Donepezil delays photoreceptor apoptosis induced by N-methyl-N-nitrosourea in mice

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Abstract. Retinitis pigmentosa (RP) is a group of inherited retinal degeneration diseases characterized by photoreceptor cell death that causes visual disturbances and eventual blindness. Intraperitoneal injection of N-methyl-N-nitrosourea (MNU) causes photoreceptor loss, and is used to create an animal model for investigating the mechanisms that cause retinal degeneration diseases. Donepezil is an acetylcholinesterase inhibitor that has a protective effect on retinal ganglion cells *in vitro* and *in vivo,* and it is understood that donepezil increases the expression of a heat shock protein 70 (Hsp70), which serves to protect neurons. Hsp70 functions as a chaperone molecule that protects cells from protein aggregation and assists in the refolding of denatured proteins. In the present study, the effects of donepezil on photoreceptor survival in mice was investigated. It was observed that donepezil upregulates the expression of Hsp70, to increase resistance to MNU-induced photoreceptor cell apoptosis by using its anti-apoptotic properties. In addition, the present study observed that Hsp70 promotes photoreceptor cell survival by upregulating the expression levels of B-cell lymphoma 2 (Bcl-2). In conclusion, the results of the present study indicate that donepezil has the potential to be used as a treatment for retinal degenerative diseases.

Introduction

Typically, genetic mutations are closely associated with retinal degenerative diseases in humans, such as retinitis pigmentosa (RP) which are a group of inherited retinal dystrophic diseases that cause retinal cell apoptosis and irreversible loss of vision (1,2). Although it is known that >50 million individuals worldwide have been affected by retinal degenerative diseases since the 1980's (3), there is currently no effective treatment method, due to the lack of understanding of their complex genetic mechanisms (4). As has been reported in a mouse model, photoreceptor cell apoptosis is a common end stage in retinal degeneration, although the detailed mechanisms of this process remain unclear (5). N-methyl-N-nitrosourea (MNU) is a strong alkylating agent that belongs to the nitrose compounds of nitrosamine, and may induce photoreceptor cell apoptosis with high selectivity and repeatability. As a result, it is frequently used to construct animal models of RP (6,7).

Donepezil is one of the second generation specific reversible central acetylcholinesterase (AChE) inhibitors, and is widely used for improving cognitive performance and global function in patients with moderate Alzheimer's disease (8). Numerous studies have demonstrated that donepezil serves a protective role in cortical neurons by acting on nicotinic acetylcholine receptors (AChR) or the toadstool cholinergic receptor pathway (9,10). In addition, research has suggested that donepezil exerts a protective effect on retinal ganglion cells (RGCs) *in vitro* and *in vivo* (11). However, Miki *et al* (12) reported that a nicotinic AChR (nAChR) inhibitor and a toadstool cholinergic receptor inhibitor did not offset the neuroprotective effect of donepezil. This implies that the precise neuroprotective mechanisms of donepezil are complex and remain controversial. Therefore, more research is required to determine the effect and mechanisms of donepezil acting on on photoreceptor cells (13). It has been demonstrated that AChE inhibitors lead to increased mRNA expression levels of heat shock protein 70 (Hsp70), resulting in enhanced cellular defenses in neurons (14). Therefore, it can be hypothesized that one of the putative neuroprotective mechanisms of AChE inhibitors is mediated through Hsp70.

HSPs are highly conserved within their family, and are stress activated proteins that participate in protein folding and repair (15). The biological functions of HSPs include acting as a molecular chaperone, providing cell protection, anti-inflammatory properties, and preventing apoptosis and cell damage. In particular, the 70-kDa HSP, Hsp70, a molecular chaperone, serves a pivotal role in protecting cells against the stresses of various types and origins. Tytell *et al* (16) observed that Hsp70 mRNA and protein levels were significantly increased in rat retinal photoreceptor cell layers following whole body hyperthermia. The work by Tytell *et al* (16), for the first time, identified that the photoreceptor cell layer was an important part of the retina for the expression of Hsp70,

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and that Hsp70 induced by hyperthermia serves a protective role by applying its anti-apoptotic properties to photoreceptors against heat-induced damage. At present, the anti-apoptotic mechanisms of Hsp70 have been drawing increasing attention, since Hsp70 was shown to interfere with apoptosis by affecting cytochrome *c* release from mitochondria and initiator caspase activation (17-19). In addition, it has been determined that the release of cytochrome *c* can be blocked by the anti-apoptotic protein Bcl-2, which is heavily involved in cell survival (20). Kelly *et al* (21) observed that viral vector-mediated Hsp70 gene transfer can increase B-cell lymphoma 2 (Bcl-2) expression in neurons in rat brains. These studies suggest that the anti-apoptotic effects of HSP 70 are closely associated with the expression of Bcl-2. In the present study, the effect of donepezil on MNU-induced photoreceptor cell apoptosis in mice was investigated and the mechanisms behind this process were explored.

Materials and methods

Materials and reagents. MNU and donepezil were purchased from Sigma-Aldrich (St. Louis, MO, USA). HSP inhibitor I was purchased from Merck Millipore (Darmstadt, Germany), ABT-199 (a Bcl-2 inhibitor) was purchased from Selleck Chemicals (Houston, TX, USA) and the Apoptosis Detection kit was provided by BD Biosciences (San Jose, CA, USA).

Animals. A total of 168 male C57BL/6 mice (age, 7-9 weeks; weight, 20-22 g; Hunan Laboratory Animal Co., Ltd, Changsha, China) were housed under standard laboratory conditions, and provided with standard rodent chow and free access to water with a 12 h light-dark cycle at 23˚C. The present study was approved by the ethics committee of the Affiliated Eye Hospital of Nanchang University (Nanchang, China).

Morphological observation. In order to investigate the loss of photoreceptor cells induced by MNU, 30 mice were intraperitoneally injected with MNU (60 mg/kg in saline) and 6 mice per day were sacrificed by cervical dislocation after 0, 1, 3, 5 and 7 days. In addition, the ability of donepezil to protect against MNU-induced photoreceptor loss was investigated. Briefly, 24 mice were divided into four groups, as follows (6 mice/group): i) Control; ii) MNU; iii) donepezil plus MNU; and iv) Hsp70 inhibitor plus donepezil plus MNU. The mice in the Hsp70 inhibitor plus donepezil plus MNU group were anesthetized by intraperitoneal injection with sodium pentobarbital (30-40 mg/kg body weight; Shanghai Qiao Xing Trading, Co., Ltd., Shanghai, China), prior to intravitreal injection with 5 μl HSP inhibitor I using a Hamilton microsyringe (Hamilton Robotics, Reno, NV, USA) with a 33 G needle. At 1 day following HSP inhibitor I administration, the mice underwent oral gavage with donepezil (10 mg/kg body weight) for 3 consecutive days, followed by intraperitoneal injection with 60 mg/kg MNU.

Following sacrifice, the mouse eyes were harvested and fixed in 4% paraformaldehyde overnight at 4˚C, then dehydrated using alcohol, made transparent using xylene (Shanghai Sheng Jun Industrial Investment, Co., Ltd., Shanghai, China) and embedded in paraffin (Shanghai Yu Jie Trade, Co., Ltd., Shanghai, China). Retinal sections were cut along the optic nerve at 12 μm thickness and mounted on Superfrost Plus glass slides (Yancheng Hongda Medical Instrument Co., Ltd., Jiangsu, China). Tissue sections were then dehydrated with 75, 95 and 100% alcohol for 1 min each, and then with xylene for 5 min. Hematoxylin and eosin (H&E; Shanghai Blue Skies Biological Technology, Co., Ltd., Shanghai, China) staining of transverse sections was used to evaluate the thickness of the retina [photoreceptor inner segment (IS), outer nuclear layer (ONL), outer plexiform layer (OPL), inner nuclear layer (INL) or ganglion cell layer (GCL)] under a microscope (BX-53; Olympus Corporation, Tokyo, Japan).

Immunofluorescence. In order to investigate the effects of donepezil on the expression of Hsp70, 18 mice were randomly divided into three groups, as follows (6 mice/group): i) Control; ii) donepezil; and iii) donepezil plus Hsp70 inhibitor groups. The mice were anesthetized by intraperitoneal injection with sodium pentobarbital (30-40 mg/kg body weight) prior to intravitreal injection with HSP inhibitor I (5 μl) and oral gavage with donepezil (10 mg/kg body weight) for 3 consecutive days. Subsequent to washing and blocking with fetal bovine serum (Sigma-Aldrich), retinal sections were incubated with rabbit anti-mouse Hsp70 monoclonal antibodies (1:50; ab45133; Abcam, Cambridge, MA, USA) at 4˚C overnight. Following incubation, the sections were washed three times for 5 min each in the dark with phosphate-buffered saline. Subsequently, the sections were incubated with biotin-conjugated mouse anti-IgG-B monoclonal antibody (1:200; sc-2491; Santa Cruz Biotechnology, Inc., Dallas, TX, USA) for 1 h at 37˚C in the dark. A fluorescence microscope (BX-53; Olympus Corporation) was used to capture and measure the retinal sections two to three disc diameters from the optic nerve. Two areas per section of ONL were randomly selected and the fluorescence intensity was measured using ImageJ software 1.46 (National Institutes of Health, Bethesda, MD, USA).

Western blot analysis. In order to investigate the concentration-dependent effect of donepezil on the protein expression levels of Hsp70, 24 mice were randomly divided into four groups, according to the dose of donepezil administered, as follows (6 mice/group): i) Control; ii) 1 mg/kg donepezil; iii) 5 mg/kg donepezil; and iv) 10 mg/kg donepezil. The mice were treated with donepezil for 3 consecutive days. Furthermore, the time-dependent effect of donepezil treatment on the protein expression of Hsp70 was investigated by oral gavage with 10 mg/kg body weight donepezil for 1, 3 or 5 days. In addition, the effect of donepezil-induced Hsp70 expression on the protein expression levels of Bcl-2 were examined. Briefly, 24 mice were divided into four groups, as follows (6 mice/group): i) Control; ii) donepezil (10 mg/kg body weight); iii) donepezil plus Hsp70 inhibitor; and iv) donepezil plus Bcl-2 inhibitor. The mice in the donepezil plus Bcl-2 inhibitor group were orally gavaged with 100 mg/kg body weight ABT-199 for 3 consecutive days prior to treatment with 10 mg/kg donepezil. All mice were sacrificed by cervical dislocation.

The protein concentration in each sample was determined using a Bradford Protein Assay. The retinas were isolated and equal quantities of total protein from each sample were

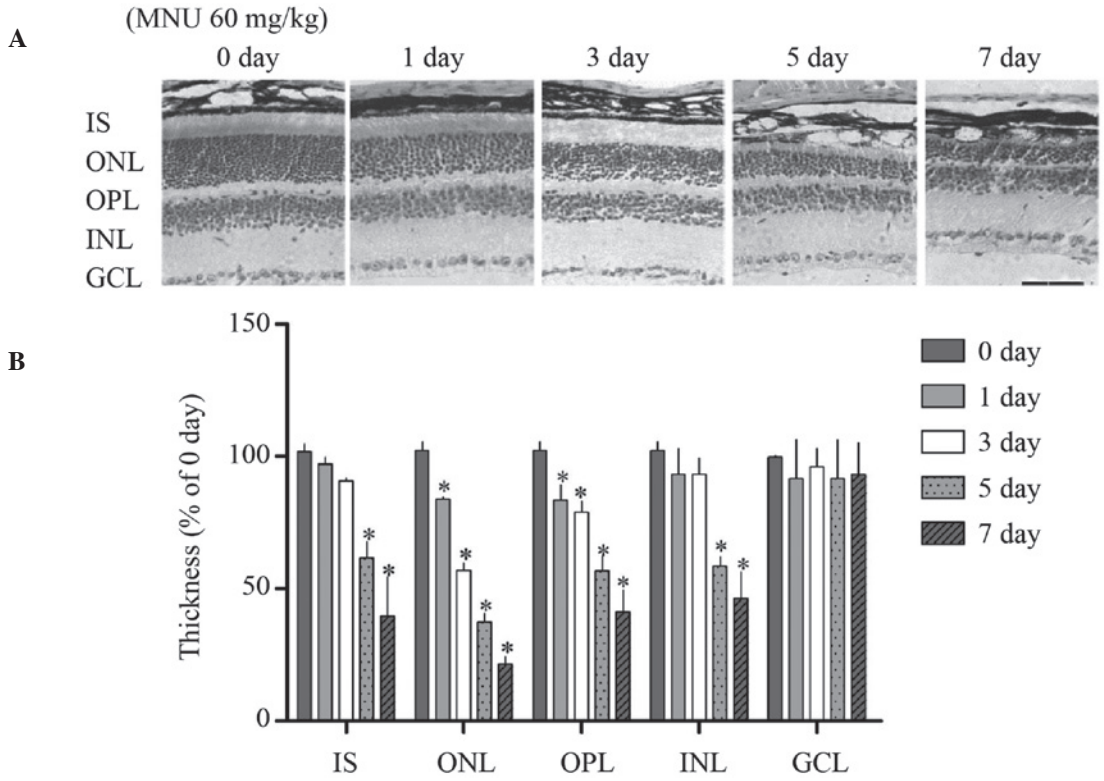

Figure 1. Loss of photoreceptor cells induced by MNU. (A) Hematoxylin and eosin staining of retinas following injection of MNU (60 mg/kg) after 0, 1, 3, 5 and 7 days. (B) Retinal degeneration was quantified by measuring the thickness of each layer of mouse retina. Scale bar = 50 μm. *P<0.01 vs. day 0 (n=6). MNU, N-methyl-N-nitrosourea; IS, photoreceptor inner segment; ONL, outer nuclear layer; OPL, outer plexiform layer; INL, inner nuclear layer; GCL, ganglion cell layer.

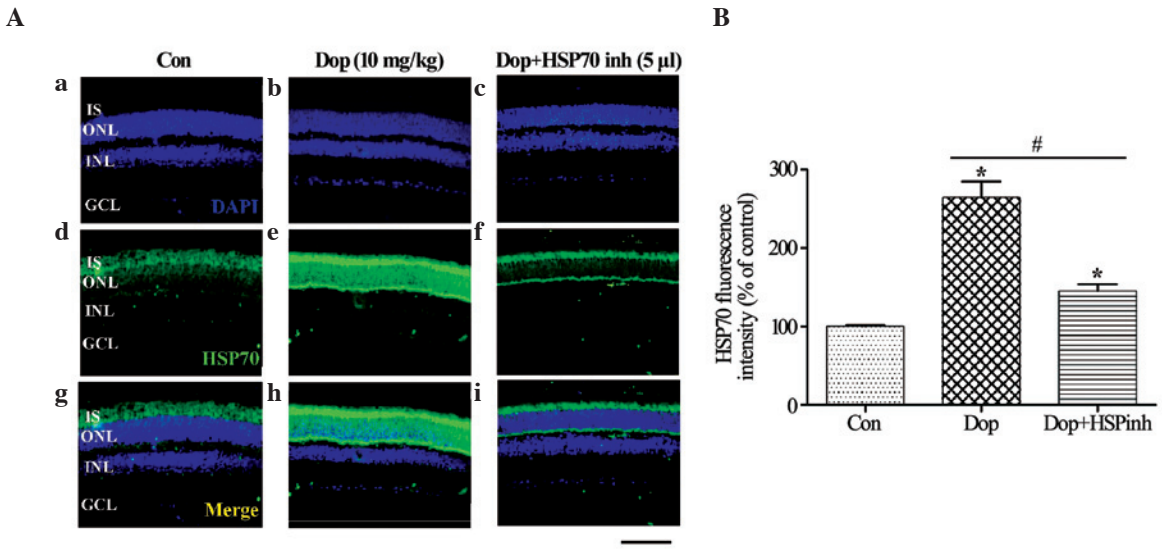

Figure 2. Influence of Dop on the expression levels of Hsp70 by immunofluorescence staining. (A) Immunohistochemistry for Hsp70 in the mouse retina. (Aa-c) DAPI (blue) staining, (Ad-f) Hsp70 (green) staining, (Ag-i) merged image of DAPI and Hsp70 [(a, d, g) vehicle Con, (b, e, h) pretreatment with 10 mg/kg body weight Dop for 3 days and (c, f, i) HSP inh + pretreatment with Dop for 3 days]. (B) Hsp70 expression quantified by analysis of fluorescence intensity. Scale bar, 50 μm, *P<0.01 vs. vehicle Con, #P<0.01 vs. donepezil. Con, control; Dop, donepezil; HSP inh, HSP inhibitor. HSP, heat shock protein; IS, photoreceptor inner segment; ONL, outer nuclear layer; OPL, outer plexiform layer; INL, inner nuclear layer; GCL, ganglion cell layer.

subjected to 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and then transferred onto a polyvinylidene fluoride membrane (Thermo Fisher Scientific, Inc., Waltham, MA, USA). Subsequent to blocking of nonspecific binding sites with 5% skimmed milk in Tris-buffered saline (TBS; pH 7.6) for 1 h, membranes were incubated with primary antibodies, as follows: Rabbit anti-Hsp70 monoclonal antibody (1:1,000; Abcam), rabbit anti-Bcl-2 polyclonal antibody (1:1,000; ab59348; Abcam) and rabbit anti-β-actin monoclonal antibody (1:1,000; ab8226; Abcam) overnight at 4˚C. Next, the membranes were washed

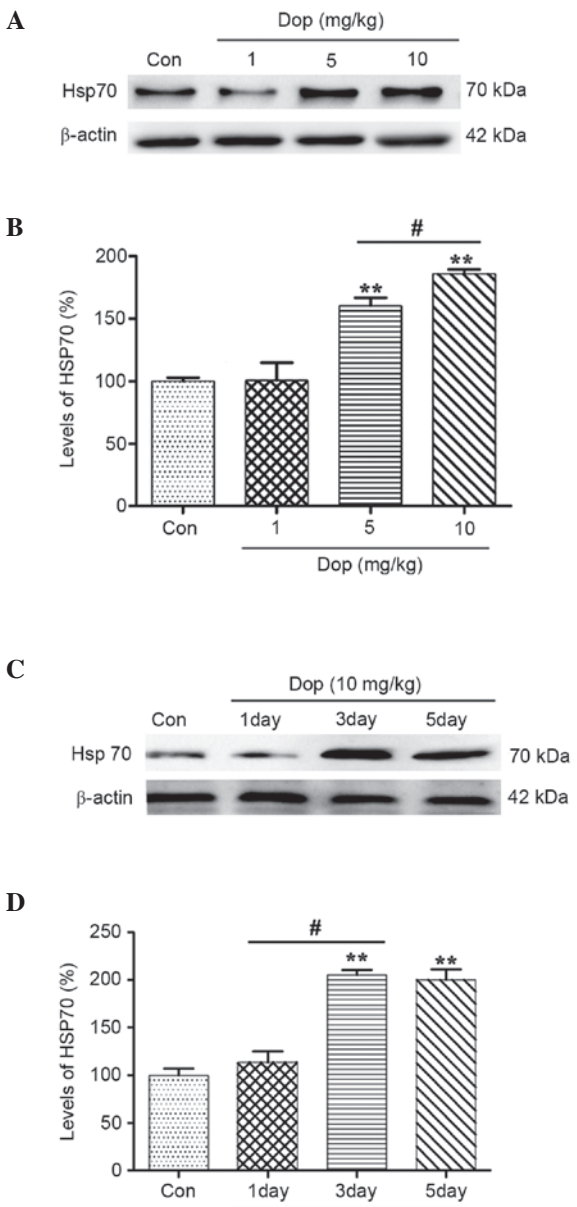

Figure 3. Time- and dose-dependent effects of Dop on the expression of Hsp70. (A) Western blot and (B) quantification of mice pre-treated with 1, 5 or 10 mg/kg Dop for 3 days. MNU was intraperitoneally injected following pretreatment with donepezil. (C) Western blot and (D) quantification of mice treated with 10 mg/kg Dop for 1, 3 or 5 days. **P<0.01 vs. vehicle con, $^{\#}$P<0.01 vs. donepezil. Dop, donepezil; Con, control.

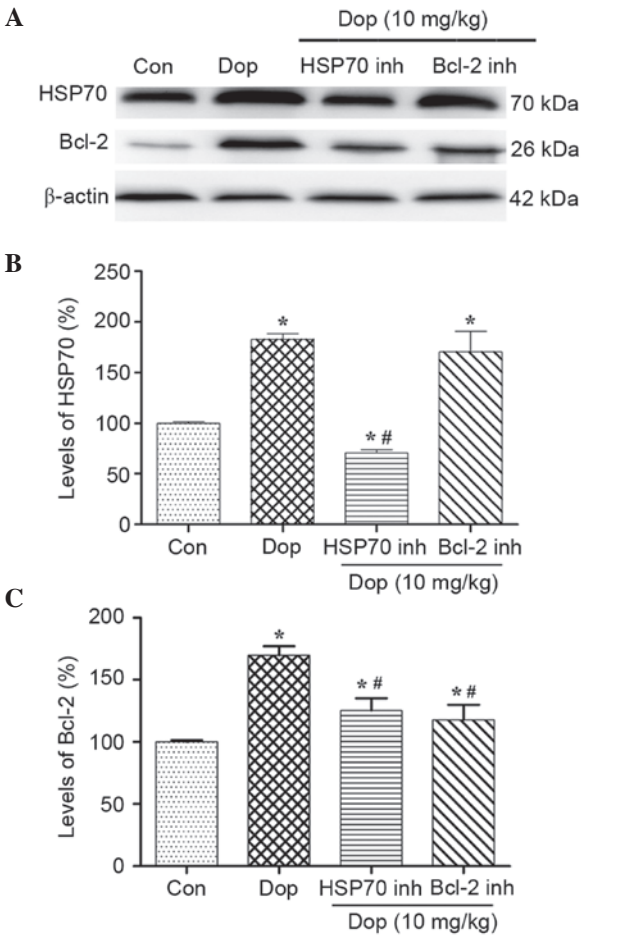

Figure 4. Expression levels of Bcl-2 were associated with Dop-induced Hsp70. (A) The expression levels of Hsp70 and Bcl-2 were analyzed by western blot analysis, in which β-actin was used as an internal control. HSP inh I was intravitreally injected 1 day prior to Dop treatment. Mice were orally gavaged with Bcl-2 inhibitor (100 mg/kg body weight) for 3 consecutive days prior to Dop treatment. (B) Graphical representation of protein expression levels of Hsp70. (C) Graphical representation of protein expression levels of Bcl-2. *P<0.01 vs. con, $^{\#}$P<0.01 vs. donepezil. Dop, donepezil; HSP70 inh, heat shock protein inhibitor; Con, control; Bcl-2 inh, B-cell lymphoma 2 inhibitor.

with 0.15% Tween 20 in TBS and incubated with horseradish peroxidase (HRP)-conjugated goat anti-rabbit (bs-0295G; BIOSS, Beijing, China) or biotin-conjugated rabbit anti-mouse (sc-358943; Santa Cruz Biotechnology, Inc.) secondary antibodies for 1 h at room temperature. After four washes, the proteins were detected using enhanced chemiluminescence (ECL kit, Thermo Fisher Scientific, Inc.). The bands on the gel were quantified using Quantity One software, version 4.6.2 (Bio-Rad Laboratories, Inc., Hercules, CA, USA). β-actin served as the internal control. All experiments were repeated a minimum of three times.

Flow cytometry. The ability of donepezil to protect against MNU-induced apoptosis of photoreceptor cells was investigated by flow cytometry. Following the morphological analysis, mouse eyes were enucleated, retinas were separated from retinal pigment epithelium and choroid and then rinsed immediately in D-Hank liquid (Beijing TransGen Biotech Co., Ltd., Beijing, China) twice. Tissue was cut using eye scissors, softly ground, and all large blood vessels were removed. The dispersed cells were filtered through a 300-mesh stainless steel sieve, and the cell suspension was cultured in an incubator at 37˚C with 25% CO_2 for 1 day. Next, 10 μl Annexin V phycoerythrin/7-aminoactinomycin (PE/7-AAD; BD Biosciences) was added into the cell suspension liquid at room temperature in the dark for 10 min. Finally, samples were analyzed using flow cytometry (FACSCalibur; Bio-Rad Laboratories, Inc.) within 1 h of staining.

Statistical analysis. Data are expressed as the mean ± standard deviation of three to five experiments. Statistical differences were analyzed by Student's t-test using SPSS Software version 17.0 (SPSS, Inc., Chicago, IL, USA). P<0.05 was considered to indicate a statistically significant difference.

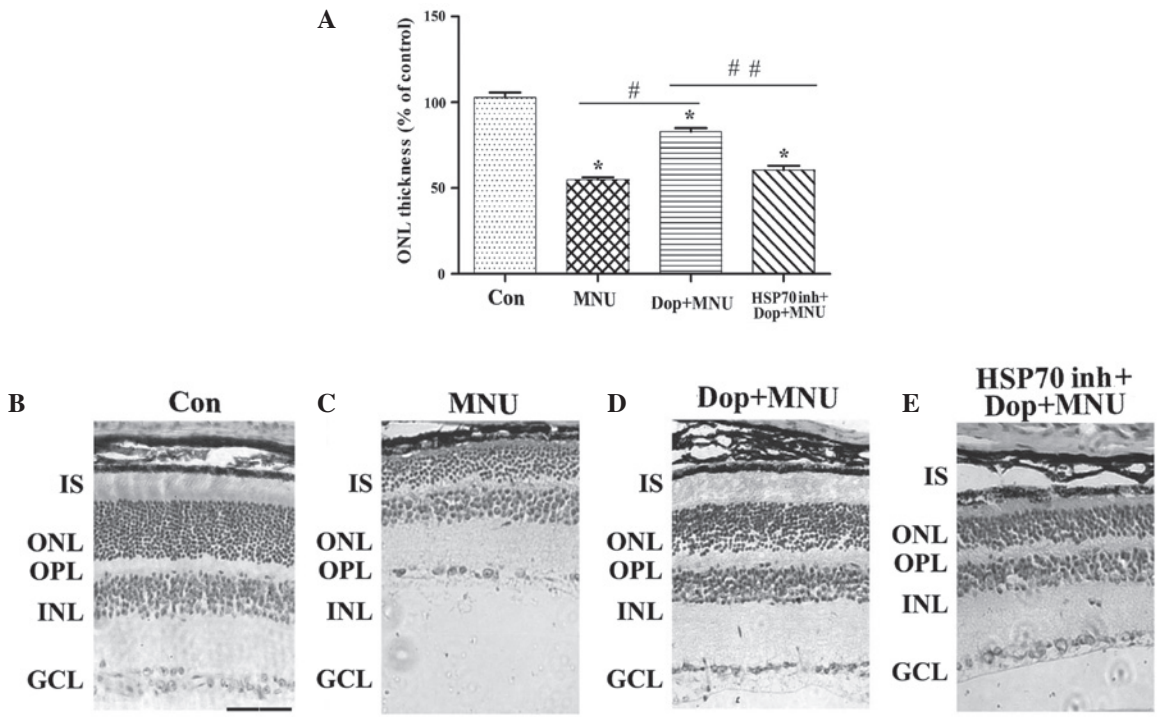

Figure 5. Dop protects photoreceptor cells against MNU in mice. (A) The thickness of the ONL in the retina of the mice. Data are presented as the mean ± standard deviation. *P<0.01 vs. con, #P<0.01 vs. MNU, ##P<0.01 vs. Dop + MNU (n=6). Microscopic images of the retina of the mice treated with (B) Con, (C) MNU (pretreatment for 3 days), (D) Dop + MNU (pretreatment for 3 days) and (E) Hsp70 inhibitor + Dop + MNU (pretreatment for 3 days). Scale bar, 50 μm. Hematoxylin and eosin staining. Con, control; MNU, N-methyl-N-nitrosourea; Dop, dopenezil; HSP70 inh, heat shock protein 70 inhibitor; IS, photoreceptor inner segment; ONL, outer nuclear layer; OPL, outer plexiform layer; INL, inner nuclear layer; GCL, ganglion cell layer.

Results

Intraperitoneal treatment with MNU induces loss of photoreceptor cells. Typical photomicrographs of mouse retinal sections treated with MNU (60 mg/kg) and stained with H&E are presented in Fig. 1A. Results demonstrated that the overall retinal thickness significantly reduced subsequent to MNU treatment as a result of the marked degeneration of ONL (P<0.01). The ONL was significantly reduced in thickness by day 3 and was further reduced by day 7 when compared with control retinas (control, day 0; P<0.01). The OPL was significantly thinner on days 1, 3, 5 and 7, whereas the INL became significantly thinner between 5 and 7 days (Fig. 1B; P<0.01), however no alterations in the ganglion cell layer were observed following MNU treatment for 7 days.

Donepezil increases the expression levels of Hsp70, as determined using immunofluorescence staining. It has been reported that Hsp70, induced by valproic acid, can protect photoreceptors against MNU-induced cell loss (19). In addition, one report demonstrated that donepezil can induce the expression of Hsp70 in neurons, creating a neuroprotective effect (22). To determine whether donepezil treatment can increase the promoter activity and the distribution pattern of Hsp70 within the retina, immunofluorescence was performed. In the control group, the expression of Hsp70 was faintly observed in the retinal IS and ONL. However, strong Hsp70 immunoreactivity was observed in the IS and ONL after donepezil pretreatment for 3 days (Fig. 2Aa-i), and the expression of Hsp70 was significantly reduced subsequent to administration of HSP inhibitor I when compared with the donepezil and control groups (P<0.01; Fig. 2A and B).

Donepezil induces Hsp 70 expression in a time- and dose-dependent manner, as determined by western blot analysis. A previous study demonstrated that an AChE inhibitor was able to induce the expression of Hsp70 in neurons, thus exerting neuroprotective effects (14). In order to determine whether administration of donepezil induces an increase in Hsp70 expression levels in the mouse retina, western blot analysis was performed. Mice were orally gavaged with donepezil at 1, 5 and 10 mg/kg for 3 consecutive days prior to MNU treatment. Results demonstrated that donepezil treatment induces an increase in Hsp70 protein expression in a dose-dependent manner. The expression of Hsp70 was significantly increased following 5 and 10 mg/kg donepezil treatment, as compared with the control (Fig. 3A and B; control, 0-fold; 5 mg/kg, 0.2-fold; 10 mg/kg, 1.9-fold; P<0.01). In addition, pretreatment with donepezil for 3 days significantly increased Hsp70 protein levels 2.1-fold in comparison with the control group (Fig. 3C and D; control, 0-fold; 3 days, 2.1-fold; 5 days, 2.0-fold; P<0.01). The observations of the current study therefore demonstrate that donepezil can induce Hsp70 expression in a dose- and time-dependent manner.

Expression levels of Bcl-2 are associated with donepezil-induced Hsp70. Although numerous studies have reported that Hsp70 serves a vital role in protecting retinal cells (23,24), the precise mechanisms of this process remain unclear. Western blotting demonstrated that the protein

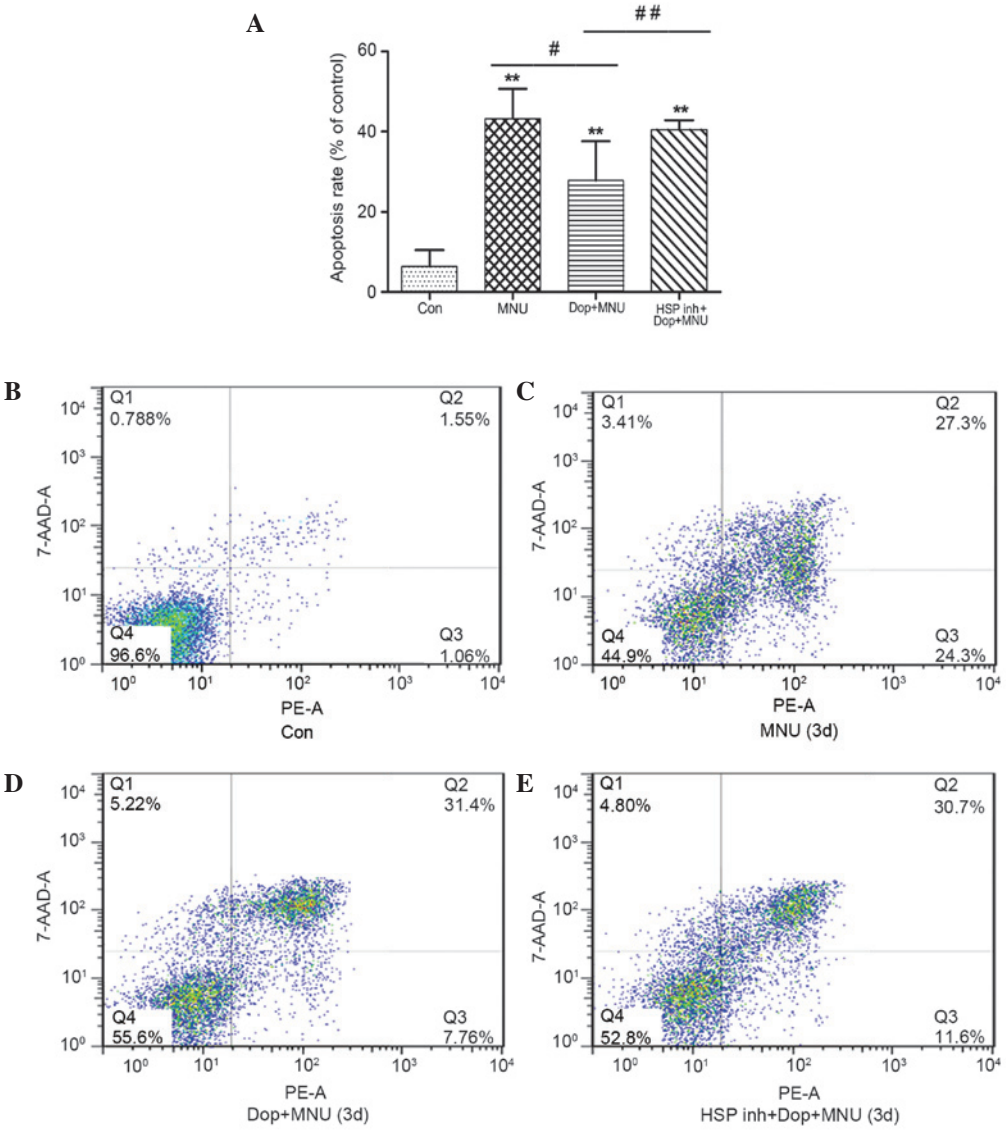

Figure 6. Annexin V-PE/7-AAD staining assay. (A) Graphical representation of the percentage of apoptotic cells. Data are presented as the mean ± standard deviation. **P<0.01 vs. Con; $^{\#}$P<0.05 vs. MNU; $^{\#\#}$P<0.05 vs. Dop+MNU. Representative graphs of apoptotic photoreceptor cells treated with (B) con, (C) MNU (pretreatment for 3 days), (D) Dop + MNU (pretreatment for 3 days) and (E) HSPinh + Dop + MNU (pretreatment for 3 days), as acquired by flow cytometric analysis subsequent to double-staining with Annexin V-PE/7-AAD. 7-AAD, 7-aminoactinomycin D; PE, phycoerythrin; Con, control; MNU, N-methyl-N-nitrosourea; Dop, donepezil; HSP inh, heat shock protein 70 inhibitor.

expression levels of Hsp70 and Bcl-2 were increased following 3 days pretreatment with 10 mg/kg donepezil (Fig. 4A), and this was shown to be significant by densitometric analyses (P<0.01; Fig. 4B and C). Furthermore, pretreatment with HSP inhibitor I resulted in a significant reduction in the expression levels of Hsp70 and Bcl-2, as compared with the donepezil group (P<0.01; Fig. 4). In addition, the Bcl-2 inhibitor could only reduce the expression levels of Bcl-2 (Fig. 4A and C), whereas the expression levels of Hsp70 were not significantly altered (Fig. 4A and B). These results suggested that Hsp70 induced by donepezil may upregulate the expression levels of Bcl-2, and that pretreatment with an HSP inhibitor was able to reduce the interaction between Hsp70 and Bcl-2.

Donepezil prevents photoreceptor cell death caused by MNU by increasing Hsp70 expression in mice. To further elucidate the role of donepezil in photoreceptor apoptosis and the possible mechanisms underlying this process, H&E staining was performed to evaluate the effect of donepezil on MNU-induced alterations in ONL thickness. Typical photomicrographs with H&E staining demonstrated that the ONL was significantly thinner in MNU-treated retinas, as compared with the control (P<0.01; Fig. 5A-C). Furthermore, pretreatment with donepezil for 3 days significantly increased the thickness of the ONL, as compared with the MNU group (P<0.01; Fig. 5A, C and D). Conversely, pretreatment with the HSP inhibitor I significantly attenuated the protective effects of donepezil (P<0.01; Fig. 5A, D and E).

The rate of photoreceptor cell apoptosis was detected by double labeling the cells with Annexin V-PE/7-AAD. The apoptotic rate in the MNU group (43.2±7.4%) was significantly increased, as compared with the control group (6.4±4.1%; P<0.01; Fig. 6A-C). Furthermore, the apoptotic rate in the donepezil plus MNU group was 29.4±4.1%, which

was significantly reduced, as compared with the MNU group (P<0.05; Fig. 6A, C and D). However, the protective effects of donepezil were partially attenuated by the administration of HSP inhibitor I; the apoptotic rate in the HSP inhibitor group was significantly increased (40.6±2.3%), as compared with the donepezil plus MNU group (P<0.05; Fig. 6A, D and E). Notably, donepezil did not affect the number of the cells in the late apoptotic stage; this may be a result of mechanical damage in the process of cell separation which led to an increased number of cells in the late apoptotic stage.

Discussion

Retinal degenerative diseases, such as inherited RP, remain a challenge for investigators and clinicians in the field of ophthalmology. Currently, it is evident that retinal degeneration is an apoptotic event involving complex crosstalk and interconnected signals (25), however, improved animal models that fully mimic human retinal degenerative diseases are required in order to thoroughly understand the mechanism. Numerous researchers have summarized that MNU causes photoreceptor cell apoptosis in golden hamsters in 7 days (7,26,27), and that injection of MNU at 60 mg/kg into mice and rats results in photoreceptor cell loss and exhaustion within one week (19). In the present study, the data indicated a similar time course of photoreceptor cell apoptosis subsequent to MNU treatment. It was observed that the thickness of photoreceptor IS and ONL in the retina were significantly reduced following intraperitoneal injection of MNU (60 mg/kg) after 3 days, with no alterations observed in the ganglion cell layer after 7 days. It can therefore be concluded that MNU selectively damages photoreceptor cells.

Donepezil is a potent AChE inhibitor that is widely used in the treatment of Alzheimer's disease, and has been reported to exert a number of beneficial functions. In previous studies, donepezil was reported to protect against cell damage induced by oxygen-glucose deprivation in rat pheochromocytoma cells (28), and increase glutathione and reduce malondialdehyde levels in a rat model of dementia (29). In addition, one study indicated that donepezil exhibits neuroprotective effects on cerebral and optic nerves by improving the blood flow in patients with normal-tension glaucoma (22). However, the mechanisms underlying the neuroprotective effects of donepezil remain unclear. Sakamoto *et al* (30) observed that the activation of AChRs and a mechanism unrelated to AChE inhibition contributes to the protective effect of donepezil. In experiments using rat cortical neurons, Takada *et al* (9) showed that the neuroprotection afforded by donepezil was prevented by methyllycaconitine (MLA), an α7-selective nAChR antagonist, but not by scopolamine, a muscarinic (m)AChR antagonist. These results were consistent with a previous study (31), which observed that the neuroprotective effect of donepezil was reversed by MLA in human neuroblastoma cells and that donepezil exerted neuroprotective effects via nAChRs. Conversely, Miki *et al* (12) reported that both mecamylamine, a nAChR antagonist, and scopolamine, were unable to affect the neuroprotective effect of donepezil on RGCs *in vitro*. These findings suggested that the activation of mAChRs or nAChRs may not be the key mechanism underlying neuroprotection in RGCs. Narimatsu *et al* (32) suggested that donepezil may improve cognitive function in mice by increasing the hippocampal production of insulin-like growth factor-I via sensory neuron stimulation. Therefore, the mechanism underlying the neuroprotective effects of donepezil is currently unclear, and remains to be clarified. An additional study demonstrated that the AChE inhibitor rivastigmine was able to enhance cellular defenses in neurons by upregulating Hsp70 mRNA levels (14). Therefore, it can be hypothesized that the mechanisms underlying the neuroprotective effects of AChE inhibitors may be mediated via the expression of Hsp70. In the present study, an increase in Hsp70 protein expression was observed following donepezil pretreatment in MNU-induced photoreceptor cell apoptosis mice.

The present study demonstrated that donepezil was able to induce the protein expression of Hsp70, which is strongly associated with cell survival. A previous study indicated that Hsp70 is critical for the photoreceptor stress response after retinal detachment (13), however further investigation is required to determine the mechanisms of interaction between Hsp70 and photoreceptor cell death. Hsp70 assists in denatured protein folding to repair DNA damage, transfers irreversibly damaged proteins to protein degradation system and allows cellular adaptation, which is necessary for cell survival (33), and numerous reports have demonstrated that Hsp70 has manifold anti-apoptotic effects both upstream and downstream of caspase activation (13,34,35). For example, upregulation of Hsp70 can increase the expression of the major anti-apoptotic protein Bcl-2 (36), which interferes with the release of cytochrome *c*, the oligomerization of apoptotic protease activating factor-1 and the activation of caspase-9, thereby preventing the aggregation of the apoptosome (37). Several studies have highlighted that the overexpression of Hsp70 is associated with reduced apoptotic cell death and a high expression level of the anti-apoptotic protein Bcl-2 (21,38,39).

Proteins of the Bcl-2 family are identified as being fundamental in the execution of the mitochondrial pathway of apoptosis (40,41). The regulation of active anti- and pro-apoptotic Bcl-2 family member proteins is critical for determining the fate of cells (40,42). Therefore, aberrant expression of these proteins can cause various diseases associated with apoptosis, such as retinal dystrophy (43,44). It is widely accepted that the Bcl-2-associated signaling pathway is involved in the apoptosis of photoreceptors in the $Rpe65^{-/-}$ murine model of Leber's congenital amaurosis (45). Eversole-Cire *et al* (46) observed that overexpression of the Bcl-2 associated X protein (Bax) protein can aggravate retinal photoreceptor cell apoptosis, however that Bcl-2 overexpression alone can inhibit apoptosis. However, the definite anti-apoptotic mechanism of the Bcl-2 family of proteins in retinal degeneration remains unclear. Further investigation has confirmed that donepezil reduces the Bax/Bcl-2 ratio during ischemia/reperfusion (47), and that Hsp70 inhibits heat-induced apoptosis upstream of mitochondria by restraining Bax translocation (39).

In the present study, a photoreceptor apoptosis mouse model was established with an intraperitoneal injection of MNU. Immunofluorescence results demonstrated that donepezil increased the expression levels of Hsp70 in the IS and ONL. Furthermore, a western blot analysis investigated the time- and concentration-dependent effects of donepezil on

the expression levels of Hsp70, and determined that donepezil (10 mg/kg) treatment results in a marked increase in the expression levels of Hsp70, when compared with lower dosage groups, and that donepezil pretreatment for 3 days leads to a significant increase in Hsp70 expression levels. In addition, it was observed that a Bcl-2 inhibitor could not significantly reduce the expression of Hsp70 induced by donepezil, but was able to reduce the expression of Bcl-2. Subsequent to binding of the Bcl-2 inhibitor (ABT-199) to Bcl-2, the sensitivity of Bcl-2 bound to primary antibodies was reduced. This may be the reason why the Bcl-2 inhibitor reduced the expression level of Bcl-2 when the western blot analysis was performed. To conclude, the results from the present study suggest that Hsp70 can promote photoreceptor cell survival which is closely associated with the expression levels of Bcl-2. Therefore, it is suggested that Hsp70, induced by donepezil, can resist MNU-induced photoreceptor cell apoptosis, which may be partially offset by an HSP inhibitor. To the best of our knowledge, this is the first study that demonstrates the key role of donepezil in inhibiting MNU-induced photoreceptor cell apoptosis associated with Hsp70.

In conclusion, the present study demonstrated that donepezil can prevent photoreceptor cell apoptosis, induced by MNU treatment, via upregulating the expression of Hsp70 and Bcl-2. Further studies are required in order to clarify the possible mechanisms of interaction between donepezil, Hsp70 and Bcl-2. The results from the present study indicate that donepezil has the potential to be used as a novel therapeutic agent for the treatment of RP.

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