Adenosine decreases oxidative stress and protects H_2O_2-treated neural stem cells against apoptosis through decreasing Mst1 expression

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Abstract. Overproduction of free radicals during oxidative stress induces damage to key biomolecules and activates programed cell death pathways. Neuronal cell death in the nervous system leads to a number of neurodegenerative diseases. The aim of the present study was to evaluate the neuroprotective effect of adenosine on inhibition of apoptosis induced by hydrogen peroxide (H_2O_2) in bone marrow-derived neural stem cells (B-dNSCs), with focus on its regulatory effect on the expression of mammalian sterile 20-like kinase 1 (*Mst1*), as a novel proapoptotic kinase. B-dNSCs were exposed to adenosine at different doses (2, 4, 6, 8 and 10 μM) for 48 h followed by 125 μM H_2O_2 for 30 min. Using MTT, terminal deoxynucleotidyl transferase dUTP nick-end labeling and real-time reverse transcription polymerase chain reaction assays, the effects of adenosine on cell survival, apoptosis and *Mst1*, nuclear factor (erythroid-derived 2)-like 2 and B-cell lymphoma 2 and adenosine A1 receptor expression were evaluated in pretreated B-dNSCs compared with controls (cells treated with H_2O_2 only). Firstly, results of the MTT assay indicated 6 μM adenosine to be the most protective dose in terms of promotion of cell viability. Subsequent assays using this dosage indicated that apoptosis rate and *Mst1* expression in B-dNSCs pretreated with 6 μM adenosine were significantly decreased compared with the control group. These findings suggest that adenosine protects B-dNSCs against oxidative stress-induced cell death, and therefore, that it may be used to promote the survival rate of B-dNSCs and as a candidate for the treatment of oxidative stress-mediated neurological diseases.

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Introduction

Numerous studies have described that oxidative stress, induced by reactive oxygen species (ROS) and mitochondrial dysfunction, is implicated in the etiology of neurodegenerative disorders (1-3). Adenosine is a naturally occurring nucleoside that is used to control heart arrhythmia (4). Adenosine is a neuromodulator that suppresses neuronal excitability through activation of the inhibitory adenosine A1 receptor (AA1R) (5). Therefore, AA1R has therapeutic potential as a neuroprotective candidate.

Apoptosis, a form of programmed cell death, serves an important role in neurodegenerative disorders (6). Studies on apoptosis and its role in neurodegenerative disorders including Alzheimer's disease, Parkinson's disease, Huntington's disease and ischemia have elucidated the possible apoptotic mechanisms underlying these disorders (7-9). Apoptosis is regulated by the expression or activation of proapoptotic genes and proteins including mammalian sterile 20-like kinase 1 (*Mst1*) (10). *Mst1* is a stress-activated, proapoptotic kinase that, following caspase-mediated cleavage, enters the nucleus and induces chromatin condensation followed by DNA fragmentation (11,12). Mst1 protein has been reported to induce the mitochondrial-dependent pathway of apoptosis as well as promote apoptosis via caspase-dependent and -independent pathways (13,14).

In the present study, the *in vitro* neuroprotective effects of adenosine in inhibiting apoptosis and promoting cell survival were investigated in bone marrow-derived neural stem cells (B-dNSCs) preexposed to hydrogen peroxide (H_2O_2). The effects of adenosine on expression of proapoptotic Mst1 and antiapoptotic nuclear factor (erythroid-derived 2)-like 2 (*Nrf2*) and B-cell lymphoma 2 (*Bcl-2*) genes in the H_2O_2-induced B-dNSCs were evaluated. Recent studies have demonstrated that Nrf1, Nrf2 and Bcl-2 family coactivators control mitochondrial transcription specificity factors (15,16). Furthermore,

Bcl-2 and Nrf2 are established as critical factors in protecting cells against H_2O_2-induced apoptosis (17). The results from this study indicated that apoptosis rate and *Mst1* expression in the adenosine-treated B-dNSCs were significantly decreased while *Nrf2* and *AAIR* mRNA levels were increased. The present study aimed to develop understanding of the neuroprotective effects of adenosin in neurological disorders.

Materials and methods

Bone marrow-derived stromal cell (BMSC) isolation and culture. In the present study, 5 male Wistar rats (weighing 150-200 g, aged 8 weeks old), purchased from Razi Vaccine and Serum Research Institute (Karaj, Iran), were sacrificed under anesthesia with 100 mg/kg ketamine and 10 mg/kg xylazine. All experimental protocols were approved by the Zanjan University of Medical Sciences (Zanjan, Iran) Ethics Committee. All rats were housed in a temperature (25-27˚C) and humidity (~50%) controlled environment under a 12-h light/dark cycle. The rat tibia and femoral bone marrow were aseptically aspirated with 2 ml Dulbecco's modified Eagle's medium (DMEM; Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) and then centrifuged for 5 min at 1,500 x g and 4˚C. The supernatant was removed, 1 ml fresh media was added and repeated pipetting was performed to create a single cell suspension. Subsequently, cells were transferred to a 25-cm^2 flask for tissue culture with 5 ml low-glucose DMEM containing 10% fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA), 100 U/ml penicillin and 100 mg/ml streptomycin (Sigma-Aldrich; Merck KGaA). The isolated cells were incubated at 37˚C in 5% CO_2 for 2 days. The adherent cells were collected and subcultured, and the culture medium was replaced every 2-3 days until cells became 70-80% confluent.

The cells were harvested with trypsin-EDTA (0.25%; Sigma-Aldrich; Merck KGaA) and passaged up to three times (P3). For identifying BMSCs, immunocytochemical evaluation was performed for cluster of differentiation (CD)-90 as a mesenchymal stem cell marker (18). Briefly, BMSCs were cultured on cover slides and fixed in 3% paraformaldehyde for 15 min at room temperature (RT), then permeabilized with 0.4% Triton X-100. Blocking was performed in 10% FBS in phosphate-buffered saline (PBS) for 30 min at RT. The cells were then incubated with anti-CD90 monoclonal antibodies (ab225; 1:200; Abcam, Cambridge, UK) overnight at 4˚C, followed by incubation with a fluorescein isothiocyanate (FITC)-conjugated rabbit anti-rat antibody (ab6730; 1:300; Abcam) for 4 h at RT. Nuclei were counterstained with ethidium bromide for 30 sec at RT.

Neurosphere formation and expansion. To form neurosphere-like structures, isolated BMSCs at P3 were seeded ($1x10^5$ cells/ml) in 25-cm^2 non-adherent plastic flasks and incubated with NSC expansion medium containing DMEM/F12 supplemented with 2% B27 (Gibco; Thermo Fisher Scientific, Inc.), 20 ng/ml basic fibroblast growth factor (bFGF; Invitrogen; Thermo Fisher Scientific, Inc.), 20 ng/ml epidermal growth factor (EGF; Invitrogen; Thermo Fisher Scientific, Inc.), 100 U/ml penicillin and 100 mg/ml streptomycin. The medium and growth factors were added every 3 days for a week. For the preparation of a single cells from the neurospheres, the floating structures were isolated by centrifuging for 5 min at 300 x g and 4˚C.

They were then dissociated enzymatically using Trypsin-EDTA (0.25%) and mechanically (by pipetting) to single cells, and subsequently expanded on 6-well adherent plates coated with poly-l-lysine (Sigma-Aldrich; Merck KGaA). The cells (10^5 cells/well) were then suspended in DMEM/F12 supplemented with 2% B27, 20 ng/ml bFGF and 20 ng/ml EGF and 5% FBS and passaged up to three times (19). For identifying B-dNSCs, immunocytochemical evaluation was performed for nestin as an NSC/progenitor cell marker (20) with corresponding antibody (ab6142; 1:300; Abcam) as above. The FITC-conjugated rabbit anti-rat antibody (ab6730; 1:300; Abcam) was used for detection (4 h at RT in the dark). Ethidium bromide (20 sec) was used for nuclei counterstaining at RT. Images were captured with an Olympus BX51 fluorescence microscope (Olympus Corporation, Tokyo, Japan).

Adenosine dose response. B-dNSC viability and the protective effects of adenosine were evaluated using the 3-(4, 5-dimethythiazol-2-yl)-2, 5-diphenyl-tetrazolium (MTT) bromide assay (21). B-dNSCs were cultured in 96-well plates (10^5 cells/well) in NSC expansion medium. The cells were then incubated with different concentrations of adenosine (0, 2, 4, 6, 8 and 10 μM) at 37˚C for 48 h. To induce oxidative stress, H_2O_2 was prepared from a 30% stock solution. The pretreated NSCs were incubated with 125 μM H_2O_2 for 30 min at 37˚C. Subsequently, the cells were incubated with 1 mg/ml MTT at 37˚C for 4 h. The culture medium was removed and 100 μl dimethyl sulfoxide was added to each well to dissolve the formazan crystals. The amount of formazan dissolved was quantified at absorbance (A)570 nm using a microplate ELISA reader. The relative cell viability in percentage was calculated as (A570 of treated samples/A570 of untreated samples) x 100 (22).

Experimental groups. Based on results of the MTT assay, cells were assigned to different experimental groups as follows: N (untreated B-dNSCs), NA (B-dNSCs treated with 6 μM adenosine), NH (B-dNSCs treated with 125 μM H_2O_2) and NAH (B-dNSCs pretreated with 6 μM adenosine followed by 125 μM H_2O_2).

Assessment of apoptosis by terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) staining. Cells were fixed with 4% paraformaldehyde in PBS for 30 min at room temperature (RT). DNA fragmentation was assessed with an *In-Situ* Cell Death Detection kit (Roche Diagnostics GmbH, Mannheim, Germany) according to the manufacturer's instructions. TUNEL-positive cells were colored using diaminobenzidine as the chromogen for 5 min at RT, and counterstained with hematoxylin for 1 min at RT. The percentage of TUNEL-positive cells was assessed in five randomly selected microscopic fields of the Olympus BX51 fluorescence microscope per culture well.

Immunofluorescence staining. Cells were cultured on cover slides at a density of $1x10^5$ cells/ml in NSC growth media as described above and fixed in 4% paraformaldehyde for 15 min

Table I. PCR primer sequences.

Gene	GenBank accession no.	Forward, 5'-3'	Reverse, 5'-3'	Product size, bp
Mst1	NM_001107800.1	GCTAAAGTGAAGTGGACGGATACC	GGAACAGTTGCTACCAGAGTGTCAG	173
Nrf2	NM_031789.2	CACCAGTGGATCTGTCAGCTACTC	GTGGTGAAGACTGAGCTCTCAACG	168
Bcl-2	NM_016993.1	GTGGCCTTCTTTGAGTTCGGTG	ATCCCAGCCTCCGTTATCCTG	147
GAPDH	NM_017008	AACCCATCACCATCTTCCAG	GTGGTTCACACCCATCACAA	197
AA1R	XM_006249861	CCACAGACCTACTTCCACACC	CTGTCTTGTACCGGAGAGGGA	136

Primers were designed with Gene Runner 3.05 software (http://generunner.net; product by info@genfanavaran.com). Mst1, mammalian sterile 20-like kinase 1; Nrf2, nuclear factor (erythroid-derived 2)-like 2; Bcl-2, B-cell lymphoma 2; AA1R, adenosine A1 receptor.

at RT, followed by permeabilization in PBS-0.1% Triton X-100 for 30 min at RT. Blocking was performed in 10% FBS in PBS for 30 min at RT. For immunofluorescence staining, cells were incubated with primary polyclonal antibodies against rat CD90 (ab225; 1:200), nestin (ab6142; 1:300; both from Abcam), Nrf2 (sc-722; 1:1,200) and Mst1 (sc-100449; 1:300; both from Santa Cruz Biotechnology, Inc., Dallas, TX, USA) individually overnight at 4˚C, then incubated with a fluorescein isothiocyanate-conjugated rabbit anti-rat antibody (ab6730; 1:300; Abcam) for 4 h at RT in the dark. DAPI (5 min) and ethidium bromide (20 sec) were used for nuclei counter-staining at RT. Images were captured with the Olympus BX51 fluorescence microscope.

Real-time reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Real-time RT-qPCR was performed with cDNA from the experimental groups following treatments. In all groups, 1,000 ng purified RNA extracted using TRIzol (Invitrogen; Thermo Fisher Scientific, Inc.) from cultured cells was used to synthesize 20 μl cDNA, using a RevertAid™ First Strand cDNA Synthesis kit (Fermentas; Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. cDNA (25 ng of RNA samples) was used to quantify *Bcl-2*, *Nrf2*, *Mst1* and *AA1R* mRNA levels. As an internal control, primers for *GAPDH* were used. The primer sequences of all primers used are listed in Table I. The PCR reaction was performed in a 12.5-μl final volume containing forward and reverse primers (200 nM each), cDNA (0.5 μl), SYBR®-Green I (6.5 μl; Fermentas; Thermo Fisher Scientific, Inc.) and nuclease-free water up to final volume for 40 cycles (Applied Biosystems 7500; Thermo Fisher Scientific, Inc.) at 95˚C for 15 sec followed by 60˚C for 1 min. For analyzing relative changes in mRNA levels, the $2^{-\Delta\Delta Cq}$ method was employed (23).

Statistical analysis. Statistical analysis of data was performed using SPSS 15.0 software (SPSS, Inc., Chicago, IL, USA) based on a minimum of three independent experiments. The results were normalized with those of the experimental controls, and were presented as the mean percentage ± standard error of the mean and analyzed by one-way analysis of variance followed by the Tukey's post hoc multiple group comparison test. A difference between groups was considered statistically significant when $P<0.05$.

Results

Isolation and culture of BMSCs. The results demonstrated that, after a 24-h culture, BMSCs successfully attached to the dish surface. When adhered, the BMSCs exhibited fibroblast-like or triangular shape (Fig. 1A). Following replacement of the medium, the majority of non-adherent cells were eliminated and adherent cells gradually proliferated. After 3-5 days, adherent cells formed clones (Fig. 1B). At 5 days the clones enlarged and fused with other clones. After 10 days, the adherent cells became confluent and could be subcultured. At 2 h after subculture the majority of BMSCs adhered, and after 3-4 days cells were confluent. The BMSCs were identified to express CD90, which served to identify cells as MSCs (Fig. 1C and D), at a positive expression rate of 97.30±0.68% (data not shown).

Induction of NSCs. Following culture in neural stem cell expansion medium, a number of the BMSCs proliferated into floating spherical aggregates, deemed as neurospheres with multipolar processes (Fig. 1E). On assessment of spheroid diameter (Fig. 1F-H), diameters at day 7 (325.61±9.09 μm) were significantly increased compared with those at 3 h (32.92±1.47 μm), 24 h (115.59±4.88 μm) and 4 days (134.69±4.21 μm; $P<0.05$; Fig. 2A). The neurospheres were passaged and induced to differentiate by neural stem cell expansion medium containing 5% FBS following plating on poly-L-lysine-coated adherent plates. To confirm the induced NSCs, the P3 cells were subjected to immunostaining analysis. The B-dNSCs from neurospheres expressed a high level of nestin (Fig. 2C) at a positive immunostaining rate of 98.00±0.35% (data not shown).

Dose response and cell viability. To evaluate the protective effect of adenosine against H_2O_2-induced cytotoxicity, B-dNSCs were treated with various concentrations of adenosine (2, 4, 6, 8 and 10 μM) for 48 h prior to H_2O_2 exposure. Cell viability was then evaluated by MTT assay (Fig. 2B). The pretreatment of B-dNSCs with adenosine increased cell viability in an apparent dose dependent manner, with the exception of 6 μM adenosine, which induced the greatest cell viability (90.02±0.24%) relative to the other concentrations tested, to a significant extent compared with 2, 4 and 8 μM adenosine ($P<0.05$). Therefore, 6 μM adenosine was selected as a suitable dose for further studies.

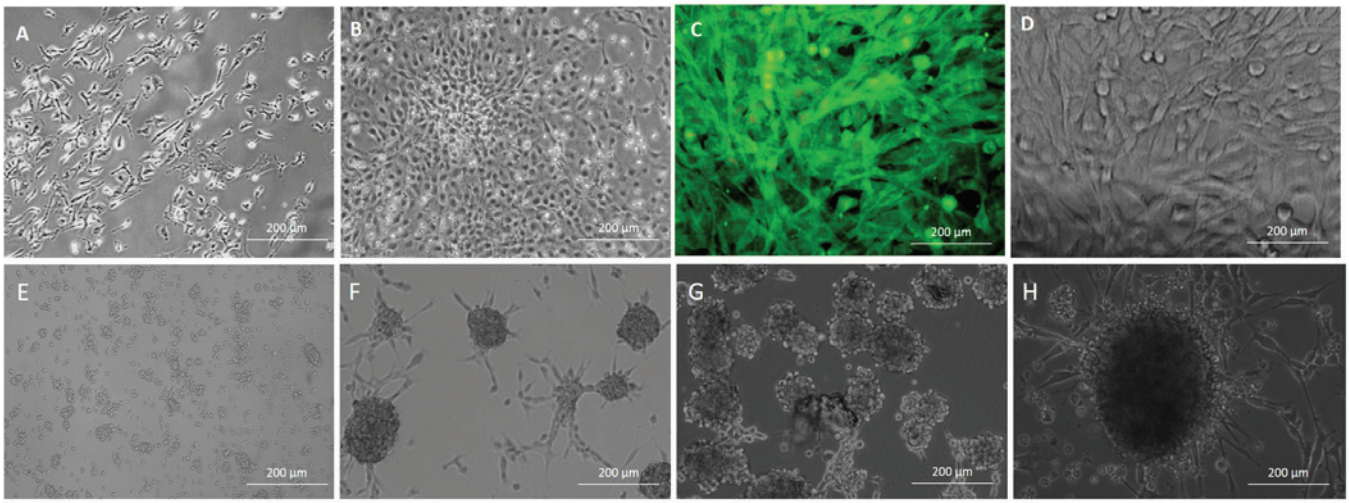

Figure 1. Representative photomicrographs of BMSCs in culture and floating neurosphere-derived BMSCs in suspension culture. (A) Cell attachments of freshly extracted BMSCs at 24 h; (B) colonies of BMSCs at day 5; (C) Immunostaining of cluster of differentiation 90 (marker for mesenchymal stem cells; green color indicates positive cells); (D) phase-contrast micrographs of the same field of view in (C); (E-H) Sequential formation of primary and clonal neurospheres at 3 and 24 h and days 4 and 7. Magnification, x200. BMSCs, bone marrow stromal cells.

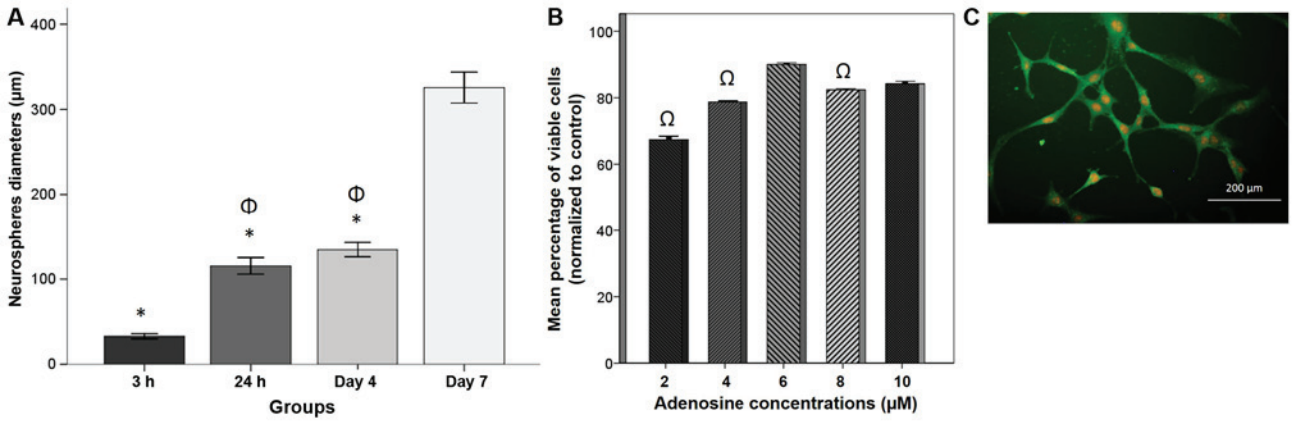

Figure 2. Neurosphere diameter and MTT assay. (A) Neurosphere diameters at different time points. (B) MTT assay graph representing the dose-response effect of adenosine pretreatment on B-dNSC viability in the presence of 125 μM H_2O_2. Results represent the mean percentage viability (relative to untreated B-dNSCs). (C) Immunostaining of nestin (marker for neural stem/progenitor cells). Magnification, x200. Primary antibody was labeled with fluorescein isothiocyanate-conjugated secondary antibody (green color indicates positive cells), while nuclei were counterstained with ethidium bromide. The bars indicate the mean ± standard error of the mean from 5 replicates. $^{\Phi}$P<0.05 vs. 3 h; $^{\Omega}$P<0.05 vs. 6 μM; *P<0.05 vs. day 7. B-dNSC, bone marrow-derived neural stem cells; H_2O_2, hydrogen peroxide.

TUNEL assay. A TUNEL assay was performed to determine the antiapoptotic effect of adenosine in B-dNSCs treated with H_2O_2. As depicted in Fig. 3A-D, TUNEL-positive nuclei staining demonstrated that H_2O_2 induced apoptotic cell death. The percentage of TUNEL-positive cells was significantly decreased in the NAH group compared with that in the NH group (P<0.05; Fig. 3E). The mean percentages of TUNEL positive cells in the N, NA, NH and NAH groups were 5.47±0.48, 9.43±0.45, 53.19±1.12 and 30.07±1.16%, respectively.

Gene expression. The changes in expression of *Bcl-2*, *Nrf2*, *Mst1* and *AA1R* in the experimental groups were examined using real-time RT-qPCR. The results were presented relative to the NH and N groups individually (Fig. 3F-G). In the groups treated with adenosine (relative to the NH group), all genes exhibited increased expression except *Mst1* and *AAR1*. The mean fold-changes relative to the NH group were as follows: For *Mst1*, NA: 0.20±0.04, NAH: 0.03±0.00; for *Bcl-2*, NA: 0.85±0.04, NAH: 1.08±0.05; for *Nrf2*, NA: 2.37±0.37.4, NAH: 1.35±0.15; and for *AA1R*, NA: 0.20±0.04, NAH: 8.50±0.57 (Fig. 3F).

The mean values of fold-change in the *Bcl-2*, *Nrf2*, *Mst1* and *AA1R* genes relative to levels in the N group are presented in Fig. 3G. mRNA expression of proapoptotic *Mst1* in the NH group was significantly upregulated compared with that in the NAH and NA groups (P<0.05). Additionally, the relative expression ratio of *AA1R* was significantly increased in the NAH and NA groups compared with that in the NH group (P<0.05). The mean fold-changes relative to the N group were as follows: For *Mst1*, NH: 1.91±0.09, NA: 0.60±0.09, NAH: 0.06±0.01; for *Bcl-2*, NH: 0.92±0.02, NA: 0.75±0.03, NAH: 0.95±0.05; for *Nrf2*, NH: 0.12±0.00, NA: 0.20±0.02, NAH: 0.13±0.01; and for *AA1R*, NH: 1.36±0.05, NA: 3.27±0.17, NAH: 3.27±0.27 (Fig. 3G).

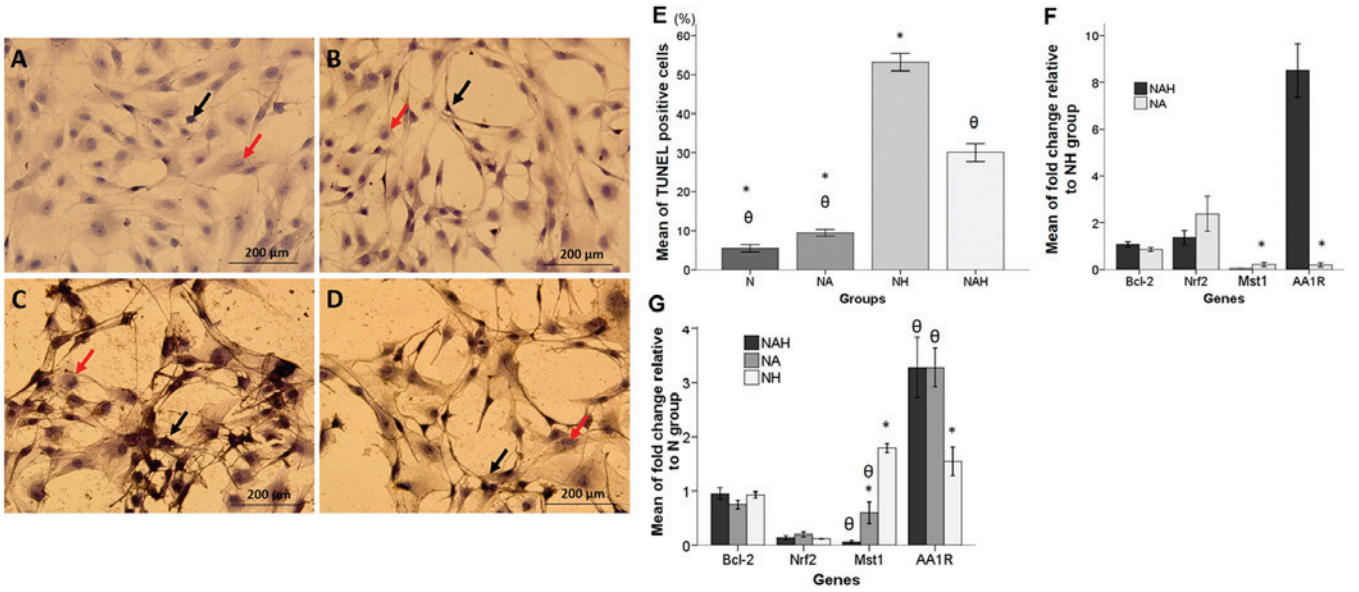

Figure 3. TUNEL assay and real-time RT-qPCR results. (A-D) Detection of apoptosis by TUNEL assay in the N, NA, NH and NAH experimental groups. TUNEL staining was performed in B-dNSCs exposed to 6 μM adenosine for 48 h then 125 μM H_2O_2 for 30 min. Photomicrographs are shown indicating the (A) B-dNSCs without treatment (N group); (B) NA group; (C) NH group; and (D) NAH group. Black and red arrows indicate TUNEL-positive and non-fragmented nuclei, respectively. (E) Histogram of the mean percentages of apoptotic cells in the experimental groups. (F and G) RT-qPCR results (F) relative to the NH group and (G) relative to the N group. mRNA level is presented as relative expression normalized to *GAPDH* mRNA amplification. The bars indicate the mean ± standard error of the mean. $^{\theta}$P<0.05 vs. NH group; *P<0.05 vs. NAH group. Magnification, x200. TUNEL, terminal deoxynucleotidyl transferase dUTP nick-end labeling; B-dNSCs, bone marrow-derived neural stem cells; N, untreated B-dNSCs; NA, B-dNSCs treated with 6 μM adenosine; NH, B-dNSCs treated with 125 μM H_2O_2; NAH, B-dNSCs pretreated with 6 μM adenosine + 125 μM H_2O_2; Bcl-2, B-cell lymphoma 2; Nrf2, nuclear factor (erythroid-derived 2)-like 2; Mst1, mammalian sterile 20-like kinase 1; AA1R, adenosine A1 receptor; H_2O_2, hydrogen peroxide; RT-qPCR, reverse transcription-quantitative polymerase chain reaction.

Mst1 and Nrf2 protein expression. To determine the neuroprotective effect of adenosine, Mst1 and Nrf2 protein expression was detected by immunocytochemistry (Figs. 4 and 5). The percentages of Nrf2 and Mst1 positive cells were calculated in five microscopic fields (Fig. 4A and B). The control group (N) exhibited Mst1 and Nrf1 positive cells at rates of 9.75±1.08 and 23.82±1.31%, respectively. By contrast, a significantly increased percentage of *Mst1* positive cells (26.4±1.56%) was identified in the NH group compared with the NAH group (P<0.05). The percentage of *Mst1* positive cells was significantly decreased in the NAH group (18.46±1.17%) compared with NH group (P<0.05). Additionally, the percentage of Nrf2 positive cells was significantly increased in the NA group (45.56±1.67) compared with the NH group (31.1±1.92; P<0.05; Fig. 5).

Discussion

The results of the present study indicate that adenosine upregulates antiapoptotic *Bcl-2* and *Nrf2* and downregulates *Mst1* in B-dNSCs, and thus protects the cells against H_2O_2-induced apoptosis. Additionally, adenosine may increase the mRNA expressions of *AA1R*, as one of the adenosine receptors activated in NSCs. The results further demonstrated that adenosine attenuated the apoptotic and necrotic effects of H_2O_2 at the cellular level. While adenosine is currently only used to treat cardiac arrhythmias, research increasingly suggests various combinations of novel clinical applications of this drug (24,25).

The current study reports a novel cytoprotective mechanism of adenosine involving *Nrf2* and *Bcl-2*-mediated induction of oxidative stress-related proteins. Oxidative damage is among the causes of neurodegenerative diseases (26). Increased production of cellular oxidants and defective protective mechanisms against oxidants are two major factors in the development of oxidative damage (21). However, physiological concentrations of free radicals in cells are essential for normal function and have key importance in the regulation of signaling pathways (14) including those associated with antiapoptotic transcription of genes and DNA repair proteins (17). The main targets of ROS during oxidative stress are considered to be DNA, RNA, proteins and lipids (22). In apoptosis, a series of events occurs systematically and repeatedly. The process is regulated by interventional genes including caspases and Fas-associated protein with death domain, and also molecular systems including Bcl-2/Bcl-2-associated X protein and Fas/Fas ligand (27). Therefore, interference in antiapoptotic and proapoptotic gene expression may be used to establish alterations in apoptosis (23).

Studies have indicated that adenosine may protect stem cells against oxidative damage and thus reduce apoptosis in these cells (28,29). The results of the current study confirmed previous findings regarding the protective effect of adenosine (30). The concept that purines, including ATP, ADP and adenosine, serve as extracellular signaling molecules was first suggested in 1929 by DeMaagd and Philip (31). Depending on the specific receptor activated, extracellular purines mediate biological functions including transmission of nerve impulses, muscle contraction, secretion from endocrine and exocrine glands and inflammation (31). Zhai *et al* (32) demonstrated that the protein level of *AA1R* was significantly increased in an intracerebral hemorrhage condition, while

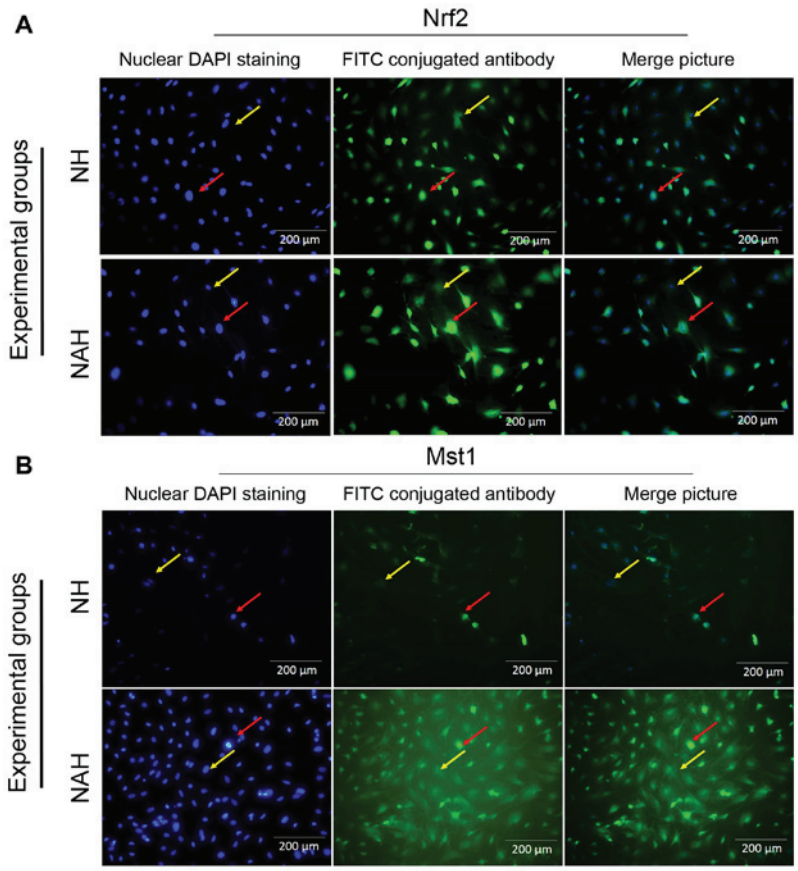

Figure 4. Nrf2 and Mst1 protein expression. Representative immunostaining photomicrographs are shown of (A) Nrf2 and (B) Mst1 immunoreactivity in the NH and NAH experimental groups after 48 h of treatments. Red and yellow arrows indicate immunopositive and negative cells, respectively. The Nrf2 and Mst1 protein marker was FITC-conjugated secondary antibody (green) and nuclei were counterstained with DAPI (blue). Magnification, x200. Nrf2, nuclear factor (erythroid-derived 2)-like 2; Mst1, mammalian sterile 20-like kinase 1; H_2O_2, hydrogen peroxide; NH, B-dNSCs treated with 125 μM H_2O_2; NAH, B-dNSCs pretreated with 6 μM adenosine + 125 μM H_2O_2; FITC, fluorescein isothiocyanate.

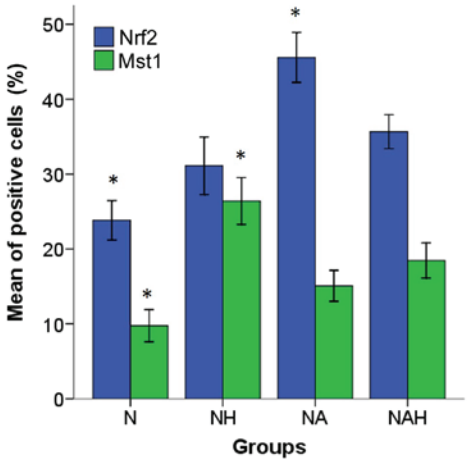

Figure 5. Histogram of the mean percentages of cells positive for Nrf2 and Mst1 protein staining in the N, NA, NH and NAH experimental groups. The bars indicate the mean ± standard error of the mean. *P<0.05 vs. NAH group. N, untreated B-dNSCs; NA, B-dNSCs treated with 6 μM adenosine; NH, B-dNSCs treated with 125 μM H_2O_2; NAH, B-dNSCs pretreated with 6 μM adenosine + 125 μM H_2O_2; Nrf2, nuclear factor (erythroid-derived 2)-like 2; Mst1, mammalian sterile 20-like kinase 1; H_2O_2, hydrogen peroxide.

there was no significant change in the protein levels of the other adenosine receptors. Furthermore, activation of *AA1R* attenuates neuronal apoptosis in cortical neurons (7). The present results demonstrated that treatment with adenosine during oxidative damage caused overexpression of the antiapoptotic *Bcl-2* and *Nrf2* genes, as well as increased expression of *AA1R* and decreased expression of proapoptotic *Mst1*.

Mst1, also known as serine/threonine kinase 4, is a stress-activated, proapoptotic kinase that is involved in the Hippo pathway (33). Despite extensive studies, the mechanisms underlying the regulation of apoptosis by *Mst1* are not well understood, though *Mst1*-induced caspase-3 cleavage may be involved (34). It has also been suggested that increased expression of *Mst1* initiates apoptosis through activation of p53, but the molecular mechanisms are not fully understood (35). The *Nrf2* gene affects cell proliferation, cell growth and regulation of cell metabolism via the phosphatidylinositol-3-kinase/Akt pathway (36). Sarkar *et al* demonstrated that *Nrf2* enhanced the expression of anti-apoptotic *Bcl-2* (37). Pingle *et al* reported that *AA1R*, protects against human immunodeficiency virus type 1 toxicity by inhibiting nu clear factor-κB and thereby reducing the expression of inducible nitric oxide (NO) synthase and NO radicals and neuronal apoptosis (38).

In addition, the use of AA1R agonist has been reported to lead to upregulation of *Bcl-2* and *Nrf2* gene expression (39). *Nrf2* expression also leads to increased expression of mitochondrial transcription factor A, as a key regulator of antioxidant signaling (40). The current results indicated that Nrf2-mediated upregulation of antiapoptotic protein Bcl-2 may lead to

decreased apoptotic cell death and increased cell survival in response to H_2O_2 exposure. *Nrf2* is a transcription factor that controls the expression of a variety of antioxidant genes (41). A previous study demonstrated that *Nrf2* was retained in the cytoplasm by the inhibitor kelch-like ECH-associated protein 1, which functions as an adapter for cullin 3/ring-box 1-mediated degradation of *Nrf2* (42). The expression of the mitochondrial protein uncoupling protein 3 is driven by the *Nrf2* transcription factor, which decreases ROS production and prevents cell death (43). Additionally, *Nrf2* may increase the expression of certain antioxidant enzymes, including heme oxygenase-1, NAD(P)H:quinone oxidoreductase 1 and manganese superoxide dismutase (44). Taken together, the *Nrf2* transcription factor may control the expression of a number of protective genes in response to oxidative stress (45). Thus, according to studies on adenosine, manipulating the adenosine system as a novel strategy for the management of brain disorders may have promise (31). However, to achieve successful clinical application in the treatment of brain disorders, further studies are required to evaluate the use of adenosine and its receptor agonists as neuroprotective drugs.

In conclusion, the results of the current study demonstrated that adenosine, as a receptor-specific agonist of *AA1R*, protected NSCs derived from BMSCs against oxidative damage. Adenosine can cross the blood-brain barrier and therefore may be considered as a suitable drug for the treatment of diseases of the nervous system caused by oxidative stress.

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Availability of data and materials

All primary data are archived at the Zanjan University of Medical Sciences and available on request.

Authors' contributions

MG performed the experiments. IJA acted as study advisor and was responsible for technical aspects in the immunocytochemistry. AT is the co-corresponding author, analyzed data regarding gene expression, was responsible for primer design and supervised the project. AA designed and directed the project, supervised the project, monitored experiments, is the co-corresponding author and wrote the manuscript. All authors discussed the results and contributed to the final manuscript.

Ethics approval and consent to participate

All experimental protocols were approved by the Zanjan University of Medical Sciences Ethics Committee.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References

1. Yeh LK, Liu CY, Chien CL, Converse RL, Kao WW, Chen MS, Hu FR, Hsieh FJ and Wang IJ: Molecular analysis and characterization of zebrafish keratocan (zKera) gene. J Biol Chem 283: 506-517, 2008.
2. Chen X, Guo C and Kong J: Oxidative stress in neurodegenerative diseases. Neural Regen Res 7: 376-385, 2012.
3. Uttara B, Singh AV, Zamboni P and Mahajan RT: Oxidative stress and neurodegenerative diseases: A review of upstream and downstream antioxidant therapeutic options. Curr Neuropharmacol 7: 65-74, 2009.
4. Liu Z, Liu Y, Gao R, Li H, Dunn T, Wu P, Smith RG, Sarkar PS and Fang X: Ethanol suppresses PGC-1α expression by interfering with the cAMP-CREB pathway in neuronal cells. PLoS One 9: e104247, 2014.
5. Cunha RA: Adenosine as a neuromodulator and as a homeostatic regulator in the nervous system: Different roles, different sources and different receptors. Neurochem Int 38: 107-125, 2001.
6. Xu L, Xu H, Cao Y, Yang P, Feng Y, Tang Y, Yuan S and Ming J: Validation of reference genes for quantitative real-time PCR during bicolor tepal development in asiatic hybrid lilies (*Lilium* spp.). Front Plant Sci 8: 669, 2017.
7. Gorman AM: Neuronal cell death in neurodegenerative diseases: Recurring themes around protein handling. J Cell Mol Med 12 (6A): 2263-2280, 2008.
8. Gibson RM: Does apoptosis have a role in neurodegeneration? BMJ 322: 1539-1540, 2001.
9. Mattson MP: Apoptosis in neurodegenerative disorders. Nat Rev Mol Cell Biol 1: 120-129, 2000.
10. Abdanipour A, Tiraihi T and Mirnajafi-Zadeh J: Improvement of the pilocarpine epilepsy model in rat using bone marrow stromal cell therapy. Neurol Res 33: 625-632, 2011.
11. Racine RJ: Modification of seizure activity by electrical stimulation. II. Motor seizure. Electroencephalogr Clin Neurophysiol 32: 281-294, 1972.
12. Collak FK, Yagiz K, Luthringer DJ, Erkaya B and Cinar B: Threonine-120 phosphorylation regulated by phosphoinositide-3-kinase/Akt and mammalian target of rapamycin pathway signaling limits the antitumor activity of mammalian sterile 20-like kinase 1. J Biol Chem 287: 23698-23709, 2012.
13. Ardestani A, Paroni F, Azizi Z, Kaur S, Khobragade V, Yuan T, Frogne T, Tao W, Oberholzer J, Pattou F, *et al*: MST1 is a key regulator of beta cell apoptosis and dysfunction in diabetes. Nat Med 20: 385-397, 2014.
14. Ura S, Masuyama N, Graves JD and Gotoh Y: MST1-JNK promotes apoptosis via caspase-dependent and independent pathways. Genes Cells 6: 519-530, 2001.
15. Strom J, Xu B, Tian X and Chen QM: Nrf2 protects mitochondrial decay by oxidative stress. FASEB J 30: 66-80, 2016.
16. Murata H, Takamatsu H, Liu S, Kataoka K, Huh NH and Sakaguchi M: NRF2 regulates PINK1 expression under oxidative stress conditions. PLoS One 10: e0142438, 2015.
17. Zhong J and Li L: Skin-derived precursors against UVB-induced apoptosis via Bcl-2 and Nrf2 upregulation. Biomed Res Int 2016: 6894743, 2016.
18. Maleki M, Ghanbarvand F, Reza Behvarz M, Ejtemaei M and Ghadirkhomi E: Comparison of mesenchymal stem cell markers in multiple human adult stem cells. Int J Stem Cells 7: 118-126, 2014.
19. Yang Q, Mu J, Li Q, Li A, Zeng Z, Yang J, Zhang X, Tang J and Xie P: A simple and efficient method for deriving neurospheres from bone marrow stromal cells. Biochem Biophys Res Commun 372: 520-524, 2008.
20. Suzuki S, Namiki J, Shibata S, Mastuzaki Y and Okano H: The neural stem/progenitor cell marker nestin is expressed in proliferative endothelial cells, but not in mature vasculature. J Histochem Cytochem 58: 721-730, 2010.

21. Albrecht-Küpper BE, Leineweber K and Nell PG: Partial adenosine A1 receptor agonists for cardiovascular therapies. Purinergic Signal 8 (Suppl 1): 91-99, 2012.
22. Abbasnia K, Ghanbari A, Abedian M, Ghanbari A, Sharififar S and Azari H: The effects of repetitive transcranial magnetic stimulation on proliferation and differentiation of neural stem cells. Anat Cell Biol 48: 104-113, 2015.
23. Lee JH, Cheon YH, Woo RS, Song DY, Moon C and Baik TK: Evidence of early involvement of apoptosis inducing factor-induced neuronal death in Alzheimer brain. Anat Cell Biol 45: 26-37, 2012.
24. Mori M, Nishizaki T and Okada Y: Protective effect of adenosine on the anoxic damage of hippocampal slice. Neuroscience 46: 301-307, 1992.
25. Cunha RA: Neuroprotection by adenosine in the brain: From A(1) receptor activation to A (2A) receptor blockade. Purinergic Signal 1: 111-134, 2005.
26. Migita H, Kominami K, Higashida M, Maruyama R, Tuchida N, McDonald F, Shimada F and Sakurada K: Activation of adenosine A1 receptor-induced neural stem cell proliferation via MEK/ERK and Akt signaling pathways. J Neurosci Res 86: 2820-2828, 2008.
27. Elmore S: Apoptosis: A review of programmed cell death. Toxicol Pathol 35: 495-516, 2007.
28. Huang NK: Adenosine A2A receptors regulate oxidative stress formation in rat pheochromocytoma PC12 cells during serum deprivation. Neurosci Lett 350: 127-131, 2003.
29. Ramkumar V, Hallam DM and Nie Z: Adenosine, oxidative stress and cytoprotection. Jpn J Pharmacol 86: 265-274, 2001.
30. Vitolo OV, Ciotti MT, Galli C, Borsello T and Calissano P: Adenosine and ADP prevent apoptosis in cultured rat cerebellar granule cells. Brain Res 809: 297-301, 1998.
31. DeMaagd G and Philip A: Parkinson's disease and its management: Part 1: Disease entity, risk factors, pathophysiology, clinical presentation, and diagnosis. P T 40: 504-532, 2015.
32. Zhai W, Chen D, Shen H, Chen Z, Li H, Yu Z and Chen G: A1 adenosine receptor attenuates intracerebral hemorrhage-induced secondary brain injury in rats by activating the P38-MAPKAP2-Hsp27 pathway. Mol Brain 9: 66, 2016.
33. Meng Z, Moroishi T and Guan KL: Mechanisms of Hippo pathway regulation. Genes Dev 30: 1-17, 2016.
34. Wrann CD, White JP, Salogiannnis J, Laznik-Bogoslavski D, Wu J, Ma D, Lin JD, Greenberg ME and Spiegelman BM: Exercise induces hippocampal BDNF through a PGC-1α/FNDC5 pathway. Cell Metab 18: 649-659, 2013.
35. Youdim MB: The path from anti Parkinson drug selegiline and rasagiline to multifunctional neuroprotective anti Alzheimer drugs ladostigil and m30. Curr Alzheimer Res 3: 541-550, 2006.
36. Alexander GE: Biology of Parkinson's disease: Pathogenesis and pathophysiology of a multisystem neurodegenerative disorder. Dialogues Clin Neurosci 6: 259-280, 2004.
37. Sarkar S, Raymick J and Imam S: Neuroprotective and therapeutic strategies against Parkinson's disease: Recent Perspectives. Int J Mol Sci 17: 17, 2016.
38. Pingle SC, Jajoo S, Mukherjea D, Sniderhan LF, Jhaveri KA, Marcuzzi A, Rybak LP, Maggirwar SB and Ramkumar V: Activation of the adenosine A1 receptor inhibits HIV-1 tat-induced apoptosis by reducing nuclear factor-kappaB activation and inducible nitric-oxide synthase. Mol Pharmacol 72: 856-867, 2007.
39. Han J, Talorete TP, Yamada P and Isoda H: Anti-proliferative and apoptotic effects of oleuropein and hydroxytyrosol on human breast cancer MCF-7 cells. Cytotechnology 59: 45-53, 2009.
40. Zhang Z, Zhou S, Jiang X, Wang YH, Li F, Wang YG, Zheng Y and Cai L: The role of the Nrf2/Keap1 pathway in obesity and metabolic syndrome. Rev Endocr Metab Disord 16: 35-45, 2015.
41. Ishikawa T: Genetic polymorphism in the NRF2 gene as a prognosis marker for cancer chemotherapy. Front Genet 5: 383, 2014.
42. Nuydens R, Dispersyn G, Van Den Keiboom G, de Jong M, Connors R, Ramaekers F, Borgers M and Geerts H: Bcl-2 protects against apoptosis-related microtubule alterations in neuronal cells. Apoptosis 5: 43-51, 2000.
43. Anedda A, López-Bernardo E, Acosta-Iborra B, Saadeh Suleiman M, Landázuri MO and Cadenas S: The transcription factor Nrf2 promotes survival by enhancing the expression of uncoupling protein 3 under conditions of oxidative stress. Free Radic Biol Med 61: 395-407, 2013.
44. Jeong YH, Park JS, Kim DH and Kim HS: Lonchocarpine increases Nrf2/ARE-mediated antioxidant enzyme expression by modulating AMPK and MAPK signaling in brain astrocytes. Biomol Ther (Seoul) 24: 581-588, 2016.
45. Birben E, Sahiner UM, Sackesen C, Erzurum S and Kalayci O: Oxidative stress and antioxidant defense. World Allergy Organ J 5: 9-19, 2012.