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Protective effects of catalpol against H_2O_2-induced oxidative stress in astrocytes primary cultures

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Abstract

It has been proposed that ROS production, including H_2O_2, may lead to neurodegenerative disorders such as Parkinson's disease and Alzheimer's disease. Catalpol, an iridoid glycoside, presents in the root of *Rehmannia glutinosa*, protects cells and mice from damage caused by a variety of toxic stimuli. In this study, we investigated whether catalpol could protect astrocytes from oxidant stress induced by H_2O_2 because of the critical role of astrocytes in the brain and found the possible mechanism of protection. The results showed that catalpol could significantly increase the cell viability and reduce the intracellular ROS formation. Furthermore, catalpol attenuated H_2O_2-induced oxidative stress via preventing the decrease in the activities of antioxidant enzymes in glutathione redox cycling such as glutathione peroxidase, glutathione reductase and glutathione content. However, the catalase activity didn't appear to be elevated by catalpol adequately. Together, the main mechanism underlying the protective effects of catalpol in H_2O_2-injured astrocytes might be related to the maintenance of glutathione metabolism balance and the decrease of ROS formation. Therefore, catalpol may be developed as a potential preventive or therapeutic drug for neurodegenerative diseases associated with oxidative stress.

Key words: catalpol; oxidative stress; neuroprotection; astrocyte; H_2O_2 cytotoxicity

Introduction

Oxidative stress, occurring as a consequence of increased intracellular levels of reactive oxygen species (ROS), such as hydrogen peroxide (H_2O_2), supposedly forms a common pathway leading to neuronal death in the conditions mentioned [1]. The balance between generation of ROS and anti-oxidative processes can become disturbed as reported for aging [2] and several neurological disorders such as Parkinson's disease (PD) and Alzheimer's disease (AD) [6].

Available literature has reviewed that a high concentration of glutathione within cells

protects against different ROS [10]. In addition to direct removal of ROS by glutathione, a number of associated enzymes confer varied roles for glutathione in living cells. For example, glutathione peroxidase, glutathione reductase, transferase and glutaredoxin all utilize glutathione in reactions that remove peroxide, as well as potential toxin, control the redox state of the cell and regulate protein function through thiolation and dethiolation. This network of enzymes using glutathione as substrate has been implicated in DNA synthesis and repair, protein synthesis, amino acid transport and enzyme activation or inactivation [15].

Brain astrocytes play a prominent role in glutathione metabolism and in the defense against ROS [6]. In culture, astrocytes are able to utilize a large variety of compounds as extracellular precursors for intracellular glutathione synthesis [5]. At the cellular level, in order to reduce the likelihood of radical formation from peroxides, astrocytes use two antioxidant systems that can rapidly inactivate H_2O_2. In a reaction catalyzed by glutathione peroxidase, the reduced glutathione (GSH) serves as an electron donor to reduce H_2O_2 to water. The product of this reaction is oxidized glutathione (GSSG) which is a substrate of glutathione reductase [6]. Furthermore, glutathione reductase is responsible for cellular glutathione redox cycling, which is also crucial for the detoxification of endogenous peroxides [7]. Besides the glutathione system, catalase (CAT) also inactivates H_2O_2, especially if this peroxide is present at high concentrations [12].

Nowadays, there is an increasing interest in focusing on natural products. Catalpol, separated from traditional Chinese herbal medicines has been shown to be neuroprotective in vitro and vivo in our laboratory [13, 17]. The aim of the present study was to explore whether catalpol could protect astrocytes from oxidative stress generated by H_2O_2 and find the possible protective mechanism.

Materials and methods

Reagents

Catalpol was separated from traditional Chinese herbal medicines *Rehmannia glutinosa* [24] and diluted in phosphate-buffered saline (PBS) for treatment.

Primary astrocyte cultures and cell treatment

Astrocytes were prepared from the whole cerebra of 1-day-old neonatal mice. Briefly, mice were decapitated and the meninges were removed. Cortices were isolated and minced. A single-cell suspension was obtained by mechanical dissociation. Then dissociated cells were seeded into poly-D-lysine-coated 35-cm^2 T-flasks at a density of $1\times10^6/cm^2$ and maintained at 37 °C in a 5% CO_2 and 95% air in Dulbecco's modified Eagle's medium/nutrient F12 (DMEM/F12) containing 10% fetal bovine serum. Microglia and other non-adherent cells were removed by mild shaking for 6 h at 180 rpm [22]. After separating, astrocytes were detached with 0.125% trypsin-EDTA and seeded in poly-D-lysine-coated plates. 2 or 3 days later, they were employed.

Astrocytes were stained with an antibody against GFAP (glial fibrillary acidic protein). Briefly, after 4% formaldehyde-fixed for 30 min, cultures were washed with PBS twice and treated with 3% H_2O_2 for another 30 min followed by sequential incubation with blocking solution for 30 min. Cells were washed with PBS and then incubated with primary antibody (mouse monoclonal anti-GFAP, 1:200, Chemicon) for 2 h at 37℃. After washing with PBS, cells were incubated with FITC-labeled secondary antibody (goat anti-mouse IgG/FITC, 1:100, Chemicon) for 1 h at 37℃. Then reaction was terminated with removing the antibody dilution and washing cells with PBS. After counting, the percentage of astrocytes was higher than 95%.

The cells were cultured in the presence or absence of 0.5mM H_2O_2 for 24 h. When the effects

of catalpol on cells were studied, the different concentrations of catalpol were added for 1 h prior to H_2O_2 treatment. One hour later, H_2O_2 was added and incubated for 24 h in growth media. The control group was treated with PBS alone in the same manner.

Cell viability assay

The viability was analyzed with the 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay [11]. After treatment, MTT (5mg/ml) was added to the astrocyte cultures and incubated at 37 °C for an additional 3 h. Then supernatants were removed and 100μl of dimethyl sulfoxide was added to each well to dissolve the formed blue formazan. Absorbance was read at 570 nm on a microplate reader.

Assay of intracellular ROS

ROS were measured with the non-fluorescent probe 2′,7′-dichlorofluorescein diacetate (DCFH-DA) as previously described [23]. DCFH-DA passively diffuses into cells and is deacetylated by esterases to form nonfluorescent 2′,7′-dichlorofluorescein (DCFH). In the presence of ROS, DCFH reacts with ROS to form the fluorescent product DCF, which is trapped inside the cells. After cell treatment, culture medium was removed and the cells were washed three times with PBS. DCFH-DA, diluted to a final concentration of 10μM with DMEM/F12, was added to cultures and incubated for 20 min at 37 °C. The fluorescence was read at 485 nm for excitation and 530 nm for emission with a fluorescence plate reader (Genios, TECAN).

Measurement of intracellular total glutathione (GStotal) and GSSG content

The GStotal and GSSG were determined using commercially available kits (Beyotime Institute of Biotechnology). All procedures completely complied with the manufacture's instructions.

Determination of glutathione reductase, glutathione peroxidase and CAT activities

Activities were determined using commercially available kits (Jiancheng Bioengineering). Cells were lysed and the lysates were collected and centrifuged at 15,000 ×g for 10 min at 4 °C. The supernatants were tested for glutathione reductase activity by measuring the decrease of absorbance at 340 nm. Glutathione peroxidase activity was assayed by quantifying the rate of oxidation of the reduced glutathione to the GSSG by H_2O_2. The CAT activity was measured in the reaction mixture contained 12μl of 3% (v/v) H_2O_2 and 100μl of cell lysates. Samples were incubated for 2 min at 37 °C and the absorbance was monitored for 5 min at 240 nm. Changes in absorbance were taken to be proportional to the breakdown of H_2O_2.

Estimation of protein content

Protein concentration was measured by the method of Bradford [3] using bovine serum albumin as standard.

Statistical analysis

Data are expressed as the means±S.D.. Statistical evaluation of the data was performed by ANOVA. All estimates were conducted in triplicate. A value of p less than 0.05 was considered statistically significant.

Results

In order to select an optimal H_2O_2 concentration to induce an oxidative stress in astrocytes, cultures were treated with different concentrations of H_2O_2 (0: control group, 0.1-0.7mM) for 24 h. Then cell viability was measured by MTT assay. As shown in Fig. 1, cell viability was significantly reduced in a H_2O_2-concentration-dependent manner. Consequently, 0.5mM H_2O_2 (56.3±1.5% of control cell viability) was chosen for subsequent experiments.

Later, we evaluated the protective effects of catalpol on MTT reduction in cultured astrocytes. The results (Fig. 2) showed that the survival rate of astrocytes was about 57.1±2.3% treated with H_2O_2 alone. However, pretreatment with different concentrations of catalpol increased the viability of astrocytes in a dose-dependant manner (viability of 63.6±1.5%, 69.4±1.9%, 83.0±2.2% and 100.5±1.9% for catalpol concentrations of 0.05mM, 0.1mM, 0.25mM and 0.5mM, respectively). Thus, it was possibly concluded that catalpol was effective for the protection of astrocytes.

ROS formation should be measured as an important biomarker of oxidative stress, because it is continuously generated during oxidative metabolism in high rates within the brain [6]. The results in Fig. 3 showed that H_2O_2 treatment significantly increased the ROS level (approximately 160% of the control group). Whereas pretreatment with catalpol (0.05-0.5mM) dose-dependently attenuated H_2O_2-induced ROS production. The main inhibition was observed at 0.25mM and 0.5mM catalpol which decreased ROS production nearly to the normal level.

As shown in Table. 1, almost all glutathione existed in the reduced form in the control group. Incubated with H_2O_2 in the absence of catalpol, GStotal decreased dramatically, only 48.9% compared with the control group. Presence of catalpol (0.05mM) didn't note a statistically significant difference on GStotal. Nevertheless, it was obviously reversed when the concentration of catalpol is higher than 0.1mM. Similar to GStotal, the GSSG:GStotal ratio also increased from 2.6±0.2% to 33.5±3.0% following incubation with H_2O_2 alone. When pretreatment with 0.05, 0.1, 0.25 and 0.5mM catalpol, the ratio of GSSG:GStotal was nicely reduced to 25.3±0.4%, 14.3±0.5%, 8.0±0.3% and 3.6±0.3%, respectively.

Previous evidence has shown that glutathione reductase is an essential enzyme in the

regeneration of GSH from GSSG and peroxides can be reduced by glutathione peroxidase using GSH as donor of reduction equivalents [4, 9]. After H_2O_2 exposure, glutathione reductase (Fig. 4A) and glutathione peroxidase (Fig. 4B) activities were markedly decreased to 3.6±0.4U mg^{-1}·protein and 53.5±2.4%, respectively. Pretreatment with catalpol notably enhanced them. In addition, the highest doses of catalpol (0.5mM) had significant higher effect than the lowest dose (0.05mM), suggesting a dose-dependent effect.

As can be seen in Fig. 5, the CAT activity also decreased sharply when treated with H_2O_2 alone (43.1±1.9% of the control group), and that there was no statistically significant improvement when pretreatment with catalpol (0.05-0.25mM). Although at the highest concentration of catalpol (0.5mM), the activity of CAT only resume to 62.7±7.4% of the control group.

Discussion

As a well-established model of oxidative stress in vitro, H_2O_2 can lead to serious oxidative injury inducing impaired ATP synthesis and free radical formation [8]. Studies of the substantia nigra after death in PD have also suggested the presence of oxidative stress and depletion of GSH [16]. Astrocytes present in higher numbers in comparison to neurons in the brain. They play a crucial role in brain homeostasis and are likely participants in information processing [19, 20]. Moreover, astrocytes also respond to prolonged oxidative stress by up-regulating plasma cystine transport activity, using this amino acid for glutathione synthesis and increasing their glutathione content [21].

Given all what has been mentioned above, the effective improvement of astrocytes function in neurodegenerative diseases is surely regarded as an important therapeutic target. Catalpol, separated from *Rehmannia glutinosa* has shown protective effects in many experimental models of

neurodegeneration [22, 24].

In the present study, exposure of astrocytes to H_2O_2 showed a significant decrease in cell viability and its cytotoxic effects could be attenuated by catalpol pre-treatment in a concentration-dependent manner. This indicated that catalpol had an ability to protect astrocytes against H_2O_2 and it might be a potential protective agent. Further experiments elucidated that when administered prior to H_2O_2, catalpol decreased the intracellular ROS formation, elevated intracellular glutathione levels and successfully reversed the depressed enzyme activities in glutathione redox cycling induced by H_2O_2. In particular, pre-treatment with catalpol dramatically enhanced glutathione reductase and glutathione peroxidase activities in this model. Nevertheless, the CAT activity was not increased markedly, even pretreated with catalpol at the highest concentration (0.5mM).

The excess of ROS may lead to peroxidative impairment of membrane lipids and consequently disrupt neuronal functions and cause cell death [16]. Furthermore, H_2O_2 can traverse cell membranes to exert its biological effects intracellularly [10]. In this study, catalpol scavenged ROS nicely which supported by the decreased DCF fluorescence. This also suggested that catalpol exerts its antioxidant effects in the intracellular compartment. Oxidative stress causes cell death when intracellular levels of metabolic and antioxidant enzymes (especially glutathione related enzymes) and substrates (glutathione) are exhausted [18]. During oxidative stress, glutathione metabolism cycling in astrocytes was significant. This process also has been reported in many other cell types and proposed as a mechanism of cellular self-defence by supporting the maintenance of a reduced thiol reduction potential [14]. Thus, in order to investigate the possible mechanism of catalpol protecting astrocytes from oxidative stress induced by H_2O_2, glutathione

and glutathione related enzymes was detected as a pivotal and interesting issue of antioxidant. The ultimate results indicated that pretreatment with catalpol caused a significant enhancement of the glutathione content, glutathione reductase and glutathione peroxidase activities. Undoubtedly, these will contribute to resuming the cell viability and the capacity of antioxidation. In addition to glutathione system, CAT is also an important antioxidant pathway in the removal of hydro [18]. However, the data showed that catapol (even at the highest concentration) could not resume CAT activity to its normal level. Thereby, it was reasonably presumed that reducing overload of ROS coupled with improving glutathione redox cycling may be the main protection mechanism of catalpol in astrocytes.

In summary, our findings indicated that catalpol is endowed with significant protective effects against H_2O_2-induced oxidative stress in astrocytes primary cultures. This protection of catalpol may be not only due to its ability to inhibit the ROS formation and cell death induced by H_2O_2, but also its possibility to improve glutathione mechanism cycling, including the glutathione content and antioxidant enzymes activities. Therefore, catalpol may be an attractive candidate for the treatment of various neurodegenerative disorders associated with oxidative stress.

References

[1] B.N. Ames, M.K. Shigenaga, T.M. Hagen, Oxidants, antioxidants, and the degenerative diseases of aging, Proc. Natl. Acad. Sci. USA. 90 (1993) 7915–7922.

[2] K.B. Beckman, B.N. Ames, The free radical theory of aging matures, Physiol. Rev. 78 (1998) 547–581.

[3] M.M. Bradford, A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein–dye binding, Anal. Biochem. 72 (1976) 248–254.

[4] R. Dringen and B. Hamprecht, Involvement of glutathione peroxidase and catalase in the disposal of exogenous hydrogen peroxide by cultured astroglial cells, Brain. Res. 759 (1997) 67–75.

[5] R. Dringen and B. Hamprecht, Glutathione restoration as indicator for cellular metabolism of astroglial cells, Dev. Neurosci. 20 (1998) 401–407.

[6] R. Dringen, Metabolism and functions of glutathione in brain, Neurobiology 62 (2000) 649-671.

[7] R. Dringen, P.G. Pawlowski, J. Hirrlinger, Peroxide detoxification by brain cells, J. Neurosci. Res. 79 (2005) 157–165.

[8] M.R. Duchen, Roles of mitochondria in health and disease. Diabetesn. 53 (2004) S96–S102.

[9] M.B. Glenda, R. Dringen, R.R. Stephen, Zinc stimulates the production of toxic reactive oxygen species (ROS) and inhibits glutathione reductase in astrocytes, Free Radical Biology & Medicine 42 (2007) 1222–1230.

[10] B. Halliwell and J.M.C. Gutteridge, Free radicals and antioxidants in the year. A historical look to the future, Ann. NY. Acad. Sci. 899 (2000) 136–147.

[11] M.B. Hansen, S.E. Nielsen, K. Berg, Re-examination and further development of a precise and rapid dye methods for measuring cell growth/cell kill, J. Immunol. Methods. 119 (1989) 203–210.

[12] J. Hirrlinger, J.B. Schulz, R. Dringen, Effects of dopamine on the glutathione metabolism of cultured astroglial cells: implications for Parkinson's disease, J. Neurochem. 82 (2002) 458–467.

[13] B. Jiang, J.H. Liu, Y.M. Bao, L.J. An, Catalpol inhibits apoptosis in hydrogen

peroxide-induced PC12 cells by preventing cytochrome c release and inactivating of caspase cascade, Toxicon. 43 (2004) 53–59.

[14] D. Keppler, Export pumps for glutathione S-conjugates, Free. Rad. Biol. Med. 27 (1999) 985–991.

[15] B.M. Lomaestro, M. Malone, Glutathione in health and disease: pharmacotherapeutic issues, Ann. Pharmacother. 29 (1995) 1263–1273.

[16] E. Manuchair, K.S. Shashi and D.B. Mayur, Oxidative stress and antioxidant therapy in parkinson's disease, Neurobiology 48 (1996) 1–19.

[17] Y.R. Mao, L. Jiang, Y.L. Duan, L.J. An and B. Jiang, Efficacy of catalpol as protectant against oxidative stress and mitochondrial dysfunction on rotenone-induced toxicity in mice brain, Environmental Toxicology and Pharmacology 23 (2007) 314–318.

[18] M.V. Naval, M.P. Gomez-Serranillos, M.E. Carretero, A.M. Villar, Neuroprotective effect of a ginseng (*Panax ginseng*) root extract on astrocytes primary culture, J. Ethnopharmacol. 112 (2007) 262–270.

[19] M. Nedergaard, Direct signaling from astrocytes to neurons in cultures of mammalian brain cells, Science 263 (1994) 1768–1771.

[20] V. Parpura, T.A. Basarsky, F. Liu, K. Jeftinija, S. Jeftinija, P.G. Haydon, Glutamate-mediated astrocyte-neuron signaling, Nature 369 (1994) 744–747.

[21] J. Sagara, N. Makino, S. Bannai, Glutathione effux from cultured astrocytes, J. Neurochem. 66 (1996) 1876–1881.

[22] Y.Y. Tian, L.J. An, L. Jiang, Y.L. Duan, J. Chen, B. Jiang, Catalpol proetects dopaminergic neurons from LPS-induced neurotoxicity in mesencephalic neuron-glia cultures, Life Science

80 (2006) 193–199.

[23] H. Wang, J. Joseph, Quantifying cellular oxidative stress by dichlorofluorescein assay using microplate reader, Free Radical Biology and Medicine 27 (1999) 612–616.

[24] X.L. Zhang, B. Jiang, Z.B. Li, S. Hao, L.J. An, Catalpol ameliorates cognition deficits and attenuates oxidative damage in the brain of senescentmice induced by D-galactose, Pharmacol. Biochem. Behav. 88 (2007) 64-72.

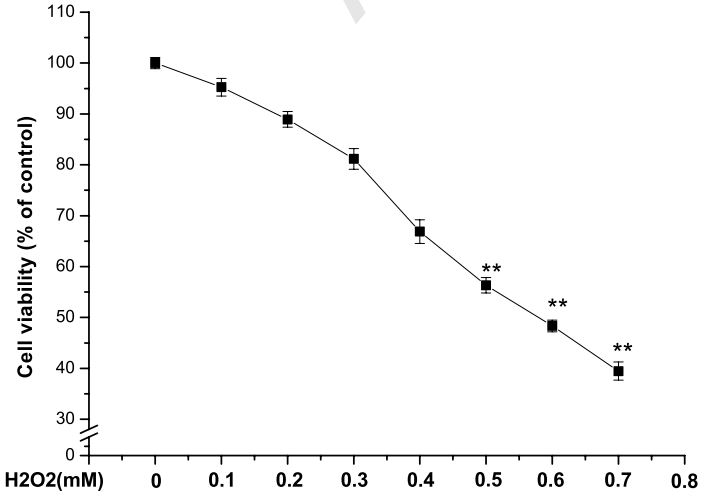
Fig. 1. Effects of H_2O_2 in different concentrations on astrocytes viability. Data are mean±S.D. (n=4) obtained from three independent experiments. $^{**}P<0.01$ compared with the control group.

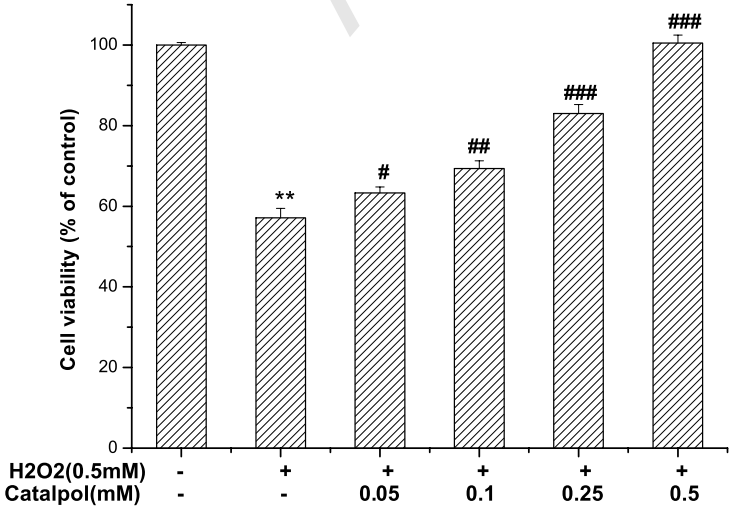
Fig. 2. Effects of catalpol on H_2O_2-induced cytotoxicity in cultured astrocytes. Data are mean±S.D. (n=4) obtained from three independent experiments. $^{**}P<0.01$ in comparison with control, $^{\#}P<0.05$, $^{\#\#}P<0.01$ and $^{\#\#\#}P<0.005$ compared with cells exposed to H_2O_2 alone.

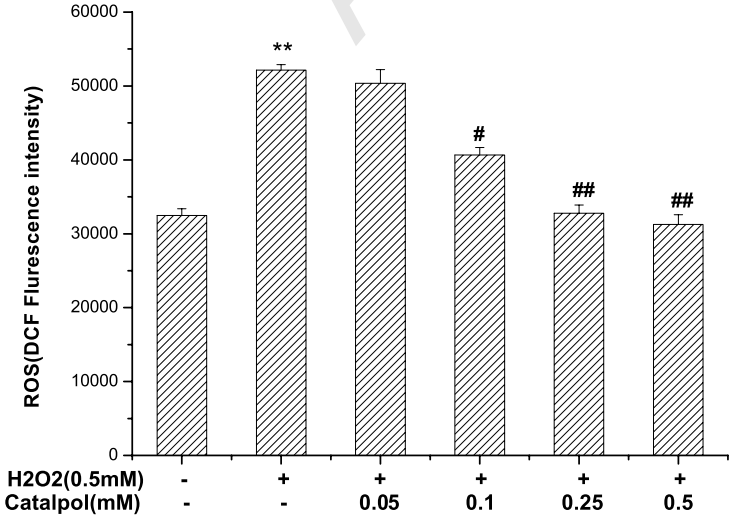
Fig. 3. Inhibitory effect of catalpol on H_2O_2-induced production of intracellular reactive oxygen species (ROS). Data are mean±S.D. (n=4) obtained from three independent experiments. $^{**}P<0.01$ in comparison with control, $^{\#}P<0.05$ and $^{\#\#}P<0.01$ compared with H_2O_2-treated cultures alone.

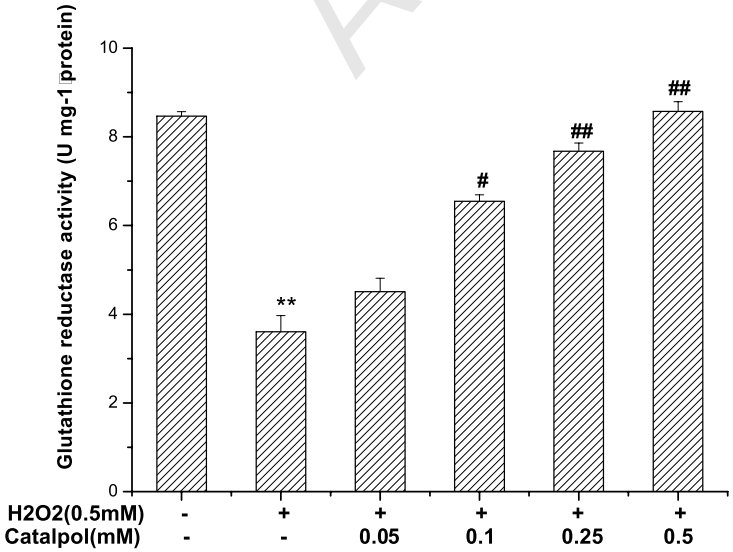
Fig. 4. Effects of catalpol on the activities of glutathione reductase (A) and glutathione peroxidase (B) in H_2O_2 -induced astrocytes. Data are mean±S.D. (n=4) obtained from three independent experiments. $^{**}P<0.01$ in comparison with control, $^{\#}P<0.05$ and $^{\#\#}P<0.01$ compared with H_2O_2-treated cultures alone.

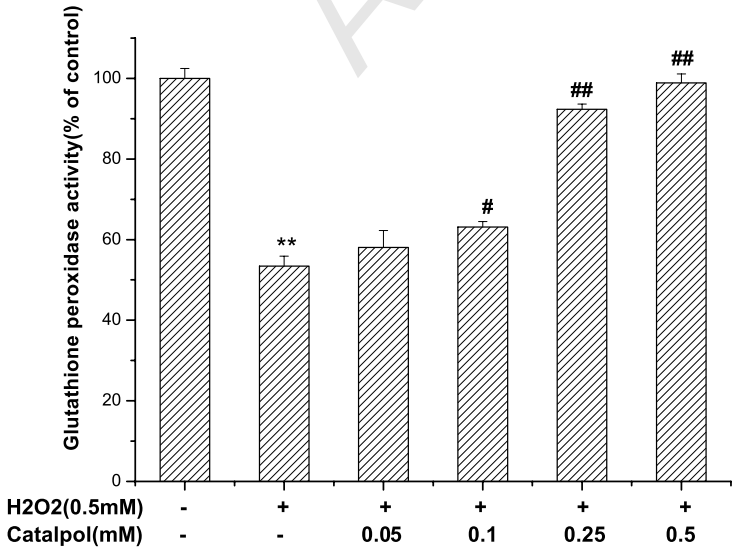
Fig. 5. Effects of catalpol on the activity of catalase (CAT) in H_2O_2-induced astrocytes. Data are mean±S.D. (n=4) obtained from three independent experiments. ***P<0.005 in comparison with control, $^{\#}P$<0.05 compared with H_2O_2-treated cultures alone.











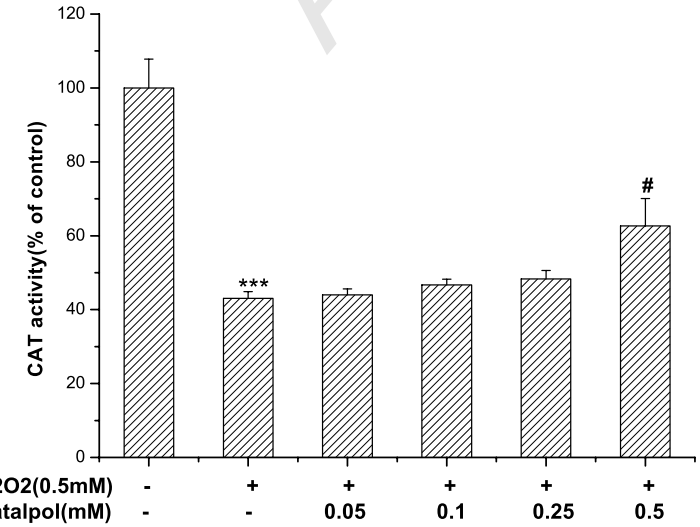


Table. 1 Effects of catalpol on the glutathione metabolism in H_2O_2 –induced astrocytes.

	GStotal (nmol/mg protein)	GSSG(nmol/mg protein)	GSSG(% of GStotal)
Control	35.3±0.8	0.9±0.05	2.6±0.2
H_2O_2-induced	17.3±1.2**	5.8±0.1***	33.5±3.0***
Catalpol(0.05mM) + H_2O_2	19.4±0.5	$4.5\pm0.05^{\#}$	$25.3\pm0.4^{\#}$
Catalpol(0.1mM) + H_2O_2	$25.0\pm0.6^{\#}$	$3.6\pm0.05^{\#\#}$	$14.3\pm0.5^{\#\#}$
Catalpol(0.25mM) + H_2O_2	$28.7\pm0.8^{\#\#}$	$2.3\pm0.1^{\#\#}$	$8.0\pm0.3^{\#\#}$
Catalpol(0.5mM) + H_2O_2	$34.0\pm1.4^{\#\#\#}$	$1.3\pm0.05^{\#\#\#}$	$3.6\pm0.3^{\#\#\#}$

Data are mean±S.D. (n=4) obtained from three independent experiments. $^{**}P<0.01$ and $^{***}P<0.005$ in comparison with control, $^{\#}P<0.05$, $^{\#\#}P<0.01$ and $^{\#\#\#}P<0.005$ compared with H_2O_2-treated cultures alone.