell-like cells were cultivated in a 96-well plate (5×10^3 cellswell). Afterward, cells were treated with several concentrations of ATRA (0, 0.1, 1, 10 µmol/L). After 24 h of incubation, MTT working solution (100 µl) was applied to each well and continues to incubate the plate for 4 h at 37 °C in a 5% CO_2 incubator.

The Supernatants was removed, and the insoluble formazan crystal was solubilized by adding of DMSO (100 µl, Carl Roth GmbH & Co. Karlsruhe, Germany) per well in a 37 °C, humidified CO2 incubator for 30 min. Next, the dissolved formazan crystals (purple color) intensity was measured by using the plate reader at 570 nm. In addition, cytotoxicity assay was performed to evaluate the contributions of ATRA treated hair cell-like cells in the tolerance to H_2O_2 (Figure 3).

Acridine orange staining

After treatment, apoptotic morphological changes were evaluated by acridine orange (AO) staining. Following ATRA and H_2O_2 co treatments, cells were rinsed three times with PBS and fixed with 4% PFA in PBS for 15 minutes, then stained with 100 µg/ml of AO (Sigma-Aldrich) for 5 minutes at room temperature. Apoptotic cells were characterized by yellow or orange-red nuclear staining (17). Apoptotic cells (per 200 cells) were counted with Image J Analyzer (18). The cells were then observed under a fluorescence microscope.

Statistical analysis

Data are presented as the mean ± SEM. One-way ANOVA was done by using GraphPad Prism version (GraphPad Software, San Diego, CA, USA). P below 0.05 is considered statistically significant.

Results

Morphological Characterization of BMSCs and Neural Stem Cells

Fourth passage BMSCs displayed typical fibroblast-like spindle shape under the phase-contrast microscope (Figure 1A). Morphological changes in BMSCs cultures during neural induction were observed on day 14. Neurite-like processes were detected after 2 weeks (Figure 1B).

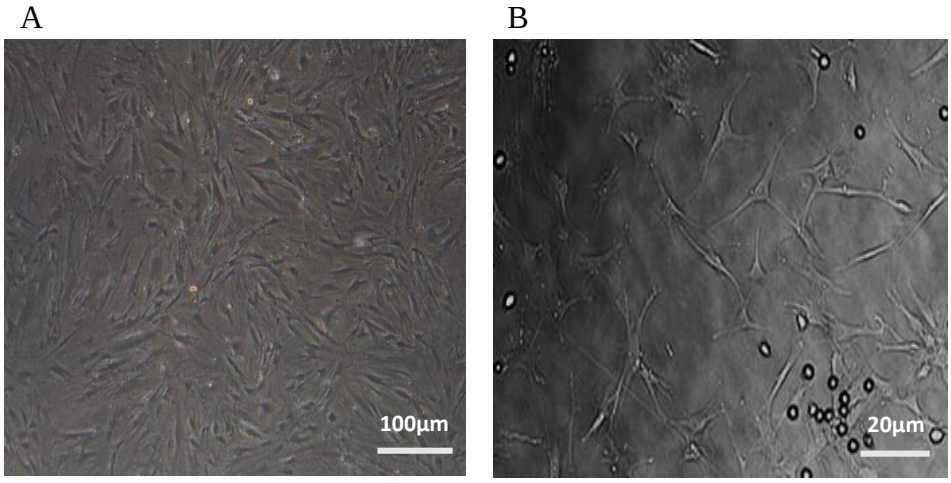

Figure 1. (A) Morphology of cultured BMSCs, (Scale bar: 100 µm) at fourth passage (B) and cultivated neural stem cell at day 14, (Scale bar: 20 µm).

Analysis of hair cell-like cells markers

The expressions of Math1 and SOX2 were assayed following 14 days of hair cell differentiation by Immunocytochemistry (ICC) technique (Figure 2). ICC analysis of hair cells related markers showed their expression in the cultured cells (Figure 2).

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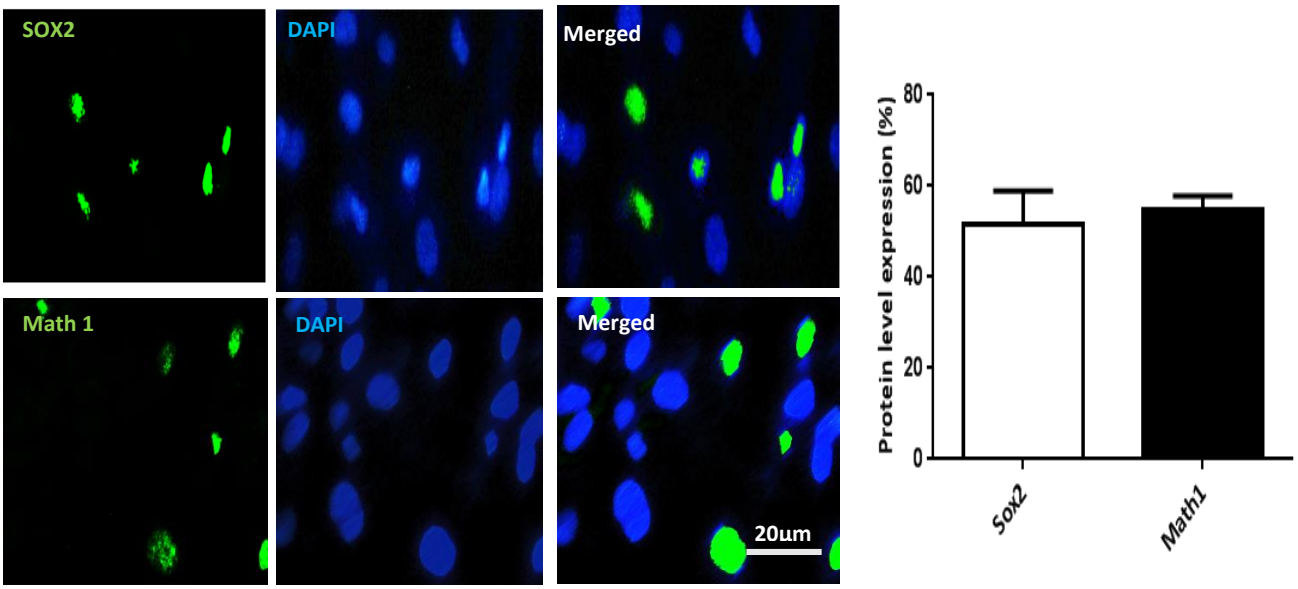

Figure 2. Evaluation of SOX2 and Math 1 expression by immunocytochemistry.
SOX2 and Math1 were detected in cultured hair cells. DAPI presented the cell nucleus in blue (Scale bar: 20 µm).

Cell viability

MTT assay was used to evaluate the cytotoxic effect of ATRA on cultured cells. In addition, cytotoxicity assay was done to evaluate the contributions of ATRA treated hair cell-like cells in the tolerance to H_2O_2. Hair cells-like cells pretreatment with 10 µmol/L ATRA for 24h significantly increased cell viability (P<0.001) (Figure 3A). Treatment with H202 inhibited growth of cultured hair cells. Hair Cells were pre-treated with ATRA for 24 h and co-incubated with H_2O_2 (500 µM) for 4 h. H202 Exposure significantly decreased cell viability percentage (P <0.001) (Figure 3B). ATRA pretreatment obviously suppressed H_2O_2-inducd cultivated hair cell death (P <0.001) (Figure 3B).

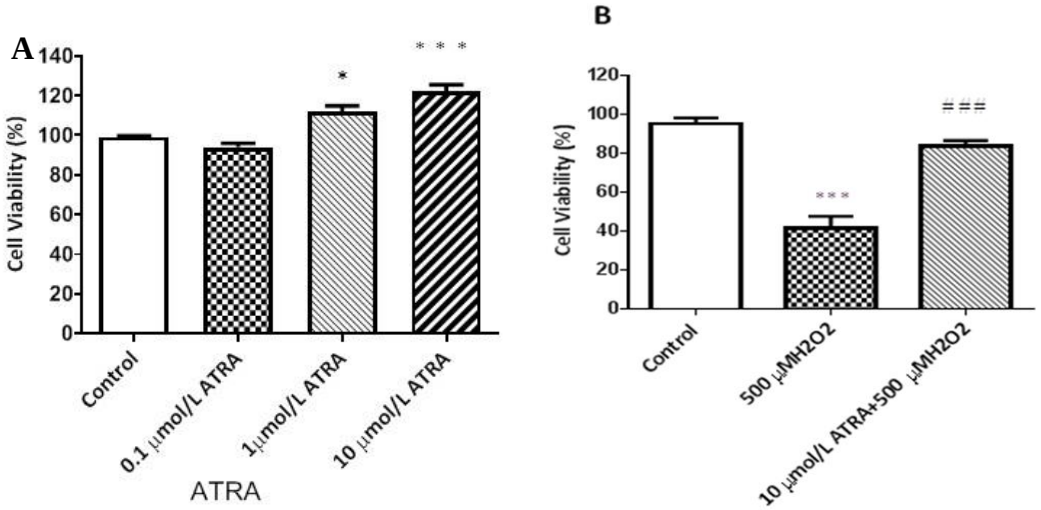

Figure 3. ATRA effect on cells viability. Several concentrations of ATRA were applied for 24h and cells viability percentage was assayed by MTT test. Results showed that viability of ATRA treated cells enhanced in comparison with the control group. ATRA decreased H_2O_2 -induced cytotoxicity in cultured hair cell. *P<0.05 and ***P<0.001 in comparison with control group. ***P < 0.001 in comparison with H_2O_2 treated cells.

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Apoptosis Assay

There were no significant apoptotic cells identified in control and 10 µmol/L ATRA treated group, respectively (Figure 4A&B). Apoptotic cell death is displayed by granular yellow-green acridine orange nuclear staining and evaluated in the H_2O_2 and ATRA+ H_2O_2 groups (Figure 4C&D). In the H_2O_2 group, apoptotic cells were significantly increased compared to control group ($P<0.001$). Percentage of apoptotic cells significantly reduced in the ATRA+ H_2O_2 co-treated group in comparison with H_2O_2 group ($P<0.001$) (Figure 4D).

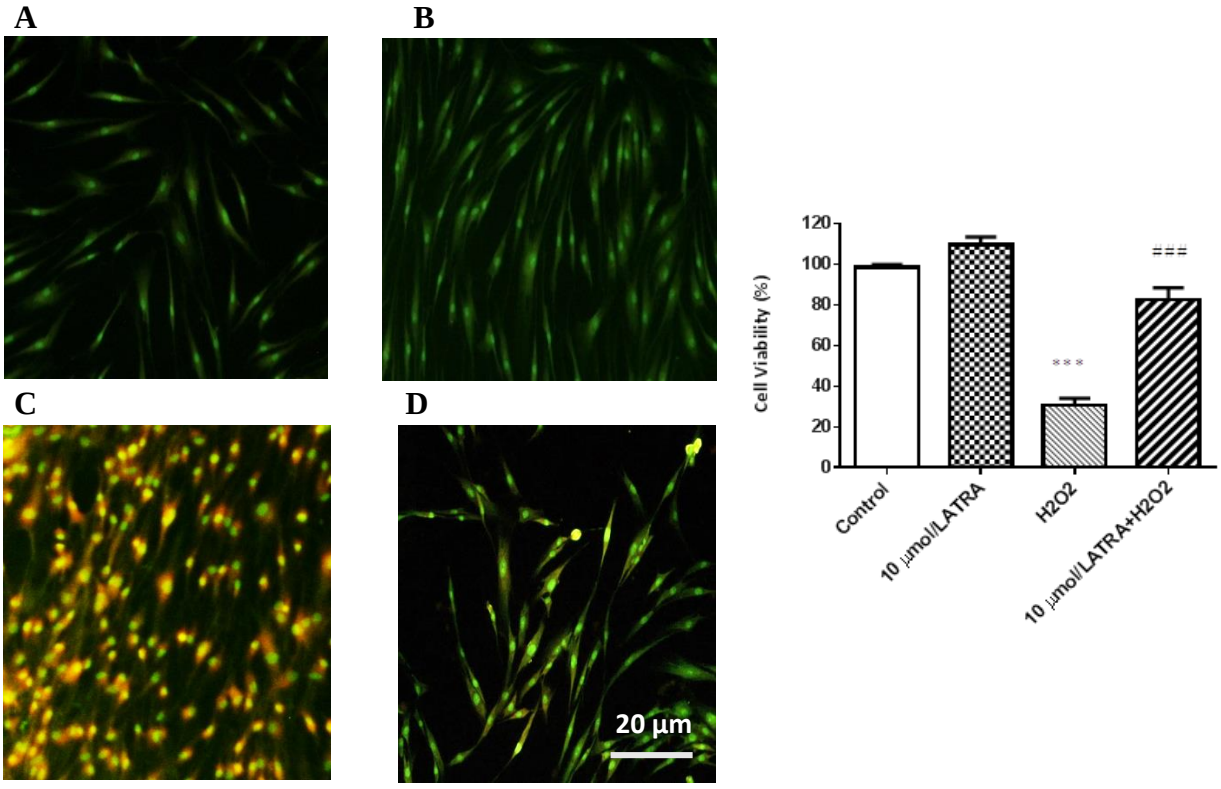

Figure 4. Apoptosis assay by AO staining. AO stained cells were observed under fluorescence microscope (A). 10 µmol/L ATRA treated group (B), 500 µM H_2O_2 treated group (C) and ATRA+ H_2O_2 co-treatment group (D). Nuclear death was detected by granular yellow-green. AO; Acridine Orange. ***P < 0.001 in comparison with the control group; ***P < 0.001 in comparison with H_2O_2 treated group. (Scale bar: 20 µm) (One-way ANOVA).

Discussion

The purpose of present study was to investigate the protective effect of ATRA preconditioning against H2O2 induced hair cells-like cells damage in vitro. Our findings revealed that ATRA can protect cultivated hair cells against oxidative stress injury by apoptosis suppression.

Our data showed that H_2O_2 could induce apoptosis in cultured hair cells. Physiological balance between ROS production and antioxidant system has a crucial role in cellular metabolism. An imbalance between ROS production and the antioxidant defenses will disrupt the homeostasis, lead to activation of apoptosis which can damage to the component of cell (DNA, proteins and lipids) (19).

RA is an essential factor for the development of the inner ear. RA can stimulate differentiation of cochlear neural progenitor cells into hair cells. ATRA is the major biological retinoic acid that has anti-cancer, anti-inflammatory and anti-oxidant properties (20). Previous works have revealed that in the neurological damage process, RA receptor-α (RAR_α) essentially advances the proliferation of astrocytes and

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oligodendrocytes, and act as a crucial function in the process of final maturation (21). Katsuki et al (29) proposed that RA may promote the brain-derived neurotrophic factor activity through RAR-α upregulation, which can protect against damage to dopaminergic neuronal in the mesencephalon (21). Ahlemeyer and Krieg Stein have revealed that RA protects against oxidative injury and apoptosis by inhibition of glutathione depletion (22). ATRA as a lipophilic molecule can be easily distributed through cell membranes and thus can reduce oxidative stress and apoptosis (23). Another study demonstrated the protective effects of ATRA on arsenic-induced estrogen signaling pathway in Sprague-Dawley female mice (24). In addition, ATRA has a protective effect on Oxygen Glucose Deprivation (OGD)-induced cell death in the hippocampus of rat by inhibiting c-jun N-terminal kinase and mitogen-activated protein kinase p38 (9). ATRA has also been shown to have a protective effect on NMDA-induced neuronal damage and apoptotic effect in the rat retina (25). Furthermore, it has been demonstrated RA induce the overexpression of p53 tumor-suppressor gene to enhance Bcl-2 expression and inhibit apoptosis in pancreatic cancer cells and thymic tumor cells (26, 27). Previous study has shown that Ad-siRAR-α could increase apoptosis in OGD-induced PC12 cells suggesting that expression of RAR-α has an anti-apoptotic effect on OGD-injured PC12 cells. The anti-apoptotic effect of ATRA on OGD-induced injury has been shown to be achieved by enhancing the anti-apoptotic Bcl2 factor expression and reducing the pro-apoptotic Bax factor expression (28).

Conclusion

In conclusion, ATRA preconditioning has protective effects on H202-induced injury in cultured hair cells by inhibiting the apoptosis.

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None to declare.

Conflicts of Interest

The authors declare no conflicts of interest.

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