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Coenzyme Q10 protects SHSY5Y neuronal cells from beta amyloid toxicity and oxygen-glucose deprivation by inhibiting the opening of the mitochondrial permeability transition pore

Geng Li[a,*], Liang-Yu Zou[a,b], Chun-Mei Cao[c] and Edward S. Yang[a]
[a]*The Jockey Club MRI Centre, The University of Hong Kong, Hong Kong, China*
[b]*Department of Neurology, The First Affiliated Hospital, Harbin Medical University, China*
[c]*Institute of Molecular Medicine, Peking University, China*

Abstract. Coenzyme Q10 (CoQ10) is an essential biological cofactor which increases brain mitochondrial concentration and exerts neuroprotective effects. In the present study, we exposed SHSY5Y neuroblastoma cells to neurotoxic beta amyloid peptides (Aβ) and oxygen glucose deprivation (OGD) to investigate the neuroprotective effect of 10 μM CoQ10 by measuring (i) cell viability by the MTT assay, (ii) opening of the mitochondrial permeability transition pore via the fluorescence intensity of calcein-AM, and (iii) superoxide anion concentration by hydroethidine. Cell viability (mean $\pm$ S.E.M.) was 55.5 $\pm$ 0.8% in the group exposed to Aβ + OGD, a value lower than that in the Aβ or OGD group alone ($P < 0.01$). CoQ10 had no neuroprotective effect on cell death induced by either Aβ or OGD, but increased cell survival in the Aβ + OGD group to 57.3 $\pm$ 1.7%, which was higher than in the group treated with vehicle ($P < 0.05$). The neuroprotective effect of CoQ10 was blocked by administration of 20 μM atractyloside. Pore opening and superoxide anion concentration were increased in the Aβ + OGD group relative to sham control ($P < 0.01$), and were attenuated to the sham level ($P > 0.05$) when CoQ10 was administered. Our results demonstrate that CoQ10 protects neuronal cells against Aβ neurotoxicity together with OGD by inhibiting the opening of the pore and reducing the concentration of superoxide anion.

Keywords: Coenzyme Q10, Beta amyloid, oxygen-glucose deprivation, mitochondrial permeability transition pore, superoxide anion

1. Introduction

Stroke and Alzheimer's disease (AD) are the most prevalent age-related neurological diseases in humans. The mechanisms of neuronal injury and neuronal death overlap in both diseases [9,21,34]. *In vivo* studies have demonstrated that susceptibility to ischemic brain damage increases in transgenic mice overexpressing amyloid precursor protein (APP) or with a presenilin-1 (PS1) mutation [31,51]. Increased

*Address for correspondence: Geng Li, The Jockey Club MRI Centre, The University of Hong Kong, Room 204, Chow Yei Ching Bldg., Pokfulam Road, Hong Kong. Tel.: +852 2293 0305; Fax: +852 2540 6215; E-mail: ligeng@eee.hku.hk.

oxidative stress and impairment of mitochondrial energy metabolism are involved in the pathogenesis of AD and cerebral ischemia [1,3,25,29,40,47,49,50]. Reduced activities of complex I, II/III and IV of the mitochondrial electron transport chain were observed in AD patients and in ischemic conditions [2,32, 49]. Coenzyme Q10 (CoQ10) is an essential cofactor produced endogenously and also provided in the food chain. Mitochondrial CoQ10 level is correlated with complex I, II/III and IV activities [43], while administration of CoQ10 increases brain mitochondrial concentration and exerts neuroprotective effects in some neurodegenerative diseases and in cerebral ischemia [10,15,30,38,39,44,48]. To date, there are few reports of therapeutic effects of CoQ10 in AD, although mitochondrial abnormalities have been found in this disease [47]. In the present study, we exposed SHSY5Y neuroblastoma cells to neurotoxic beta amyloid peptides (Aβ) and oxygen-glucose deprivation (OGD) to build an *in vitro* model of AD with ischemia [8,13], and investigated the neuroprotective effect of the antioxidant CoQ10 by measuring cell viability, opening of the mitochondrial permeability transition pore (abbreviated to "the pore" below) and the concentration of the superoxide anion radical.

2. Material and methods

2.1. Materials and chemicals

All culture medium supplements (minimum essential medium (MEM), Dulbecco's modified Eagle medium with high glucose (DMEM), glucose-free DMEM, penicillin, streptomycin, fetal bovine serum (FBS), Ham's F12 medium, 0.5% trypsin, 0.53 M ethylenediaminetetraacetic acid (trypsin-EDTA) and trypan blue) were obtained from Life Technologies (Gibco BRL, MD, USA). Dimethyl sulfoxide (DMSO), poly-L-lysine, paraformaldehyde, Aβ1–42, atractyloside (Atr), the lactate dehydrogenase (LDH) kit and coenzyme Q10 were obtained from Sigma-Aldrich (St. Louis, MO, USA). Hydroethidine (HEt) was purchased from Molecular Probes and calcein-AM was from Biotium Inc. The cell proliferation kit with 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) was obtained from Roche Diagnostics. The human neuroblastoma cell line SH-SY5Y was purchased from ATCC (VA, USA).

2.2. Cell culture

The stock vial of the SHSY5Y cell line was kept in liquid nitrogen at vapor phase. The vial of cells was first thawed at 37°C, then transferred to a 75 cm^2 culture flask containing 12.15 mL of culture medium and cultured for 1 to 2 weeks [37]. When the cells became confluent, they were dissociated using trypsin-EDTA and sub-cultured at a density of 10^5 cells/well. The culture medium was changed twice a week. For the cell viability experiment, cultured cells were reseeded into 96-well culture plates at a density of 10^5 cells/well. All cultures were incubated at 37°C under 5% CO_2 and 95% air (v/v) at 90% humidity.

2.3. Experimental protocols

Aβ1–42 was dissolved at 50 μM in sterile ultra-pure water (Milli-Q standard; Millipore, Watford, UK) and kept frozen until use; the final concentration of Aβ was 5 μM [46]. CoQ10 was dissolved at 1.0 M in 100% (w/v) DMSO and kept at 4°C until use; the final concentration was 10 μM [33]. Atr was dissolved at 1.0 M in sterile ultra-pure water and kept at 4°C; the final concentration was 20 μM [6].

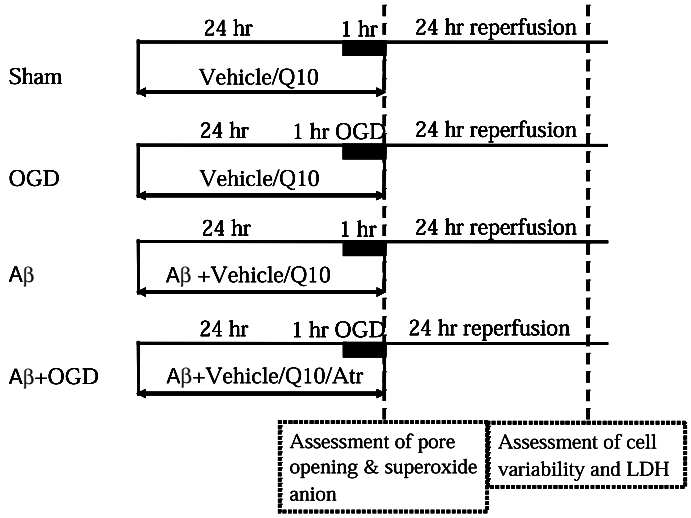

Fig. 1. Experimental protocols. Atr: 20 μM atractyloside; Aβ: 5 μM beta amyloid; OGD: oxygen-glucose deprivation; Q10: 10 μM CoQ10; LDH: lactate dehydrogenase.

The experimental groups and protocols are summarized in Fig. 1. Cells were first exposed to 5 μM Aβ (Aβ and Aβ + OGD groups) or to sterile ultra-pure water (sham and OGD groups) for 24 hr. Then cells in the OGD and Aβ + OGD groups were exposed to OGD (glucose-free DMEM plus anoxia) for 1 hr, while the sham and Aβ groups were left alone. During the process of OGD, Aβ was added to the Aβ and Aβ + OGD groups, and sterile ultra-pure water was added to the sham and OGD groups. Pore opening and superoxide anion concentration were measured at the end of OGD exposure. After 1 hr of OGD, all cells were returned to normal, normoxic medium and incubated for a further 24 hr, at which time cell viability was measured. For the CoQ10 treatment, all the above procedures were followed, except that vehicle (2% DMSO), 10 μM CoQ10 or 10 μM CoQ10 + 20 μM Atr were added to the four groups from the beginning of exposure to Aβ to the end of OGD.

2.4. In vitro OGD model of ischemia

Oxygen-glucose deprivation was achieved using published methods [13]. Briefly, 4 days after subculture, pretreatments (described in the experimental protocols) were carried out for 24 hr. Then the culture medium of cells in the OGD and Aβ + OGD groups was changed to glucose-free DMEM containing the drugs and placed in an anaerobic chamber flushed with 5% CO_2 and 95% N_2 (v/v). The same anaerobic gas mixture was bubbled through the glucose-free DMEM to "deoxygenate" the medium. The cell cultures within the anaerobic chamber were kept in a humidified incubator at 37 °C for 1 hr. The oxygen concentration was $<$ 0.8% throughout OGD, as monitored by an oxygen meter (OX 2000, Oldham, Arras Cedex, France). To terminate OGD, the culture medium was changed to normal medium before returning the cells to the normoxic incubating conditions. In the sham and Aβ groups, the cell cultures were subjected to the same experimental procedures, but were not exposed to glucose-free DMEM plus anoxia.

2.5. Assessment of cell viability

Cellular viability was assessed using the mitochondrial assay kit according to the manufacturer's instructions (Boehringer Mannheim) and published methods [18]. Briefly, 10 μl of the MTT labeling

reagent at a final concentration of 0.5 mg/mL was added to each well 24 hr after the termination of OGD and the cells were incubated at 37°C under 5% CO_2 and 95% air (v/v) at 90% humidity for 4 hr to allow formation of purple formazan crystals. Four hours later, 100 μl of the solubilization reagent was added to each well before overnight incubation. Finally, the spectrophotometric absorbance of the solubilized purple formazan crystals was measured using a microplate reader (Model 550, Bio-Rad, Hercules, CA, USA) at an absorbance wavelength of 570 nm and a reference wavelength of 670 nm [18]. All MTT results were normalized and expressed as percentages of the average optical density reading from the sham-OGD group.

2.6. Determination of neuronal injury by LDH efflux

The culture medium was collected after 24 hr of normoxia, and LDH was spectrophotometrically assayed using a kit. LDH activity was expressed as units per milliliter [6].

2.7. Measurement of mitochondrial permeability transition pore opening and superoxide anion concentration

Cells were counted and plated at a density of 2×10^5 cells per well on 24-well plates with 12 mm glass coverslips precoated with poly-L-lysine (10 μg/mL). Cells were pretreated as described above. After pretreatment, culture medium was removed and cells were washed in PBS. Then the cells were incubated with 10 μM HEt [22] or 2 μmol/L calcein-AM in the presence of 4 mmol/L of cobalt chloride (CoCl2) [12] for 30 min at 37°C in a dark and humidified incubator under an atmosphere of 5% CO_2 to measure the intracellular concentration of superoxide anion radicals or pore opening. After loading, the cells were washed three times with medium, followed by 1 hr of OGD. As a negative control, cells were incubated in medium lacking the fluorescent dye. After OGD, the cells were incubated for 1 hr in 4% (wt/vol) paraformaldehyde in PBS for fixation [28]. After washing, the cells on the coverslips were mounted onto glass slides using permount. These slides were examined with a microscope (Axioskop 2 plus, Zeiss, Thornwood, NY, USA) under fluorescent light. The fluorescence (red signal) from superoxide anions was examined at an excitation wavelength of 510–550 nm and an emission wavelength > 580 [4]. The calcein-AM was exited with 488 nm light and the emitted fluorescence (green signal) was measured at 510–525 nm; increased fluorescence intensity indicated enhanced opening of the pore [19]. After preparation, digital images of cells were taken using a video camera connected to the microscope. All the images from each experiment were captured under identical settings of exposure time, gain and offset, and further analyzed using an image analysis system. The fluorescence intensity values from three different fields of view per slide were determined using MetaMorph software (MetaMorph imaging system 4.01) and the mean values were calculated [22]. The mean intensity represents the total intensity minus background, divided by the total target area. Background was calculated as the mean of all pixels outside a cell with the lowest optical density. Values are the result of the analysis of at least six images per experimental condition, with at least 20 cells per experimental condition. Each condition was repeated twice. The results are expressed as relative fluorescence intensity, which represent the percentages of optical densities relative to sham control.

2.8. Data analysis

Numerical data are expressed as mean $\pm$ S.E.M. Data were pooled from five independent sets of studies for cultured SHSY5Y neuroblastoma cells. The data were analyzed using one-way ANOVA followed by the Student-Newman-Keuls' *post hoc* test. A two-tailed P value of 0.05 or less was taken to indicate statistical significance.

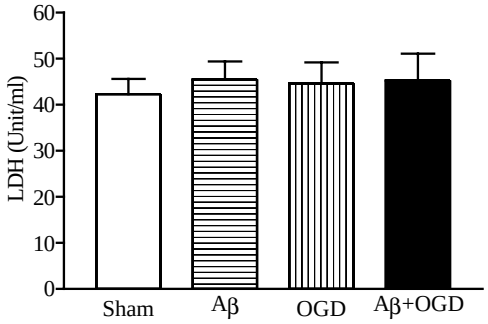

Fig. 2. LDH measurements in SHSY5Y cells. Aβ: 5 μM beta amyloid; Q10: 10 μM CoQ10; OGD: oxygen-glucose deprivation.

3. Results

3.1. Neuroprotective effect of CoQ10 against OGD and Aβ neurotoxicity in SH-SY5Y cells

Compared with the sham group, Aβ, OGD and Aβ + OGD had no effect on LDH release from neuronal cells ($P > 0.05$) (Fig. 2). However, Aβ, OGD, and Aβ + OGD decreased the cell viability (as measured by the MTT value) to 63.0 $\pm$ 3.9%, 68.3 $\pm$ 6.6% and 55.5 $\pm$ 0.8%, respectively. Compared with the groups exposed to either Aβ or OGD, OGD combined with Aβ induced more neuron death ($P < 0.01$) (Fig. 3A). When 10 μM CoQ10 was administered to the Aβ + OGD group, the cell viability was 57.3 $\pm$ 1.7%, higher than that in the vehicle group (54.6 $\pm$ 0.8%, $P < 0.05$). Nevertheless, the cell viability decreased to 54.9 $\pm$ 0.9% when 20 μM atractyloside was administered to the Aβ + OGD group together with 10 μM CoQ10, i.e. not different from vehicle control ($P > 0.05$) (Fig. 3B).

3.2. Effect of CoQ10 on pore opening (calcein-AM fluorescence) in SH-SY5Y cells

Compared with sham control, Aβ or OGD had no effect on the relative calcein-AM fluorescence intensity ($P > 0.05$), while Aβ + OGD significantly increased the intensity ($P < 0.01$) (Fig. 4A, B). However, when 10 μM CoQ10 was administered, the relative calcein-AM fluorescence intensity in the Aβ + OGD group and vehicle control decreased to the level of the sham control ($P > 0.05$) (Fig. 5A, B).

3.3. Effect of CoQ10 on superoxide anion concentration (HEt fluorescence) in SH-SY5Y cells

Compared with sham control, Aβ + OGD significantly increased the relative HEt fluorescence intensity ($P < 0.01$). Whereas when 10 μM CoQ10 was administered, the intensity decreased to the level of the sham control ($P > 0.05$) (Fig. 6A, B).

4. Discussion

In vivo studies have shown that susceptibility to cerebral ischemia increases in transgenic mouse models of AD [31,51]. Mattson and colleagues also found that the presenilin-1 mutation increased neuronal vulnerability to hypoxia and glucose deprivation in cell culture [31]. In the present study, an *in vitro* model of AD combined with ischemia was developed by exposing SHSY5Y human neuroblastoma

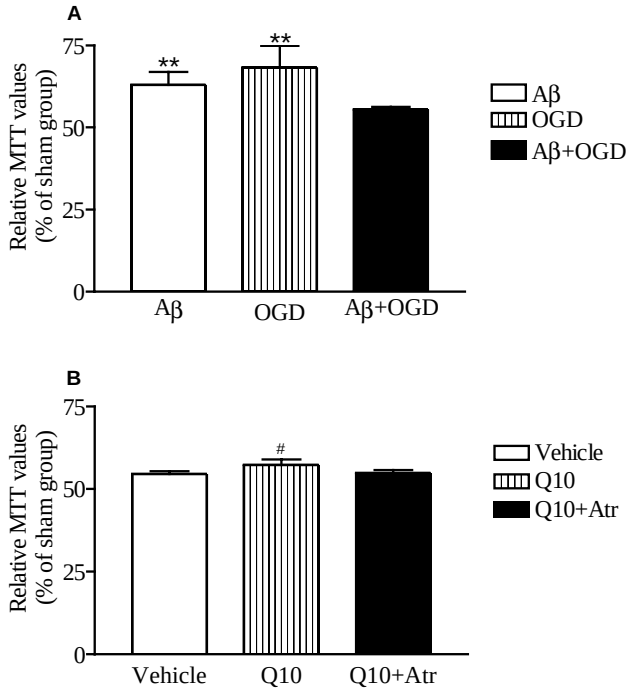

Fig. 3. Cell viability. A, SHSY5Y cells treated with Aβ, OGD or Aβ + OGD relative to sham control = 100%. $^{**}P < 0.01$, as compared with the Aβ + OGD group. B, SHSY5Y cells treated with Aβ + OGD and vehicle, Aβ + OGD and CoQ10, or Aβ + OGD with CoQ10 + Atr relative to sham. $\#P < 0.05$, as compared with the vehicle group. Atr: 20 μM atractyloside; Aβ: 5 μM beta amyloid; Q10: 10 μM CoQ10.

cells to neurotoxic beta amyloid peptides and oxygen-glucose deprivation [8,13]. Similar to the previous study, Aβ or OGD alone did not enhance LDH release from neuronal cells [14,42]. In the present study, a combination of the Aβ and OGD also had no effect on LDH release. Aβ increased the vulnerability of the cells to ischemia as manifested by reduced viability. LDH is a cytoplasmic enzyme whose presence in the culture medium reflects the loss of plasma membrane integrity [24], while MTT assay is to detect the metabolic activity of cells, resulting in different observation of the cell damage caused by a combination of the Aβ and OGD. Possible mechanisms include increased oxidative stress and impairment of mitochondrial energy metabolism [1]. Since CoQ10 is neuroprotective against external oxidative stress [45], it was administered to cells undergoing Aβ treatment and/or OGD to investigate its potential for neuroprotection in AD with cerebral ischemia. In the present study, although CoQ10 did not protect SHSY5Y neuroblastoma cells against either Aβ neurotoxicity or OGD alone, the viability of cells exposed to Aβ + OGD was significantly increased by treatment with 10 μM CoQ10. The mechanism of this neuroprotective effect was investigated by measuring the opening of the mitochondrial permeability transition pore. Pore opening limits mitochondrial calcium load, mediates mitochondrial reactive oxygen species (ROS) signaling [12], and triggers apoptosis/necrosis [7,27]. We measured pore opening using the calcein/Co^{2+} imaging technique. Previous studies have investigated the occurrence and the mode of opening of the MPTP directly in intact cells by monitoring the fluorescence of mitochondrial entrapped calcein-AM when the cells are coloaded with cobalt chloride and calcein-AM, and exposed to cobalt chloride throughout the experiment [12]. In the current study, the cells were first coloaded with cobalt chloride and calcein-AM to quench the cytoplasmic and nucleic fluorescence. After washing, the cells were then exposed to OGD and/or Aβ treatment for 1 hour, which caused the calcein-AM remained in

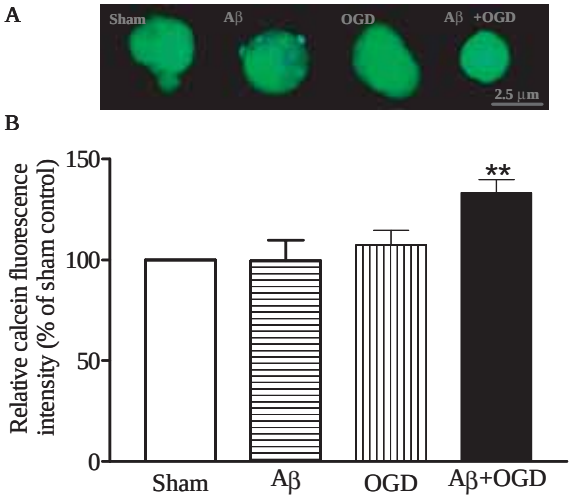

Fig. 4. Calcein fluorescence intensity. A, Photomicrographs showing calcein fluorescence intensity in SHSY5Y single cells from sham control, 5 μM beta amyloid (Aβ), oxygen-glucose deprivation (OGD), and Aβ + OGD groups. B, Calcein fluorescence intensity measurements from the groups in A relative to sham control. $^{**}P < 0.01$, as compared with the sham group.

the mitochondria to outflow into the cytoplasm. In the sham group, self-quenching induced outflowing of calcein-AM from the mitochondria to the cytoplasm [41]. Warm incubation with calcein-AM can lead to almost exclusive cytosolic distribution of calcein in both rat hepatocytes and rabbit cardiacmyocytes, and under this condition, mitochondria are visualized by confocal microscopy as round and oval voids about a micron in diameter in images of green calcein fluorescence [35,36]. Therefore the calcein-AM fluorescence intensity measured after OGD and/or Aβ treatment was the calcein-AM signal in the cytoplasma which had outflown from mitochondria during treatment. The difference of the calcein-AM fluorescence intensity between the treatment and sham group was used to measure the opening of MPTP caused by treatment. Aβ or OGD alone did not alter the calcein-AM fluorescence intensity, while Aβ + OGD increased it. Moreover, the rise in fluorescence intensity decreased significantly when CoQ10 was administered. This suggests that inhibition of pore opening is a key point for the neuroprotective effect of CoQ10. Previous studies on heart have showed that the pore opens during the first few minutes of reperfusion and results in myocardial injury [16,17,19,20]. The pore opening was measured at the end of the OGD in this study, thereafter, OGD alone did not enhance pore opening. However, pore opening occurred earlier in OGD if Aβ was present (Fig. 4). At the end of OGD, the CoQ10 treatment was completed, so it had no effect on the pore opening caused by the return to normoxia (analogous to reperfusion), but did affect the earlier opening of the pore caused by Aβ + OGD. This may explain why CoQ10 was not neuroprotective against ischemia, but did protect the cells against OGD + Aβ. In order to test the hypothesis that CoQ10 protects cells from Aβ + OGD via inhibition of pore opening, we treated the cells undergoing this protocol with 20 μM atractyloside, the pore opener. Opening the pore with Atr abolished the neuroprotective effect of CoQ10 (Fig. 3B), supporting the hypothesis.

Disruption of the mitochondrial respiratory chain induces overproduction of reactive oxygen species (ROS), leading to oxidative stress and the activation of apoptotic mediators [5]. The overproduced ROS, including the superoxide anion, are involved in neuronal cell death in acute injury of central nervous system and in chronic neurodegenerative diseases [11,26]. In the present study, we observed an increase in superoxide anion production with Aβ + OGD, and this overproduction decreased significantly by CoQ10 administration. Past research has shown that mitochondria actively generate ROS such as superoxide

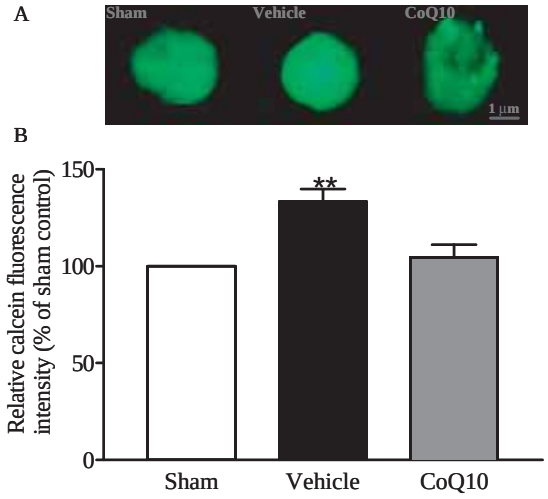

Fig. 5. Calcein fluorescence intensity and CoQ10. A, Photomicrographs showing calcein fluorescence intensity in sham control, 5 μM beta amyloid (Aβ) with oxygen-glucose deprivation (OGD) and vehicle, and Aβ + OGD with 10 μM CoQ10 groups. B, Calcein fluorescence intensity values from the groups in A relative to sham control. $^{**}P < 0.01$, as compared with sham group.

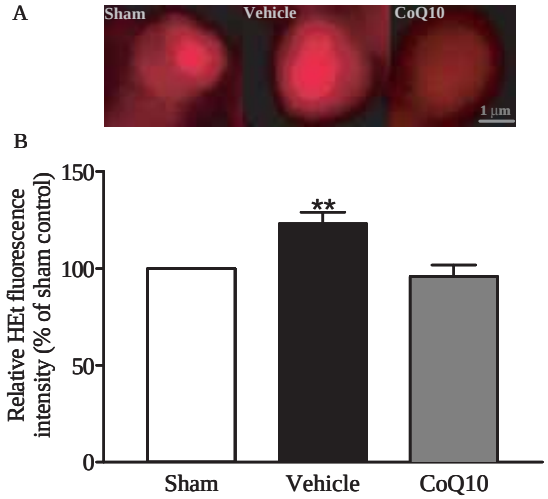

Fig. 6. Hydroethidine (HEt) fluorescence intensity. A, Photomicrographs showing calcein fluorescence intensity in single SHSY5Y cells from sham control, 5 μM beta amyloid with oxygen-glucose deprivation (Aβ + OGD) and vehicle, and Aβ + OGD with 10 μM CoQ10 groups. B, Calcein fluorescence intensity values from treatment groups in A relative to sham control. $^{**}P < 0.01$, as compared with the sham group.

anions and hydrogen peroxide [23], and CoQ10 protects cells at the mitochondrial level [45]. Therefore, CoQ10 may protect cells from Aβ + OGD by inhibiting pore opening, which reduces the production of superoxide anions and thereafter attenuates the oxidative stress and the activation of apoptotic mediators.

In summary, CoQ10 offers neuroprotection at the mitochondrial level against beta amyloid neurotoxicity and ischemia-like damage caused by oxygen-glucose deprivation. The underlying mechanisms include inhibition of the opening of the mitochondrial permeability transition pore, which reduces the

production of superoxide anion. CoQ10 can therefore be considered as a potential therapeutic agent for AD with cerebral ischemia.

Acknowledgments

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