Protective effect of oyster extract on apoptosis of cerebral neural stem cells induced by hyperthermia*★

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Abstract

BACKGROUND: Previous results of our study show that oyster extract has some protective effects on apoptosis of the neuroepithelium in neural tube defects induced by hyperthermia in vivo. It remains unclear whether the extract also protects in vitro cultured neural stem cells.

OBJECTIVE: To investigate the protective effect of oyster extract on apoptosis of cerebral neural stem cells induced by hyperthermia.

METHODS: The cerebral neural stem cells of embryonic mice of 13 days were cultured in vitro. Nestin expression was detected by immunofluorescence method to identify neural stem cells. The neural stem cells of passage 3 were divided randomly into 4 groups: hyperthermia control group and oyster treated I, II, III groups (mass concentration 2.5, 5, 10 g/L oyster extract solution). In addition, culture solution control group (no cells), and culture solution+ oyster extract control group (no cells) were designed. All oyster extract groups and control groups were treated by hyperthermia over 39 °C. The survival rate and the vitality of neural stem cells were detected by trypan blue staining and MTT assay. Western-blotting was employed to explore the expression of p53 in cerebral neural stem cells of each group.

RESULTS AND CONCLUSION: The survival rate and the value of MTT assay in oyster treated groups II and III were significantly greater than hyperthermia control group ($P < 0.05$), but the expression of p53 in oyster treated groups II and III were weaker than hyperthermia control group. Oyster extract plays an important protective role in the apoptosis of neural stem cells induced by hyperthermia.

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INTRODUCTION

Embryonic neural tube defects (NTDs) may associate with syndromes, disorders, and maternal factors[1]. In animals, excessive core body temperatures have been documented to cause malformation, NTDs are the most frequently reported. Annually, there are 80 000–100 000 neonates with NTDs in China[2]. Hyperthermia during early pregnancy can induce NTDs in embryos[3].

Hyperthermia, a common physical injury factor, could induce several kinds of apoptosis[4-5]. Results of our previous study has shown that oyster extract had protective effect on apoptosis in NTDs by hyperthermia[6], but there is no report showing that it has protective effect on apoptosis of neural stem cells in vitro. The present study investigated the protective effect of oyster extract on primary cultures of neural stem cells exposed to hyperthermia.

METERIALS AND METHODS

Design

In vitro cytology study.

Time and setting

The experiment was performed at Shandong University from January 2005 to December 2006.

Materials

Female Kunming mice, gestational 13.0–14.0 days, were provided by the Laboratory Animal Center of Shandong University (Jinan, China). The experimental procedures were performed in accordance with the *Guidance Suggestions for the Care and Use of Laboratory Animals*, formulated by the Ministry of Science and Technology of the People's Republic of China[7].

Oyster extract (32.5% protein, 63.7 g/kg taurine, 228 mg/kg Zn) was provided by Penglai Marine Organisms Limited Company (Penglai, China). Require concentration of extract was prepared with PBS, autoclaved and stored at 4 ℃.

Reagents and instruments are listed as follows:

Reagent and instrument	Source
Photomicrograph system	Olympus Corporation, Japan
MT-1 transfer electrophoresis bath	Xinzhi Scientific Instrument Institute, Ningbo, China
Superclean bench	Sujing Clique
CO_2 incubator	HERAEUS Corporation, USA
DMEM/F12, B27, epidermal growth factor (EGF), basic fibroblast growth factor (bFGF)	Gibco Corporation, USA
Fetal calf serum (FCS)	Sijiqing Corporation, Hangzhou
nestin monoclonal antibody	Abcam Corporation, USA
FITC signed II antibody	Southern Biotenich
Polyclone rabbit-p53 antibody, biotinylation II antibody, ECL kit	Sigma Corporation, USA

Methods

Cell culture

Neural tube tissue from mice embryos of early pregnancy was soaked in D-Hank's solution. The cerebral cortex of embryos was harvested, and meninges were removed. The cortex was minced, centrifuged at 1 000 r/min for 5 minutes, washed with DMEM for 3 times. The neural stem cells at a concentration of $(2.0–3.0)\times10^8$/L were cultured with

growth medium containing DMEM/F12, EGF (20 μg/L), bFGF (20 μg/L), 2%B27, at 37 °C in 95% air and 5% CO_2. Nestin was detected by immunofluorescence staining to identify the neural stem cells. Cells of passage 3 to 7 were used in the experiments.

Cell grouping

The cells after 3 passages were seeded into 96-well plate and divided randomly into 4 groups, including hyperthermia control group, oyster treated I, II, III groups, culture solution control group (no cells), and culture solution+ oyster extract control group (no cells). All oyster extract groups and control groups were treated by hyperthermia.

Measurement of survival rate and viability

The neural stem cells of oyster treated groups were incubated with 10 μL oyster extract solution at a mass concentration of 2.5, 5 and 10 g/L, respectively. The cells were cultured for 36 hours, incubated 30 minutes at 39 °C and then cultured for 2 hours, followed by trypan blue staining and MTT assay to examine cell survival and viability, respectively.

MTT assay: 20 μL MTT(5 g/L) was added to each well and incubated for 4 hours, followed by the addition of 150 μL dimethyl sulfoxide. The absorbance of samples was measured at 570 nm. Each assay was performed in triplicate.

Trypan blue staining: 0.4% trypan blue was added to each well and incubated for 15 minutes. The number of death cells and viable cells was quantified under the microscope

Western-blotting

Protein concentration of cell extracts was determined using the bicinchoninic acid kit according to the manufacture's protocol. Total protein was applied to each lane on 10% SDS-polyacrylamide gels, and transferred to polyvinylidene fluoride membranes. The membranes were blocked with 5% milk, and incubated with anti-p53 antibody as primary antibody (1:100) overnight at 4 °C. The membranes were incubated with secondary antibody and the ECL western blotting analysis system according to the manufacture's protocol was used. The filter was stripped the probed with a goat polyclonal antibody against β-action to determine whether the amount of proteins in each lane was comparable.

Main outcome measures

Cell survival rate, cell proliferation activity, and p53 expression.

Design, enforcement and evaluation

The study was designed by Song Haiyan and Wu Yuling, performed by Song Haiyan, Song Yongli and Yu Qingmei, and evaluated by Song Haiyan and Wu Yuling with a blind method. All authors received training.

Statistical analysis

All statistical analyses were performed by the first author with SPSS 12.0 software package for Macintosh. The data were expressed as Mean±SD. The survival rate and the value of MTT assay among groups were analyzed by *t*-test. $P < 0.05$ was considered significant, and $P < 0.01$ remarkably significant.

RESULTS

Trypan blue staining

The number of viable cells in the oyster treated II and III were greater than the hyperthermia control group ($P < 0.05$), but in oyster treated group I, there was no significantly change compared with hyperthermia control group. The results showed that oyster extract could protect the cell growth doe-dependently (Figure 1).

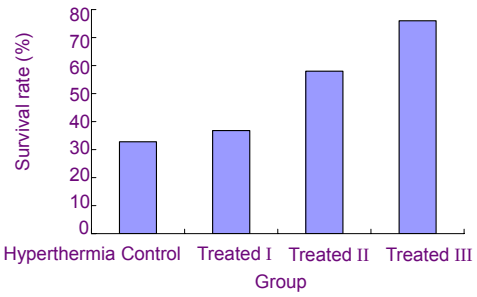
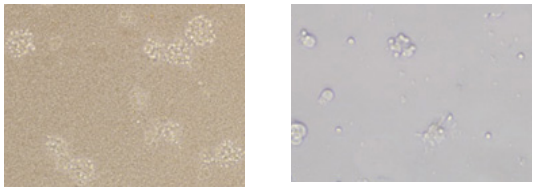


Figure 1 Cell survival rate in hyperthermia control group and oyster treated groups I, II, III

MTT assay

Compared with hyperthermia control group, the neurosphere did not exhibit significant changes in oyster treated group I. But in the oyster treated II and III, there were more neurospheres with large volume, lace rim, clear cell boundary compared with hyperthermia control group (Figure 2).



a: Treated group III b: Hyperthermia control group (×100)

Figure 2 Growth state of neural stem cells after treated by hyperthermia.

Compared with the hyperthermia control group, the value of MTT assay in the oyster treated II and III were greater ($P < 0.05$, Table 1).

Table 1 Effects of different concentrations of oyster extract on apoptosis of neural stem cells ($\bar{x}\pm s$)

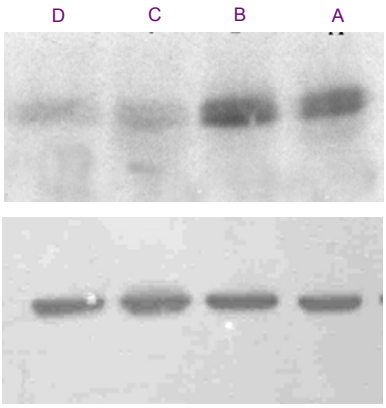
Group	Value of MTT
Hyperthermia control	0.877±0.042
Treated I	0.878±0.052
Treated II	0.941±0.044[a]
Treated III	0.991±0.056[a]
Control solution	0.325±0.026
Control solution+oyster extract	0.344±0.023

[a]$P < 0.05$, *vs*. hyperthermia control group

Expression of p53 by Western blotting

Western blotting results showed that the expression of p53 was decreased significantly in neural stem cells of oyster treated II

and III compared with hyperthermia control group, suggesting that the cell growth protection and apoptosis induced by oyster extract were correlated with this gene expression (Figure 3).



A: Hyperthermia control group; B: Treated group I; C: Treated group II; D: Treated group III

Figure 3 Expression of p53 in neurons analyzed by Western-blotting

DISCUSSION

NTDs are generally believed to result from failure of fusion of the neural tube during early embryogenesis. Epidemiological and experimental studies on NTDs provide some evidence that a host of physical agents (*e.g*. x-irradiation, hyperthermia), drugs (*e.g*. thalidomide folate antagonists), substance abuse (*e.g*. alcohol), chemical agents (*e.g*. organic mercury), maternal infections, and maternal metabolic conditions, are capable of causing congenital malformations of the central nervous system structures[8-10].

Hyperthermia has emerged as a common risk factor for NTDs. In a study of the effect of hyperthermia on rat embryos in culture by completely removing the embryo from maternal influence and exposing rat embryonic explants to mild hyperthermic insults during the onset of neural tube closure, nearly one-half of exposed rat embryos were microcephalic[11]. In addition, the parameters determining hyperthermia-induced heat defects in the rat showed a dose-response manner of temperature and duration of exposure in the induction of NTDs in the experimental animals[12]. Numerous experimental, clinical and epidemiological studies have demonstrated that maternal heat stress during pregnancy can produce a high frequency of fetal malformation[13-14].

Based on results of experiments in laboratory animals, several mechanism of NTDs induced by hyperthermia have been hypothesized including reduced rates of cell proliferation, inhibition of protein synthesis, apoptosis[15-16]and upregulation of p53[17]. Recent in vitro studies show that heat-induced cell death in susceptible tissue of mouse embryos involves mitochondria-mediated apoptotic pathway[18]. In addition, neuroepithelium of heat-stressed embryos have shown apoptotic figures[19]. Results from the present study have demonstrated neuroepithelium of rat embryos was sensitive to maternal hyperthermia, and there were numerous apoptotic cells.

At present there is no effective treatment for NTDs once the neural tube has failed to close, and preventive therapy must be targeted to early pregnancy. The present study showed that oyster extract had protective effect on apoptosis of the neuroepithelium in NTDs induced by hyperthermia *in vivo*. The goal of the present study was to investigate whether oyster extract could protect apoptosis of neural stem cells cultured in vitro induced by hyperthermia.

Oyster is an ocean seashell animal with abundant nutritional ingredient, such as taurine, zinc which has effect of antioxygen and protecting tissues from the injury by oxidant and free radicals. Taurine is widely distributed in brain and can promote the growth, proliferation and differentiation of nervous system. High concentration of zinc has been demonstrated to reduce apoptosis[20].

This experiment investigated the reproductive activity of neural stem cells treated with oyster extract by MTT. The result showed that measurement value of hyperthermia control group was significantly lower than oyster extract treated II and III. In order to avoid the effect of color of oyster extract on measurement value, differences between hyperthermia control group and culture solution control group, and differences between each experimental group and culture solution+ oyster extract control group were measured, followed by the statistical analysis between them. The result showed there were significant differences, suggesting oyster extract had protective effect on neural stem cells treated by hyperthermia.

Animal study reported that expression of p53 in embryos which maternal treated by hyperthermia was upregulated[17]. P53 can promote apoptosis as the major regulatory in the pathological condition, such as DNA injury, ischemia and excitotoxicity. Studies showed that the injury of DNA could cause the increase of p53 level to terminate proliferation and obtain more time for DNA repair, but if the DNA could not been repaired, the level of p53 would increase constantly until apoptosis[21-22].

In the present study, the expression of p53 was greater in hyperthermia control group, indicating that p53 took part in the apoptosis of neural stem cells treated by hyperthermia. The level of p53 in oyster extract treated II and III was lower than that in hyperthermia control group, showing oyster extract could reduce the apoptosis of neural stem cells induced by hyperthermia. Results from the present study further indicated that oyster extract had protective effect on apoptosis of nerve cells induced by hyperthermia. However, investigations are required to explore the mechanisms, which might relate with the taurine and zinc that can clear free radical, reduce the peroxidation and attenuate DNA injury.

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牡蛎提取物在高温诱导皮质神经干细胞凋亡中的保护作用*★

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摘要

背景：课题组前期研究表明，牡蛎提取物对高温所致神经管畸形中的凋亡细胞有一定的保护作用，该提取物对体外培养的神经干细胞的凋亡是否也有保护作用，尚未见报道。

目的：观察牡蛎提取液在高温所致神经干细胞凋亡中的保护作用。

方法：取孕 13 d 小鼠胚胎大脑皮质细胞培养，采用免疫荧光法检测巢蛋白的表达以鉴定神经干细胞，将培养 3 代的神经干细胞随机分为 4 组：高温对照组和牡蛎保护Ⅰ、Ⅱ、Ⅲ组(分别加入质量浓度 2.5，5，10 g/L 的牡蛎提取液)，同时设立培养液对照组(其中无细胞)、培养液+牡蛎提取液对照组(其中无细胞)，各牡蛎保护组及对照组均经过 39 ℃高温处理。锥虫蓝染色法检测神经干细胞存活率；MTT 法检测神经干细胞的增殖活性；蛋白质印迹方法检测神经干细胞凋亡相关蛋白 p53 的表达。

结果与结论：牡蛎保护Ⅱ、Ⅲ组中细胞的存活率及 MTT 值明显高于高温对照组($P < 0.05$)；而 p53 的表达则明显低于高温对照组。提示牡蛎提取液对高温所致神经干细胞的凋亡具有一定的保护作用，这种作用有浓度依赖性。

关键词：牡蛎；神经干细胞；高温；小鼠；凋亡
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