Original Article

Effects of repeated high dosage of chloral hydrate and pentobarbital sodium anesthesia on hepatocellular system in rats

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Abstract: This study aims to investigate the possible effects of repeated high dosage of chloral hydrate and pentobarbital sodium anesthesia on hepatocellular system in rats. Thirty Sprague Dawley rats were randomly divided into 3 groups: control group (group A), chloral hydrate group (group B) and pentobarbital sodium group (group C). Antioxidant enzymes superoxide dismutase (SOD), glutathione peroxidase (GSH-Px), glutathione transferase (GST) and catalase (CAT) activities and thiobarbituric acid-reactive substances (TBARS) level as well as serum biochemical parameters alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP) and total bilirubin (T-BIL) were determined. Liver histopathological examinations were performed at termination. Furthermore, Bax and Bcl-2 expression, and caspase-3 activity were also evaluated. The SOD, GSH-Px, GST and CAT activities significantly decreased but TBARS levels increased in group B and C compared with group A. Hepatic injury was evidenced by a significant increase in serum ALT, AST and ALP activities in group B and C, which also confirmed by the histopathological alterations. Moreover, administration of chloral hydrate and pentobarbital sodium could induce certain hepatic apoptosis accompanied by the upregulated Bax expression, the downregulated Bcl-2 expression and Bcl-2/Bax ratio, and the increase of caspase-3 activity. Repeated high dosage of chloral hydrate and pentobarbital sodium anesthesia could produce hepatotoxicity.

Keywords: Chloral hydrate, pentobarbital sodium, anesthesia, hepatotoxicity, oxidative damage, antioxidant enzymes, serum biochemistry, histopathological alterations, apoptosis, Bcl-2/Bax ratio

Introduction

A large number of evidences in the research area of laboratory animals suggest that different experimental methods can cause animal stress response [1]. If not control or intervene in time, it will cause negative effects on the body and even affect the scientific results. Chloral hydrate is frequently used as a hypnotic agent at low dosage, it also acts as an anesthetic agent at higher dosages [2]. Side effects of chloral hydrate with high dosage mainly occurred in abdominal organs. Many investigators have confirmed that chloral hydrate given at a high dosage can cause inflammation of the splenic capsule, gastric ulcers [3], severe adynamic ileus [4], peritonitis, and even death [5, 6]. Besides, it also has a certain inhibitory effect on respiratory system and can cause great damage to the body of laboratory animals [1]. Pentobarbital sodium, a barbiturate, also acts as an important anesthetic agent in animal experiment. Previous reports have demonstrated that it provide a “protective” effect for the brain in regional [7-13], as well as global ischemia [14, 15]. Moreover, it also acts as an important anesthetic agent in animal experiment. Owing to its high pH value, pentobarbital sodium given with high dosage has a low safety margin due to the potent respiratory and cardiovascular depressant effects [1], and recovery might be prolonged, with convulsive movements and “paddling”, severe tissue reactions [16].

Although chloral hydrate and pentobarbital sodium are not the first-choice anesthetic for surgical procedures and negative effects were
Chloral hydrate and pentobarbital sodium anesthesia on hepatocellular system

also observed, laboratories have been using these drugs to establish their research model. Recent observations demonstrated a complex influence of chloral hydrate and pentobarbital sodium on the immune status, on the integrity of various organ systems and on the post-anaesthetic outcome of different diseases, especially at high dosages or concentrations [17]. However, up to now there were no controlled studies focused on repeated high dosages of chloral hydrate and pentobarbital sodium anesthesia on the hepatocellular systems. Therefore, present study aims to explore the possible effects of repeated high dosage of chloral hydrate and pentobarbital sodium anesthesia on hepatocellular system by evaluating antioxidant enzymes, serum biochemical parameters and histopathological changes and some apoptosis factors.

Materials and methods

Animals

Sprague Dawley rats (330 ± 20 g, male) were purchased from the Experimental Animal Center of Suzhou Aiermaite technology Co. Ltd. (SPF grade, Certificate No. SCXK20140007). All rats were housed in groups of five with free access to food and water and kept in a regulated environment with a 12 h light/dark cycle at 23 ± 3°C and 40-70% humidity. Each rat was examined for clinical signs of ill health on receipt and observed within 7 days of arrival. All procedures were in accordance with the Guidelines of the Animal Care and Use of Laboratory Animals from the Association of Laboratory Animal Science and the Center for Laboratory Animal Science of Yantai University.

Experimental design

Thirty Sprague Dawley rats were randomly divided into three groups according to different methods of anesthesia: control group (group A, n = 10), chloral hydrate intraperitoneal injection group (group B, n = 10) and pentobarbital sodium intraperitoneal injection group (group C, n = 10). Chloral hydrate and pentobarbital sodium were all purchased from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). Nothing was done for group A. All rats were fed with the same laboratory diet during the study. Rats in group B were treated with intraperitoneal injection of 10% chloral hydrate anesthesia according to the rat weight at 4.5 mL/kg, group C were anesthetized by intraperitoneal injection of 1% pentobarbital sodium according to the rat weight at 6 mL/kg. The animals in group A were administered with 6 mL/kg normal saline. All the above administrations were repeated for 5 consecutive days. After the last administration the rats were sacrificed by cervical dislocation. They were disinfected abdominally, and then the abdomen was cut open to expose abdominal veins, 2.0 mL blood was collected into ethylenediamine tetraacetic acid (EDTA) treated tubes. The livers of the rats were quickly removed which was immediately divided into two portions. Part of each liver was immediately placed in 10% (v/v) formaldehyde solution for electron microscopic examination and the rest of the livers were kept at -80°C for subsequent assays.

SOD, CAT, GSH-Px and GST activities and TBARS level

Antioxidant components in the hepatic tissue samples were analyzed in the experimental rats. Liver tissues were washed with cold deionized water to discard blood and then homogenized in 50 mM Tris-HCl, pH = 7.4 (1:10, w/v). After centrifugation at 2400 × g for 15 min at 4°C, the upper clear layer was taken and used for the analyses. In this fraction thiobarbituric acid-reactive substances (TBARS) levels were determined by using the thiobarbituric acid method [18]. A part of the homogenate was extracted in ethanol/chloroform mixture (5:3, v/v) to discard the lipid fraction, which caused interferences in the activity measurements of superoxide dismutase (SOD), glutathione peroxidase (GSH-Px), glutathione s transferase (GST) and catalase (CAT). The SOD, GSH-Px, GST and CAT activities which reflected as common indexes of antioxidant status of tissues were determined with the corresponding ELISA kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) according to the manufacturer’s instructions. The results were expressed as U/mg protein, IU/mg protein, IU/mg protein and IU/mg protein, respectively.

Serum biochemistry

Blood samples were centrifuged at 3000 × g for 10 min to separate plasma. Serum biochemical parameters including alanine aminotransferase (ALT), aspartate aminotransferase
Chloral hydrate and pentobarbital sodium anesthesia on hepatocellular system

(ALT), alkaline phosphatase (ALP) and total bilirubin (T-BIL) were analyzed using a biochemical analyzer (TBA-FR40; TOSHIBA Co., Ltd., Osaka, Country). All the kits mentioned above were purchased from Zhongsheng Beikong Biotechnology and Science Inc. (Beijing, China). The results were expressed in U/L.

Histopathological examination

Histopathological analysis was performed from three animals per group and was randomly selected the picture of one animal per group. Livers were fixed with 10% neutral buffered formalin solution, and were routinely processed (embedded in paraffin and sectioned at 3-5 μm). The sections were stained with hematoxylin and eosin (H&E) stain for microscopic examination.

Western blot analysis

The levels of Bcl-2 and Bax were detected by western blot analysis. The hepatic tissue samples were homogenized in lysis buffer containing complete protease inhibitor cocktail (1 M Tris-HCl (pH 8.0), 5 M NaCl, 10% Nonidet P-40 and 1 M 1,4-dithio-dl-threitol (DTT)). After quan-
titrated with the bicinchoninic acid (BCA) protein assay kit (Beyotime Biotech, Shanghai, China), the total proteins were electrophoresed in 12% SDS-PAGE gels, transferred to polyvinylidene fluoride (PVDF) membranes. After the blots were blocked with 5% fat-free dried milk for 2 h at room temperature, the membranes were incubated overnight at 4°C with corresponding primary antibodies. Subsequently, the membranes were incubated with the corresponding secondary antibodies at room temperature for 2 h. The blots were visualized with enhanced chemiluminescence (ECL) detection system (Amersham), and the results were analyzed by LabImage version 2.7.1 (Kapelan GmbH, Halle, Germany). Bax, Bcl-2 and β-actin antibodies were purchased from Cell Signaling Technology (Danvers, MA, USA).

Measurement of caspase-3 activity

The caspase-3 activity of hepatic tissue samples were detected using Caspase-Glo 3/7 assay kit (Promega, Madison, WI, USA) in the present study according to the kit instruction.

Statistical analysis

The data was expressed as mean ± SD values. All data were analyzed by SPSS 19.0 statistics software. One-way analysis of variance followed by Dunnett’s post-hoc test was used to compare treatment and control group data. Statistical significance was set at a level of P < 0.05.

Results

Effects of different treatment on hepatic SOD, CAT, GSH-Px, GSH, TBARS levels

Figure 1A-E demonstrated the effects of repeated high dosage of chloral hydrate and pentobarbital sodium treatment on SOD, GSH-Px, GST and CAT activities as well as TBARS levels in hepatic tissues. As displayed in Figure

![Figure 1A-E](image-url)
1A-D, the hepatic SOD, GSH-Px, GST and CAT activities were significantly ($P < 0.05$) decreased from $16.98 \pm 1.61$, $57.06 \pm 4.42$, $2.58 \pm 0.42$ and $15.84 \pm 2.62$ IU/mg in group A to $11.36 \pm 1.32$, $48.22 \pm 3.81$, $1.82 \pm 0.32$ and $10.67 \pm 1.77$ IU/mg in group B, respectively. Obviously, pentobarbital sodium seems to induce more damage to the activities of antioxidant enzymes SOD, GSH-Px, GST and CAT, which were significantly lower ($P < 0.01$) in group C ($9.64 \pm 1.27$, $39.72 \pm 3.49$, $1.63 \pm 0.35$ and $9.64 \pm 1.18$ IU/mg, respectively) than these in group A. TBARS level (Figure 1E) of rats exposed to chloral hydrate and pentobarbital sodium presented an increasing tendency from $1.06 \pm 0.26$ nmol/mg in group A to $1.88 \pm 0.37$ nmol/mg in group B and $2.23 \pm 0.51$ nmol/mg in group C, respectively, suggesting that chloral hydrate and pentobarbital sodium anesthesia could significantly cause lipid peroxidation.

**Effects of different treatment on serum biochemistry**

Effects of chloral hydrate and pentobarbital sodium on the enzymatic activities of serum ALT, AST, ALP and T-BIL are displayed in Figure 2A-D. Application of chloral hydrate led to a significant elevation of serum ALT, AST and ALP activities from $58.2 \pm 4.8$, $38.8 \pm 5.6$ and $116.2 \pm 11.3$ U/L in group A to $148.7 \pm 12.2$, $128.9 \pm 13.8$ and $332.4 \pm 35.2$ U/L in group B, respectively. Pentobarbital sodium anesthesia led to a similar but less pronounced effect, with activities of $121.2 \pm 11.3$, $118.9 \pm 12.4$ and $278.6 \pm 29.3$ U/L in group C, respectively. T-BIL levels also showed a trend towards elevation, from $5.2 \pm 0.5$ mg/dL in group A to $6.8 \pm 0.7$ mg/dL in group B and $8.3 \pm 0.9$ mg/dL in group C, respectively.

**Figure 3.** Effects of different treatment on hepatic histological injury in rats. A portion of the liver tissues was stained with hematoxylin and eosin (H&E), and histological assessment was performed under a microscope. Group A: control group, Group B: repeated high dosage chloral hydrate anesthesia, Group C: repeated high dosage chloral hydrate anesthesia.
Chloral hydrate and pentobarbital sodium anesthesia on hepatocellular system

53.7 ± 7.7 and 142.4 ± 6.8 U/L in group B ($P < 0.01$), respectively. Similarly, rats exposed to pentobarbital sodium suffered more severe hepatic injury as evidenced by a significant increase in serum ALT, AST and ALP activities to 96.2 ± 6.7, 63.1 ± 9.8 and 179.1 ± 7.9 U/L ($P < 0.01$) in group C, respectively. However, there was no statistical significance of serum T-BIL activities of group B and C compared with group A.

Histopathological examination

As depicted in Figure 3, histopathological observations of H&E staining of the livers were performed to further support the results that the hepatic antioxidant enzymes and serum biochemical analysis indicated. H&E stained analysis of liver from group A showed intact central vein and hepatic cords with healthy hepatocytes and thin sinusoidal spaces. In comparison with the hepatic cellular architecture of rat tissues from group A, the liver sections in the group B showed relatively intact central vein, moderate hepatocytes hypertrophy and swollen in partial region around the central vein. As expected, rats in group C suffered more severe hepatic alterations than these in groups A and B. H&E stained analysis of liver from group C exhibited hepatocytes hypertrophy, swollen, ballooned lipid laden hepatocytes and dilated sinusoidal spaces in most regions, revealing extensive liver lesions.

Discussion

Chloral hydrate and pentobarbital sodium are commonly used anesthetic agents in animal experiment. However, previous studies have reported that negative effects of these two anesthetic agents usually occur at higher dosages or concentrations in laboratory animals [1, 3-6, 16]. In this study, we for the first time evaluated the possible effects of repeated high dosage of chloral hydrate and pentobarbital sodium anesthesia on rat hepatocellular system. The present data revealed that repeated high dosage of chloral hydrate and pentobarbital sodium anesthesia could produce hepatotoxicity and induce oxidative damage, lipid peroxidation and cause hepatic cell damage and even apoptosis.

Although no specific radical molecule has been confirmed for chloral hydrate or pentobarbital sodium hepatotoxicity, indirect effects such as...
the alterations in the levels of antioxidant enzymes might be related to their hepatotoxic effects. Oxidative stress due to overproduction of reactive oxygen species (ROS) might be one of the main mechanisms underlying chloral hydrate and pentobarbital sodium toxicity. Live tissue is thought to be involved in congenital antioxidant defense mechanisms, and the endogenous antioxidant enzymes, including SOD, GSH-Px, GST and CAT, are considered to constitute a mutually supportive team of defense against ROS [19] and are important indicators which indirectly determine the tissue oxidative damage and lipid peroxidation. GSH-Px acts as an enzymatic antioxidant both intracellularly and extracellularly in conjunction with various enzymatic processes that reduces hydrogen peroxide (H$_2$O$_2$) and hydroperoxides [20]. SOD can catalyze the clearance of the superoxide anion radicals and prevents the formation of H$_2$O$_2$ [21]. A decrease in the activity of these enzymes leads to deleterious effects, such as loss of integrity and function of cell membranes, which is regarded to connect with the accumulation of highly ROS [22]. The results of our study demonstrated that rats exposed to repeated high dosage of chloral hydrate and pentobarbital sodium suffered an inhibition in liver SOD and GSH-Px activities, indicating these two anesthetics could induce hepatotoxic effects and cause oxidative damage of liver. Moreover, the oxidative damage of liver induced by these two anesthetics can be also explained since this metal alters the activities of several hepatic antioxidants enzymes GST and CAT. In the current study, rats exposed to these two anesthetics suffered a decrease in liver GST activity, an enzyme plays an important role in the antioxidant response against lipid peroxidation, attenuating this process by reducing hydroperoxides and protects cells from toxic end products of lipid peroxidation [23]. CAT, another enzyme that was considered as part of the system for ROS detoxification [24], was inhibited by these two anesthetics. In our experiment, we observed that the activities of the antioxidant enzymes SOD, GSH-Px, GST and CAT in group C were slightly lower than these in group B. It means that the liver antioxidant capacity was lower in group C than in group B. Against all the protective enzymatic mechanisms, cellular damage that supports the elevation of lipid peroxidation was observed. As expected, the TBARS levels, an important index that indirectly reflect of cell oxidative damage, were significantly increased(5,6),(996,993)
Chloral hydrate and pentobarbital sodium anesthesia on hepatocellular system

Sonably suggested that these two anesthetics could induce the apoptosis of hepatic cell. The activation of caspase-3 is a vitally important step in the execution phase of apoptosis and the inhibition hinders apoptosis [28]. Moreover, caspase-3 activity can be induced by Bax and inhibited by Bcl-2, and Bax can neutralize Bcl-2 actions by forming heterodimers with Bcl-2 [29]. In this study, the caspase-3 activity was detected to further support our findings. The results showed that the caspase-3 level was significant higher in groups B (P < 0.01) and C (P < 0.01) compared with the group A. Since the Bcl-2/Bax ratio was low, a compensatory induction of Bcl-2 was not sufficient to overcome the proapoptotic actions of Bax on caspase-3 activation.

Conclusions

In conclusion, the present study clearly shows that repeated high dosage of chloral hydrate and pentobarbital sodium anesthesia can induce hepatoxotoxic which can cause the liver damage and even apoptosis. The underlying mechanisms might be attributed to increasing oxidative stress, reducing Bcl-2 family protein translocation and inducing caspase-3 activation. Overall, the degree of hepatic injury in pentobarbital sodium anesthesia group was more severe than these of choral hydrate anesthesia group. Enzymatic findings and histopathological changes etc. may be important signs of toxic effects of administrations of repeated high dosage of chloral hydrate and pentobarbital sodium. Therefore, the reasonable selection and control of anesthetics are very important in order to avoid the experimental errors caused by anesthesia. The study could provide an experimental basis and a useful reference for the animal experiment and anesthesia method.

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Disclosure of conflict of interest

None.

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Chloral hydrate and pentobarbital sodium anesthesia on hepatocellular system


