Doxepin protects cultured neurons against oxidative stress-induced injury

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ABSTRACT

AIM: To investigate protective effects of doxepin (Dox) on cultured neuronal injury induced by oxidative stress. **METHODS**: Cytotoxicities of glutamate (Glu 0.5 mmol/L, 15 min), sodium dithionite (0.5 mmol/L, 24 h) or hemoglobin (Hb100 mg/L,24 h)and the protective effects of Dox were observed. **RESULTS**: Exposure of cultured neurons to Glu, sodium dithionite and Hb developed a neurotoxicity expressed in the thiazol blue tetrazolium bromide (MTT) assay, the increase of lactate dehydrogenase (LDH) leakage, malondialdehyde (MDA) content and intracellular $[Ca^{2+}]_i$ accumulation, as well as the decrease of superoxide dismutase (SOD) activity. DOX 1-100 nmol/L significantly inhibited all above changes. **CONCLUSION**: Dox protects cultured neurons against oxidative stress-induced injury by suppressing intracellular $[Ca^{2+}]_i$ accumulation, decreasing lipid peroxide generation and stimulating antioxidant enzyme.

INTRODUCTION

Oxidative stress is defined as loss of balance in the body for the generation of oxygen free radicals, or as cytotoxicity induced by accumulation of reactive oxygen species due to overuptake of exotic oxidizing agent [1]. It is recognized that some central nervous diseases such as cerebral ischemia, Parkinson's disease and Alzheimer's disease are related to accumulation of free radicals[2]. Doxepin (Dox), a tricycline anti-depressant, is known to have protective effects on acute cerebral ischemia by attenuating exhaustion of monoamine transmitter and suppressing generation of lipid peroxide *in vivo*[3,4]. In this study, by technique of cell culture, the effects of Dox *in vitro* on neuronal injury induced by oxidative stress were observed and its possible mechanisms were investigated.

MATERIALS AND METHODS

Drugs and reagents Dox was obtained from Zhedong Pharmaceutical Factory (batch No 95118); Glutamate (Glu), sodium dithionite, hemoglobin (Hb), Fura-2/AM, Triton-X100, egtazic acid, and thiazol blue tetrazolium bromide (MTT) were purchased from Sigma. The assay kit of lactate dehydrogenase (LDH), superoxide dismutase (SOD) and malondialdehyde (MDA) were purchased from Nanjing Jiancheng Co; Dulbecco's modified Eagle's medium (DMEM) was Gibco product. All other reagents were analytical reagents (AR).

Neuronal cultures Cortical neurons were isolated from embryonic (gestation of 16-18 d old) Sprague-Dawley rats. The dissected hemisphere was

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rinsed with ice-cold Ca^{2+}-and Mg^{2+}-free Hanks' solution (pH 7.2-7.4). Meninges and blood vessels were meticulously removed. Following a wash step with Ca^{2+}- and Mg^{2+}-free Hanks' solution, the brain was mechanically dissociated by gentle triturate 10-15 times with a polished pipette. The isolated brain cells were filtered through nylon sieve (200 mesh, hole width 95 μm) and collected in a cup. The cells were resuspended in DMEM supplemented with 10 % bovine serum and 10 % horse serum. Cells were seeded onto *L*-polylysin-coated 24-well plate at 37 ºC , the density of cell was 2×10^9/L, the medium containing unattached cell was removed and fresh medium was changed every 3-4 d. Cytarabine (10 μmol/L) was added into the medium to inhibit the growth of astrocytes

Neuronal injury induced by Glu[5] The cells were cultured for 10-12 d, then the medium was removed and the cells were rinsed with Mg^{2+}-free Eagle's solution for two times . Glu 0.5 mmol/L was added and incubated at 37 ºC in 5 % CO_2 atmosphere for 15 min. After rinsed with DMEM, the cells were incubated with serum-free DMEM at 37 ºC in 5 % CO_2 atmosphere for 24 h. Dox was added into the medium in different concentrations 24 h before the Glu was added respectively. The same treatment happened in control group except adding Glu.

Neuronal injury induced by sodium dithionite[6] The cells were cultured for 10-12 d, then the medium was removed , the cells were incubated with glucose-free Eagle's solution containing sodium dithionite (0.5 mmol/L), an O_2 scavenger, at 37 ºC in 5 % CO_2 atmosphere for 24 h. Dox was added into the medium in different concentrations 24 h before the sodium dithionite was added respectively. The same treatment happened in control group except adding sodium dithionite.

Neuronal injury induced by Hb[7] The cells were cultured for 10-12 d , then the cells were rinsed with serum-free DMEM twice and incubated with serum-free DMEM containing Hb (100 mg/L) for 24 h. After rinsing with serum-free DMEM, the cells were incubated with serum-free DMEM at 37 ºC in 5 % CO_2 atmosphere for 24 h. Dox was added into the medium in different concentrations 24 h before Hb was added. The same treatment happened in control group except adding Hb.

Measurement of cell death Cell death was evaluated by MTT assay according to previous report[8] , the absorbance was measured at 570 nm.

LDH efflux assay LDH efflux assay and protein content of sample were determined according to direction of assay kit.

Determination of MDA and SOD[9] Cells were rinsed with PBS solution for two times, every well was added with 1 % Triton-X100 50 μL. The protein was precipitated by adding 25 % H_3PO_4 100 μL. The homogenate was centrifuged at 10 000×*g* at 4 ºC for 1 h. SOD, MDA, and content of protein were determined according to direction of assay kit.

Measurement of intracellular $[Ca^{2+}]_i$[10] The suspended cells were loaded with Fura-2/AM (5000 nmol/L) at 37 ºC for 45 min, then rinsed with Hanks' solution containing 0.2 % bovine serum albumin. The density of cell was 2×10^9 /mL. The intracellular $[Ca^{2+}]_i$ was determined by fluorescene measurement (MPF-4 Spectrofluorometer, HITACHI). The excitation wave length was 345 nm (bandwidth,5 nm),the emission wave length was 500 nm (bandwidth,10 nm). $[Ca^{2+}]_i$ was calculated according to formula: $[Ca^{2+}]_i = K_d \times (F-F_{min})/(F_{max}-F)$.

Statistical analysis All data were presented as mean±SD and analyzed using analysis of variance (ANOVA) followed by *q* test .

RESULTS

Effects of Dox on injury induced by Glu There were marked increase of cell death, LDH efflux, content of MDA and intracellular $[Ca^{2+}]_i$, while the activity of SOD decreased after Glu was incubated at 37 ºC in 5 % CO_2 atmosphere for 15 min. Dox could attenuate the injury at different concentrations respectively (Tab 1). MTT assay of mixed cell culture was not modified when control group was pretreated with Dox 100 nmol/L compared to vehicle-treated control group (0.57±0.04 *vs* 0.58±0.11).

Effects of Dox on injury induced by sodium dithionite After the cells were incubated with sodium dithionite at 37 ºC in 5 % CO_2 atmosphere for 24 h, obvious increase of cell death, LDH efflux, content of MDA and intracellular $[Ca^{2+}]_i$ and a decrease of SOD activity were observed. Dox could inhibit all above changes in different concentrations respectively (Tab 1). The MTT assay of mixed cell culture was not modified when control group was pretreated with Dox 100 nmol/L compared to vehicle-treated control group (0.43±0.05 *vs* 0.43±0.05).

Effects of Dox on injury induced by Hb After the cells were incubated with Hb at 37 ºC in 5 % CO_2

Tab 1. Effects of doxepin (Dox) on neuronal injury induced by glutamate (Glu, 0.5 mmol·L^{-1}) , sodium dithionite (0.5 mmol·L^{-1}), and hemoglobin (100 mg·L^{-1}). *n*=6 wells. Mean±SD. $^{c}P<0.01$ *vs* Glu. $^{f}P<0.01$ *vs* sodium dithionite. $^{i}P<0.01$ *vs* hemoglobin.

Drugs/nmol·L^{-1}	$A_{570\ nm}$	LDH/kU·g^{-1} Pro	SOD/kU·g^{-1} Pro	MDA/μmol·g^{-1} Pro	$[Ca^{2+}]_i$/nmol·L^{-1}
Control	0.58±0.11	20±5	2.1±0.4	3.0±0.7	129±29
Glu	0.16±0.04	60±7	0.62±0.13	13±4	594±98
Dox 1	0.28±0.04^{c}	47±9^{c}	0.88±0.09^{c}	10.0±1.2^{c}	450±55^{c}
10	0.42±0.05^{c}	39±9^{c}	1.13±0.25^{c}	8.1±0.9^{c}	405±31^{c}
100	0.43±0.07^{c}	28±4^{c}	1.5±0.4^{c}	5.1±0.9^{c}	300±46^{c}
Control	0.4±0.05	26±5	2.0±0.4	3.3±0.6	128±1
Sodium dithionite	0.17±0.05	53±8	0.51±0.10	12.3±2.8	460±54
Dox 1	0.26±0.04^{f}	44±5^{f}	0.73±0.08^{f}	8.9±1.4^{f}	371±33^{f}
10	0.31±0.023^{f}	37±6^{f}	0.79±0.021^{f}	7.2±0.9^{f}	311±33^{f}
100	0.37±0.03^{f}	32±7^{f}	1.15±0.24^{f}	6.1±0.8^{f}	275±44^{f}
Control	0.60±0.05	24±6	2.9±0.5	2.8±0.6	140±12
Hemoglobin	0.17±0.04	57±6	0.57±0.13	13.1±0.9	422±42
Dox 1	0.31±0.06^{i}	43±4^{i}	0.96±0.27^{i}	9.9±1.0^{i}	365±26^{i}
10	0.41±0.04^{i}	36±5^{i}	1.25±0.28^{i}	7.0±1.0^{i}	330±20^{i}
100	0.46±0.06^{i}	31±3^{i}	1.9±0.6^{i}	5.9±1.3^{i}	310±16^{i}

MTT: thiazol blue tetrazolium bromide; LDH: lactate dehydrogenase; SOD: superoxide dismutase; MDA: malondialdehyde; Pro: protein.

atmosphere for 24 h, the numbers of cell death, LDH efflux, and the content of MDA increased, while the activity of SOD decreased. Dox could suppress all above changes in different concentrations respectively (Tab 1). MTT assay of mixed cell culture was not modified when control group was pretreated with Dox 100 nmol/L compared to vehicle-treated control group (0.61±0.02 *vs* 0.60±0.05).

DISCUSSION

Oxidative stress is a characteristic feature in a number of neurodegenerative disorders such as stroke, Parkinson's disease, and Alzheimer's disease. It is mediated by reactive oxygen species (ROS), including superoxide anion radicals, hydrogen peroxide, and hydroxyl radicals, which are accumulated due to the imbalances in the production of ROS and the activity of protection mechanisms. Therefore, therapeutic strategies aimed at removal of free radicals or prevention of their formation might be a reasonable choice for these diseases.

MTT is a substrate for intracellular and plasma membrane oxidoreductase, its reduction is an indication of cellular metabolic activity. Active mitochondria of living cells can cleave MTT to produce formazan, the amount of which is directly related to the number of living cells[11]. LDH normally existing in the cell is often released into the medium from damaged neurons. Therefore, neurotoxicity was estimated by the MTT assay and LDH activity released into the medium from damaged neurons. Our study showed that Dox (1-100 nmol/L) significantly inhibited the increase of cell death and LDH efflux, suggesting that Dox has significant protective effects against oxidative stress-induced injury on neurons.

The damage caused by oxidative stress is accompanied by increased lipid peroxides. Intracellular MDA, an important indicator for lipid peroxidation was elevated after the cultured neurons were exposed to Glu, sodium dithionite, and hemoglobin. It was markedly attenuated when the cells were pretreated with Dox in different concentrations. SOD, along with glutathione peroxidase, catalase and other non-enzymatic antioxidants, such as α-tocopherol, ascorbate, glutathione, and cysteine, serves as a detoxifying system to prevent damage caused by reactive oxygen species, among which, antioxidant enzymes play a pi-votal role. The present studies showed that the decrease of intracellular SOD induced by oxidative stress was mitigated by Dox. These results were consistent with the previous report[3], demonstrating that the neuroprotective effects

of Dox against oxidative stress-induced injury might be involved in decreasing lipid peroxide generation and stimulating antioxidant enzyme.

Calcium plays a key role in the cerebral oxidative stress-induced injury[12]. Oxidative stress increases intracellular $[Ca^{2+}]_i$ levels and activates Ca^{2+}-dependent enzymes such as phosphataseA_2, xanthine oxidase (XO) and nitric oxide synthase (NOS), which lead to generation of large amounts of arachidonic acid and oxygen free radicals. Overload of calcium is well known as a final way for neuron death. The present study showed that accumulation of intracellular $[Ca^{2+}]_i$ in the presence of Glu, sodium dithionite and hemoglobin was significantly suppressed when the cells were pretreated with Dox, indicating that Dox might act as a calcium antagonist. However, further studies are needed to identify precise mechanisms of this accumulation and the effect of Dox.

In conclusion, the neuroprotective effect of Dox against oxidative stress-induced injury might be attributed to its suppressing intracellular $[Ca^{2+}]_i$ accumulation, decreasing lipid peroxide generation and stimulating antioxidant enzyme.

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