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Original Article

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

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Abstract: The present study was intended to explore the possible effect of the repeated dose of the sevoflurane and chloral hydrate anesthesia on the hepatocellular system in the rats. The Sprague Dawley [30 rats] were used for the experimental study, and the rats were randomly divided into following groups; group I: normal control, group II: chloral hydrate and group III: sevoflurane. The biochemical parameters such as aspartate aminotransferase [AST], alanine aminotransferase [ALT], total bilirubin [T-BIL] and alkaline phosphatase [ALP]; antioxidant parameters such as glutathione peroxidase [GSH-Px], superoxide dismutase [SOD], catalase [CAT] and glutathione transferase [GST] were also estimated. At end of the study, the rats were sacrificed and the liver was subjected to the histopathological examination. Additionally, the level of Bcl-2 and Bax and caspase-3 were also scrutinized. Results suggest that, the serum level of ALP, AST and ALT in the group II and III, which was significantly modulated in comparison with the control for its possible involvement in liver damage. This was proved by the histopathological evaluation of liver with the presence of abnormal microstructure. The antioxidant marker such as GSH-Px, SOD, CAT and GST activity was found to be significantly reduced, whereas, the level of the TBARS was found to be significantly increased in the group II and III as compared to group I. Moreover, the administration of the sevoflurane and chloral hydrate induces the hepatic apoptsis accompanied by the down-regulation of the expression of Bcl-2 and unregulated Bax expression together with Bcl-2/Bax ratio. The expression of caspase-3 was also found to be elevated. Therefore, it has been suggested that, sevoflurane and chloral hydrate anesthesia in repeated dose could generate the considerable hepatotoxicity.

Keywords: Sevoflurane, chloral hydrate, caspase-3, Bcl-2

Introduction

Several scientific reports from across the globe have indicated the influence of diverse experimental methods on the stress level of the animals [1]. Thus, if these responses are not adequately controlled, then will have a possibility to interfere with the experimental results and have significant impact on the study. The pharmacological activity of many agents have specially tuned with the help of the dose, chloral hydrate is one of the such chemical that at low dose was used as a hypnotic and at high dosage used as anesthetic agent [2]. Due to the low dose, chloral hydrate exhibits fewer side effects as compared to the high dose which causes serious destruction to the abdominal organs. It has been reported that, at higher dose, it causes gastric ulcer, inflammation of the splenic capsule, peritonitis, severe adynamic ileus and even causes the death [2-6]. Moreover, it also showed certain inhibitory effect on the respiratory system and able to damage the different organ and body of the laboratory animal [1].

Sevoflurane, is an another very common and important anesthetic agent used in the animal experimental model. Several research reports have clearly showed that, it exert protective effect against the global ischemia. Moreover, owing of the high pH value of the sevoflurane, it was given in the high doses, which have low safety profile due to the cardiovascular depressant and potent respiratory effects and the recovery of the subject may be prolonged, along

with the paddling and convulsive movements, severe tissue reactions [2-4].

Although sevoflurane and chloral hydrate are not used as the first choice anesthetics for the surgical procedures because of negative effects, but at certain instances, it was used. The current investigation showed a complex influence of sevoflurane and chloral hydrate on the immune system and the integrity of various organ status, especially at high concentration or doses. The present study was undertaken, prompted by the fact that, till now, no study has claimed the effect of the high doses of sevoflurane and chloral hydrate on the hepatocellular systems. Therefore, the aim of the current investigation was to explore the possible effects of the repeated doses of sevoflurane and chloral hydrate on the hepatocellular system in the light of various biochemical parameters, antioxidant marker, histopathological parameters and apoptosis factors [4, 6].

Material and methods

Animals

The Sprague Dawley [320±25 g, male] rats were used for the study. The rats were procured from the Department animal house of the university. All the rats were kept in the house in the group of six, with free access to water and food. All the rats were stored in the normal environment with a 12 h light/dark cycle at 25±5°C with relative humidity. The rats were physically scrutinized after one week of arrival for the identification of the any clinical sign of the ill health. All procedures for the animal handling and experiment were performed in accordance with the Guidelines of the Animal Care for Laboratory Animals form the Association of Laboratory Animal Science.

Experimental study

Sprague Dawley rats [30] were randomly divided into three groups according to the following method of anesthesia: Group I: normal control, Group II: treated with chloral hydrate intraperitoneal injection, Group III: treated with the Sevoflurane intraperitoneal injection. Group I received the 6 mL/kg normal saline at end of the experimental study. All groups rat received the normal laboratory diet during the experimental study. Group II rats treated with the intraperitoneal injection of the chloral hydrate 10% prepared to the rat weight at 4.5 mL/kg, Group III rats treated with the intraperitoneal injection of the sevoflurane 1% prepared to the rat weight at 6 mL/kg. All group rats received the predetermined treatment for 5 days. After the day five, they received the last dose of the treatment and the rats were scarified by cervical dislocation. After sacrificing, rats were disinfected abdominally, and then the abdomen was cut and open to expose abdominal veins for collecting the blood sample [2 mL] into the ethylenediamine tetraacetic acid [EDTA] containing tubes. The liver of the rats were immediately removed and divided into two parts. The each portion of the liver was immediately kept into the formaldehyde solution [10%, v/v] for electron microscope examination and remaining portion of the livers were kept at the -80°C for subsequent assays.

Serum biochemistry

After collecting the blood samples, tit was centrifuged at 3000 rpm for 15 min to separate to the plasma. Biochemical parameters such as total bilirubin [T-BIL], alanine aminotransferase [ALT], aspartate aminotransferase [AST] and alkaline phosphatase [ALP] were analyzed using a biochemical analyzer.

Effect on the antioxidant markers

The antioxidant level of the hepatic tissue samples were evaluated in the experimental rats. The liver tissues which were removed previously and washed with the cold deionized water to remove the blood and the liver sample were homogenized in Tris-HCl [50 mM], pH=7.4 [1:10 w/v]. The samples were centrifuged at 2400 rpm for 15 min at 4°C and supernatant was collected for further used. The level of the thiobarbituric acid-reactive substances [TBARS] was estimated by the previously reported method [8]. The homogenate was extracted in the chloroform/ethanol system [3:7, v/v] and the lipid fraction was discarded form the homogenate, which might cause interferences in the activity of the glutathione peroxidase [GSH-Px], catalase [CAT], superoxide dismutase [SOD] and glutathion transferase [GST]. the level of GSH-Px, SOD, CAT and GST was identified using the instruction of the ELISA kit manufacture.

Histological examination

After sacrificed the rats, the liver tissue was immediate removed and process for the histo-

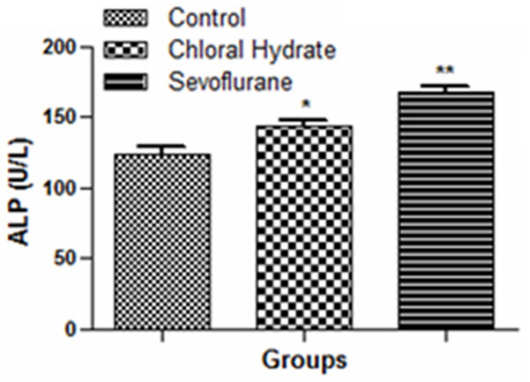

Figure 1. Effects of different treatment on serum ALP level of rats. Data are presented as mean ± SD for 10 rats in each group. *P < 0.05 and **P < 0.01, compared to the control group.

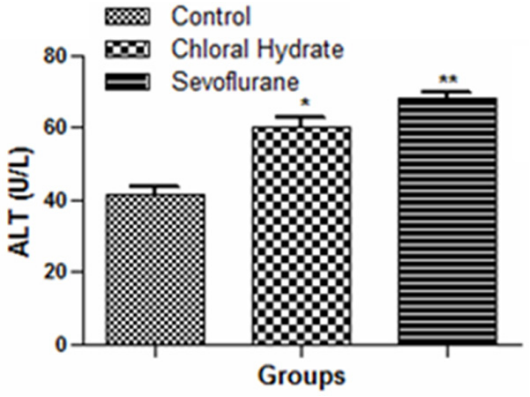

Figure 2. Effects of different treatment on serum ALT level of rats. Data are presented as mean ± SD for 10 rats in each group. *P < 0.05 and **P < 0.01, compared to the control group.

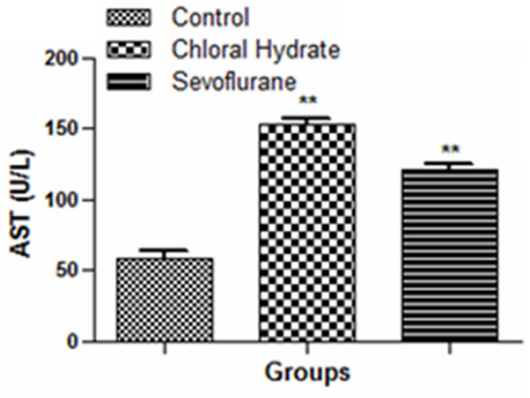

Figure 3. Effects of different treatment on serum AST level of rats. Data are presented as mean ± SD for 10 rats in each group. *P < 0.05 and **P < 0.01, compared to the control group.

pathology. The liver section of the rats were randomly selected and fixed in the 10% natural buffered formaldehyde solution and cut the sections in 3-5 μm. Hematoxylin and eosin [H&E] was used as the stainining agent for the microscopial evaluation.

Western blot

For the estimation the molecular mechanism, we have estimated the Bcl-2 and Bax level using the western blot analysis. The hepatic tissues of the rats were homogenized in lysis buffer, which contain the complete protease inhibitor cocktail [5 M NaCl, 1 M Tris-HCl [pH 8.0], 1 M 1,4-dithio-dl-threitol [DTT] and 10% Nonidet P-40]. After quantification with bicinchoninic acid protein kit [BCA] assay, the quantity of total proteins were estimated using the 12% SDS-PAGE gel and shifted to polyvinylidene fluoride [PVDF]. After blocking the blot the dried milk was used to prepare the fat free [5%] dried milk at room temperature [2 h], the membranes were incubated overnight maintain the temperature 4°C for using the corresponding antibodies. Consequently, the membrane was also incubated for 2 h at room temperature using the secondary antibodies. The blots were visualized with the enhanced chemiluminescence [ECL] detection system [Amersham], and the results were examined through LabImage.

Estimation of caspase-3 activity

The caspase-3 activitiy in the hepatic tissues of the rats were estimated using the available Caspase-Glo 3/7 assay kit with following the instruction provided by the manufacture.

Statistical analysis

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

Results

Effect of the sevoflurane and chloral hydrate on serum biochemistry

Figures 1-4 showed the effect of the repeated dose of the sevoflurane and chloral hydrate on

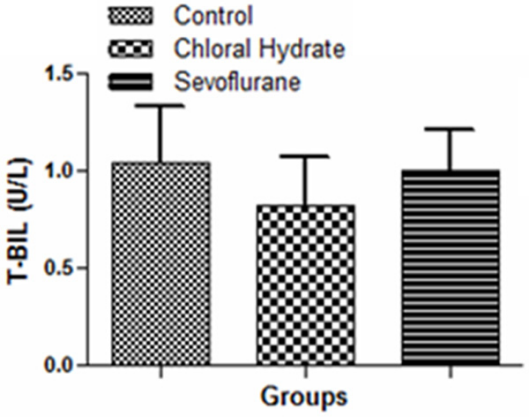

Figure 4. Effects of different treatment on serum T-BIL level of rats. Data are presented as mean ± SD for 10 rats in each group. *P < 0.05 and **P < 0.01, compared to the control group.

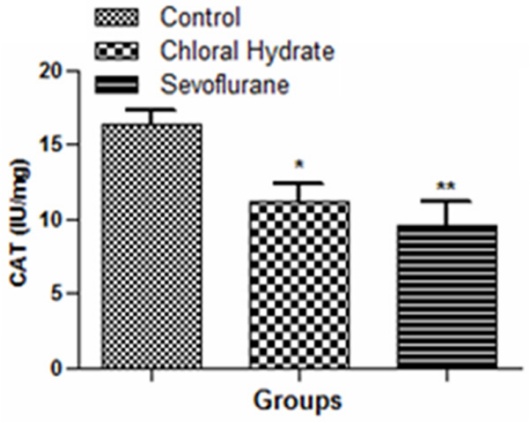

Figure 5. Effects of different treatment on CAT level of rats. Data are presented as mean ± SD for 10 rats in each group. *P < 0.05 and **P < 0.01, compared to the control group.

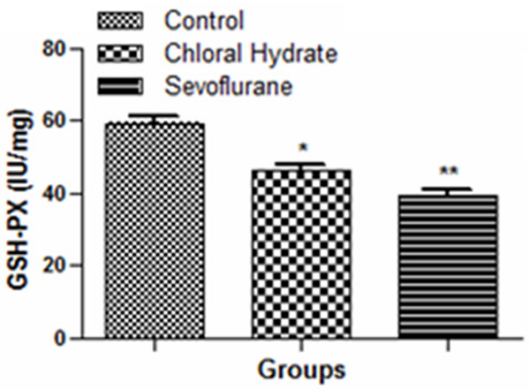

Figure 6. Effects of different treatment on serum GSH-PX level of rats. Data are presented as mean ± SD for 10 rats in each group. *P < 0.05 and **P < 0.01, compared to the control group.

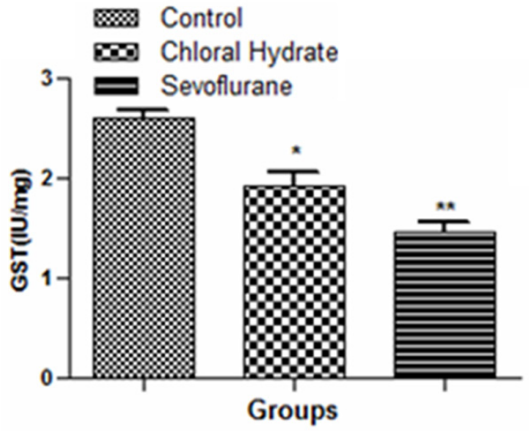

Figure 7. Effects of different treatment on serum GST level of rats. Data are presented as mean ± SD for 10 rats in each group. *P < 0.05 and **P < 0.01, compared to the control group.

the serum level of ALT, ALP, AST and T-BIL. It was suggested that, the level of the ALP [143.8±4.35] [**Figure 1**], ALT [60±3.76] [**Figure 2**], AST [153.2±9.83] [**Figure 3**] was found to be significantly elevated and reduction in the level of the T-BIL [0.82±0.002] [**Figure 4**], respectively as compared to control group [P < 0.01]. Similarly, the rats were treated to sevoflurane suffered more serve hepatic damage as evidenced by a significant increase in the serum level of ALP [167.6±8.43] [**Figure 1**], ALT [68.1±4.87] [**Figure 2**], T-BIL [0.1±0.004] and AST [121.8±6.54] [**Figure 3**] as compared to normal control group rats. However, the data of the T-BIL activity was found not significant as compared to the normal control group.

Effect of sevoflurane and chloral hydrate on antioxidant markers

Figures 5-9 demonstrated the effect of the repeated dose of the sevoflurane and chloral hydrate on the antioxidant markers such as CAT, SOD, GST and GSH-PX as well as TBARS level in the hepatic tissues. As showed in the figure, the level of the CAT [**Figure 5**], GSH-PX [**Figure 6**], GST [**Figure 7**] and SOD [**Figure 8**] activities were found to be significantly [P < 0.05] reduced from 16.4±1.83, 59.5±3.23, 2.6±0.43 and 15.98±1.26 IU/mg in normal control to 11.2±1.21, 46.4±2.37, 1.92±0.63 and 8.2±0.63 IU/mg in sevoflurane group, respectively. Obviously, chloral hydrate seems to induce more injury as evident from the antioxidant parameters, which was found to be sig-

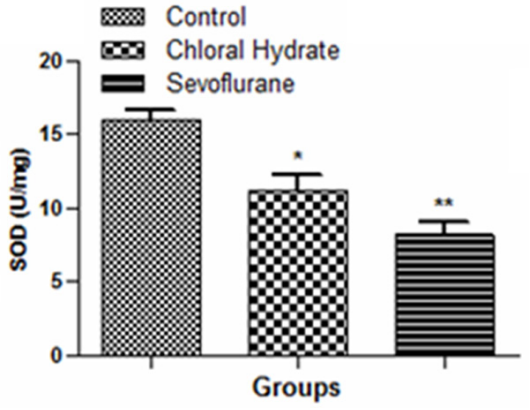

Figure 8. Effects of different treatment on serum SOD level of rats. Data are presented as mean ± SD for 10 rats in each group. *P < 0.05 and **P < 0.01, compared to the control group.

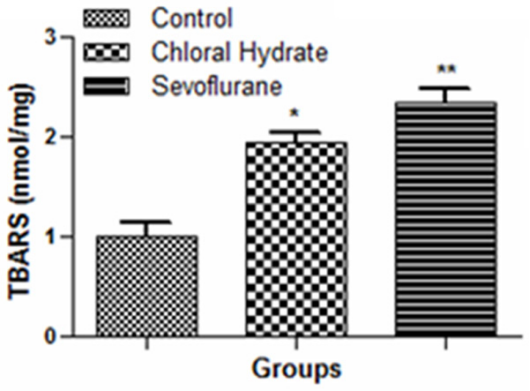

Figure 9. Effects of different treatment on serum TBARS level of rats. Data are presented as mean ± SD for 10 rats in each group. *P < 0.05 and **P < 0.01, compared to the control group.

nificantly [P < 0.01] lowered [9.6±1.18, 39.4±2.54, 1.46±0.63, 8.2±0.92 IU/mg, respectively] as compared to the normal control group. The level of the TBARS of rats treated with the sevoflurane and chloral hydrate presented the enhanced tendency, i.e. its level has been enhanced form 1±0.03 nmol/mg in control group to 1.94±0.08 nmol/mg in sevoflurane group and 2.34±0.12 nmol/mg in chloral hydrate group rats, respectively, suggesting that the sevoflurane and chloral hydrate anesthesia could significantly induce the lipid peroxidation.

Effect of sevoflurane and chloral hydrate on Bcl-2/Bax ratio and caspase-3 activation

Figures 10 and **11** showed the expression of the Bax, Bcl-2 and caspase-3 activation. In the current investigation, we have found that the apoptosis level was found to be increased in the chloral hydrate and sevoflurane group along with a significant [P < 0.01] downregulation of the Bcl-2 expression and unregulated in the Bax expression and the Bcl-2/Bax ratio [P < 0.01] compared to the normal control group. As presented in the **Figure 11**, the activity of the caspase-3 considered as a marker of the cell apoptosis, which was found to be considerably increased in the chloral hydrate [131.6±4.3%, P < 0.01] and sevoflurane B [149.6±5.6%, P < 0.01] than these in normal control in the liver tissues.

Histopathological examination

As showed in the **Figure 12**, the histopathological observation of H&E staining of the livers was performed to further confirm the results observed in the serum biochemical estimation and hepatic antioxidant enzymes. The control group rat's histopathology showed the hepatic cords along with strong hepatocytes with intact central vein and skinny sinusoidal spaces. The chloral hydrate treated rats showed the moderate hepatocytes hypertrophy, relatively intact central vein and swollen in partial region around the central vein in comparison with the hepatic cellular architecture of the rat tissue from the normal control. As expected, rats in sevoflurane suffered more with serve hepatic alterations as compared to control and chloral hydrate. The sevoflurane rats showed the swollen, hepatocytes hypertrophy, dilated sinusoidal spaces in most regions, ballooned lipid laden hepatocytes revealing extensive liver lesions.

Discussion

Sevoflurane and chloral hydrated are very commonly used anesthetic agents in the animal experimental model. However, previous results have reported that the negative effect of sevoflurane and chloral hydrate agents usually occur at higher concentration or doses in laboratory animals [1, 6, 7]. In the current investigation, we are the first to make effort to scrutinize the possible effect of repeated higher dosage of sevoflurane and chloral hydrate anesthesia on rat hepatocellular system. The current result showed that the repeated high dosage of the sevoflurane and choral hydrate anesthesia could cause the oxidative damage, hepatotoxic-

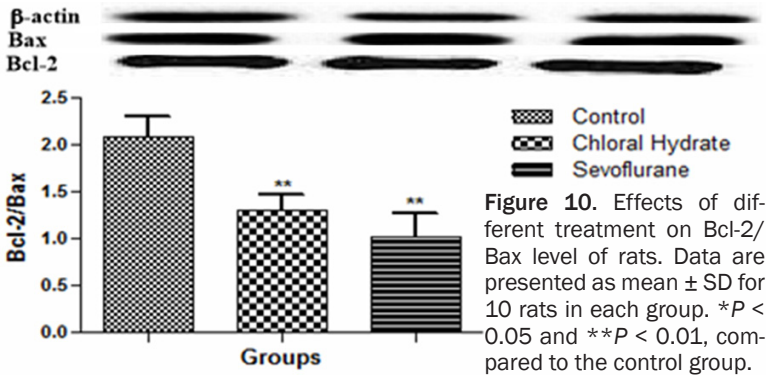

Figure 10. Effects of different treatment on Bcl-2/Bax level of rats. Data are presented as mean ± SD for 10 rats in each group. *P < 0.05 and **P < 0.01, compared to the control group.

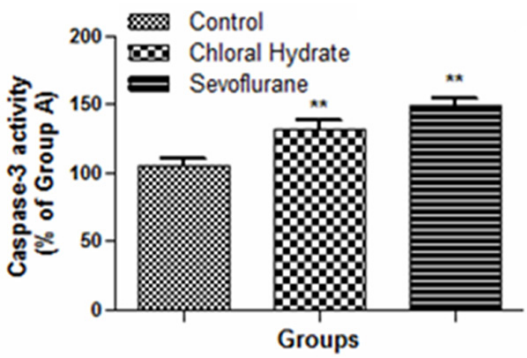

Figure 11. Effects of different treatment on Caspase-3 activity of rats. Data are presented as mean ± SD for 10 rats in each group. *P < 0.05 and **P < 0.01, compared to the control group.

ity, lipid peroxidation and induces the hepatic cell damage and even apoptosis.

While no specific reason have been figure-out to explain the effect of sevoflurane and chloral hydrate induced hepatotoxicity, instead, they will act via indirect effect, such as, modulation of the level of the antioxidant enzymes which causes induction of the hepatotoxic effect. Free radical or reactive oxygen species [ROS] induces the oxidative stress, which might be the possible mechanism of action to induce the sevoflurane and chloral hydrate toxicity. Liver tissue is thought to be protected by its antioxidant protection methods, and the endogenous antioxidant marker such as GSH-Px, SOD, CAT, GST, are jointly able to provide protection against ROS [9]. Thus, these antioxidant markers were used as an indicator which can indirectly suggest about the lipid peroxidation and tissue oxidation. GSH-Px acts as an enzymatic antioxidant both extracellular and intercellular in conjucation with different enzymatic process, which can able to reduce the hydroperoxides and hydrogen peroxide [H_2O_2] [10]. SOD radical can catalyze the superoxide anion radicals [O_2^-] clearance and prevent the H_2O_2 generation [11]. Thus, decline in the level of the SOD leads to injurious effects, such as failure of the cell membrane function and integrity, which clearly indicates the presence of high level of ROS [12]. The data of the study clearly showed that the rats exposed to the repeated dose of sevoflurane and chloral hydrate showed significant inhibition of the level of GSH-Px and SOD activities, confirming that the both anesthesia could cause the hepatotoxic effect and induces the hepatic oxidative damage. In the current study, the rats were exposed to the sevoflurane and chloral hydrate also showed reduced level of the hepatic GST activity. GST play an important role in the antioxidant retorts against lipid peroxidation, thus, attenuating this process by inhibiting the hydroperoxides may prevent the cells from the toxic end products of LPO [13]. CAT, is an another enzyme that was considered to play part in the ROS detoxification [14], and in the present study, its level was found to be significantly attenuated by sevoflurane and chloral hydrate. In the current investigation, we find that the level of the antioxidant enzymes GSH-Px, SOD, CAT and GST in sevoflurane was found to be slightly decreased as compared to the chloral hydrate group rats. The result clearly showed that the hepatic antioxidant activity was lowered in sevoflurane than chloral hydrate group rats. We also observed the cellular damage that supports the enhanced level of lipid peroxidation. As expected, the level of the TBARS, used as an important index, which indirectly reflect the cell oxidative damage, were found to be considerably enhanced in sevoflurane and chloral hydrate group as compared to the control group, suggesting that the administration of the sevoflurane and chloral hydrate can significantly cause lipid peroxidation. The current result showed alteration of the hepatic enzymatic antioxidant protection methods of the sevoflurane and chloral hydrate anesthetics, via disturbance in hepatic oxidative status, which could share to the hepatic damage.

The underlying molecular mechanism of repeated high doses of sevoflurane and chloral

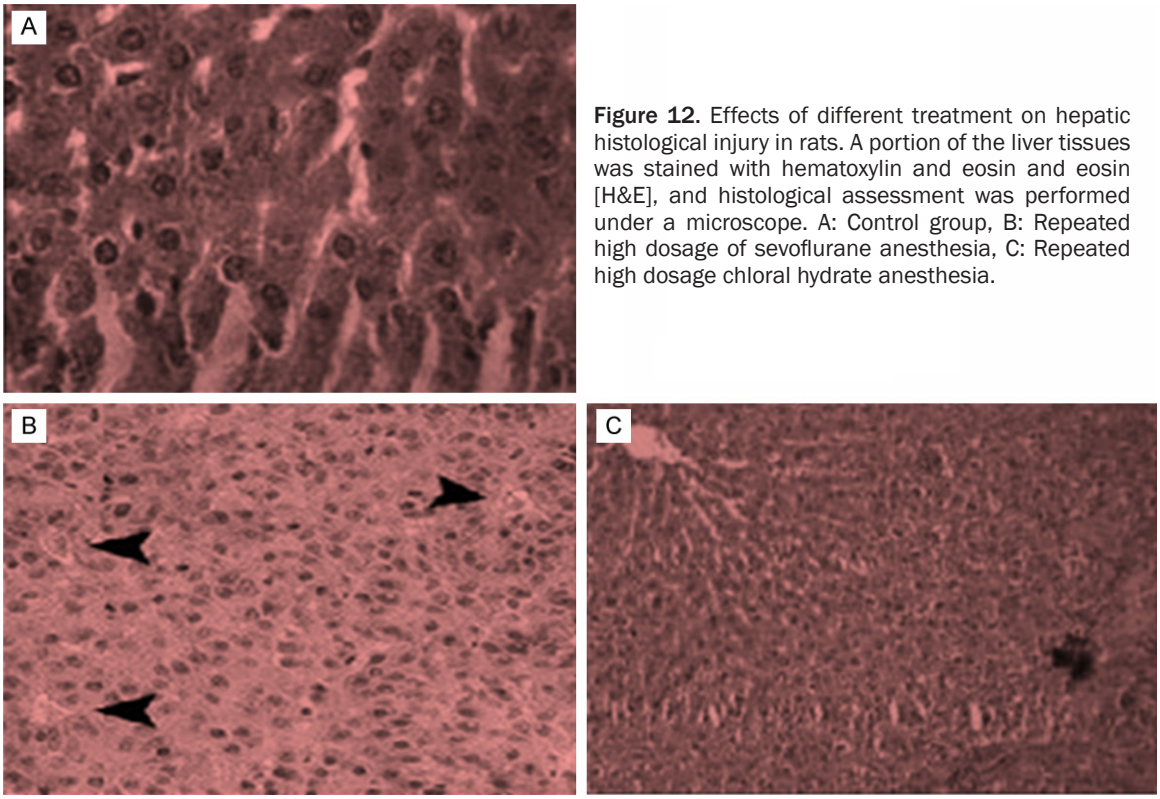

Figure 12. Effects of different treatment on hepatic histological injury in rats. A portion of the liver tissues was stained with hematoxylin and eosin and eosin [H&E], and histological assessment was performed under a microscope. A: Control group, B: Repeated high dosage of sevoflurane anesthesia, C: Repeated high dosage chloral hydrate anesthesia.

hydrate anesthesia still remain to be elucidated. Bcl-2 and Bax are involved with apoptosis under pathological and physiological conditions, which are considered as major indicators in estimation the death or cell survival after apoptosis inducement [16]. It is widely known that increased level of Bax will induce the cell death, whereas if Bax heterodimerization predominates and Bcl-2, the cell will survive [17]. In the current investigation, we have found enhanced level of apoptosis in sevoflurane and chloral hydrate treated group along with a significant enhancement in the expression of Bax [$P < 0.01$] and reduction in the expression of Bcl-2 [$P < 0.01$] and the ratio of Bcl-2/Bax [$P < 0.01$] as comparised with the normal control group. It might be reasonably suggested that the sevoflurane and chloral hydrate could cause the apoptosis of liver cell. Caspase-3 activation is a vitally important step in the execution phase of the apoptosis and hinders apoptosis [16]. Furthermore, the Bax causes induction, while, Bcl-2 inhibited the caspase-3 activity. The Bax neutralize the Bcl-2 activity via the generation of heterodimers with Bcl-2 [18]. In the current study, the estimation of the caspase-3 activity was found in aggrement of our investigation. The data of the manuscript demonstrated that the level of caspase-3 was found to be significantly [$P < 0.01$] increased in sevoflurane and chloral hydrate as compared to the control group. Since the data of the study confirmed the Bcl-2/Bax low ratio, a compensatory initiation of Bcl-2 was not enough to conquer the proapoptotic actions of Bax on caspase-3 activation.

Disclosure of conflict of interest

None.

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