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Role of oxidative/nitrative stress in hepatic encephalopathy induced by thioacetamide

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The study was designed to reveal the pathogenic mechanism of peroxynitrite in hepatic encephalopathy (HE), assess oxidative/nitrative stress in relation to HE induced by thioacetamide (TAA) and provide new ideas and scientific basis for the etiology and treatment of HE. Male Wistar rats were divided into four groups randomly: A (control), B (model), C (ebselen) and D (solvent). All the groups were treated with TAA by intraperitoneal (i.p.) except group A (treated with saline i.p.) to manufacture the model of HE. When rats treated with TAA came to the second stage of HE the four groups were administered intragastrically (i.g.) with saline (A, B), ebselen (C) and dimethyl sulfoxide (DMSO) (D), respectively. Plasma was collected to detect the levels of 3-nitrotyrosine (3-NT), NO, T-SOD and MDA. The results showed that the levels of 3-NT, NO, MDA significantly increased and T-SOD decreased obviously in rats suffering from HE. With the development and progression of HE the extent of oxidative/nitrative stress increased. When treated with ebselen the symptoms of HE mitigated and the levels of biochemical indicators ameliorated significantly. This indicates that oxidative/nitrative stress is involved in the mechanisms of hepatic encephalopathy.

1. Introduction

Hepatic encephalopathy (HE) is a serious cerebral complication of both acute and chronic liver failure. However, the precise mechanisms of HE are still not known exactly. The general agreement is that ammonia plays a key role (Butterworth 2002). But by clinical observations, the plasma ammonia of some HE patients remained at normal levels. This suggests that the ammonia poisoning hypothesis is not the only way to explain HE (Li 2008). Researchers suggested that protein tyrosine nitration might have a role in the pathogenesis of HE by culturing rat astrocytes (Boris et al. 2006) and oxidative/nitrosative stress may be one of the pathogenic mechanisms in HE (Jiang et al. 2009a, b). It is well known that in HE patients the levels of endotoxin, and tumor necrosis factor are higher than normal, and they can induce NOS expression which promotes to the generation of nitric oxide ($\cdot\text{NO}$). Besides, the decreased antioxidant defenses of HE patients lead to a high levels of superoxide anion ($\text{O}_2\cdot^-$). Nitric oxide ($\cdot\text{NO}$) reacts with superoxide anion ($\text{O}_2\cdot^-$) at a diffusion-controlled rate to form peroxynitrite (ONOO^-). As a strong oxidant and nitrating agent, ONOO^- whose cytotoxicity is higher than that of $\cdot\text{NO}$ and $\text{O}_2\cdot^-$, may oxidize and nitrate some major types of biomolecules including enzymes, proteins (Boris et al. 2006; Hüsamettin et al. 2007), lipids and DNA (Wayenberg et al. 2009; Chen et al. 2010), as well as various low-molecular-weight biomolecules. Researches have shown that ONOO^- could cause hepatocyte DNA damage and apoptosis (Wag et al. 2004). Studies also showed that ONOO^- could damage neurons in rats and induce apoptosis of brain cells (Jiang et al. 2009a). Thus, a role of ONOO^- in the pathogenesis of HE cannot be excluded.

Ebselen, 2-phenyl-1,2-benzisoxselenazole-3(2H)-one, a synthetic seleno-organic compound, is well known by its antioxidant and anti-inflammatory activities. It has been shown to act as GPx mimic and as a scavenger of ONOO^- . Research has shown that ebselen pretreatment significantly suppressed cisplatin-induced increases in intracellular reactive nitrogen species (RNS) and reactive oxygen species (ROS) levels (Yolanda et al. 2009). And it may be used for the treatment of oxidative/nitrative tissue stress.

The objective of the present study was to reveal the pathogenic mechanism of ONOO^- in hepatic encephalopathy (HE) and assess oxidative/nitrative stress in relation to HE induced by thioacetamide (TAA). The changes of symptoms were observed and the concentrations of ONOO^- and other related biochemical markers such as NO, T-SOD and MDA of plasma were measured as the extent of oxidative/nitrative stress in HE rats. In a second series of studies, ebselen was administered to reduce the level of ONOO^- Whether ONOO^- participated in the pathogenesis of HE was studied by comparing the changes of symptoms and concentrations of ONOO^- and other related biochemical markers in HE rats and HE rats treated with ebselen.

Because of the short half-life of ONOO^- (Kamat 2006), it is hard to measure its concentration directly in biological samples. ONOO^- can nitrate tyrosine residue to form 3-nitrotyrosine (3-NT) which is very stable and has been used as a biomarker of NO/ ONOO^- (Kamat 2006; Pacher et al. 2007; Peluffo et al. 2007). 3-NT in plasma was detected with a HPLC method (Guo et al. 2009) because of its low detection limit, good accuracy and precision.

Table 1: Levels of 3-NT, NO, T-SOD and MDA in plasma at the first stage

Groups	n	3-NT(nmol/l)	NO(μ mol/l)	T-SOD(U/ml)	MDA(μ mol/l)
Control	10	1.27 \pm 0.32	21.31 \pm 4.48	139.39 \pm 17.43	6.43 \pm 1.45
Model	10	2.07 \pm 0.48*	27.86 \pm 4.63*	114.14 \pm 15.82*	11.16 \pm 3.05*
Solvent	10	2.16 \pm 0.62*	29.04 \pm 5.66*	110.48 \pm 17.70*	12.35 \pm 2.62*
Ebselen	10	2.11 \pm 0.54*	28.22 \pm 5.16*	106.45 \pm 19.07*	12.02 \pm 2.91*

Values are shown as mean \pm standard deviation

* $P < 0.01$, compared to the corresponding value of control group

Table 2: Levels of 3-NT, NO, T-SOD and MDA in plasma at the second stage

Groups	n	3-NT(nmol/l)	NO(μ mol/l)	T-SOD(U/ml)	MDA(μ mol/l)
Control	10	1.32 \pm 0.46	22.05 \pm 4.87	142.71 \pm 24.04	6.23 \pm 1.87
Model	8	3.29 \pm 0.96*	43.51 \pm 7.11*	85.96 \pm 17.72*	15.73 \pm 3.59*
Solvent	9	3.24 \pm 0.87*	41.63 \pm 6.35*	95.09 \pm 18.76*	14.86 \pm 3.71*
Ebselen	10	1.85 \pm 0.67#	23.34 \pm 4.66#	132.09 \pm 28.15#	7.72 \pm 1.60#

Values are shown as mean \pm standard deviation

* $P < 0.01$, compared to the corresponding value of control group

$P < 0.01$, compared to the corresponding value of model group

2. Investigations and results

We have successfully established a rat model of HE using TAA. In this progress, the movement of rats was obviously reduced and rats came to HE after two or three days. In the following days the rats in the model and in the DMSO group showed symptoms of incontinence, nasal bleeding and developed HE or even came to death. However, the health condition of rats treated with antioxidant ebselen turned better as times went by.

The injection of TAA in rats induced significant changes of 3-NT and some other plasma markers. In Table 1, the levels of 3-NT in model, solvent and ebselen groups were higher than in control rats ($P < 0.01$) and among the three groups there were no significant differences. During the second stage of the experiment, the content of 3-NT also significantly increased in model group compared with control ($P < 0.01$) (Table 2). As can be seen in Fig. 1, with the development of the disease, the change of 3-NT was statistically significant compared to the first stage with the second in model group ($P < 0.01$). Likewise, the level of NO and MDA increased in model rats at the first stage of the experiment ($P < 0.01$) and continued to rise at the second stage ($P < 0.01$) (Tables 1, 2 and Figs. 2 and 3). The level of T-SOD was significantly decreased ($P < 0.01$) (Tables 1, 2 and Fig. 4). In HE rats, the plasma markers changed significantly as mentioned above. However, the administration of ebselen retarded everything. At the second stage, the level of 3-NT in blood was significantly decreased in the ebselen group versus model rats ($P < 0.01$) (Table 2). Compared with themselves in the

first stage, the decrease (Fig. 1) was not statistically significant ($P > 0.05$). Compared with the control group, the level of 3-NT in ebselen group was still higher but it also had no statistically significance ($P > 0.05$) (Table 2). While the administration of DMSO to the rats suffered from HE did not produce an obvious change in blood 3-NT levels compared with group model ($P > 0.05$) (Table 2). This suggested that not DMSO but ebselen significantly decreased the level of 3-NT in the blood of HE rats. Changes were also seen in the levels of NO, T-SOD and MDA. At the second stage, the levels of NO, T-SOD and

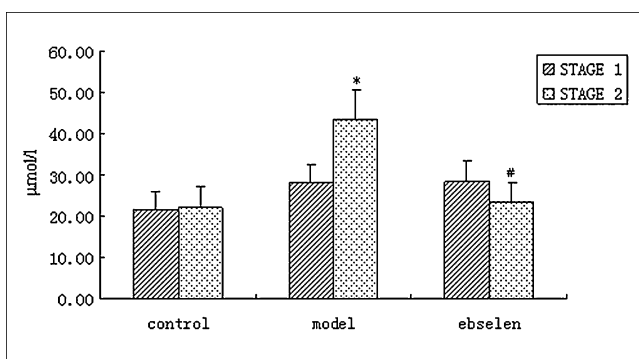


Fig. 2: Levels of NO in control, model and ebselen group at two stages of the experiment. *, $P < 0.01$, compared to the corresponding value of the first stage; #, $P < 0.05$, compared to the corresponding value of the first stage

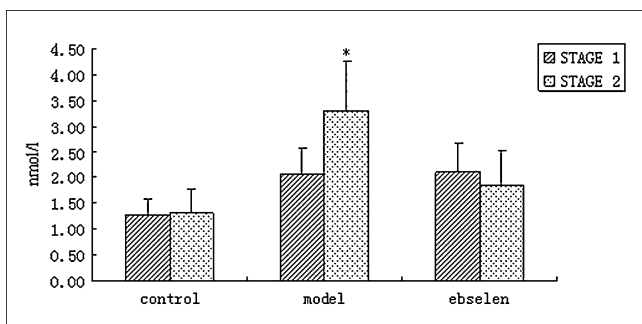


Fig. 1: Levels of 3-NT in control, model and ebselen group at two stages of the experiment. *, $P < 0.01$, compared to the corresponding value of the first stage

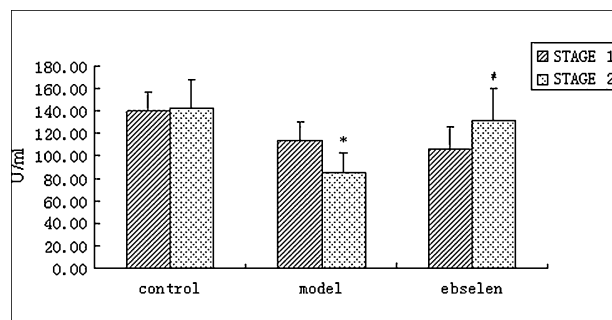


Fig. 3: Levels of T-SOD in control, model and ebselen group at two stages of the experiment. *, $P < 0.01$, compared to the corresponding value of the first stage; #, $P < 0.05$, compared to the corresponding value of the first stage

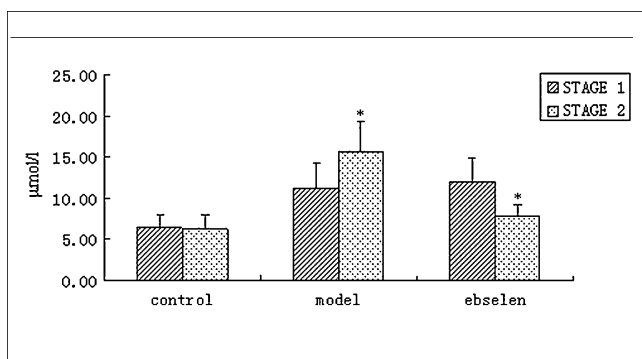


Fig. 4: Levels of MDA in control, model and ebselen group at two stages of the experiment. *, $P < 0.01$, compared to the corresponding value of the first stage

MDA in ebselen group changed significantly compared with the model group ($P < 0.01$) but have no obvious changes in contrast to control group (Table 2). In the ebselen group, there were significant changes of NO ($P < 0.05$), T-SOD ($P < 0.05$) and MDA ($P < 0.01$) levels before and after administration of ebselen (Figs. 2–4).

From the results above, with the development of HE, the levels of 3-NT, NO and MDA increased significantly and the T-SOD levels decreased in the model group in contrast to the group treated with antioxidant ebselen. This suggested that oxidative/nitrative stress may play an important role in the development of HE.

3. Discussion

TAA, a selective hepatotoxin, is used to induce hepatic failure. After administration, TAA is metabolized to thioacetamide-S-dioxide, a highly reactive compound which binds to the tissue macromolecules and might induce hepatic necrosis (Reddy et al. 2004). In this study, some changes have been made in the dose and the duration of administration of TAA based on former research and our experimental purpose. Rats treated with TAA alone showed symptoms of brain injury such as lethargy, lack of spontaneous movement, loss of righting reflex, lack of response to pain, Coma et al.

Results of the present study showed that the level of 3-NT in plasma of rats under basal conditions is low, but in morbid states it increased significantly, and this suggested that the ONOO⁻ level in HE rats was higher than in normal rats and so was the extent of nitrative of tyrosine. The increase of MDA, commonly used as oxidative stress markers, suggests that in rats suffering from HE, the lipid peroxidation aggravated. The decrease of the ability of T-SOD suggested the ability of removing O₂⁻ decreased and caused the accumulation of O₂⁻. The increase in O₂⁻ and the aggravated lipid peroxidation deteriorated the membrane damage and induced liver and brain cells damage at last. Many studies have shown that excessive uncontrolled NO formation is the cause of many common diseases or important contributing factors. In the present experiment, the level of NO in the HE rats was higher than normal rats. They were in accordance with prior research that NO level in HE patients were significantly higher than in normal (Shao et al. 2001). The excessive O₂⁻ reacted speedily with diffuse NO to form ONOO⁻, which can react with tyrosyl residues in proteins. Tyrosine-nitrated proteins were considered to be oxidative/nitrative stress indicators (Boris et al. 2010). 3-NT, the product of nitration of tyrosine, is often detected as the marker for generation and distribution of ONOO⁻. The nitration of tyrosyl residues in proteins may disturb signaling pathways relying on tyrosine phosphorylation/dephosphorylation and regulate cellular proliferation and apoptosis (Wu et al. 2005). The oxidation and nitration of pro-

teins mediated by ONOO⁻ may disturb the tertiary structure of proteins and at higher degrees of damage, induce and render them inaccessible for degradation by the proteasome. It can be conjectured that oxidative/nitrative stress belongs to the mechanisms of development and progression of HEs.

Ebselen is used to prevent HE as it is a scavenger of ONOO⁻ and has neuroprotective effects in brain (Damanjit et al. 2010). Ebselen pretreatment significantly inhibited increases in intracellular RNS and ROS levels. Under this condition, when rats suffering from HE treated with ebselen the level of 3-NT decreased, accompanied with the decrease of NO and MDA and the increase of T-SOD simultaneously. However, the level of 3-NT in the ebselen group was still higher than that in control group although there was no statistical significance. This may be on account of the stability of 3-NT and the mechanism of ebselen acting on HE. This results suggested that oxidative/nitrative stress may contribute to the formation and development of HE and ebselen may have some protective effects on HE. The exact mechanism by which ebselen is acting on HE need to be further studied.

In summary, the study shows that: (i) Evidence that oxidative/nitrative stress is implicated in the pathogenesis of HE is provided by the findings of the present study of the increases in expression of indicator of oxidative/nitrative stress (3-NT) and other plasma biochemical markers (NO, T-SOD and MDA). (ii) As a scavenger of ONOO⁻, ebselen can alleviate liver and brain damage to some extent by ameliorating oxidative/nitrative stress. This suggests that oxidative/nitrative stress may play an important role in the mechanism of the formation and development of HE. Ebselen may have some effect in the prevention of the formation and development of HE. It may be a potential medicine for the therapy of HE.

4. Experimental

4.1. Chemicals

The standard of 3-NT was obtained from Sigma-Aldrich (St. Louis, MO, USA). 4-Fluoro-7-nitrobenzofurazan (NBD-F), used as the fluorescent agent, was purchased from TCI (Tokyo, Japan). Analytical grade thioacetamide (TAA) was obtained from Shanghai Chemical Reagent Center. Ebselen was purchased from Biochemical Technology Co., Ltd. Chengdu Jing Peng. Dimethyl sulfoxide (DMSO) was purchased from Beijing Tong Guang Fine Chemical Co., Ltd. Enzymes such as nitric oxide (NO), total superoxide dismutase (T-SOD), and malondialdehyde (MDA) were purchased from the Research Institute of Nanjing Jiancheng Bio-engineering. Acetonitrile of HPLC grade was purchased from J. T. Baker (Phillipsburg, NJ), potassium tetraborate, trifluoroacetic acid (TFA), potassium dihydrogen phosphate of analytical grade were purchased from Sinopharm Chemical Reagent Co. (Shanghai, China). Ultrapure water was prepared using a Millipore (Bedford, MA, USA) Milli-Q water system.

4.2. Animals

Male Wistar rats (180–240 g) obtained from the Experimental Animal Center of Shandong University were used. The animals were kept at a constant temperature ($23 \pm 1^\circ\text{C}$) on a 12 h light/dark cycle with free access to food and water. The study was performed in accordance with the University Ethics Committee Guidelines for experiments with animals.

4.3. Establishment of the model of HE

HE is divided into four stages by a neurobehavioral: the first stage: lethargy; the second stage: mild ataxia; the third stage: lack of spontaneous movement and loss of righting reflex, but still responsive; the fourth stage: coma and lack of response to pain. To establish the model of HE, TAA was administered intraperitoneally (i.p.) at a single dose of 300 mg/kg (in physiological saline solution) for the first day and 150 mg/kg for the following days.

4.4. Experimental procedures

A total of 40 rats were used to perform the study. They were randomly divided into four groups with 10 rats in each group: A (control), B (model), C (ebselen) and D (solvent). The first stage the control rats received normal saline (12 ml/kg for the first day and 6 ml/kg for the following days)

by intraperitoneal (i.p.) injection through a 5 ml syringe while other groups were treated with intraperitoneal injections of TAA until the rats came to the second stage of HE. At the second stage group A and B received normal saline (10 ml/kg) by gavages, the rats of C and D groups were treated intragastrically with ebselen (5 mg/ml, diluted in 0.5% DMSO) and DMSO (10 ml/kg, in normal saline solution), respectively, until symptoms changed. Blood samples were collected when rats treated with TAA came to each of the first three stages of HE by cutting the tails of rats about 1 cm. At the end of the experiment when the rats in group B reached to the fourth stage of HE, all rats were put to death and blood samples were obtained from the vascular trunk of the neck. Bloods collected at the first two stages and the last two stages of HE were combined for further analysis.

4.5. Evaluation of liver injury induced by TAA

4.5.1. Preparation of plasma before detection

Blood samples collected were put aside for 30 min at 4 °C, and then centrifuged at 2500 rpm for 20 min at room temperature to obtain the plasma. The plasma samples were labeled and stored at -20 °C until being analyzed.

4.5.2. Determination of 3-NT, NO, T-SOD and MDA

3-NT (nmol/l) was detected with HPLC. Plasma NO, T-SOD and MDA levels were examined using reagent boxes purchased from the Research Institute of Nanjing Jiancheng Bio-engineering. NO, T-SOD and MDA were expressed as micromoles per liter ($\mu\text{mol/l}$), units per milliliter (U/ml) and micromoles per liter ($\mu\text{mol/l}$) respectively.

4.5.2.1. Determination of 3-NT by high performance liquid chromatography (HPLC) with fluorescence detector. In previous studies, the levels of 3-NT in biological samples were often measured by Western blot or ELISA (Ren et al. 2008; Sumanta et al. 2009). These are semi quantitative methods and so the accuracy, precision and limit of detection of the results were worse than with HPLC though it can describe the location of the nitration occurred. In this experiment we adopted the HPLC method to detect the 3-NT in blood (Guo et al. 2009). It gave a low detection limit, good accuracy and precision.

The determination was performed by a Shimadzu model 10ATvp LC system (Tokyo, Japan) and fluorescence detector (RF-10AXL). The system consisted of a column (Nova-Pak C18, 3.9 mm \times 150 mm; 4 μm particle size) and a Dikma SecurityGuard™ guard cartridge (C18, 4 mm \times 3.0 mm). The mobile phase consisted of a mixture of acetonitrile-phosphate buffer (0.02 mol/l, plus 500 $\mu\text{l/l}$ TFA, pH 3.0) (36:64, v/v). The fluorescence detector was set at 470 nm and 530 nm for excitation and emission wavelengths, respectively. And the column temperature was 35 °C. Data were collected and analyzed using a LC-solution workstation with a data processing system (Shimadzu, Kyoto, Japan) installed on it.

The preparation of samples includes three stages. After the precipitation of proteins with acetonitrile, we used a SPE cartridge to clean and concentrate the samples and then proceeded the derivatization reaction of 3-NT with fluorescent agent NBD-F.

4.5.2.2. Determination of NO. NO is metabolized in the body very fast. Thus we measured $\text{NO}_2^-/\text{NO}_3^-$ which indirectly represent the levels of NO. In the acidic environment, the adduct product of NO_2^- with sulfanilic acid could react with N-(1-naphthyl)-ethylene diamine, and the reaction products had absorption peaks at 550 nm.

4.5.2.3. Determination of T-SOD. T-SOD was determined according to the enzymatic of xanthine oxidase. method Superoxide anion radicals were produced through the reaction between xanthine and xanthine oxidase. They oxidized hydroxylamine to form nitrite, in the presence of fuchsia as a chromogenic agent. And then absorbance was detected by a visible spectrophotometer. When the samples contained T-SOD, it would inhibit superoxide specifically to reduce the formation of nitrite. As a result the value of absorbency in measured tube was lower than the control tube. So we could calculate the activity of T-SOD in samples measured using formulas given.

4.5.2.4. Determination of MDA. MDA was determined by the thiobarbituric acid (TBA) method based on the red condensation product which is produced from the reaction between TBA and MDA. It shows maximum absorption at 532 nm.

4.6. Statistical analysis

Statistical analysis of the data was accomplished by means of a statistical software package (SPSS 16.0 for windows). The Shapiro-Wilk test did

not show a significant deviation from normality in the distribution of variance values. To evaluate variations in data, a one-way analysis of variance (one-way ANOVA) was performed among the four groups and independent-samples t test was performed intra-group at the two stages. The level of statistical significance was set at $P < 0.05$. All results are expressed as mean \pm standard deviation.

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