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Original Article

MPEG-SOD inhibits cerebral hemorrhage induced neural cell apoptosis and c-myc upregulation

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Abstract: Studies indicated that oxidative stress induced by cerebral hemorrhage might affect the neuronal cell apoptosis and c-myc protein overexpression. 100 rats were random equally to two groups-test and control. Each group was set 5 time points at 6 h, 12 h, 24 h, 48 h and 72 h after administration, respectively. After hydraulic brain injury rat model was successfully established, animals in test group were given a single intravenous injection with 5 mg/L/kg mono-methoxy polyethylene glycol modified-SOD (MPEG-SOD), while animals in control group were injected with isotonic saline. The rate of neuronal cell apoptosis, the expression level of c-myc protein, and the correlationship between them were analysed. Cell edema and widened gap occurred in brain at 6 h, and the most obvious brain damage appeared at 48 h after cerebral hemorrhage in test group. The number of TUNEL-positive cells in test group at each time point were significantly less than those in control group ($P<0.01$). The gray values of c-myc protein in test group at each time point were lower than those in control group ($P<0.01$). The number of TUNEL-positive nerve cells and the gray level of c-myc protein were correlated (test group $R^2 = 0.857$, control group $R^2 = 0.654$, $P<0.01$). The cell apoptosis rates in test group were significantly lower than those in control group ($P<0.01$). In onclusion, while oxidative stress plays a key role in neuronal death after traumatic brain injury, MPEG-SOD inhibits the increase the overexpression of c-myc protein, and prevents neuronal cell apoptosis.

Keywords: Cerebral hemorrhage, oxidative stress, neural cell apoptosis, c-myc protein expression

Introduction

Recently, cerebral hemorrhage has become the most common accident in emergency neurology, of which clinical mortality rate is very high, and even if they can survive, but suffered neurological brain damage. Most patients survive life without ability to take care of themselves, which cause a huge burden to the society. There is a high incidence of spontaneous intracerebral hemorrhage in China, and the age of pathogenesis tends to much younger [1-3]. Currently, while the pathological mechanism of cerebral hemorrhage was complicated, studies indicated that the leading cause of secondary neurological injury after cerebral hemorrhage was nerve cell apoptosis, namely oxidative stress occurs after nerve cell apoptosis, resulting in edema or even necrosis of nerve cells, abnormal expression of c-myc protein caused imbalance in normal cell cycle regulation, leading to nerve cell apoptosis. But studies on whether c-myc protein overexpression and reactive oxygen species involved in nerve cell apoptosis after cerebral hemorrhage was less [4]. It was found that as a purified SOD and MPEG combination product, mono-methoxy polyethylene glycol-superoxide dismutase (MPEG-SOD) could reduce the content of free radicals in tissue, and protect nerve cells [5]. But there were few studies on whether MPEG-SOD could inhibit expression of the c-myc protein in hematoma surrounding tissue of patients with intracerebral hemorrhage and the relationship with the death of nerve cells [6]. Therefore, the present study uses hydraulic shocks to establish cerebral hemorrhage model, in order to study relationship between neuronal apoptosis and c-myc protein expression in rats with cerebral hemorrhage, as well as the influence of MPEG-SOD on both phenomenon.

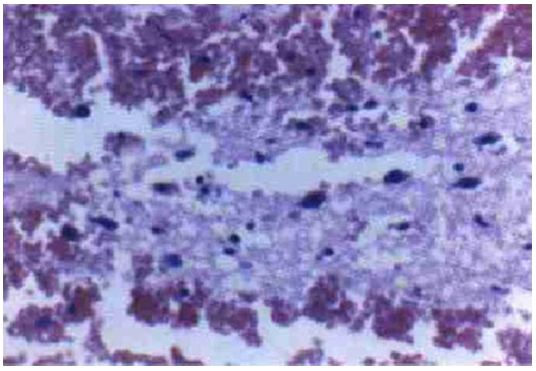

Figure 1. Apoptotic cells were exhibited with cells nuclear condensation and deeply stained around cerebral hemorrhage edema area.

Materials and methods

Animals

100 healthy male SD rats, weighing 220-280 g, provided by Shanghai Chris Laboratory Animal Co., license number: SCXK (Shanghai) 2014-0010, housed separately, animal laboratory temperature (20-25)°C, relative humidity (50 to 60)%.

Rats were used for all experiments, and all procedures were approved by the Animal Ethics Committee of Shenzhen Chinese Traditional Medical Hospital.

Agents and facilities

Mono-methoxy polyethylene glycol-superoxide dismutase (MPEG-SOD) were purchased from Shijiazhuang City Hysen Chemical Co.; situ cell death detection kit purchased from Beijing Zhongshan Biotechnology Limited; animal hydraulic injury means available from the US Department of Biomedical Engineering at Northwestern University; pressure sensors purchased from American Gloud companies.

Hydraulic model

The 100 rats were divided randomly into test and control groups, and each group was set 6 h, 12 h, 24 h, 48 h and 72 h time-point, leading to divide into five phase subgroups. According to McIntosh et al. [7], hydraulic strike force of 2.3 KPa in the hydraulic model of brain injury. After the modeling, the rats appeared short limbs, stiffness, increase in breathing and heart rate, but returned to normal at 1 min after brain injury. While the test group was administered intravenously 5 mg/L/kg MPEG-SOD, the control group received the same amount of intravenous isotonic saline after the modeling 30 min. Tissue isolation was conducted in test group and control group at 6 h, 12 h, 24 h, 48 h and 72 h after brain injury. Remove brain tissue in rats under anesthesia, embed with paraffin, slice in serial sections, 5 μm. Select three cerebral hemorrhage tissue slices from each animal randomly, 10 vision per slice, HE staining in situ, label with TUNEL marks and immunohistochemical staining.

Assay of TUNEL and c-myc protein

Expression of c-myc protein: SP detection after immunohistochemical staining, positive cells: cell cytoplasm brown, using computer MIAS-300 analysis c-myc protein gray scale. TUNEL tags: apoptosis detection follow the TUNEL kit instruction after staining, apoptosis is the brown staining of cells; apoptosis rate: clean the cerebral hemorrhage area and surrounding brain tissue in rats with cerebral hemorrhage, cut the brain tissue and digest with trypsin, about 30 min, using a 200 mesh strainer to filter twice. Then, add ribonuclease, 37°C, 30 min, add propidium iodide solution, detect the degree of apoptosis with flow cytometry, then calculate the apoptotic index (AI): three slices per animal, each slice calculated in accordance with 10 high-power fields, AI = (apoptosis of nerve cell count/total number of nerve cells)%.

Statistical analysis

Experimental data was expressed as mean ± standard deviation, analyzed with statistical software SPSS17.0, using the T-test.

Results

HE staining of tissue from rat bleeding brain

Cerebral tissue edema and brain tissue gap widened showed up at 6 h after cerebral hemorrhage in rats; at 48 h, there were brain injury, brain hemorrhage surrounding cells nuclear condensation, stained, and cell membrane integrity, such nerve cells namely apoptotic cells (**Figure 1**).

TUNEL positive staining in rat brain

Experimental results were shown that TUNEL positive stain was detected 6 h after cerebral hemorrhage, and the number of positive cells

Table 1. The percentage of TUNEL positive cell between the two groups

Group	TUNEL positive cell				
	6 h	12 h	24 h	48 h	72 h
Test	9.23±1.23	27.21±5.17	42.16±10.53	50.32±11.54	39.21±8.65
Control	16.33±1.54	41.43±8.11	66.53±11.59	75.17±11.65	59.43±11.91
t	8.765	11.543	10.876	10.987	11.876
P	0.009	0.006	0.007	0.007	0.006

Table 2. Cell apoptosis rates between the two groups

Group	n	Cell apoptosis rate (%)				
		6 h	12 h	24 h	48 h	72 h
Test	50	5.43±1.54	10.31±2.12	12.13±3.51	12.21±3.12	3.21±1.12
Control	50	10.32±2.12	21.32±3.34	26.32±4.22	27.21±4.32	9.43±1.81
t		10.732	11.541	11.876	12.912	13.816
P		0.006	0.006	0.005	0.005	0.004

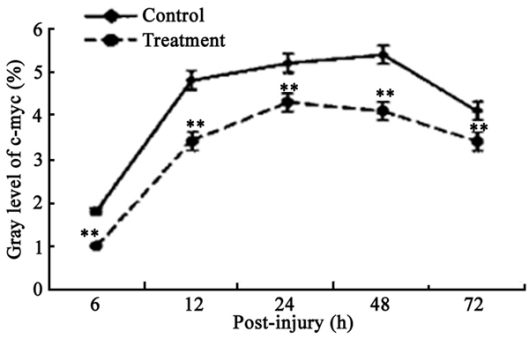

Figure 2. Percentage of gray level of c-myc protein in different time points belong two groups.

increased at 24 h and reached a peak at 48 h, but started to decrease at 72 h. The number of TUNEL-positive cells in the test group was significantly less than that in the control group (P<0.01) (**Table 1**).

Neural cell apoptosis rate

Apoptosis peak shown up at 6 h after cerebral hemorrhage, reached a peak at 48 h. Apoptosis rate at 6 h, 12 h, 24 h, 48 h and 72 h subgroups was significantly reduced (P<0.01), while compared with the control group (**Table 2**).

Inmmunohistochemistry staining of c-myc protein

Expression of c-myc protein at 6 h after cerebral hemorrhage was mainly in brain damage, areas surrounding edema, peripheral edema hippocampus region, and gradually moving into the nucleus. At 24 h and 48 h after cerebral hemorrhage, the expression of c-myc protein mainly expressed in the nucleus, and the level was higher according to time. But, the level of c-myc protein expression decreased at 72 h after cerebral hemorrhage. The gray value of c-myc protein was significantly less than that in the control group at 6 h after cerebral hemorrhage, and the difference was statistically significant (P<0.01). The gray value of c-myc protein was significantly less than that in the control group at 12 h after cerebral hemorrhage, and the difference was statistically significant (P<0.01). The gray value of c-myc protein was significantly less than that in the control group at 24 h after cerebral hemorrhage, and the difference was statistically significant (P<0.01). The gray value of c-myc protein was significantly less than that in the control group at 48 h after cerebral hemorrhage, and the difference was statistically significant (P<0.01). The gray value of c-myc protein was significantly less than that in the control group at 72 h after cerebral hemorrhage, and the difference was statistically significant (P<0.01). Consequently, There was correlationship between the number of TUNEL-positive nerve cells and the gray value of c-myc protein (test group R^2 = 0.857, group R^2 = 0.654, P<0.01) (See **Figures 2**-**4**).

Discussion

Histopathological damage after cerebral hemorrhage was classified two types-primary injury and secondary injury. Primary injury was mainly caused by the hematoma pressing the surrounding brain tissue, so that it was unavailable to interfere; but the secondary injury is the emergence of a radical loss and the inflammatory response around the hematoma brain tissue, and then accompanied by inflammation, ischemia and edema, leading to accelerate further deterioration of nerve function [8, 9]. There were regional cerebral edema and tissue dam-

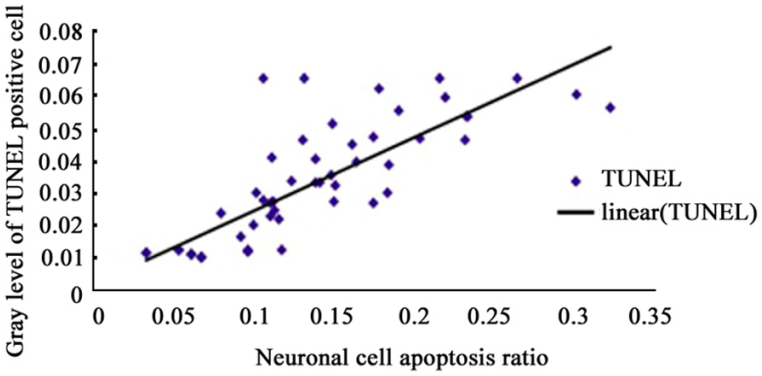

Figure 3. Immunohistochemical analysis of c-myc protein: gray level of TUNEL-positive cells and neuronal apoptosis in test group and control group, as well as correlation analysis of the data.

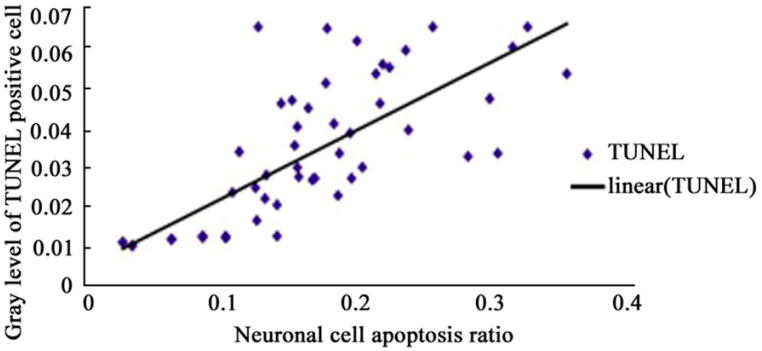

Figure 4. Immunohistochemical analysis of c-myc protein: gray level of TUNEL-positive cells and neuronal apoptosis in test group and control group, as well as correlation analysis of the data.

age zone in the brain tissue around the hematoma being able to exacerbate the disease, but this kind of injury cells still have the possibility of recovery. Therefore, the function of neural cell suffered from damage being able to significantly improve if the brain was actively dealt with during the stage. But with the continued development of lesions reaction, brain tissue damage would become irreversible, causing permanent damage [10]. Such kind of secondary brain injury after cerebral hemorrhage still has a possibility of being able to restore within a specific period. Moreover, reduction in hazard of secondary brain damage and improving recovery penumbra around the hematoma brain tissue function has become the research hotspot recently [11, 12].

Recently, studies have shown that MPEG-SOD, as purified SOD and MPEG combination product, could reduce the level of radicals, protect nerve cells, slow down the speed of lipid peroxidation, improve microcirculation, prevent cell edema and maintain nerve cell excitability [13, 14]. Clinical research trial F conducted by Qureshi et al. [15] has found that apoptotic cells appeared in the brain tissue of patients with at 24 h after intracerebral hemorrhage. Matsushita et al. [16] labeled the apoptotic cells with the TUNEL staining, most of which were neurons and astrocytes, indicating that cell apoptosis and tissue damage is closely related. Results suggested that cell edema occurred and brain tissue gap widened along with the rat brain hemorrhage, while the number of positive cells reached to a peak at 24-48 h after cerebral hemorrhage. The results of measurement of the cell cycle by flow cytometry suggested that cell apoptosis peak occurred at 6 h after cerebral hemorrhage in rats. The apoptosis reached to peak at 48 h after cerebral hemorrhage, and the incidence of apoptosis decreased. Immunohistochemical staining results suggested that c-myc protein expression was mainly in the nucleusin at 24 h and 48 h after cerebral hemorrhage, and the degree of expression gradually increased; while the level of expression decreased at 72 h after brain hemorrhage. The gray levels of c-myc protein in five time-point 6 h, 12 h, 24 h, 48 h and 72 h belong to the test group were lower than those belong to the control group ($P<0.01$). Results suggested that the expression of c-myc protein occurred both in the test group and the control group of rats suffered from intracerebral hemorrhage, indicating that c-myc protein expression along with the occurrence of cell apoptosis, and there was correlationship between TUNEL-positive nerve cells and the gray level of c-myc protein. Results of gray level of c-myc protein in the test group lower than that in the control group indicated that the test group MPEG-SOD could inhibit the expression

of c-myc protein. More recently, reports from intracerebral hemorrhage related rat models from foreign labs showed that the intervention of NF-κB activation could reduce neural cell apoptosis after cerebral hemorrhage in rats, and the active oxygen could activate NF-κB (NF-κB) under the condition of oxidative stress. Furthermore, NF-κB could activate apoptotic gene c-myc, and the change in the intensity of expression of c-myc gene accompanied by the transfer of NF-κB. When NF-κB transferred into the nucleus and reach to the peak, the expression of c-myc gene enhanced, indicating that apoptosis-related genes c-myc participated in and promoted the neural cell apoptosis induced by oxidative stress. Max binding to c-myc gene to form a heterodimer. Then it could bind to DNA core sequence in a dimer form again, and control the DNA transcription and neural cell apoptosis [17-21].

To sum up, the oxidative stress plays an important role in neural cell apoptosis after traumatic brain injury, but MPEG-SOD could inhibit the increase in TUNEL-positive neurons and the expression of c-myc protein after cerebral hemorrhage, thereby reduce the neural cell apoptosis.

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Disclosure of conflict of interest

None.

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