



Possible effects of some anaesthetic agents in rat hepatotoxicity: Histological and biochemical study

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Received: 25.05.2021

Accepted/Published Online: 26.10.2021

Final Version: 01.01.2022

Abstract

Selectively steroidal neuromuscular blocking agents, such as rocuronium, are required to maintain surgical procedures. It is a sugammadex γ -cyclodextrin-derived drug that encapsulates these drugs and reverses its effects. In the present study, it was aimed to investigate the effects of sugammadex rocuronium complex on liver and possible toxic effects on hepatocytes using histopathological and biochemical methods. Thirty-two adult Sprague - Dawley male rats were used. Four groups were designed as pure control, control group, sugammadex and sugammadex-rocuronium. Following the experimental procedures, liver tissues extracted from rats were stained after routine histological procedures. The images were taken from the sections for histopathological evaluation. For biochemical analysis, glutathione (GSH) and malondialdehyde (MDA) enzymes were analyzed in the liver tissues. In the group treated with the sugammadex-rocuronium complex, caspase-3 expressions were observed to be higher than in other groups. In addition, while the amount of GSH belonging to sugammadex and sugammadex-rocuronium groups decreased compared to the control group, an increase in MDA amount was found. Sugammadex-rocuronium can lead to oxidative stress in the hepatocytes. However, more studies are needed to reveal the toxic effects of sugammadex and sugammadex and rocuronium complex in the liver.

Keywords: sugammadex, rocuronium, oxidative stress, immunohistochemistry, liver histology, rat

1. Introduction

Most patients who undergo surgical procedures require general anesthesia. For this purpose, in addition to neuromuscular blockers, various analgesic and anesthetic agents are used (1). Muscle relaxants can cause many side effects such as atelectasis, pneumonia, pulmonary complications, hypercapnia, and hypoxemia in the postoperative period (2,3). Rocuronium is one of the muscle relaxants commonly used in general anesthesia and provides neuromuscular junction blockade (4). In general anesthesia applications, neuromuscular blocking agents are used to facilitate airway intubation, to provide mechanical ventilation and to adapt surgical operation conditions (5). In this regard, it is necessary to eliminate the effect of muscle relaxants to improve muscle function in the patient and prevent neuromuscular blockage (6). Steroidal neuromuscular blocking drugs, such as rocuronium, are widely used in clinical anesthesia to facilitate tracheal intubation and to allow surgical access to body cavities. Residual neuromuscular block and subsequent respiratory insufficiency are associated with substantial morbidity and mortality, although the quality of tracheal intubation has significantly improved because of the clinical use of neuromuscular blocking agents (NMBAs), resulting in a reduction of pharyngo-laryngeal lesions. Therefore, many anesthetists

routinely reverse neuromuscular block to facilitate rapid and complete recovery after surgery to prevent residual block (7-10).

Sugammadex is a γ -cyclodextrin designed to reverse the effects of steroidal neuromuscular blocking agents such as rocuronium and vecuronium (11). The mechanism of action of sugammadex is different from other reversing agents. Since sugammadex is a molecule-binding agent, it does not act on acetic cholinesterase and other receptor systems (12). The sugammadex-rocuronium complex formed by the binding of a sugammadex molecule target agent rocuronium in plasma, quickly and safely restores the rocuronium-induced deep neuromuscular blockade (13). The complex formed by the pharmacokinetic properties of sugammadex is inactivated and excreted from the body through the kidneys (12). In addition, it has been suggested that this complex remained in circulation for a long time. For this reason, it has been reported that it causes histopathological findings such as skeletal muscle myopathy, vacuolization, pyknotic nuclei clumps, and thus causes disorders such as weakening of muscle fibers and hypertrophy (14). Since sugammadex is excreted from the kidneys and is not metabolized in the liver (15), there are a limited number of studies including its effect on liver morphology and enzymes (16). Clarke et al. (17)

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reported that a cutaneous anaphylaxis effect in patients sensitive to rocuronium was alleviated with sugammadex after two minutes. It has been demonstrated that sugammadex reduces the rocuronium-induced increase in tryptase concentration caused by rocuronium in the rat liver after five minutes (17). In this context, sugammadex is suggested to reduce the activation of mast cells (18). In the light of this information, studies on the role of rocuronium and sugammadex on anaphylaxis-related liver enzymes are limited (17-19). There are no morphological studies hepatopathologically. Based on this point, we aimed to investigate the effects of sugammadex and sugammadex-rocuronium complex on liver morphology in our study by using histopathological, immunohistochemical and biochemical methods. The presented study contributes to the literature in terms of explaining the effect of sugammadex on the liver in detail using immunohistochemical, histopathological and biochemical methods.

2. Material and Methods

2.1. Animals and study design

The experimental procedures of the present study were approved by the Adiyaman University, Animal Experiments Local Ethics Committee with the report of ADYU-HADYEK 2019/032. In our study, 32 adult *Sprague - Dawley* male rats weighing 300-350 g were used (20,21). The rats were monitored daily under favorable conditions (Room temperature: 22-25 °C and humidity: 50-55%) with standard pellet diet and water ad libitum. 12-hour light-dark cycle using cold white fluorescent lamps (06.00-18.00) was provided. The rats were randomly divided into four groups (n=8): Pure control group, control group, sugammadex group, sugammadex-rocuronium group. The pure control group consisted of animals that did not have any surgical treatment and that comply with the routine care rules to obtain basal values. Rats of the control group were given 16 mg / kg intravenous (IV) 0.9% isotonic saline. Rats of the sugammadex group were given 16 mg / kg IV sugammadex (BridionR; Schering - Plow Corporation, Oss, The Netherlands). After three minutes following the administration of 16 mg/kg IV sugammadex (the bridion; Schering - Plow Corporation, Oss, The Netherlands), 1 mg/kg IV rocuronium (ESMERON is, Organon, Istanbul, Turkey) was injected to sugammadex + rocuronium group. All drugs were administered intravenously over the tail vein. Doses administered Bostal et al. (22) and Pühringer et al. (23) was determined by taking the studies conducted as a guide. Following experimental procedures, liver tissues under ketamine/xylazine anesthesia were dissected from animals under ketamine/xylazine anesthesia.

2.2. Tissue processing, sectioning, and staining procedures

Liver samples obtained for the purpose of histopathological examinations were placed in 10% formaldehyde solution in separate groups. After a week-long fixation, paraffin blocks were prepared by routine histological tissue follow-up. 5 µm

thick sections were taken from paraffin blocks. The sections taken were stained with hematoxylin-eosin, Masson trichrome and Toluidine blue dyes. Images were obtained from liver slides of each group using a microscope with a digital camera attachment, Axiocam ERc5 model of Carl Zeiss. Histopathological evaluation was performed on the images obtained.

2.3. Immunohistochemical staining

For immunohistochemical analysis, primary antibody Caspase-3 (Thermo Fisher Scientific; cat no: PA5-16335) diluted 1/200 with the commercial kit Thermo Scientific™ TP-015-HA was used. From the blocked tissues, 5 µm thick sections were taken into adhesive slides and deparaffinized. Streptavidin-biotin-peroxidase complex method was used in staining. Positive and negative controls were performed as recommended by the manufacturers. After applying AEC Chromogen, reverse staining was performed with Mayer's hematoxylin. Histopathological evaluation was performed on the images obtained. The degree of staining was determined as 0: no, +0.5: extremely low, +1: low, +2: moderate, +3: severe (24). The removed liver tissues were used for histopathological and biochemical analysis

2.4. Biochemical tests

The dissected liver samples were washed with saline at a temperature of +4°C, placed in ependorhic tubes according to the cold chain principles and kept at -70°C until examination. Tissue homogenates for malondialdehyde (MDA) and reduced glutathione (GSH) measurements were prepared cold using 0.15 M KCl with 10%, (w/v) homogenizer. The Mihara and Uchiyama method used for the determination of lipid peroxidation and MDA, which is a marker of free oxygen radical amount, is based on the reading of the pink colored product from N-butanol phase at 535 and 520 nm because of reacting with the thiobarbituric of the pink colored product at 95 °C (25). Liver tissue was centrifuged by homogenizing in 10% trichloroacetic acid. After the supernatant was mixed with an equal volume of 0.67% thiobutyric acid, it was incubated in boiling water for 15 minutes at 90 ° C. Following incubation, it was centrifuged by cooling. Tissue MDA concentrations were measured in nmol /g tissue under 532 nm absorbance. GSH is an antioxidant enzyme that catalyzes the reduction of harmful peroxides such as lipid peroxide and hydrogen peroxide. During this reduction, reduced glutathione is converted to oxidized glutathione. In the GSH analysis performed by the method described by Elman (26), glutathione in the analysis tube reacts with 5i-dithiobis2-nitrobenzoic acid to give a yellow-greenish color. The light intensity of this color was measured with a spectrophotometer at a wavelength of 410 nm.

2.5. Statistical analysis

In the statistical analysis of the data obtained, the Kolmogorov-Smirnov test was used to determine whether the data was normally distributed. One Way ANOVA test was

used for normally distributed data and Tukey test, post-hoc test for multiple comparisons. The Kruskal-Wallis test was used for non-normally distributed data and the Mann Whitney-U test for multiple comparisons. The results were evaluated within the mean \pm standard deviation (SD) of 95% confidence interval. P value <0.05 was considered statistically significant. SPSS program (Chicago, IL, USA; version 22.0) was used for statistical analysis.

3. Results

3.1. Light microscopic findings

When hematoxylin eosin-stained sections of tissues belonging to pure control and control groups were examined, it was observed that hepatocyte cords extending from v. centralis to periphery in the middle of the liver lobule and sinusoids located between these cords were normal (Figs. 1a and 2a). The presence of polygonal shaped liver cells was monitored. It was determined that hepatocyte cytoplasm showed acidophilic staining feature, which varies in density according to the activity status of the cells. The nuclei of these cells were found to be centrally located, large, round and euchromatic, some hepatocytes have bi-nucleus and in normal morphology (Figs. 1b and 2b). In the rat liver tissues belonging to pure control and control group, which was applied by Masson's trichrome staining method to show the connective tissue density, Intense connective tissue was observed around the v. centralis and periportal area (Figs. 1c and 2c). The density of mast cells in the vascular connective tissue was normal (Figs. 1d and 2d). When hematoxylin eosin-stained sections of rat liver tissues belonging to the group treated with sugammadex were examined, hepatocytes that formed the parenchyma in small magnification compared to the group treated with Rocuronium. It was observed that it showed a slightly more regular structure around v. centralis (Fig. 3a). It was noted that the hepatocytes that make up the liver parenchyma are of normal structure and maintain their acidophilic structure in large enlargements of the same group. Among the hepatocytes, it was determined that there were degenerated hepatocytes, and dilatation in sinusoids (Fig. 3b). The connective tissue density was observed to be normal around the v. centralis and periportal areas in the Masson's trichrome staining method (Fig. 3c). Mast cell density was low compared to sugammadex and rocuronium applied group (Fig. 3d). When the sections of rat liver tissues belonging to the group treated with sugammadex and rocuronium were examined with hematoxylin-eosin, at small magnification, a liver structure was observed in which there was a disruption in the arrangement of hepatocyte cords formed by hepatocytes around the v. centralis, and the lobule structure and boundaries were not clearly distinguishable (Fig. 4a). It is noteworthy that in the large enlargements of the same group, the cytoplasmic boundaries of the hepatocytes that make up the parenchyma are not clearly distinguished, the polygonal shapes disappear, the size differences and degenerative changes between the cells are broken. These were observed to

have a dark pyknotic core in some areas. In addition, in this group, an increase in dilatation was observed in sinusoid structures (Fig. 4b). To show the connective tissue density, Masson's trichrome staining method has been applied in rat liver tissues, compared to other groups around the v. centralis and periportal areas, it was observed that the connective tissue density increased (Fig. 4c). The density of mast cells in the vascular connective tissue was significantly increased compared to the pure control and control groups (Fig. 4d).

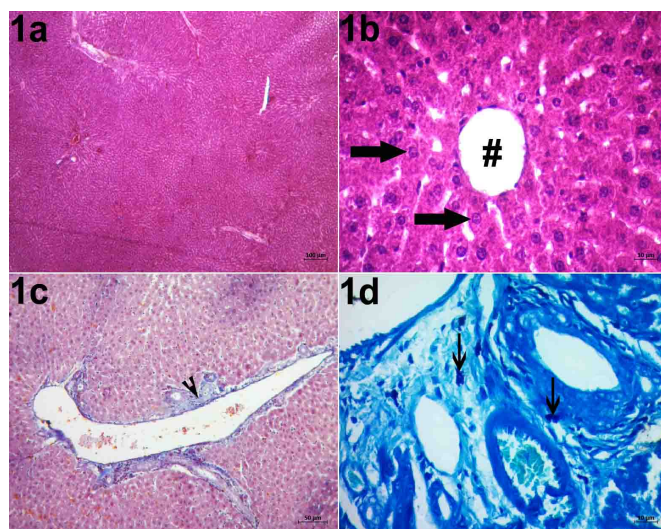


Fig. 1. (1a-1d pure control group) 1a, 1b, 1c and 1d: Images of the group in magnification x4, x40, x10 and x40 respectively; (Hematoxylin-Eosin staining, Masson' trichrome staining and Toluidine blue staining); #, central vein; thin arrow, mast cell; thin arrowhead, connective tissue; thick arrow, healthy hepatocyte

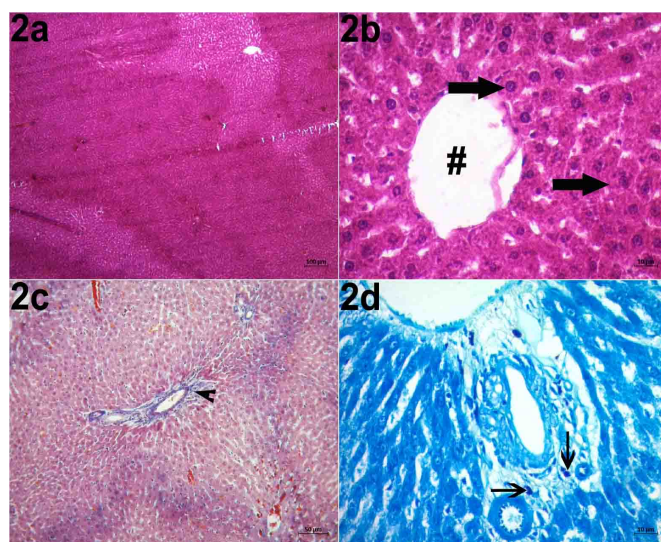


Fig. 2. (2a-2d control group) 2a, 2b, 2c and 2d: Images of the group in magnification x4, x40, x10 and x40 respectively; (Hematoxylin-Eosin staining, Masson' trichrome staining and Toluidine blue staining); #, central vein; thin arrow, mast cell; thin arrowhead, connective tissue; thick arrow, healthy hepatocyte

3.2. Histopathological results

When the cellular degeneration, lobular degeneration, fibrosis, sinusoidal dilatation, mast cell density, mast cell density parameters of the sugammadex+rocuronium group were examined by histopathological scoring, it was determined that there was a highly significant increase in the pure control and control groups. Statistical data are given in

Table 1.

3.3. Immunohistochemical findings

Caspase-3 immunoreactivity was extremely low in hepatocytes in the hepatic cords in the pure control and control groups (Figs. 5a and b). The involvement in the sugammadex group increased slightly compared to the control and control groups (Fig. 5c). In the sugammadex + rocuronium group, positively stained cells were higher than in the other groups (Fig. 5d).

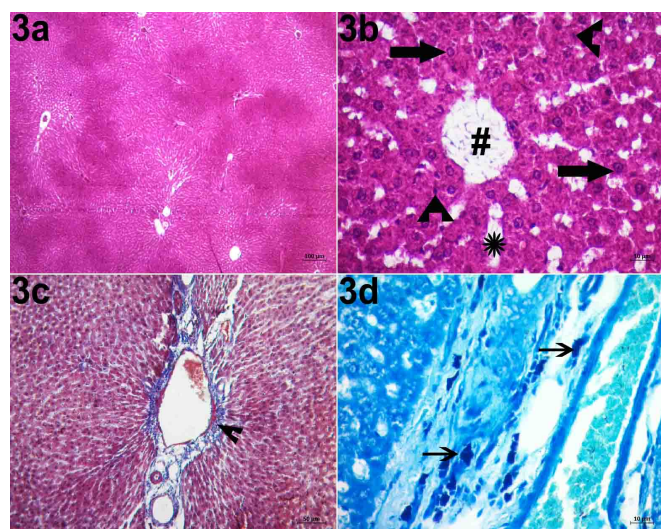


Fig. 3. (3a-3d sugammadex group) 3a, 3b, 3c and 3d: Images of the group in magnification x4, x40, x10 and x40 respectively; (Hematoxylin-Eosin staining, Masson' trichrome staining and Toluidine blue staining); #, central vein; thin arrow, mast cell; thin arrowhead, connective tissue; thick arrow, healthy hepatocyte; thick arrowhead, degenerated hepatocyte cells; star, dilated sinusoid

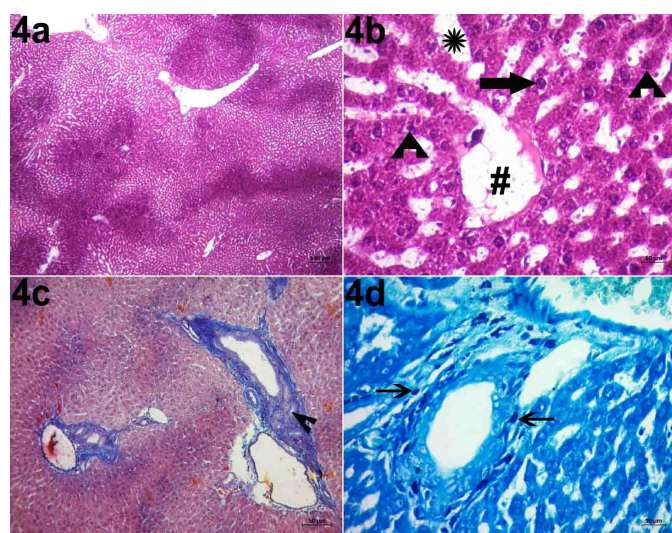


Fig. 4. (4a-4d sugammadex + rocuronium group) 4a, 4b, 4c and 4d: Images of the group in magnification x4, x40, x10 and x40 respectively; (Hematoxylin-Eosin staining, Masson' trichrome staining and Toluidine blue staining); #, central vein; thin arrow, mast cell; thin arrowhead, connective tissue; thick arrow, healthy hepatocyte; thick arrowhead, degenerated hepatocyte cells; star, dilated sinusoid

When the degree of immunostaining of the sugammadex+rocuronium group were examined by histopathological scoring, it was determined that there was a highly significant increase in the pure control and control

groups. Statistical data are given in Table 1.

3.4. Biochemical analysis results

There was no statistically significant difference between pure control and control groups at the MDA level, which is defined as the indicator of free radical-induced damage in tissues ($p > 0.05$). However, sugammadex and sugammadex + rocuronium groups showed a significant increase in MDA levels compared to pure control and control groups ($p < 0.01$). The data of the groups are shown in Table 2 and Fig. 6.

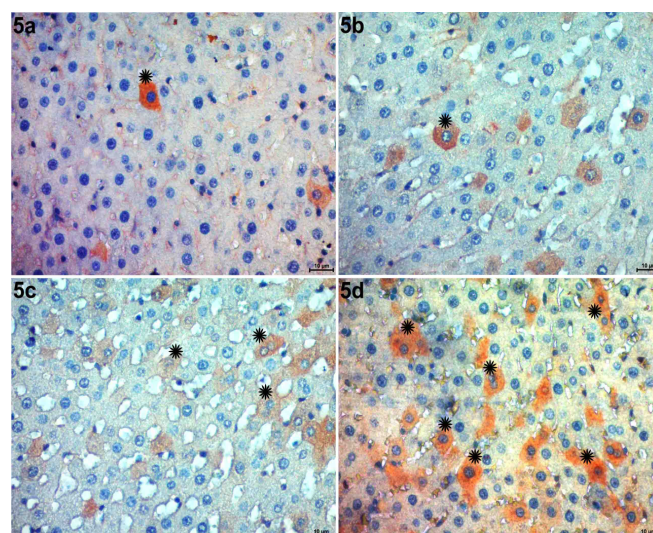


Fig. 5. 5a, 5b, 5c and 5d: Images at x40 magnification of groups (Pure control, control, sugammadex and sugammadex + rocuronium group respectively); * Caspase 3 positive cells exposed to apoptosis

Table 1. Histopathological scoring in all groups

Groups	Pure Control	Control	Sugammadex x	Sugammadex + Rocuronium
Cellular degeneration	0.75 ± 0.71	0.50 ± 0.53	1.14 ± 0.70	2.12 ± 0.83*
Fibrosis	0.50 ± 0.75	0.62 ± 0.74	0.71 ± 0.75	2.01 ± 0.78*
Sinusoidal dilation	0.62 ± 0.74	0.65 ± 0.51	1.42 ± 0.78	1.98 ± 1.09*
Lobular degeneration	0.62 ± 0.74	0.50 ± 0.75	0.85 ± 0.69	2.10 ± 1.12*
Mast cell density	0.75 ± 0.70	0.75 ± 0.70	1.57 ± 0.53	2.09 ± 1.24*
The degree of immunostain	0.81 ± 0.65	0.50 ± 0.46	1.21 ± 0.56	2.37 ± 0.91*

It was found that GSH level, which plays a role in preventing free radical-induced damage in tissues, decreased in the group treated with sugammadex + rocuronium compared to other groups ($p < 0.01$). The data of the groups are shown in Table 2 and Fig. 6.

Table 2. Liver biochemical parameters in all groups (n=8)

Groups	Pure Control	Control	Sugammadex	Sugammadex + Rocuronium
MDA (nmol/g)	630.22 ± 70.23	553.09 ± 46.95	889.85 ± 78.77*	901.99 ± 73.09*
GSH (nmol/g)	2277.15 ± 104.72	2999.48 ± 221.66	2358.08 ± 105.59	1721.89 ± 153.51#

Valus are expressed as mean±SE; n=8 for each treatment group, * Statistical significance compared to the Pure Control and Control groups ($p < 0.01$), # Statistical significance compared to the other groups ($p < 0.01$)

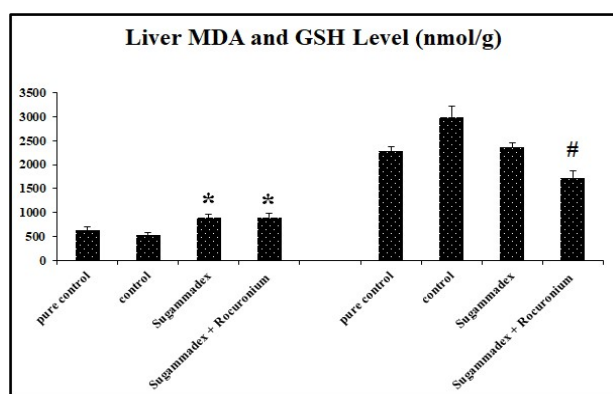


Fig. 6. Biochemical data which resulted from these groups are presented in this graph. Values are expressed as means \pm SD; $n=8$ for each treatment group. Statistical significance compared to the pure control and control group: *: $p<0.01$. Statistical significance compared to the control group: #: $p<0.01$

4. Discussion

In current anesthesia applications, rocuronium, which is in the aminosteroid structure, is a muscle relaxant that is frequently used before intubation and is safe for surgical maintenance. The return of this neuromuscular blockade is at least as important as its maintenance. In this regard, various agents, such as sugammadex, are needed to restore the patient's airway control. These agents play an important role in reducing mortality and morbidity due to respiratory complications in the postoperative period. Sugammadex-rocuronium complex is eliminated by the kidneys. In this regard, prolonged effect of rocuronium in patients with renal failure is observed (15). As a result, depending on the elimination time of sugammadex-rocuronium complex, it is not recommended for use in patients with severe renal disease, since the decrease in sugammadex in plasma concentration varies (27). Tomak *et al.* (28) observed that the number of mast cells increased in rats treated with sugammadex compared to the control group. In this case, they suggested that treatment with sugammadex increased susceptibility to allergic reactions. However, the susceptibility to rocuronium-induced allergic reaction has been observed to a greater extent than susceptibility to sugammadex in the liver. The results show that sugammadex may inhibit rocuronium-induced mast cell degranulation depending on the dose (28). However, according to Clarke *et al.* (28), sugammadex is not sufficiently effective in changing the clinical reflection of the allergic reaction caused by rocuronium use (17). In histopathological examination, we found less mast cells in sugammadex group compared to sugammadex-rocuronium group. This finding supports the suppression feature of sugammadex anaphylaxis. There are no adequate studies on the effects of sugammadex on the liver. However, the effects of cyclodextrins on the liver have been studied (29-31). There are three types of cyclodextrin, α , β and γ cyclodextrins consist of six, seven and eight α - (1,4) linked glycosyl units, respectively. Although three types of cyclodextrin have water solubility feature, γ cyclodextrins with the highest water dissolution rate (32). In one study, the

oral combination of a β -cyclodextrin group drug with clotrimazole, an antimycotic agent, induces fat accumulation in the liver compared to clotrimazole and can lead to liver hypertrophy. Although the resulting complex is promising in treatment due to its oral bioavailability, the hepatotoxicity it causes cannot be excluded (29). On the other hand, in another study, it is noted that β cyclodextrins can affect the hepatoprotective effect positively as carbon tetrachloride-induced acute hepatotoxicity increases the water solubility of the proactive detective hydrophobic flavone compounds (23). In this case, it can be concluded that the β cyclodextrin group may vary depending on the complex it constitutes regarding the hepatotoxic activity. Based on this point, we examined the effects of sugammadex alone, γ gamma cyclodextrin, and sugammadex and rocuronium complex on the liver using histopathological and biochemical methods. Redox signal transmission pathways play an important role in the regulation of cell functions. Oxidative stress is an important part of the pathogenic processes that occur in relation to redox homeostasis disorder. In case of cell damage, a high concentration of redox signal is observed by DNA degeneration (33). Pathological processes caused by oxidative stress can be reversible or irreversible (34). In addition, liver is the primary organ sensitive to pathological cascades caused by oxidative stress, and especially parenchymal cells are very vulnerable in the oxidative environment. Abundant reactive oxygen radicals are produced from microsomes in mitochondria and parenchymal cells (35). Simultaneously, the hepatic content of increased MDA, decreased hepatic superoxide dismutase, glutathione peroxidase, GSH indicates hepatic oxidative damage. In addition to these signs, increased caspase 3 mRNA expression in the cell indicates this (36). In this context, the increase in MDA levels in the groups belonging to sugammadex and sugammadex-rocuronium complex in our study proves oxidative stress occurring in liver tissues of both groups. However, the simultaneous decrease in GSH levels in the sugammadex and sugammadex-rocuronium groups is also accompanied. Especially the decrease in GSH level of sugammadex-rocuronium group is higher compared to sugammadex group. Therefore, oxidative stress damage occurring in the group treated with Sugammadex-rocuronium complex is higher compared to sugammadex group. Similarly, Palanca *et al.* (37) examined the neurotoxic effects of sugammadex and suggested that sugammadex-induced changes were associated with oxidative stress and apoptosis activation. sugammadex predominantly causes cell death in neurons, and induced apoptosis induction has been associated with a change in neuronal cholesterol homeostasis (37). The immunohistochemical analyzes also indicated this. Caspase 3 is expressed more in the sugammadex-rocuronium group compared to the sugammadex group. Caspase 3 is considered as an important biomarker in the mitochondrial apoptotic pathway (38). Based on this point, the sugammadex-rocuronium complex can trigger the apoptotic process in

hepatocytes.

In conclusion, sugammadex is γ gamma cyclodextrin group drug that can reverse the effects of rocuronium, a neuromuscular blocker. There are no adequate publications on the effects of sugammadex on the liver. In addition, sugammadex can inhibit rocuronium-induced anaphylaxis by reducing mast cell count. However, its toxic effect on hepatocytes has not been studied. In this context, in the present study, it has been demonstrated by biochemical methods that sugammadex and sugammadex-rocuronium complex cause hepatic oxidative stress. In addition, sugammadex-rocuronium complex was shown to increase induction of apoptosis and increase caspase 3 expression in hepatocytes compared to sugammadex. Furthermore, several morpho-quantitative studies are needed to reveal the toxic effects of the sugammadex-rocuronium complex.

Conflict of interest

None to declare.

Acknowledgments

None to declare.

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