



## Prevention of rocuronium induced mast cell activation with prophylactic oleuropein rich diet in anesthetized rabbits<sup>1</sup>

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### Abstract

**Purpose:** The effect of a prophylactic oleuropein-rich diet before anesthesia accompanied by the widely-used steroid-based neuromuscular drug rocuronium on mast cell activation was investigated in the study.

**Methods:** 14 rabbits used in the study. The rabbits in the oleuropein group were given oleuropein-rich extract added to the animals' water at doses of 20 mg/kg oleuropein for 15 days orally. After 15 days, all rabbits in the two groups were given general anesthesia with rocuronium of 1 mg/kg. After 1 day, animals were sacrificed and the liver tissue sections stained with H&E, toluidine blue and tryptase for immunohistochemical study.

**Results:** There was no statistically significant difference between ALT, AST and albumin averages of the oleuropein and control groups ( $p > 0.05$ ). The tryptase average of the control group was higher than the tryptase average of the oleuropein group and this difference was statistically significant ( $p = 0.003$ ). The T. blue average in the oleuropein group was higher than the control group. However, there was no statistically significant difference between groups ( $p = 0.482$ ).

**Conclusions:** Rocuronium adverse effects, like hypersensitivity and anaphylaxis, may limit routine use of this substance. The use of oleuropein reduced the number of inflammatory cells and prevented degranulation.

**Key words:** Anesthesia. Mast Cells. Rabbits.

## ■ Introduction

The olive tree contains phenolic compounds with important biological properties and oleuropein is considered the most important of these phenolic compounds<sup>1</sup>. The pharmacological benefits of oleuropein in preventing and protecting against diseases are known. Oleuropein has been shown in a variety of scientific studies to have anti-inflammatory effects in addition to antioxidant, antihistaminic, cardioprotective and hypolipidemic effects, the effect of inhibiting thrombocyte aggregation, anti-atherogenic effect, anticarcinogenic effect, antimicrobial properties against gram-positive and gram-negative bacteria, neuroprotective effect and antiviral effects<sup>2-8</sup>. Previous studies have shown that hydroxytyrosol and oleuropein are the most important phenolic components in olive plants<sup>9</sup>. These phenolic components inhibit mast cell degranulation via induced immune and non-immune pathways. It has been reported that hydroxytyrosol and oleuropein may shed light on researches in terms of preventing mast cell mediated disorders and developing useful tools for their treatment<sup>10</sup>.

Mast cells play an important role in the development of allergic reactions due to the release of histamine, cytokines and proteolytic enzymes<sup>11</sup>. As mast and T cells interact with each other, mast cells especially increase proliferation of T cells and secretion of cytokines<sup>12</sup>. Thus, events leading up to the reactions that result in anaphylaxis.

Rocuronium is a widely used steroidal neuromuscular muscle relaxant that is used to ease endotracheal intubation and mechanical ventilation during general anesthesia<sup>13</sup>. Rocuronium often causes mast cell mediated hypersensitivity reactions. Neuromuscular blocker (NMB) drugs are the most important cause of anaphylactic reactions (38.1% of all cases) during general anesthesia. Rocuronium

(50% of cases) is responsible for most anaphylactic reactions related to NMB drugs<sup>14</sup>.

The aim of this study is to demonstrate the effect of prophylactic administration of oleuropein for the prevention of mast cell aggregation and degranulation after rocuronium administration given for neuromuscular blockage during general anesthesia.

## ■ Methods

### *Study groups and diet protocol*

All the protocols described here are in accordance with the European Communities Council Directive (86/609/EEC). Ethics committee approval was gained from the local animal experiment.

Fourteen adult male New Zealand rabbits weighing between 2.7 and 3.8 kg were used for the study. The rabbits were clinically evaluated for behavior, respiratory and cardiovascular system problems and no negative result was found for animals included in the study. All experiments took place between 09.00 and 16.00. During the experiments the animals were fed with standard rabbit food and were given continuous access to water. The temperature of the shelter was kept at  $21 \pm 2^\circ\text{C}$ .

The animals were randomly divided into two groups: Group C (Control, n:7) were fed with normal diet and water, and group O (Oleuropein, n:7) were fed with normal diet and water containing 20 mg/kg/day of oleuropein. Both groups were fed for 15 days. Oleuropein was extracted from olive leaves as described by Simsek *et al.*<sup>15</sup>.

### *Analysis of oleuropein in olive leaf extract (OLE)*

A HPLC (High Pressure Liquid Chromatography) assisted method was used

for analysis of oleuropein content of OLE<sup>16</sup>. OLE sample was diluted (1:10, v:v) with ultrapure water and filtered through 0.45µm PTFE syringe filter before injection to HPLC system. With a sample loop of 20µL OLE injected to a photodiode array detector (SPD-M20A, Shimadzu, Japan). Separation of oleuropein was carried on Inertsil ODS 3 (250mm, 4.6ID, 5µ) reverse-phase column under isocratic condition with 80:20 v/v water:acetonitrile (adjusted to pH 3 with acetic acid) as a mobile phase. Column was maintained at 35°C during chromatographic run. Flow rate was 1 mL/min and the DAD detector was set at 200-400 nm to analyze the spectra. Quantification was made at 280 nm using external standard method. Results were expressed as mg of oleuropein/L of OLE based on seven-point standard curve prepared by the oleuropein standard (Sigma-Aldrich, Germany) solutions range in 10-1000 mg/L concentration.

#### *General anesthesia protocol*

Rabbits in the study were fasted for 8 hours before the general anesthesia. All rabbits were administered 10 mg/kg ketamine for premedication before the general anesthesia. After 15 minutes, the animals were monitored with ECG. Then a vein was opened in the ear with 26 G branula and 4 L/min O<sub>2</sub> was administered through the mask.

During the general anesthesia, pulse and oxygen saturation measured from the ear by a pulse oximetry device. Body temperature was monitored and held at 37-39°C during the anesthesia.

V-Gel Rabbit (V-gel rabbit R-3 Docsinnovent® Ltd. London, UK) supraglottic airway was inserted to provide reliable airway after the intravenous administration of 2mg/kg ketamine and 1 mg/kg rocuronium bromide. Later the animals were linked to an anesthetic device (Anesthesia Machine w/

O<sub>2</sub> Flush Model M3000PK Parkland Scientific Lab And Research Equipment. Florida, USA) and ventilated manually. 1 MAC isoflurane used to maintain anesthesia and 50% oxygen, 50% air mix was used with. The rabbits were manually ventilated by the same anesthetist to a respiration count of about 40/minute and pressure of 15 cmH<sub>2</sub>O (about 10ml/kg) appropriate for rabbit physiology. The animals were also monitored with pulse oximeter during the anesthesia procedure. General anesthesia duration determined as 30 minute to ensure the data integrity. V-Gel Rabbit was removed and animals were taken to recovery after the observation of spontaneous respiration at sufficient levels.

#### *Euthanasia and sample collection*

All animals were observed for 24 hours following rocuronium administration and then underwent euthanasia with remifentanyl anesthesia. After euthanasia, blood samples were taken for AST, ALT, and albumin analyses and the liver tissues were harvested and stored in 10% neutral formalin solution. Tissues underwent routine tissue processing protocols for light microscopy and were embedded in liquid paraffin. Tissue sections were cut to a thickness of 4 µm with a microtome (Leica RM2255; Leica Microsystems, Wetzlar, Germany) and were stained with routine hematoxylin-eosin (HE) and toluidine blue. HE stained sections were graded using the following scoring system for inflammatory cell infiltration; 0, no infiltration; 1, mild infiltration; 2, moderate infiltration; 3, severe infiltration. Tryptase positive cells were investigated using the immunohistochemical method. Two slides from each rabbit were prepared and five portal triads were examined on each slide. Total and tryptase-positive mast cells in all groups were counted as described by Tomak *et al.*<sup>17</sup>.

### Statistical analysis

Statistical analysis was performed by using the SPSS software version 20 (SPSS 20 for Windows, IBM, Chicago, Illinois, USA). Mann-Whitney U test used to compare the differences between groups. Data were expressed as the mean  $\pm$  standard deviation. P value less than 0.05 considered as statistically significant.

### ■ Results

None of the rabbits had signs and symptoms of any infection, anaphylactic or allergic reactions during the study period.

The average weight of rabbits in the control group was 3541 $\pm$ 150g and the average

weight of OLE group rabbits was 3288 $\pm$ 428g with no statistically significant difference. The oxygen saturation levels, mean arterial pressure and heart rate values in both groups were similar during the observation.

The rabbits in study had daily water consumption of 80-100 ml/kg/day calculated according to weight. The amount of oleuropein in OLE was calculated as 4.31 $\pm$ 0.18 mg/ml with the amount of OLE found according to rabbit weight for an oleuropein dose of 20 mg/kg.

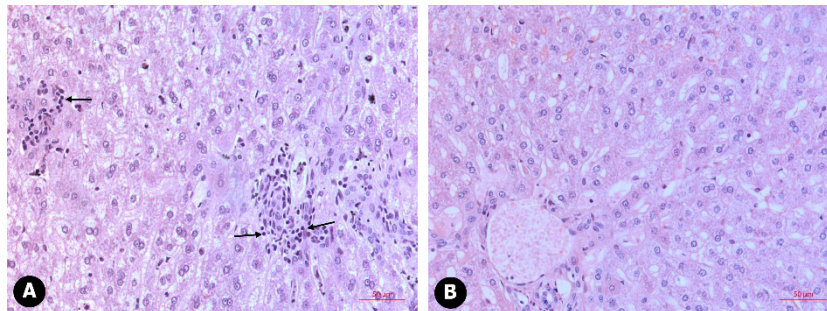
There were no significant differences between the 2 groups for alanine aminotransferase (ALT), aspartate aminotransferase (AST) and albumin (ALB) mean levels ( $p>0.05$ ) which were obtained from blood samples (Table 1).

**Table 1** - Comparison of the groups for ALT, AST, ALB.

Variables	Group O (n=7)		Group C (n=7)		p
	Mean $\pm$ SD	Median (min/max)	Mean $\pm$ SD	Median (min/max)	
ALT	32.5 $\pm$ 12.4	30.0 (15.4-54.8)	27.0 $\pm$ 7.6	28.0 (18.4-39.6)	0.443
AST	21.7 $\pm$ 13.3	15.9 (10.8-46.1)	14.3 $\pm$ 3.4	15.1 (8.3-17.9)	0.654
ALB	4.3 $\pm$ 0.9	4.2 (3.0-5.9)	4.4 $\pm$ 1.2	4.5 (2.2-6.1)	0.848

HE stained sections showed preserved histological tissue architecture in both control and OLE groups, with normal histological structure of hepatocyte cords and vena centralis on microscopic examinations. The histological appearance of portal triads regions was normal and no destruction was

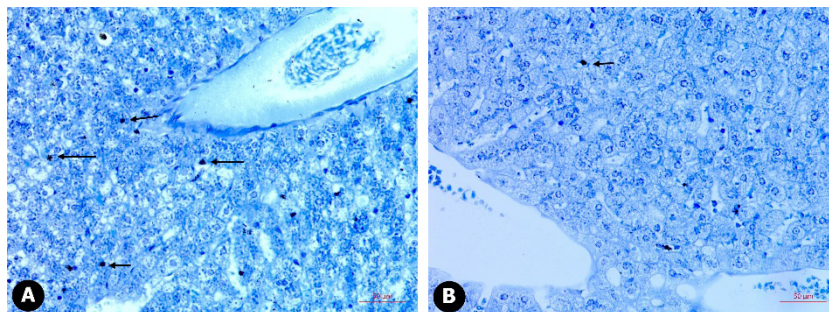
found. However, inflammatory cell infiltrations were found especially in portal areas in control group rather than the OLE group. According to scoring system, grade 1 inflammatory cell infiltration observed in OLE group when compared to grade 3 infiltration in control group (Figure 1).



**Figure 1** - Microscopic comparison of routine histopathologic changes with Heatoxylin & Eosin staining shown in **A** (group C) and **B** (group O) (Magnification x4). Inflammatory cell infiltrations are significant in **A** than **B**.

Mean mast cell numbers in Group O were significantly higher than Group C for toluidine blue staining (1774.4±336.0 vs. 1673.6±291.4, respectively), though there was no significant difference between the two groups statistically (p=0.482) (Figure 2).

Tryptase positive cell mean value in group C was significantly higher than group O and the difference was statistically significant (1169.6±137.5 vs. 775.1±180.0, respectively, p=0.003) (Table 2, Figure 3).

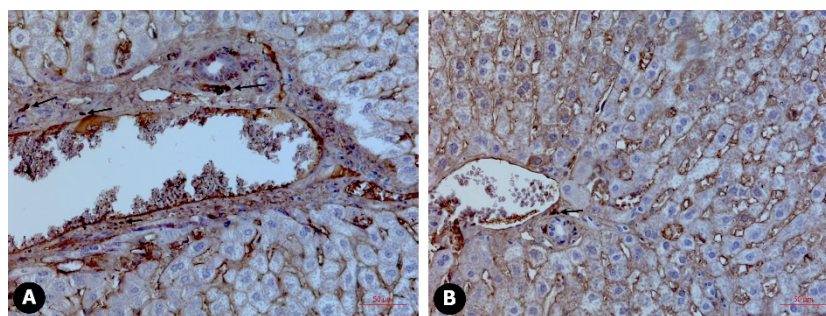


**Figure 2** - Microscopic comparison of Toluidine blue staining was performed in both groups for evaluating the mast cell numbers. **A** (group C) and **B** (group O) (Magnification x10) are Toluidine blue stainings of group C and group O sections, respectively. Mast cell accumulation is significant in **A**.

**Table 2** - Comparison of the groups in Tryptase and Toluidine Blue.

Variables	Group O (n=7)		Group C (n=7)		p
	Mean±SD	Median (min/max)	Mean±SD	Median (min/max)	
<b>Triptase</b>	775.1±180.0	792.0 (548.0-1053.0)	1169.6±137.5	1149.0 (949.0-1361.0)	<b>0.003</b>
<b>T-Blue</b>	1774.4±336.0	1860.0 (1304.0-2147.0)	1673.6±291.4	1645.0 (1201.0-2062.0)	0.482

SD: Standard Deviation  
 p: Mann Whitney U Test



**Figure 3** - Microscopic comparison of immunohistochemical evaluation for triptase positive cells is shown in **A** (group C) and **B** (group O) (Magnification x20). Group O demonstrated less count of triptase positive cells.

## ■ Discussion

Mast cell aggregation and degranulation effects of rocuronium are known from

previous research<sup>18</sup>. Sugammadex can be used to reverse the effects of rocuronium. Tomak *et al.*<sup>17</sup> stated that sugammadex is beneficial for the treatment or prevention of

rocuronium-induced anaphylaxis. However Menéndez-Ozcoidi *et al.*<sup>19</sup> reported an allergic reaction after the administration of sugammadex (3.2 mg/kg) to reverse neuromuscular block induced by rocuronium. Tomak *et al.*<sup>17</sup> found that sugammadex could increase the numbers of mast cells. This situation can lead to a predisposition for allergic reactions. Nevertheless, the rocuronium-induced increase in mast cells is significantly higher than the sugammadex-induced increase in mast cells. According to these findings a preoperative diet formulation could be used to prevent allergic reactions and anaphylaxis rather than drugs which could induce histamine discharge. Previous research has indicated the antiallergic and anti-histaminic effects of different medicinal plants. Brazilian pepper tree (*Schinus molle* Raddi), *Ximenia Americana* and Oleuropein are some of the flavonoids previously studied for the inhibition of mast cell degranulation<sup>20-22</sup>. Oleuropein is one of the most studied flavonoid for different medical conditions. The extract is derived from *Olea Europea* tree leaves which is containing the phenolic compound Oleuropein in high density<sup>1</sup>.

Recent studies related to Oleuropein is especially focused on its anti allergic and anti inflammatory effects<sup>23</sup>. Using this phenolic compound for the prevention of rocuronium induced mast cell activation before the general anesthesia and surgical operation process is a new and continuing stage of the Oleuropein studies.

A variety of studies were taken as reference for the dose of oleuropein used in rabbits. Different animal studies have reported a range of calculations related to the dose of oleuropein that should be administered<sup>24,25</sup>. According to these calculations, as anti-oxidant, anti-inflammatory and vasodilatory effects are clearly observed with doses of 20

mg/kg, we chose to use an OLE formulation containing the same dose of oleuropein. Olive leaf extracts (OLEs) were stored at -20°C to prevent degradation of oleuropein during the experiment. All rabbits in the OLE group consumed the extract mixed with water. Oral tolerance of OLE appeared successful. A study of rats reported the intestinal absorption of oleuropein was rapid with a 20 mg/kg oral dose reaching peak plasma concentration in 2 hours<sup>26</sup>.

At non-toxic doses oleuropein inhibits the release of hexosaminidase from peritoneal mast cells playing a role as mast cell stabilizer and additionally some in vitro studies have shown that a variety of phenolic diets inhibit mast cell activation<sup>27,28</sup>. When cells are exposed to calcium ionophore A23187, it is reported that oleuropein inhibits mast cell degranulation more efficiently than sodium cromoglycate and hydroxytyrosol. It is reported that polyphenols dissolve proteins or form insoluble complexes and change the structure of allergenic proteins or reduce their bioavailability to render them hypoallergenic<sup>22</sup>. In terms of the effect mechanism of oleuropein, a different study reported that oleuropein blocked the phosphorylation of p38 mitogen-activated protein kinase through the up-regulation of annexin A1. It is a potent anti-inflammatory calcium-dependent phospholipid binding protein also an endogenous mediator of glucocorticoid signaling downstream signal pathways of systolic calcium increase<sup>29</sup>.

Animals were sacrificed and livers were harvested for tissue preparation 24 hours after general anesthesia according to the pharmacokinetics of rocuronium. As reported in the literature, rocuronium is primarily eliminated by the liver. Plasma concentration decreases according to hepatic intake and bile excretion after the administration of rocuronium and nearly a third of the

rocuronium in circulation is expected to be excreted in urine in 24 hours<sup>30,31</sup>.

IgE receptors are present on the mast cell membranes. When IgE-coated antigens bind to surface receptors, mast cell degranulation occurs. Increased mast cell tryptase has a central role in inflammatory and immediate allergic reactions initiated by immunoglobulins IgE<sup>32</sup>.

IgE antibodies are generated by B-lymphocytes in response to the exposure to a specific antigen like rocuronium molecule. IgE molecules bind to high affinity IgE receptors situated on the surface of mast cells and leading to mast cell degranulation, releasing both preformed and newly synthesised mediators. Under normal circumstances, these mediators help for the development of defensive acute inflammatory reactions. When there is massive release of these mediators, bronchoconstriction and vasodilation predominate<sup>32</sup>. In our study evaluation with toluidine blue staining in all rabbits within the two groups showed significantly increased mast cell counts in hepatic portal areas. This finding supports the results of previous studies about rocuroniums' mast cell aggregation pattern. Our results found that all rabbits in group C had higher numbers of mast cells in hepatic portal areas, in contrast there was no significant difference between the two groups in statistical analysis due to the limited number of rabbits.

Generally, it has been accepted that mast cell tryptase levels show the activation and degranulation of mast cells<sup>24,25</sup>. For that reason, immunochemical staining methods were used to determine tryptase-positive mast cell counts. Tryptase-positive mast cell numbers were significantly lower in animals receiving rocuronium after an oleuropein-rich diet compared with Group C and this data demonstrates that oleuropein can decrease

mast cell aggregation and degranulation.

Acute toxicity study in rabbits fed with OLE was performed in order to explore any adverse effect resulted from OLE. OLE administered orally revealed no sign of poisoning in rabbits of OLE group. After 15 days of the treatment, no rabbits died and no significant change of weight between the both groups. Several studies have demonstrated that the elevated liver enzymes AST and ALT indicate hepatocellular plasma membrane damage, and these two enzymes are considered good markers for liver inflammation and necrosis. Marmugi *et al.*<sup>33</sup>. There was no significant difference between two groups for ALT, AST and ALB levels in biochemical evaluation. This means that neither rocuronium nor oleuropein has any toxic effects on hepatic functions.

Previous research has illustrated the antiallergic effects of oleuropein<sup>14</sup> via the inhibition of mast cell degranulation. Our results support these previous studies with the decrease in tryptase-positive mast cell counts in rabbits fed with an oleuropein rich diet.

## ■ Conclusions

This study assessed the histological and immunological effects of a prophylactic oleuropein-rich diet in terms of mast cell numbers and degranulation levels in liver cells developing after intravenous administration of rocuronium in rabbits undergoing general anesthesia. Our findings show that the use of oleuropein-rich diet reduced the number of inflammatory cells and prevented degranulation. These findings show that using prophylactic oleuropein-rich diet would be useful to improve preoperative anesthesia approaches to prevent anaphylactic reactions according to the administered neuromuscular

blockers as rocuronium during the general anesthesia.

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