Potassium aspartate inhibits SH-SY5Y cell damage and apoptosis induced by ouabain and H_2O_2

XUEFEI SUN[1], DONGYU MIN[2], YAN WANG[1] and LIYING HAO[1]

[1]Department of Pharmaceutical Toxicology, School of Pharmaceutical Science, China Medical University, Shenyang, Liaoning 110001; [2]The Experimental Center of Traditional Chinese Medicine, The Affiliated Hospital of Liaoning University of Traditional Chinese Medicine, Shenyang, Liaoning 110032, P.R. China

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Abstract. The present study aimed to investigate the effects of L-aspartic acid potassium salt (potassium aspartate, K-asp) on SH-SY5Y cells treated with ouabain and H_2O_2. An 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was performed to investigate the effects of K-asp on SH-SY5Y cell death induced by ouabain. Nissl staining was used to demonstrate the morphological changes of the SH-SY5Y cells. Light microscopy and 4',6-diamidino-2-phenylindole (DAPI) staining were performed to visualize apoptosis in SH-SY5Y cells incubated with ouabain for 6, 24 and 48 h. Transmission electron microscopy was used to observe the effect of K-asp on ultrastructural changes of the SH-SY5Y cells following incubation with ouabain for 24 and 48 h. An annexin V-fluorescein isothiocyanate/propidium binding assay and flow cytometry were performed successively to investigate how K-asp affected the H_2O_2-induced cell apoptosis. The MTT assay demonstrated that K-asp attenuated the cytotoxicity of the SH-SY5Y cells following treatment with ouabain, in a dose-dependent manner. The cell survival rates following 48 h incubation in the K-asp (15 mM) and K-asp (25 mM) groups were higher compared with the KCl and MK801 groups. Nissl staining demonstrated that the severity of cell injury in the KCl and K-asp (25 mM) groups were alleviated. In the DAPI staining and transmission electron microscopy analyses, KCl and K-asp (25 mM) reduced the rate of ouabain-induced apoptosis. Flow cytometry revealed that K-asp (25 mM) reduced H_2O_2-induced apoptosis. These results demonstrated that K-asp (25 mM) inhibited the ouabain and H_2O_2-induced SH-SY5Y cell damage and apoptosis, possibly by supplementing levels of intracellular K^+.

Correspondence to: Dr Liying Hao, Department of Pharmaceutical Toxicology, School of Pharmaceutical Science, China Medical University, 92 Beier Road, Shenyang, Liaoning 110001, P.R. China
E-mail: lyhao@mail.cmu.edu.cn

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Introduction

The potassium ion (K^+) is an important ion, which is involved in the metabolism of sugar and protein in a variety of cell types, and maintains the balance between the pH and osmolarity of cells. It is essential in the formation of resting potential, neuromuscular excitability and the maintenance of normal myocardial diastolic movement coordination (1). Hypokalemia is a common disease in which abnormal concentrations of K^+ in the blood cause severe pathological changes, including muscle weakness, intestinal paralysis, sinus tachycardia, ventricular fibrillation and other arrhythmias (2). Hypokalemia is caused by various factors, including insufficient food, vomiting, severe diarrhea, kidney disease, digitalism and long-term use of glucocorticoids (3). The major treatment for hypokalemia is K^+ supplementation. L-aspartic acid potassium salt (K-asp) is commonly used in the clinical treatment of hypokalemia (4,5), in which L-aspartic acid is used as the carrier to transport K^+ into the cells (6). However, the protective effect of K-asp in nerve cells surrounded by a low potassium environment remains to be elucidated.

Several neurodegenerative diseases, including Alzheimer's disease, Parkinson's disease, amyotrophic lateral sclerosis, ischemia and excitotoxicity are associated with oxidative injury (7). Previous findings have indicated that oxidative damage may be associated with reactive oxygen species (ROS) and manifested by cell lysis, oxidative bursting or excessive quantities of free transition metals (8,9). H_2O_2 is one type of ROS, which has been used as an important reagent to establish an *in vitro* model of oxidative stress injury (10). Ouabain is an Na^+ and K^+-adenosine triphosphate (ATP)ase inhibitor, which can induce concentration-dependent neuronal cell death. Neuronal cell swelling is followed by cell shrinkage, which is accompanied by an increase in intracellular Na^+ and decrease in K^+ (11). In the present study, the anti-apoptotic effect of K-asp was investigated in ouabain-treated and H_2O_2-treated human SH-SY5Y cells.

Materials and methods

Cells and drugs. Human SH-SY5Y cells were obtained from American Type Culture Collection (Rockville, MD, USA) and were cultured in Dulbecco's modified Eagle's medium

(Invitrogen Life Technologies, Carlsbad, CA, USA), supplemented with 10% fetal bovine serum (Gibco Life Technologies, Carlsbad, CA, USA) and 100 U/ml penicillin/streptomycin (Sigma-Aldrich, St. Louis, MO, USA). The cells were incubated in a humidified incubator at 37˚C with 5% CO_2, and the medium was replaced every 2 days. Ouabain (Sigma-Aldrich, St. Louis, MO, USA), H_2O_2 (Sigma-Aldrich), 25 mM KCl, 2 μM MK801 (Sigma-Aldrich) and 15, 25, 50 or 75 mM K-asp (Liaoning Union Pharmaceutical Co., Ltd., Liaoning, China) were dissolved in distilled water. KCl, MK801 and K-asp were added 4 h prior to treatment with either ouabain or H_2O_2. The present study was approved by the ethics committee of China Medical University (Shenyang, China).

Analysis of cell death using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. The human SH-SY5Y cells were plated into 96-well plates at a concentration of $5x10^3$ cells/well and were incubated with KCl (25 mM), MK801 (2 μM) or K-asp (15 mM, 25 mM, 50 mM or 75 mM), at 37˚C for 4 h, prior to the addition of ouabain (100 μM). Following treatment, the cells were incubated for 24 and 48 h. The cells were treated with MTT (0.5 mg/ml/well) for 4 h, prior to the MTT being replaced with 150 μl dimethyl sulfoxide (DMSO) and the absorption was determined at 570 nm using a spectrophotometric plate reader (680; Bio-Rad Laboratories, Inc., Hercules, CA, USA).

Nissl staining. The human SH-SY5Y cells ($5x10^4$) were plated into 24-well plates and incubated, as described above. Following incubation with 100 μM ouabain for 6, 24 or 48 h, the cells were fixed with 4% paraformaldehyde (Sinopharm Chemical Reagent Co., Ltd., Shanghai, China) and subsequently incubated in Nissl solution (1%) at room temperature for 12 min. The stained cells were observed under a light microscope (CK X41; Olympus, Tokyo, Japan).

Analysis of apoptosis using 4',6-diamidino-2-phenylindole (DAPI) staining. The human SH-SY5Y cells ($5x10^4$) were plated onto a cover slip and incubated as described above. Following incubation with 100 μM ouabain for 24 or 48 h, the cells were fixed with 4% paraformaldehyde and incubated in DAPI solution (100 ng/ml) for 1 min in the dark. The stained cells were observed under a fluorescence microscope (BX61/DP71; Olympus).

Transmission electron microscopy. The cells were incubated as described above. Following incubation with 100 μM ouabain for 24 and 48 h, the cells were fixed using 4% gluteraldehyde (Sinopharm Chemical Reagent Co., Ltd.) in 0.1 M phosphate buffer (pH 7.4; Sinopharm Chemical Reagent Co., Ltd.), and postfixed with 1% osmioum tetroxide (Sinopharm Chemical Reagent Co., Ltd.) in 0.1 M cacodylate (Sinopharm Chemical Reagent Co., Ltd.) buffer for 1 h at 4˚C. The cells ($5x10^4$) were subsequently dehydrated using ethanol (50, 70 and 90%), infiltrated using acetone and epoxy resin (Structure Probe, Inc., West Chester, PA, USA), and finally embedded in capsules (Agar Scientific, Essex, UK). Polymerization was performed at 60˚C for 48 h. Thin sections were cut with a diamond knife on an ultramicrotome and mounted onto slot grids (Ted Pella, Inc., Redding, CA, USA). The unstained sections were observed under a Hitachi H-600 transmission electron microscope (Hitachi, Tokyo, Japan).

Flow cytometry. The cells ($1x10^6$) were incubated, as described above. Following incubation with 100 μM H_2O_2 for 48 h, the cells were collected into tubes and washed twice with 10 ml phosphate-buffered saline (PBS; Sinopharm Chemical Reagent Co., Ltd.). The cells ($1x10^6$ cells/sample) were stained with annexin V-fluorescein isothiocyanate (FITC)/propidium iodide (PI; BD Biosciences, San Jose, CA, USA), according to the manufacturer's instructions (BD Biosciences, San Jose, CA, USA), and subsequently analyzed on a fluorescence-activated cell sorting instrument (FACSAsia; Becton Dickinson, Franklin Lakes, NJ, USA). The PI- and annexin V-negative cells (lower left quadrant) were considered to be normal, PI-negative and annexin V-positive cells (lower right quadrant) were considered early apoptotic cells, PI- and annexin V-positive cells (upper right quadrant) were considered late apoptotic cells, and the PI-positive and annexin V-negative cells (upper left quadrant) were considered mechanically injured. All experiments were performed in triplicate and representative figures produced.

Statistical analysis. The results were analyzed using SPSS 13.0 software (SPSS, Inc., Chicago, IL, USA). The data are expressed as the mean ± standard deviation. Two groups of mean values were compared using Student's t-test. $P<0.05$ was considered to indicate a statistically significant difference.

Results

K-asp attenuates the cytotoxicity of ouabain on SH-SY5Y cells in a dose-dependent manner. To investigate the effect of K-asp on ouabain-induced cell death, an MTT assay was performed. The viability of the SH-SY5Y cells exposed to ouabain for 24 h was 65.89±3.41% of that in the control group. The viabilities of the cells treated with KCl, MK801, K-asp (25 mM) and K-asp (50 mM) were 93.21±3.67 ($P<0.01$), 84.49±1.89 ($P<0.01$), 91.32±1.75 ($P<0.01$) and 74.19±0.82% ($P<0.05$), respectively (Fig. 1A). The cells exposed to ouabain for 48 h was 88.37±9.08% of that of the control group. The viabilities of the cells treated with K-asp (15 and 25 mM) were 101.20±19.07 ($P<0.05$) and 101.40±12.54% ($P<0.01$), respectively (Fig. 1B). The data suggested that K-asp attenuated the ouabain-induced cytotoxicity of the SH-SY5Y cells in a dose-dependent manner.

K-asp decreases the severity of ouabain-induced necrosis in the SH-SY5Y cells, in a dose-dependent manner. To observe the morphological changes of the SH-SY5Y cells, Nissl staining was performed. Following incubation with ouabain for 6, 24 and 48 h, the control group exhibited few injured cells, and the visual field was predominantly clear and intact cells without cell necrosis. However, a significant proportion of the cells in the ouabain group were damaged, exhibiting extensive degenerative changes, including sparse cell arrangements, loss of integrity, a shrunken cytoplasm and swollen cell bodies (Fig. 2). The cells of the 48 h incubation group exhibited more severe injury compared with those in the 6 and 24 h incubation groups. By contrast, the severity

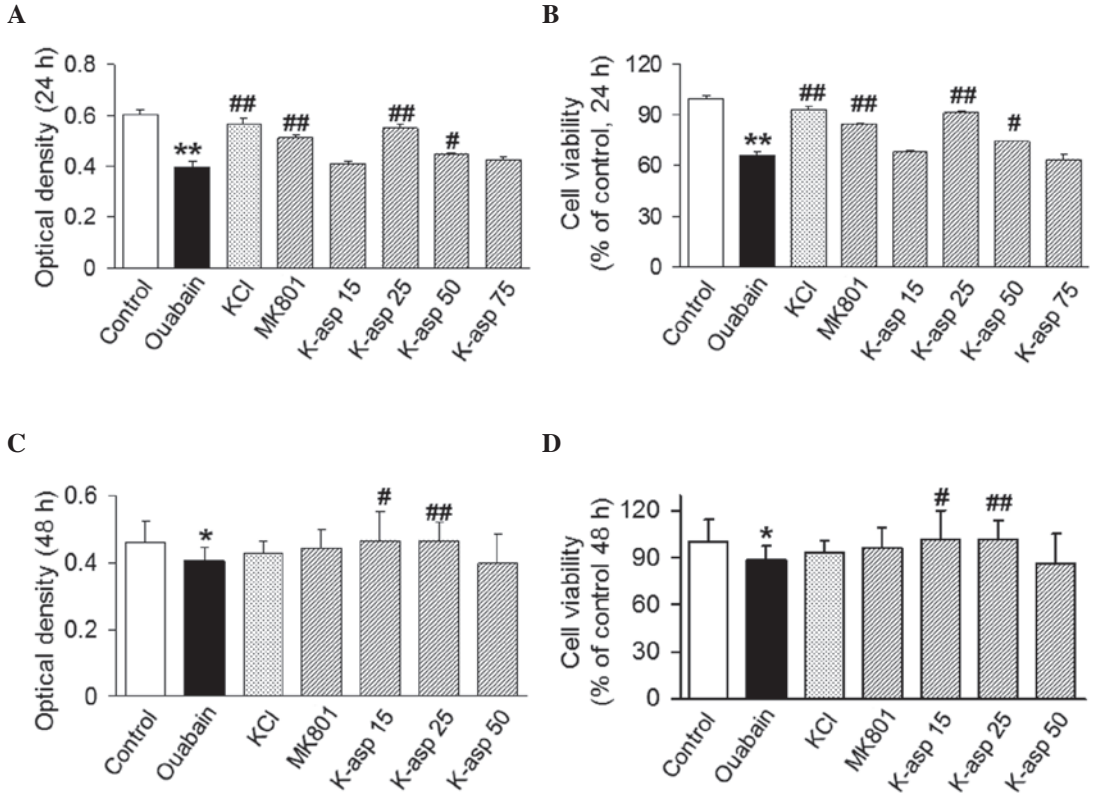

Figure 1. Effect of K-asp on SH-SY5Y cell death induced by ouabain. (A) Optical density of SH-SY5Y cells treated with 100 μM ouabain for 24 h. (B) Viability of SH-SY5Y cells treated with 100 μM ouabain for 24 h, expressed as the percentage of the control. (C) Optical density of SH-SY5Y cells treated with 100 μM ouabain for 48 h. (D) Viability of SH-SY5Y cells treated with 100 μM ouabain for 48 h, expressed as the percentage of the control. KCl (25 mM), MK801 (2 μM) or K-asp (15 mM, 25 mM, 50 mM or 75 mM) were added 4 h prior to treatment with ouabain. Independent experiments were repeated three times. The data are expressed as the mean ± standard error of the mean. $P<0.05$ and $^{**}P<0.01$, compared with the control; $^{\#}P<0.05$ and $^{\#\#}P<0.01$, compared with the ouabain-treated cells. K-asp, potassium aspartate.

of cell necrosis in the KCl, MK801, K-asp (15 mM) and K-asp (25 mM) groups was alleviated, whereas cell necrosis in K-asp (50 mM) and K-asp (75 mM) groups was not. These results suggested that K-asp alleviated the severity of ouabain-induced necrosis in the SH-SY5Y cells, in a dose-dependent manner.

K-asp ameliorates ouabain-induced apoptosis of SH-SY5Y cells, and 25 mM K-asp is the most effective concentration. To examine the apoptotic response of the SH-SY5Y cells incubated with ouabain for 6, 24 or 48 h, light microscopy and DAPI staining were performed. In the control group, the cell structures were clear, synapses were complete and large quantities of the stained nuclei were uniform and oval in shape. In the ouabain-treated group, the cell structures were unclear and the number of viable cells were reduced, with a large quantity of cell fragments. The nuclei were unevenly stained, their shape and size were irregular, and their number was significantly reduced. The number of cells in the ouabain-treated group, following incubation for 24 and 48 h, was significantly reduced compared with the control group. In the KCl group, light microscopy revealed that the cell structures were clear and few cells were round, and the results of the DAPI staining demonstrated that the number of the cells was increased. In the MK801 group, light microscopy revealed that the cell structures were clear and only a few cell fragments were observed, with the DAPI staining demonstrating few cell shape abnormalities. In the K-asp (25 mM) group, light microscopy revealed higher numbers of cells and fewer cell fragments, compared with the KCl, MK801, K-asp (15 mM) and K-asp (50 mM) groups (Fig. 3). These data demonstrated that K-asp ameliorated the ouabain-induced apoptosis of the SH-SY5Y cells, with K-asp (25 mM) being the most effective.

K-asp (25 mM) reduces cellular ultrastructure changes, induced by ouabain. To determine the effect of K-asp on ultrastructure changes of the SH-SY5Y cells following incubation with ouabain for 24 and 48 h, the cells were visualized using transmission electron microscopy. In the control group, the cell membrane and nuclear membrane were intact, and the structures of the mitochondria and endoplasmic reticulum were clear. The ouabain group exhibited incomplete cell membranes, cell shrinkage, nuclear cleavage fragments, sparse necrotic cell chromatin, irregular granular distribution, cell swelling and damage to the organelle structures. By contrast, the KCl group and K-asp (25 mM) group exhibited nuclear chromatin condensation, crescent- or ring-shaped nuclear membrane bodies and clear organelle structures (Fig. 4). These data suggested that KCl and K-asp (25 mM) alleviated the cellular ultrastructure changes, which were induced following treatment with ouabain.

K-asp (25 mM) ameliorates the apoptosis of SH-SY5Y cells induced by H_2O_2. To determine how K-asp affected H_2O_2-induced cell apoptosis, an annexin V-FITC/PI binding

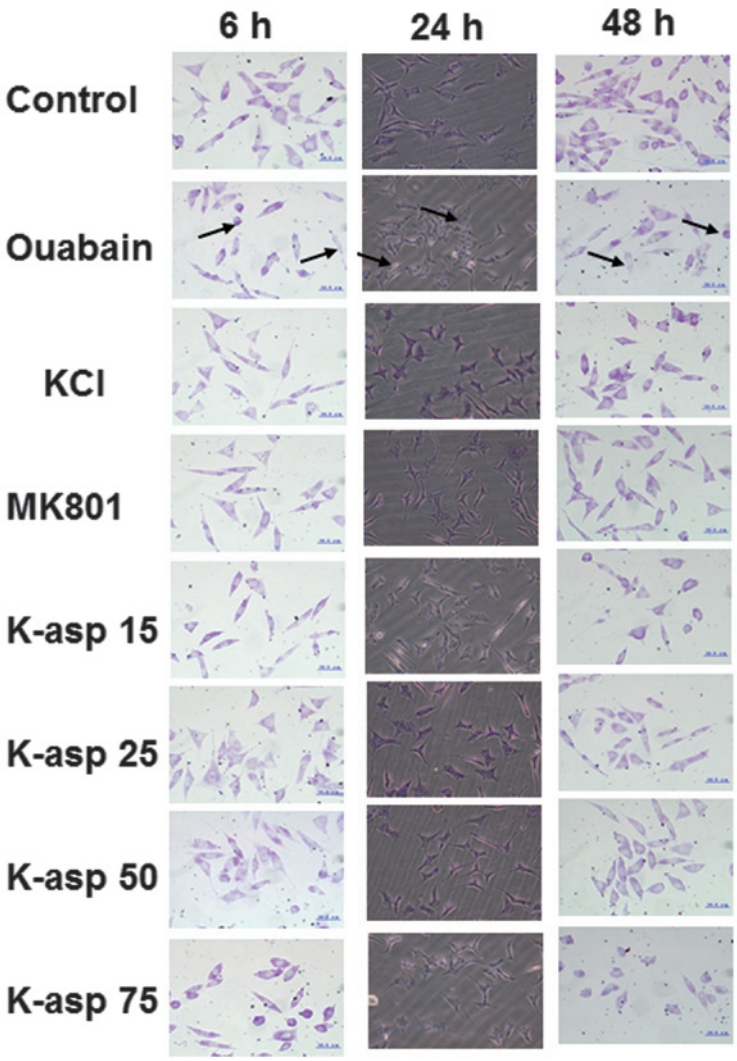

Figure 2. Effect of K-asp on the cytotoxicity of SH-SY5Y cells following incubation with ouabain for 6, 24 and 48 h. Images were captured (magnification, x200) following Nissl staining. KCl (25 mM), MK801 (2 μM), K-asp 15 (15 mM), K-asp 25 (25 mM), K-asp 50 (50 mM) or K-asp 75 (75 mM) were added 4 h prior to treatment with ouabain (100 μM). The stained Nissl bodies are demonstrated in the images of SH-SY5Y cells. Nissl bodies are indicators for nerve cell functions. Arrows indicate the damaged cells (scale bar=50 μm). K-asp, potassium aspartate.

assay and flow cytometry were performed to detect cell apoptosis. In the double parameter dot plots (Fig.5), an increased number of cells in the LR area were indicative of early apoptotic cells and those in the UR area were indicative late apoptotic cells (Fig. 5A). In the control group, the early apoptotic population of the SH-SY5Y cells was 1.38±0.50% and the late apoptotic population was 3.69±0.98%, combining to a total apoptotic population 5.07±0.87%. In the H_2O_2 group, the early apoptotic population of cells was 10.29±3.96% and the late apoptotic population was 10.09±0.85%, combining to a total apoptotic population 20.38±3.89%. The total number of apoptotic population of the cells in H_2O_2 group was significantly higher than that in the control group ($P<0.001$). In the K-asp (25 mM) group, the early apoptotic population of cells was 0.77±0.45% and the late apoptotic population was 4.89±1.56%, combining to a total apoptotic population of 5.66±1.98%. The total apoptotic population of the cells in K-asp (25 mM) group was significantly decreased, compared with the H_2O_2 group ($P<0.001$; Fig. 5E). These data demonstrated that K-asp (25 mM) ameliorated the apoptotic response of the-H_2O_2-induced SH-SY5Y cells.

Discussion

Ouabain is a Na^+-K^+-ATP enzyme inhibitor, which increases the levels of Na^+ and Ca^{2+} and decreases the levels of K^+ under intracellular conditions (11). In the early period of ouabain treatment (2-6 h), SH-SY5Y cells swell and the cell volume decreases gradually, resulting in cell apoptosis and necrosis (11). Previous studies have demonstrated that intracellular K^+ concentrations are reduced to 80% in neurons treated with ouabain for 12 h (12-14). The present study demonstrated that, following treatment with ouabain for 24 h, the number of cells was significantly decreased and the cell survival rate was significantly increased in the KCl, MK801 and K-asp (25 mM) groups. No difference in cell survival rate was observed between the KCl and K-asp (25 mM) groups, and the cell survival rates in these two groups were higher compared with that in the MK801 group. The cell survival rates following incubation for 48 h in the K-asp (15 mM) and K-asp (25 mM) groups were higher compared with the KCl and MK801 groups. These results indicated that KCl had a short-term protective effect against cellular damage caused by

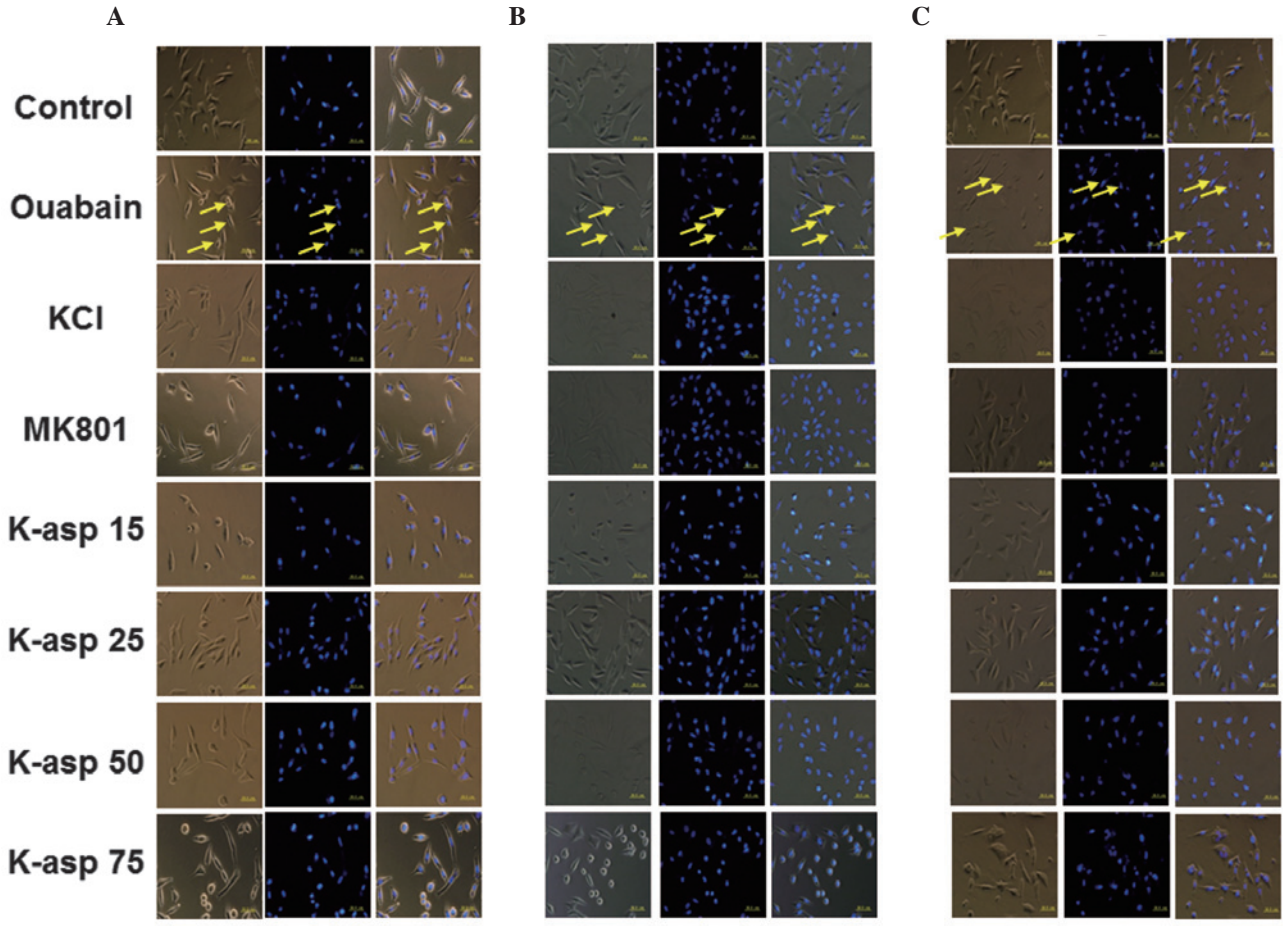

Figure 3. Effect of K-asp on the apoptosis of SH-SY5Y cells following incubation with ouabain for (A) 6, (B) 24 and (C) 48 h. The left column of images were captured under light microscopy (magnification, x200) with no staining, the middle column of images were captured following DAPI staining, and the right column of images represent a merge of the respective left and middle columns of images. KCl (25 mM), MK801 (2 μM), K-asp 15 (15 mM), K-asp 25 (25 mM), K-asp 50 (50 mM) or K-asp 75 (75 mM) were added 4 h prior to treatment of the cells with ouabain (100 μM). Arrows indicate damaged cells (scale bar=0 μm). K-asp, potassium aspartate.

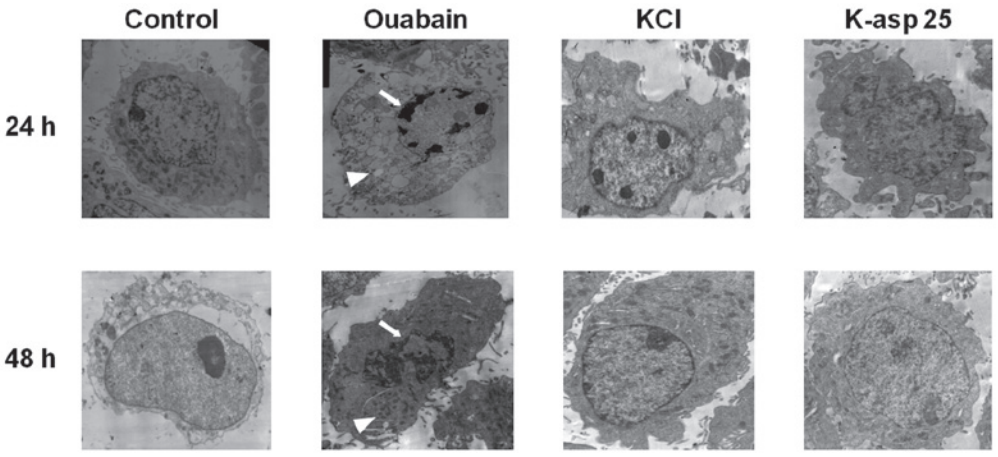

Figure 4. Effect of K-asp on the apoptosis of SH-SY5Y cells following incubation with ouabain for 24 and 48 h. Images were captured under a transmission electron microscope. KCl (25 mM) or K-asp 25 (25 mM) were added 4 h prior to treatment of the cells with ouabain (100 μM). Arrows indicate nuclear cleavage fragments; triangles indicate organelle structural damages. K-asp, potassium aspartate.

low potassium, however, K-asp exhibited a longer duration of protective effects compared with KCl.

Nissl bodies indicate nerve cell functions (12). The results of the Nissl staining in the present study demonstrated that the cells in the KCl, MK801 and K-asp (25 mM) groups exhibited darker staining, compared with those in the ouabain group, with the number of cells being increased. This observation indicated that K-asp and KCl protected against ouabain-induced cell damage.

As a well-established model of *in vitro* SH-SY5Y cell oxidative stress, H_2O_2 can readily pass through the cell

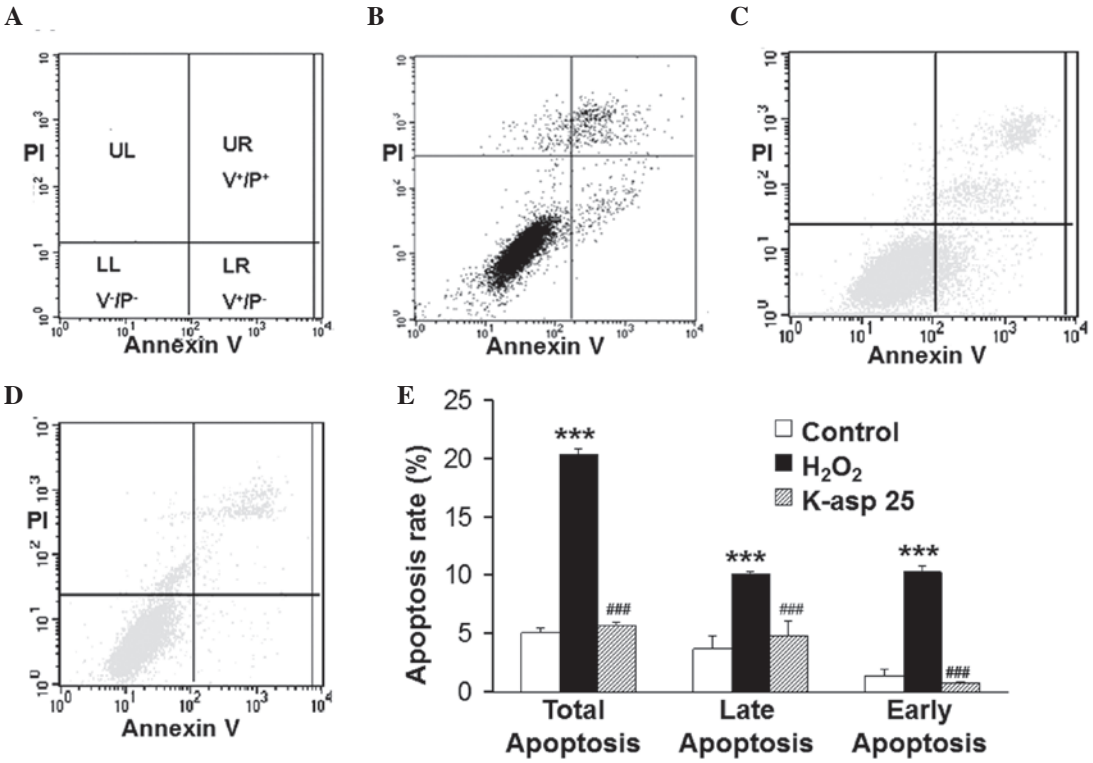

Figure 5. Effect of K-asp on the apoptosis of SH-SY5Y cells following incubation with H_2O_2 for 48 h. Apoptosis was determined using an annexin-V fluorescein isothiocyanate/propidium iodide binding assay and flow cytometry. K-asp (25 mM) was added 4 h prior to treatment of the cells with H_2O_2 (100 μM). (A) Normal cells (V^-/PI^-) were indicated in the LL quadrant of the dot plot, early apoptotic cells (V^+/P^+) were indicated in the UR dot quadrant and late apoptotic cells (V^+/P^-) were indicated in the LR quadrant. (B) Dot plot of cells in the control group. (C) Dot plot of cells in the H_2O_2 group. (D) Dot plot of cells in the K-asp group. (E) Histograms of the percentages of apoptosis, based on the accumulation of annexin-V FITC positive cells in UR and LR quadrants. The data are expressed as the mean ± standard error of the mean ($^{***}P<0.001$, compared with the control group; $^{###}P<0.001$, compared with the H_2O_2 group). K-asp, potassium aspartate; V, annexin-V fluorescein isothiocyanate; P, propidium iodide, $^+$, positive; $^-$, negative; UL, upper left; UR, upper right; LL, lower left; LR, lower right.

membrane and cause cell damage, resulting in the disturbance of ion homeostasis (15,16). H_2O_2 inhibits Na^+-K^+-pump activity and decreases intracellular levels of K^+. A previous study demonstrated that apoptotic cell dehydration was caused by the loss of K^+ (17). In cancerous tissues, fewer $K_V1.1$ and $K_V1.3$ potassium channels are expressed and the cell apoptosis is abnormal, indicating that potassium channels may contribute to apoptosis (18). Another previous study demonstrated cerebellar granule neuron apoptosis following the transfer of cells from different extracellular potassium concentrations (19). In the present study, K-asp (25 mM) protected the SH-SY5Y cells from the induction of apoptosis following incubation with H_2O_2 48 h. The total apoptotic population of the cells in the H_2O_2 group was significantly increased compared with the control group, however, this effect was suppressed following treatment with K-asp (25 mM). Subsequent analysis revealed that the protective effect of K-asp (25 mM) was persistent in the early and late periods of apoptosis. These results demonstrated that K-asp (25 mM) significantly reduced the apoptotic rate of the cells, however, excessively high K^+ concentrations (≥50 mM) resulted in apoptosis. These results were consistent with those reported in previous studies (20,21).

It has been reported that ouabain inhibits the reduction in Bcl-2 and the increases the phosphorylation of the pro-apoptotic factor, p53, in SH-SY5Y cells (13). Ouabain, a Na^+-K^+-ATP enzyme inhibitor, increases levels of intracellular Na^+ and promotes Na^+-Ca^{2+} exchange, which causes intracellular calcium overload and activates caspase-3 and endogenous nucleases, leading to apoptosis and irreversible damage (11). In the present study, analysis using DAPI staining and transmission electron microscopy revealed that KCl and K-asp (25 mM) reduced the level of apoptosis induced by ouabain. The annexin V-FITC/PI binding assay indicated that K-asp (25 mM) also reduced apoptosis, which was induced by H_2O_2. In the present study, MK801, a NMDA receptor antagonist, reduced ouabain-induced cell damage caused.

Decreased intracellular ions lead to decreased intracellular osmotic crystals and the outflow of water molecules, causing a reduction in cell volume (19). This is partly consistent with the results of the present study. K-asp, a novel energy type potassium agent, has a high affinity towards cells. Aspartic acid contributes to the citric acid cycle to provide ATP for the body and to assist in Na^+-K^+-ATP enzyme recovery (22). In the present study, K-asp (15 mM and 25 mM) had a better protective effect compared with KCl, whereas the cells in the K-asp (75 mM) group exhibited severe damage, caused by the high concentration of K^+ (23,24).

In conclusion, the present study demonstrated that K-asp (25 mM) had protective effects on the SH-SY5Y cells, with superior effects compared with KCl, following incubation for 48 h. This suggested that K-asp supplemented the levels of intracellular K^+ and inhibited the apoptosis of the SH-SY5Y cells.

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