

Full Length Research Paper

Protective effect of L-3-n-Butylphthalide on the brain of ethanol dependence rats

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This study was carried out to explore the protective effect of L-3-nbutylphthalide (NBP) on the brain of ethanol dependence rats. With the administration of different dosage of NBP, the variation of withdrawal scores, hydrogen sulfide (H₂S) contents, cystathionine β-synthase (CBS) activities and expression N-methyl-D-aspartate (NMDA) receptor 2B subunit (NR2B) in the hippocampus tissue of ethanol dependent rats were discussed. Results showed that most significant difference in withdrawal symptom score, H₂S content, CBS activity and the expression of NR2B mRNA between normal and ethanol dependence model group rats ($P < 0.01$) was observed. There were no significant differences in withdrawal symptom score, H₂S content, CBS activity and the expression of NR2B mRNA between low dose NBP group and experiment control group rats ($P > 0.05$). However, withdrawal symptom score, H₂S content, CBS activity and NR2B mRNA expression in both middle and high dose NBP group rats were much lower than those of experiment control group ($P < 0.05$). With increasing concentrations of NBP, it could reduce withdrawal symptoms of ethanol dependence rats to different extent. The alleviation of ethanol dependence symptoms mediated by NBP may be related to down-regulation in NR2B mRNA expression.

Key words: Ethanol dependence, L-3-nbutylphthalide, hydrogen sulfide (H₂S), cystathionine β-synthase activity, N-methyl-D-aspartate (NMDA) receptor 2B subunit (NR2B).

INTRODUCTION

Ethanol dependence is one of the most important worldwide public health problems. The discontinuation of chronic administration of ethanol is associated with excitatory withdrawal signs called ethanol withdrawal syndrome (EWS). EWS is the most important evidence indicating the development of physical dependence on ethanol (Jaffe, 1990). Although, attenuating the severity of EWS is very important, current treatment choices are very limited except for the use of benzodiazepines. Acamprosate (a glutamate antagonist), naltrexone (an opioid antagonist) and disulfiram (an aldehyde dehydrogenase blocker) are approved for the treatment

of ethanol dependence, but these medications are effective in attenuating ethanol cravings and consumption rather than treatment of EWS (Heilig and Egli, 2006). New approaches and new drug choices are necessary for treatment of EWS.

L-3-n-Butylphthalide (NBP) was extracted as a pure component from seeds of *Apium graveolens* Linn, Chinese celery. Afterward, L-3-n-butylphthalide was synthesized and approved by the State Food and Drug Administration of China for clinical use in stroke patients in 2002. It has been shown to have multiple neuro protective effects by reducing oxidative stress (Dong and Feng, 2002), improving mitochondrial function (Li et al., 2009), blocking inflammatory reactions, and reducing neuronal apoptosis (Chang and Wang, 2003). NBP also suppresses the production of peroxynitrite, superoxide, and nitric oxide (Li et al., 2009). Therefore, there is a

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growing interest in NBP on pharmaceutical effects. Unfortunately, there is no information about the effect of NBP on ethanol dependence rats.

In this paper, we evaluated the effect of NBP on ethanol dependence in rats. Simultaneously, the mechanisms underlying the protective efficiency of NBP on physical dependence to ethanol or the ethanol withdrawal syndrome were explored.

MATERIALS AND METHODS

L-NBP was supplied by the Shijiazhuang Pharma Group NBP Pharmaceutical Co. Ltd (Shijiazhuang, China) with a purity of more than 99%. L-Cysteine was purchased from Sigma Chemical Co. (USA). Trizol reagent was obtained from Invitrogen (USA). All other reagents were of analytical grade.

Animals and treatment

Male Sprague-Dawley rats (120- 160 g) from Henan Experimental Animal Center were used in the study. They were placed in a quiet, temperature and humidity-controlled room ($21 \pm 4^\circ\text{C}$ and $60 \pm 9\%$, respectively) in which 12-24 h light-dark cycle was maintained (08:00-20:00 h light). The Medical Experimental Animal Administrative Committee of Henan approved all experiments. Animal experiments were carried out in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals. All efforts were made to minimize animal suffering and reduce the number of animals used.

Rats were divided into six experimental groups randomly ($n=28$ for each group): normal group (normal rats), ethanol dependence model group (ethanol-treated rats), control group (vegetable oil and ethanol-treated rats), and three NBP-treated groups (Low, middle, and high dosage). Except for normal group, other groups were subjected to a modified liquid diet of ethanol (6%, V/V) for 28 days as previously described with minor modification (Uzbay and Kayaalp, 1995). Liquid diet was prepared daily and given to the rats at the same time of day (10:00 h). The weights of the rats were recorded every day, and the daily ethanol intake was measured and expressed as grams per kilogram per day. NBP was dissolved in vegetable oil at a concentration of 40 mg/ml, and was prepared fresh on the morning of each experiment.

Drug intervention began at the 14th days, control group rats were treated with vegetable oil, the low dose group rats were treated with NBP with a concentration of 10 mg/kg body weight (BW), the medium dose group rats were treated with NBP with a concentration of 20 mg/kg BW, high dose group rats were treated with NBP with a concentration of 40 mg/kg BW. Normal group received normal diet, and was not subjected to any treatment. At the end of the exposure to 6% ethanol-containing liquid diet, ethanol was withdrawn from the diet by replacing the diet with one that did not contain ethanol (10:00 h).

Ethanol withdraw scales

Rating scales were determined according to the published method with minor modification (Gray et al., 2010). Ethanol withdraw scales was carried to evaluate the rats withdrawal symptom at 6 h after termination of ethanol drinking administration and last for 18 min. Rats of the dead and with a large size were excluded.

At the sixth hour of the withdrawal testing, rats were exposed to an audiogenic stimulus (100 dB) for 1 min. The intensity of the

seizures was scored as follows: seizures were rated on a five-point scale ranging from 1 to 5. A score of 1 was assigned to rats showing only wild running. The rats showing tonic and tonic-clonic seizures in addition to wild running were given scores of 2 and 3, respectively. A score of 4 was assigned to the rats with longer lasting periodic (>90 s) tonic-clonic seizures. A score of 5 was given if mortality occurred. The intensity of the parameters was expressed as a median value. To calculate the total ethanol withdrawal score, the behavioral parameters were expressed as percent incidence and converted into scores ranging from 1 to 5 (10- 20%: 1; 30-40%: 2; 50- 60%: 3; 70- 80%: 4; 90- 100%: 5). Then, the median values of each behavior were summed for an individual observation period.

Determination of hydrogen sulfide content in the hippocampus of ethanol dependence rats

Hydrogen sulfide (H_2S) content was determined according to the methods of Ren et al. (2010). After ethanol withdraw scales, brain tissues of eight rats in each group were removed immediately and then homogenized in ice-cold 50 mmol/L potassium phosphate buffer, 120 mg/ml pH 8.0 with a Polytron homogenizer. The homogenate was centrifuged at 4°C , 47000 g for 10 min and 75 μl of the supernatant was collected for testing H_2S levels. The supernatant or plasma was mixed with 0.25 ml of 10 mg/ml zinc acetate and 0.45 ml of distilled water for 10 min at room temperature. 0.25 ml of 100 mg/ml trichloroacetic acid (TCA) was then added, centrifuged at 4°C , 14 000 g for 10 min, and the clear supernatant was collected and mixed with 133 μl of 20 mmol/L N,N-dimethyl- p-phenylenediamine sulfate in 7.2 mol/L HCl and 133 μl of 30 mmol/L FeCl_3 in 1.2 mol/L HCl. After 20 min, absorbance at 670 nm was measured with a microplate reader (Elx-800, Bio-Tex). The H_2S concentration was calculated against the calibration curve of the standard H_2S solutions.

CBS activity in cortical homogenate

CBS activity in cortical homogenates was studied as described by Ren et al. (2010) with some modification. Briefly, rat cerebral cortex was homogenized in ice-cold 50 mmol/L potassium phosphate buffer, 120 mg/ml pH 8.0, with a Polytron homogenizer. Homogenate (0.9 ml), pre-incubated at 37°C with or without an inhibitor for 5 min in a 20 ml glass vial, and then was cooled on ice for 10 min before L-Cys (10 mmol/L final concentration) and pyridoxal 5'-phosphate (2 mmol/L) were added. The final volume was 1 ml. A 2 ml tube containing a piece of filter paper (0.5x1.5 cm) soaked with 0.3 ml of 10 mg/ml zinc acetate was put inside the vial. The vial was then flushed with a slow stream of nitrogen gas for 20 s and then capped with an airtight serum cap. The vials were then transferred to a 37°C shaking water bath. After 90 min, 0.5 ml of 500 mg/ml TCA was injected into the reaction mixture through the serum cap. Another 60 min was allowed for the trapping of evolved H_2S by the zinc acetate solution as zinc sulfide. Then the serum cap was removed and 50 μL of 20 mmol/L N, N-dimethyl-p-phenylenediamine sulfate in 7.2 mol/L HCl and 50 μL of 30 mmol/L FeCl_3 in 1.2 mol/L HCl were added to the inner tube. After 20 min, absorbance at 670 nm was measured with a microplate reader (Elx-800, Bio-Tex). CBS activity was calculated using H_2S as standard and expressed as nmol H_2S of production / (h-g tissue).

RT-PCR analysis of expression of NR2B mRNA

Total mRNA from brain tissues was extracted using TriZol reagent (Invitrogen). RNA (5 μg) was reversely transcribed into cDNA using

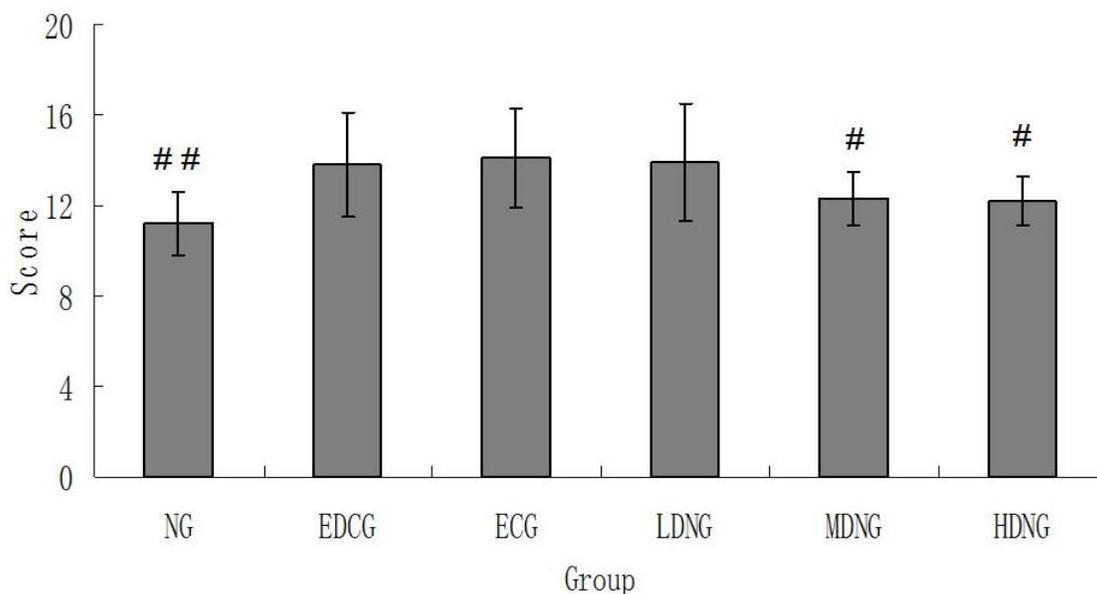


Figure 1. Withdrawal syndrome scores of ethanol dependence rats. (NG: Normal group; EDCG: Ethanol dependence model group; ECG: experiment control group; LDNG: low dosage NBP group; MDNG: middle dosage NBP group; HDNG: high dosage NBP group. Data were expressed as means \pm standard deviations (n=22). #, compared with the experiment control group, the differences are significantly ($P<0.05$). ##, compared with the experiment control group, the differences are extremely significantly ($P<0.01$).

Avian Myeloblastosis Virus reverse transcriptase (Promega) and oligo dT12 as primers. The sequences of primers, annealing temperature/time, and PCR cycle were as follows: β -actin (internal standard; 242 bp; 55°C/1 min; 25 cycles), sense: 5'-ATG GAT GAC GAT ATC GCT GCG -3', antisense: 5'-TCG TCC CAG TTG GTG ACA ATG -3'; CBS (572 bp; 50°C/1 min; 30cycles), sense: 5'-GAA CCA GAC GGA GCA AAC AG-3', antisense: 5'-TGT AGA GGA CTT TGC AGA CT -3'. PCR amplification products were analyzed with a 2.0% agarose gel. Bands were visualized with an ultraviolet transilluminator. The optical density of mRNA band was measured. The ratio of NR2B mRNA/actin mRNA was considered as the relative amount of NR2B mRNA.

Statistical analysis

Data were expressed as means \pm standard deviations (SD) and then analyzed by SPSS 11.5 software (version 11.5, SPSS Inc., USA). One way analysis of variance (ANOVA) and LSD multiple-range test were used to determine the differences of means. Differences between the means at the 0.05 level were considered to be significant, and differences between the means at the 0.01 level were considered to be extremely significant.

RESULTS

Withdraw scales for some behavior signs and audiogenic seizures induced by ethanol withdrawal in rats

The results showed that the rats in ethanol dependence model group, experiment control group and low dosage NBP showed spontaneous vocalization and very

aggressive, sniffing, increase of the frequency and severity of audiogenic epilepsy seizure. As shown in Figure 1, the ethanol withdrawal score of rats in normal group was significantly lower than that of control group at 6 h after termination of ethanol drinking administration ($P<0.05$). The ethanol withdrawal score of rats treated with middle (20 mg/kg) and high (40 mg/kg) concentration of NBP were much lower than that of control group (14.05 ± 2.03) ($P<0.05$). While, the ethanol withdrawal score of rats treated with low concentration of NBP changed slightly and compared with experiment control group, it was not significantly different ($P>0.05$).

Effect of NBP on H₂S content in hippocampus of ethanol dependence rats

H₂S is a gaseous messenger and serves as an important neuro modulator in central nervous system (Eto and Kimura, 2002), so the effect of NBP on H₂S content in hippocampus of ethanol dependence rats was explored. H₂S content in hippocampus of rats in normal group (26.91 ± 5.51 nmol/g tissue) was extremely significantly lower than that of ethanol dependence model group (43.19 ± 7.70 nmol/g tissue) ($P<0.01$). And H₂S contents in hippocampus of rats in middle and high dosage NBP treated group (30.81 ± 7.84 and 33.56 ± 4.13 nmol/g tissue, respectively) were significantly lower than that of experiment control group (44.16 ± 5.70 nmol/g tissue) ($P<0.05$). While the difference of H₂S content in hippocampus between ethanol dependence model group

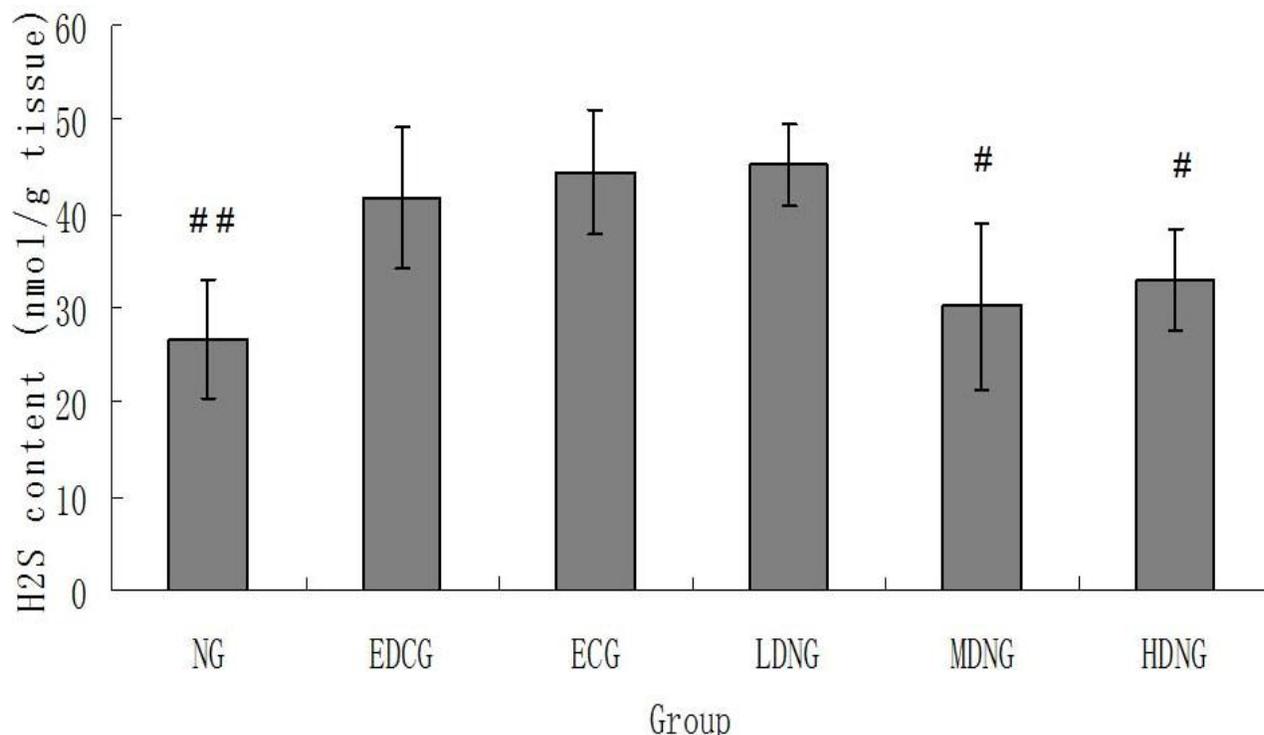


Figure 2. Effect of NBP on H₂S content in hippocampus of ethanol dependence rats. NG: Normal group; EDCG: ethanol dependence model group; ECG: experiment control group; LDNG: low dosage NBP group; MDNG: middle dosage NBP group; HDNG: high dosage NBP group. Data were expressed as means \pm standard deviations (n=16). #, Compared with the experiment control group, the differences are significantly ($P < 0.05$). ##, Compared with the experiment control group, the differences are extremely significantly ($P < 0.01$).

and experiment control group was not significant ($P > 0.05$), and the same had happened not only between low dosage NBP treated group and experiment control group, but also between middle and high dosage NBP treated group (Figure 2).

Effect of NBP on CBS activity in hippocampus of ethanol dependence rats

CBS is one of the most important enzymes in the brain, which could produce H₂S (Eto et al., 2002). And the production of H₂S could be modulated with the change of CBS activity (Ren et al., 2010). CBS is mainly expressed in the brain, peripheral nervous system, liver and kidney, whereas CSE is mostly found in the liver, vascular smooth muscle and endothelial cells (Eto and Kimura, 2002; Zhao et al., 2001), so we investigated the change of CBS. CBS activity in hippocampus of ethanol dependence rats in normal group (71.97 ± 2.03 nmol/ (h·g tissue)) was extremely significantly lower than that of experiment control group (79.06 ± 3.18 nmol/ (h·g tissue)) ($P < 0.01$) (Figure 3). Additionally, CBS activity in hippocampus of rats in middle and high dosage NBP treated group (72.75 ± 6.13 and 68.69 ± 8.02 nmol/ (h·g tissue), respectively) were significantly lower than that of

experiment control group ($P < 0.05$). However, the difference of CBS activity in hippocampus between ethanol dependence model group and experiment control group was not significant ($P > 0.05$), and the same had happened between low dosage NBP treated group and experiment control group.

Effect of NBP on the expression of NR2B mRNA in hippocampus of ethanol dependence rats

Results were shown in Table 1 and Figure 4. The expression of NR2B mRNA in hippocampus of rats in normal group (19.60 ± 0.89 of gray value) was different significantly from the expression level of experiment control group (29.27 ± 1.69 of gray value) ($P < 0.01$). And expression of NR2B mRNA in the hippocampus of rats in middle and high dosage NBP treated group (19.11 ± 1.17 and 22.46 ± 1.49 of gray value, respectively) were significantly lower than that of experiment control group (29.27 ± 1.69 of gray value) ($P < 0.05$). While the difference of expression of NR2B mRNA in the hippocampus between ethanol dependence model group and experiment control group was not significant ($P > 0.05$), and the same result was obtained between low dosage NBP treated group and experiment control group.

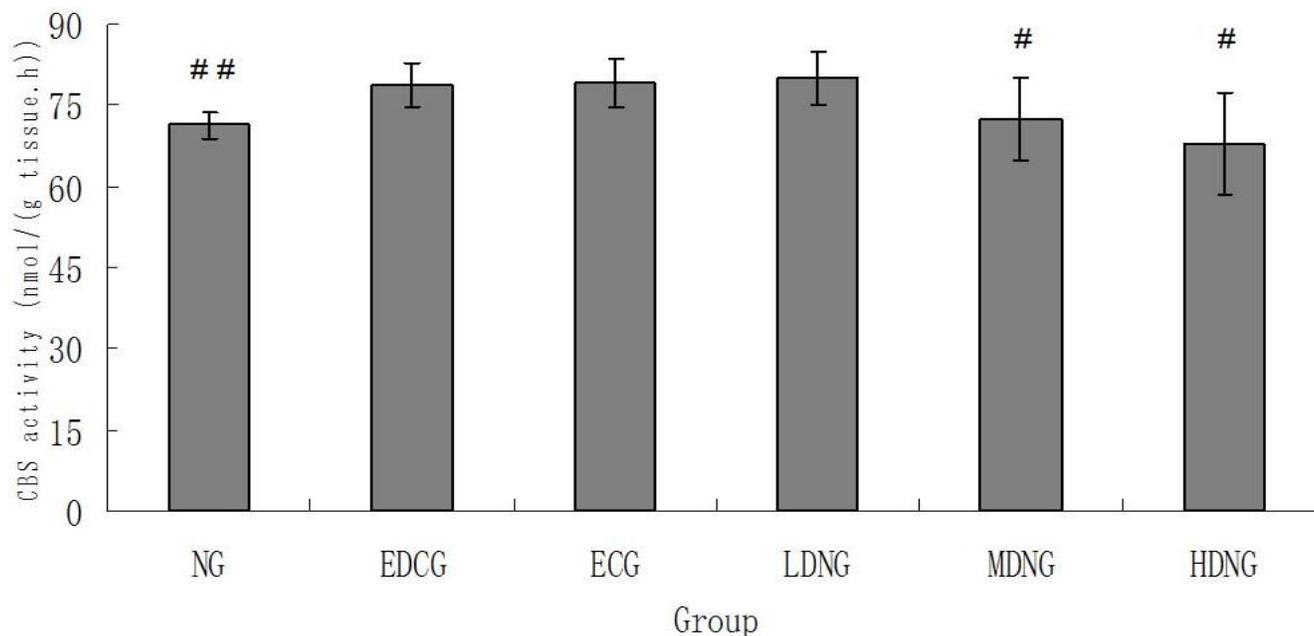


Figure 3. Effect of NBP on CBS content in hippocampus of ethanol dependence rats. NG: Normal group; EDCG: ethanol dependence model group; ECG: experiment control group; LDNG: low dosage NBP group; MDNG: middle dosage NBP group; HDNG: high dosage NBP group. Data were expressed as means \pm standard deviations ($n=16$). #, Compared with the experiment control group, the differences are significantly ($P<0.05$). ##, Compared with the experiment control group, the differences are extremely significantly ($P<0.01$).

Table 1. Effect of NBP on gray value changes in NR2B mRNA in hippocampus of ethanol dependence rats.

Group	NR2B mRNA gray value ^A
Normal group	19.60 \pm 0.89 ^{##}
Ethanol dependence model group	29.56 \pm 0.90
Experiment control group	29.27 \pm 1.69
Low dosage NBP group	30.26 \pm 0.94
Middle dosage NBP group	19.11 \pm 1.17 [#]
High dosage NBP group	22.46 \pm 1.49 [#]

^A Data were expressed as means \pm standard deviations ($n=3$). #, Compared with the experiment control group, the differences are significantly ($P<0.05$). ##, Compared with the experiment control group, the differences are extremely significantly ($P<0.01$).

DISCUSSION

Ethanol abuse and dependence was one of the most important worldwide public health problems, and led to tolerance, dependence, and memory impairments (Celikyurt et al., 2011). In addition, ethanol withdrawal symptoms were most severe at 6 h after ethanol withdrawal (Li and Yuan, 2003). In this paper, the model of ethanol dependence rats was built up according to the method of that ethanol was administered in drinking water at the concentration of 6% (V/V) for 28 d. And the ethanol withdrawal scores of ethanol dependence model group and experiment control group were much higher

than that of control group at 6 h after ethanol withdrawal treated ($P<0.05$). This indicated that the model of ethanol dependence rats was good. The score of some behavior signs and audiogenic seizures in rats treated with middle and high dose NBP was much lower than that of experiment control group ($P<0.05$). It indicated that NBP could alleviate the symptom of ethanol withdrawal.

Some research indicated that Ca^{2+} could increase the formation of H_2S . Particularly, signal transduction of calcium/calmodulin-mediated could quickly adjust the amount of H_2S formation (Qin and Liang, 2009). Long-term ethanol consumption could increase Ca^{2+} influx of hippocampus cell, and combine with one 19 amino acid

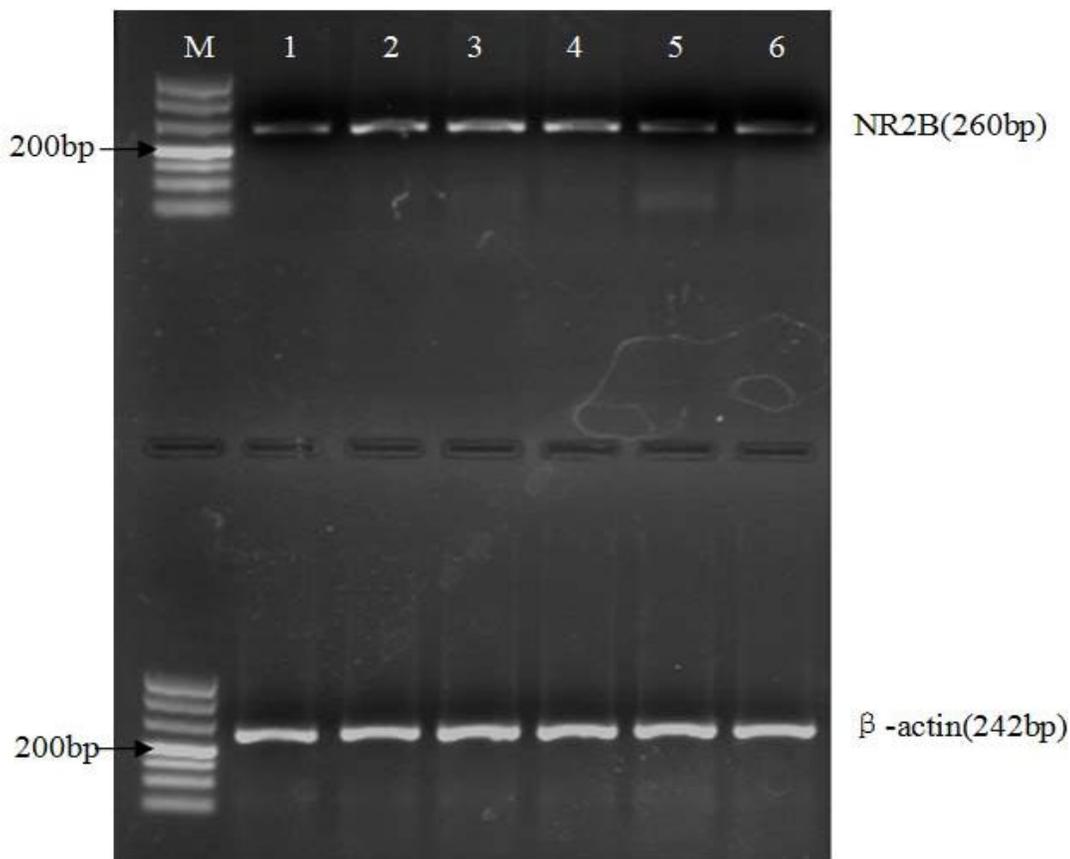


Figure 4. Hippocampus of NR2B mRNA RT-PCR products of polyacrylamide gel electrophoresis (M: Mark; 1: normal; 2: alcohol dependence model group; 3: experimental control group; 4: NBP low dose group; 5: NBP middle dose group; 6: NBP high dose group).

fragment. Then, CBS was activated and it could catalyze the production of H_2S , which indicated that H_2S formation could be a response to neuronal excitability (Samudio-Ruiz et al., 2010). The production of H_2S increased with the increasing activities of CBS in mice treated with chronic consumption of ethanol (Narita et al., 2000), this was also observed in this paper. When H_2S concentration was higher than that of physiological concentration, it could probably inhibit cellular respiration by affecting the activity of cytochrome C Oxidase, so that it had toxicity to the nerve cells (Lin and Feng, 1996). In addition, we found NBP could reduce H_2S content and CBS activities of hippocampus cell in rats treated with chronic ethanol consumption in this paper.

Several studies showed that ethanol was a potent and selective inhibitor of the N-methyl-D-aspartate (NMDA) receptors and prolonged ethanol exposition lead to a compensatory "up-regulation" of these receptors resulting in an enhanced NMDA receptor-mediated functions after removal of ethanol (Nagy, 2004). NMDA receptor consisted of two families, the NR1 and NR2 (R2A-2D) subunits. And NR2B subunit played a key role in the structure and function of NMDAR. Up-regulation of NR2B expression could lead to excessive ethanol intake and

relapse (Wang et al., 2010). The expression of NR2B mRNA in experiment control group increased extremely significant than that in normal group ($P < 0.01$), the same had been obtained in the study of Li et al. (2006). The expression of NR2B mRNA in middle dose NBP group and high dose NBP group were much lower than that of experimental control group ($P < 0.05$).

From the above assays, it can be concluded that NBP could reduce withdrawal symptoms of ethanol dependence rats. It may be related to the down-regulation in NR2B mRNA expression. Our study could put forward an important role of NBP in the treatment of ethanol dependence. Having established the protective effect of NBP in ethanol dependence rats, the protective mechanisms of NBP will be further investigated.

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Key lab of Biological Psychiatry.

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