

# Effects of Sevoflurane and Adenosine Receptor Antagonist on the Sugammadex-Induced Recovery from Rocuronium-Induced Neuromuscular Blockade: An Ex-vivo Study

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## Research article

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# Abstract

**Background:** Sevoflurane affects on the A1 receptor in the central nervous system (CNS) and potentiates the action of neuromuscular blocking agents. In the present study, we investigated whether sevoflurane (SEVO) has the ability to potentiate the neuromuscular blocking effect of rocuronium and if the specific antagonist of adenosine receptor (SLV320) can reverse this effect.

**Methods:** Phrenic nerve–hemidiaphragm tissue specimens were obtained from forty Sprague-Dawley (SD) rats. The specimens were immersed in an organ bath filled with Krebs buffer and stimulated by a train-of-four (TOF) pattern using indirect supramaximal stimulation at 20 s intervals. The specimens were randomly allocated to control, 2-chloroadenosine (CADO), SEVO, or SLV320+SEVO groups. In the CADO and SLV320+SEVO groups, CADO and SLV320 were added to the organ bath from the start to a concentration of 10  $\mu$ M and 10 nM, respectively. We then proceeded with rocuronium-induced blockade of >95% depression of the first twitch tension of TOF (T1) and TOF ratio (TOFR). In the SEVO and SLV320+SEVO groups, SEVO was added to the Krebs buffer solution to concentration of 400 - 500  $\mu$ M for 10 min. Sugammadex-induced T1 and TOFR recovery was monitored for 30 min until >95% of T1 and >0.9 of TOFR were confirmed, and the recovery pattern was compared by plotting these data.

**Results:** There were no significant differences in the recovery pattern between the control and SEVO groups. However, there were significant differences between the SEVO and SLV320+SEVO groups.

**Conclusion:** Sevoflurane potentiates of rocuronium-induced neuromuscular blocking effect and delays sugammadex-induced recovery from neuromuscular blockade.

## Background

Sevoflurane is a commonly used inhalation anaesthetics in clinical settings. Although the effects of inhalation anaesthetics cannot be explained by a single molecular mechanism, it is known that sevoflurane has a tendency to activate adenosine A1 receptor [1]. The hypnotic or soporific effects of adenosine have been described in animals [2] and humans [3]. Furthermore, specific antagonists of the adenosine receptor, such as theophylline, can decrease the sedation effect of propofol [4] and sevoflurane [5]. Such effects are due to the blockade of effects of sevoflurane or propofol on adenosine receptors. At the neuromuscular junction, adenosine receptors, together with presynaptic muscarinic acetylcholine receptors (mAChRs), modulate acetylcholine (ACh) release upon neuronal firing and regulate each other as a facilitatory or inhibitory receptor [6–8]. When these receptors are modulated and their functions are affected by specific agonists and antagonists, spontaneous and evoked release of ACh at the neuromuscular junction has been observed. These changes, in turn, affect the responses of muscle tension to variable neuronal stimulation [9].

Sugammadex, a  $\gamma$ -cyclodextrin derivative, can reverse the effect of aminosteroidal neuromuscular blocking agents by encapsulating and inactivating them [10–12]. This effect does not influence the amount of ACh at the neuromuscular synaptic junction and this is different from the mechanism of

classic reversal strategy to increase ACh using cholinesterase inhibitors such as neostigmine [13]. We hypothesized that, during sugammadex-induced recovery from neuromuscular blockade, the muscle tension might be influenced by modulation of the presynaptic release of ACh. We also hypothesized that the modulation of ACh release might be responsible for the potentiation of the neuromuscular blockade effect of sevoflurane. To confirm these hypotheses, here, we performed *ex-vivo* experiments using rodent phrenic nerve/hemidiaphragm tissue specimens. We investigated the time course of sugammadex-induced recovery from rocuronium-induced neuromuscular blockade in tissue specimens in an organ bath with adenosine A<sub>1</sub> receptor agonist, sevoflurane, or sevoflurane and adenosine A<sub>1</sub> receptor specific blocker.

## Methods

### Basic study design and sample preparation

The study protocol was approved by the Ethics Committee of the Laboratory of Animal Research, Asan Institute of Life Science (Seoul, Republic of Korea) on September 1, 2018 (Protocol No.2018-13-198). Forty male Sprague-Dawley (SD) rats with average weight of 245.45 g (range 225.0–296.9 g) were used in the study. All rats were bred at a constant temperature of 22 °C under a regular diurnal cycle. They were provided food and water ad libitum. We performed surgical procedures for harvesting tissue specimen after confirming that the rats didn't respond by pinching their hindlimb. Additional sacrifice methods for SD rats were not necessary in this study because rats were expired as soon as the thoracic cages were harvested during the preparation. Inclusion criteria was that the initial T1 twitch tension showed more than 100 mN. Data were excluded when the T1 twitch tensions were gradually decreased before adding study drugs or when the maximum recovery of T1 twitch tension was less than 50 mN. Urethane, 2-chloroadenosine (CADO), and SLV320 was purchased from Sigma-Aldrich Korea Ltd, (Gyeonggido, Republic of Korea). CADO stock solution was stored at -20 °C in a refrigerator and thawed before used. Stock solutions were discarded 2 weeks after preparation if unused.

### Study protocol

Each rat was anaesthetized with intraperitoneal injection of urethane (500 µg/kg). The thoracic cage was immediately isolated, and the phrenic nerve/hemidiaphragm tissue was obtained. The sampled tissues were immersed in Krebs buffer solution (120 mM NaCl, 2.5 mM CaCl<sub>2</sub>, 4.7 mM KCl, 1.2 mM MgSO<sub>4</sub>, 1.2 mM KH<sub>2</sub>PO<sub>4</sub>, 25 mM NaHCO<sub>3</sub>, and 11 mM α-D-glucose) maintained at 35 °C and 95% O<sub>2</sub> and 5% CO<sub>2</sub> with continuous bubbling to ensure tissue viability throughout the experimental session. Sizes and weights of each tissue were measured and compared between groups (Table 1). The tissues were fixed to a frame with electrodes and then immersed in a 100 ml organ bath containing 75 ml of oxygenated Krebs buffer solution. Subsequently, 40 mN resting tension was applied to the tendinous portion of the diaphragm of each sample hooked to a Grass FT03 Force Transducer (Grass Technologies, West Warwick, RI, USA). The phrenic nerve was fixed to a platinum bipolar electrode and stimulated using a Grass S88 Stimulator (Grass Technologies). Supramaximal stimulation with a square wave pulse of

0.2 ms was administered every 20 s at trains of 2 Hz for 2 s (train of four (TOF) stimulation). All waveforms were acquired and stored using the PowerLab 4/26 Data Acquisition System (AD Instruments, Sydney, Australia) and LabChart 7 Software (AD Instruments, Colorado Springs, CO, USA), respectively.

Table 1  
Characteristics of Sprague-Dawley rats and tissue specimen

	<b>Control</b> (n = 10)	<b>SEVO</b> (n = 10)	<b>SEVO + SLV320</b> (n = 10)	<b>CADO</b> (n = 10)
<b>BW (g)</b>	246.36 ± 13.78	239.38 ± 5.94	255.70 ± 16.26	240.48 ± 6.23
<b>wWt (mg)</b>	123.91 ± 18.53	123.08 ± 18.34	121.00 ± 13.70	122.78 ± 18.64
<b>Size (mm<sup>2</sup>)</b>	133.04 ± 28.94	125.38 ± 24.63	127.20 ± 33.93	128.51 ± 23.52
Data are expressed as mean ± SD. There were no significant differences among the groups (P > 0.05).				
Abbreviations: BW, body weight of rats; CADO, 2-chloroadenosine group; SEVO, sevoflurane; wWt, weight of the hemidiaphragm; Size, size (width x length) of the hemidiaphragm.				

The phrenic nerve/hemidiaphragm tissues were randomly allocated to the control, CADO (CADO 10 µM), SEVO (sevoflurane 400–500 µM), or SLV320 group (sevoflurane 400–500 µM + SLV320 10 nM) after generating random number by using following equation in Microsoft Excell 2010 program (Microsoft Office®, Microsoft Corporation, USA);  $f_x = (INT(RAND()*4)) + 1$ . Group blinding was not available because SEVO is volatile and its scent is pungent. Twitch tensions were serially monitored during a 30 min-stabilization time. Thereafter, Krebs buffer solution, SLV320, or CADO stock solution, of same volume, was added to the organ bath. A 500 µg loading dose of rocuronium (Esmeron®; MSD Korea, Seoul, Republic of Korea) and 250 µg boost dose of rocuronium were subsequently added into the organ bath. The subsequent boost dose was added 10 min after the previous dose or when five consecutive T1 twitch tension depressions were less than 3% of the previous T1 twitch tension. The boost doses were stopped when 95% or more depression of the T1 was achieved. The loading dose was set as the dose at which the T1 twitch tension did not change after adding the loading dose and the TOF ratio (TOFR) changes were within 3% of those achieved before adding the loading dose. The boost dose was set as the first dose at which the T1 twitch tension changed and the total boost doses were within 10. After confirming that the T1 twitch tensions disappeared, sevoflurane was added to the SEVO and SLV320 groups for 10 min to a concentration of 400–500 µM, whereas the other groups were allowed to rest for 10 min. After 10 min of reaction, equimolar doses of sugammadex, which were to react 1:1 to the rocuronium used to create > 95% depression of T1 twitch tension, were added to each group to recover from rocuronium-induced neuromuscular blockade. Recovery patterns were monitored and plotted for 30 min or until the T1 twitch tension recovery was > 95% and TOFR was ≥ 0.9. The study protocol is summarized in Fig. 1.

## Statistical analysis

The first aim of this study was to compare the time required in each group to attain > 95% recovery in T1. To achieve this goal, the T1 twitch tensions were serially monitored and recorded; these responses were plotted as regression curves and compared among the groups. T1 recovery and TOFR recovery were achieved simultaneously until TOFR was > 0.9. The second aim was to compare the variables obtained during sugammadex-induced recovery from neuromuscular blockade. In a clinical practice, the recovery index (RI, the interval of T1 recovery from 25–75%) is a good marker for comparing the recovery rates from neuromuscular blockade. We performed inter-group comparisons of the time interval of T1 twitch tension recovery at 10%, 25%, 50%, 75%, and 95%.

There are many issues with performing sample size estimation in *ex-vivo* neuromuscular studies. Previous studies have suggested that about 10 animals per group are needed to achieve statistically significant results [14, 15]. In the present study, the sample size was calculated based on the previous experiment and pilot study, which suggested that 10 samples per group were sufficient at  $\alpha = 0.05$ , power = 0.80, and a dropout rate of 10%.

Results are expressed as mean  $\pm$  standard deviation (SD). All doses were converted and expressed in  $\mu\text{mol/L}$  ( $\mu\text{M}$ ). Time is expressed in min. Graphs were plotted, and statistical analyses were carried out using SPSS 13.0 software (SPSS Inc., Chicago, IL, USA). Rocuronium  $\text{EC}_{50}$  and  $\text{EC}_{95}$  values for twitch tension were calculated by fitting nonlinear regression curves to group data. We used the following equation for T1 depression;  $y = 50 + 50\sin(\Omega x + b)$ , where  $y$  represents the T1 tension,  $x$  represents the recovery time,  $b$  represents half of the time required to achieve T1 > 95%, and  $\Omega$  represents the slope of the regression curve ( $R^2 = 0.87$ ). Start points were set as the time of administration of sugammadex and the time when T1 twitch tension recovered to 10%, and the slopes are represented as  $\Omega_1$  and  $\Omega_2$ , respectively. We considered the RI as the time interval from 25–75% of T1 twitch tension recovery, and compared the RI among the groups. We also used the following equation for TOFR;  $y = 1 - \lambda x^2$ , where  $y$  and  $x$  represent TOFR and recovery time, respectively, while  $\lambda$  represents the slope of the regression curve ( $R^2 = 0.83$ ). Differences in continuous variables among the groups were analysed using analysis of variance, followed by the Bonferroni method for multiple pairwise comparisons. The mean group values of  $\Omega$ s and  $\lambda$  were compared using the Mann Whitney U test. Statistical significance was set at  $p < 0.05$ .

## Results

There were no differences in the average body weight of rats, wet weight of tissue specimens, and dose of rocuronium used among the groups (Table 1). We plotted the recovery data of T1 and TOFR of each group and compared their slopes (represented as  $\Omega$  and  $\lambda$ , respectively). When T1 twitch tension recovery was plotted with the start point as the time of sugammadex administration, there were no significant differences on  $\Omega_1$  between groups (Fig. 2A, B). The data of the CADO group were not included in this comparison because most of the T1 twitch tensions in the CADO group were reappeared 5 min after the administration of sugammadex and did not recover within 30 min. When the start point was set as the time when the T1 twitch tension recovered to 10%, there were significant differences in  $\Omega_2$  of the CADO

and SEVO groups compared with that of control and SLV320 + SEVO groups (Fig. 2C, D). We also compared the recovery time interval from 25–75% recovery of T1 (RI) among the groups. The RIs of the CADO and SEVO groups were significantly different from those of the other groups, although there were no significant differences between the control and SEVO + SLV320 group (Table 2,  $P < 0.05$ ). A comparison of TOFR recovery revealed no significant differences among the groups with the equation that we adopted, but the TOFRs of control and SEVO + SLV320 groups were well above 0.7 from the start, showed temporary decrease to about 0.5, and then recovered again. In the CADO and SEVO groups, although some patterns of TOFR recovery progression were similar to those of the control and SEVO + SLV320 groups, some patterns showed a linear progression (Fig. 3).

Table 2  
Comparison of recovery progression and recovery index.

	Control (n = 10)	SEVO (n = 10)	SEVO + SLV320 (n = 10)	CADO (n = 10)
$\Omega_1$	0.975 ± 0.237	1.027 ± 0.104	0.954 ± 0.234	-
$\Omega_2$	0.877 ± 0.202	1.002 ± 0.092*	0.898 ± 0.202	0.999 ± 0.056*
RI (min)	6.034 ± 1.791	8.531 ± 2.113 <sup>†</sup>	5.534 ± 1.852	14.949 ± 4.498 <sup>†</sup>
Data are expressed as mean ± SD. *: $P < 0.050$ compared with the control or SEVO + SLV320 group, †: $P < 0.050$ compared with the control or SEVO + SLV320 groups.				
Abbreviations: CADO, 2-chloroadenosine group; SEVO, sevoflurane; $\Omega_1$ , regression slope when the zero point was set as the time of sugammadex administration; $\Omega_2$ , regression slope when the zero point was set as the time of 10% T1 twitch tension recovery.				

## Discussion

In the present study, we demonstrated that sevoflurane affects the  $A_1$  receptor at the neuromuscular junction and delays sugammadex-induced recovery from rocuronium-induced neuromuscular blockade. Adenosine acts as an  $A_1$  agonist at a low concentration (300 nM), and as an  $A_{2A}$  agonist at high concentration ( $> 1 \mu\text{M}$ ) [16]. Furthermore, it has been demonstrated that enflurane and sevoflurane have the ability to activate adenosine  $A_1$  receptors in an *in-vitro* culture of rat hippocampus [1]. Aminophylline is a nonselective antagonist of the adenosine receptor [17], and it can decrease the sedation effects of sevoflurane [5, 18], but not the anaesthesia induced by desflurane [4]. Sevoflurane is one of the most potent volatile anaesthetics that potentiates the effect of neuromuscular blocking agents [19]. The present study results are in consistent with these findings. Sugammadex has no effect on the neuromuscular junction and ACh release. Sugammadex-induced recovery from rocuronium-induced neuromuscular blockade is dependent on the relative concentration of rocuronium and ACh at the neuromuscular junction. Thus, the T1 twitch response to the indirect nerve stimulation reappears in the presence of a large amount of ACh molecules at the neuromuscular junction, because the ACh molecules

have a greater chance to attach to AChR than rocuronium. When the action of the receptor or channel related to the release of ACh was modulated and the release of ACh in the neuromuscular presynaptic membrane was reduced, there was a delay in recovery from neuromuscular blockade [14]. In the present study, the modulation of adenosine receptor with CADO and sevoflurane, delayed T1 recovery and resulted in a low recovery index compared with those of the control (no receptor modulation). We speculated that the modulation of the A<sub>1</sub> receptor at the presynaptic membrane of the neuromuscular junction by sevoflurane could partially induce delayed recovery from neuromuscular blockade. We did not change the current for phrenic nerve stimulation and thus, the magnitude of indirect stimulation to the phrenic nerve might not have changed. However, in this study, the amount of ACh released per indirect stimulation might have reduced during sugammadex-induced recovery from neuromuscular blockade because of the activation of the A1 receptor in the neuromuscular junctions by sevoflurane. Furthermore, sugammadex binds to rocuronium only outside the neuromuscular junction, and there is a concentration difference in rocuronium between the neuromuscular junction and organ bath; consequently, rocuronium is removed from the neuromuscular junction. In the present study, the decrease in rocuronium concentration at the neuromuscular junction was thought to be similar among all groups because we used equimolar dose of sugammadex and rocuronium in the organ bath. Rocuronium molecules were able to bind to the AChRs at the postsynaptic membrane because the amount of ACh released was less at the same stimuli that caused presynaptic A1 receptor modulation by sevoflurane. This might have resulted in the delayed recovery from rocuronium-induced neuromuscular blockade in the present study.

The present study had some limitations. First, this was an *ex-vivo* study. We extracted phrenic nerve-hemidiaphragm tissue specimens after sacrificing SD rats. During this phase, although we handled the specimen in the Krebs buffer solution with 95% O<sub>2</sub>/CO<sub>2</sub> gas bubbling, transient hypoxemia and tissue damage were inevitable. Furthermore, pharmacokinetic component was abolished during all phases of the experiment. To compensate for these limitations, we cautiously performed the following steps. (1) tissue specimens were extracted and immersed in a Petri dish and organ bath containing oxygenated Krebs buffer solutions throughout the experiment to minimize tissue hypoxia. (2) Maintaining the concentration of sevoflurane in the organ bath was another challenge. In the clinical setting, sevoflurane is supplied using an exclusive vaporizer and a closed circuit system, and its concentration is expressed as vol%. However, in the present study, it was difficult to develop a closed circuit system because frames, electrodes, and strings connected to the force transducer were out from the orifice of organ bath. Furthermore, scavenging system of our laboratory was not suitable for use with volatile anesthetics. Instead, to minimize air pollution in the laboratory, we had to find alternative method simulating a closed circuit system to apply sevoflurane. We sealed the organ bath with a flexible film and added sevoflurane intermittently to achieve the desired concentration in the Krebs buffer solution. In a clinical experiment, it took about 40 min for sevoflurane to equilibrate between blood and muscle component and make effect on muscle [20, 21]. However, in some studies, sevoflurane was only needed 10 min for sevoflurane to make effect [21]. In our experiment, we shortened this reaction time to 10 min by applying sevoflurane directly to the environment of tissue specimen. This was quite different approach comparing with the clinical setting. Moreover, as sevoflurane is volatile, it is very difficult to maintain the designated

concentration of sevoflurane in the organ bath, and fluctuation in its concentration was inevitable. The dose of sevoflurane was selected as 400–500  $\mu\text{M}$  at the point of initiation of recovery. This was determined before the main experiment by performing a pilot study and by referring to a previous study [22]. Second, this was a functional study, not an immunochemistry study. We performed this experiment by measuring the tension generated by diaphragm contraction. That is, we deduced the results in an indirect manner, as the tension is thought to be driven by the ACh concentration differences at the neuromuscular junction. For accurate data, timely measurement of the amount of ACh released during serial indirect stimulation is required. However, we could not find ideal method for this. The ACh concentrations should be measured repeatedly at 20 s interval. We performed a conventional functional study that is commonly used in neuromuscular studies. To obtain more convincing results, a more suitable immunohistochemical study should be conducted in the future.

## Conclusion

sevoflurane potentiates the effects of neuromuscular blocking agent partially by modulating the adenosine  $A_1$  receptor at the neuromuscular junction. Furthermore, it affects sugammadex-induced neuromuscular recovery after rocuronium-induced neuromuscular blockade. Although the results were obtained in an *ex-vivo* environment, they suggest that if sevoflurane is used as the main anaesthetic drug, recovery from neuromuscular blockade can be delayed at the end of surgery and, unlike it is expected, that sugammadex-induced recovery from neuromuscular blockade also be delayed.

## Abbreviations

Acetylcholine (ACh), acetylcholine receptor (AChR) CADO, 2-chloroadenosine; SEVO, sevoflurane; SD, Sprague-Dawley rats; TOF, train-of four stimulation. muscarinic acetylcholine receptors (mAChRs).

## Declarations

## Ethics approval and consent to participate

The study protocol was approved by the Ethics Committee of the Laboratory of Animal Research, Asan Institute of Life Science (Seoul, Republic of Korea) on September 1, 2018 (Protocol No.2018-13-198).

## Consent for publications

N/A

## Availability of data and material

Raw data in this study will not be shared. If there are anyone who need the raw data of this study, please contact the corresponding author (fred0314@gilhospital.com).

## Competing interests

The authors have no potential conflicts of interest to declare in association with this work.

## Funding

There are no fundings in the present study.

## Authors contributions

YBK and HSY designed the study, wrote the manuscript, YBK, JC, HC and HSY analyzed and interpreted the data. YBK, JC, HC, JI and CP collected and statistical analyses the data. YBK wrote manuscript and all authors read and revised manuscript body. JC and HSY performed political and external relation work.

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# Figures

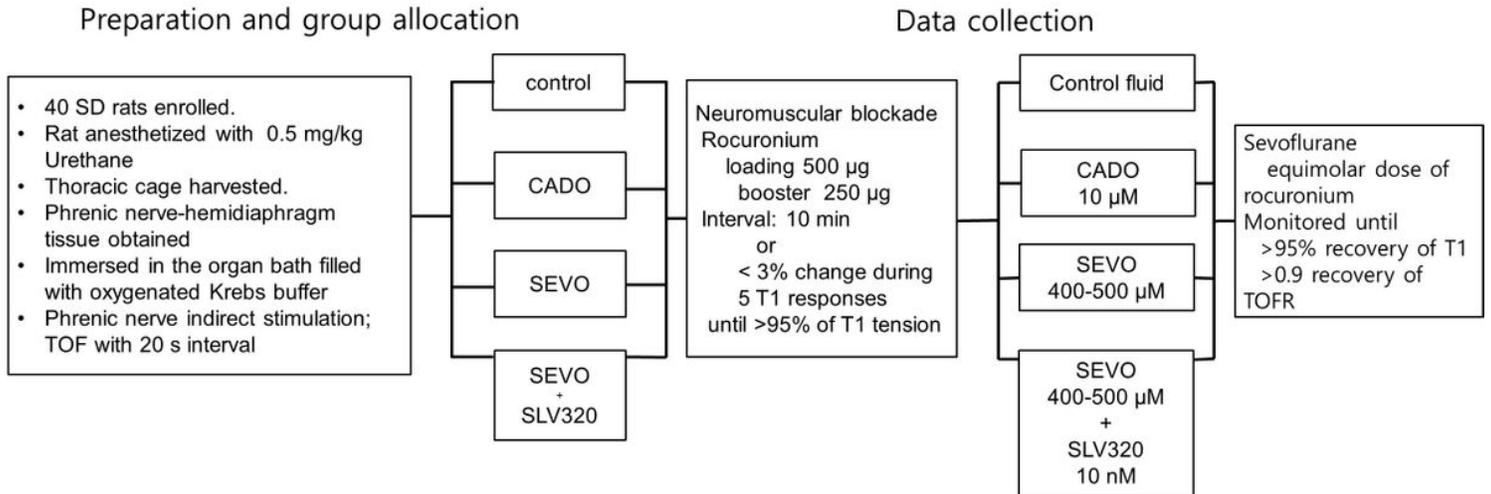
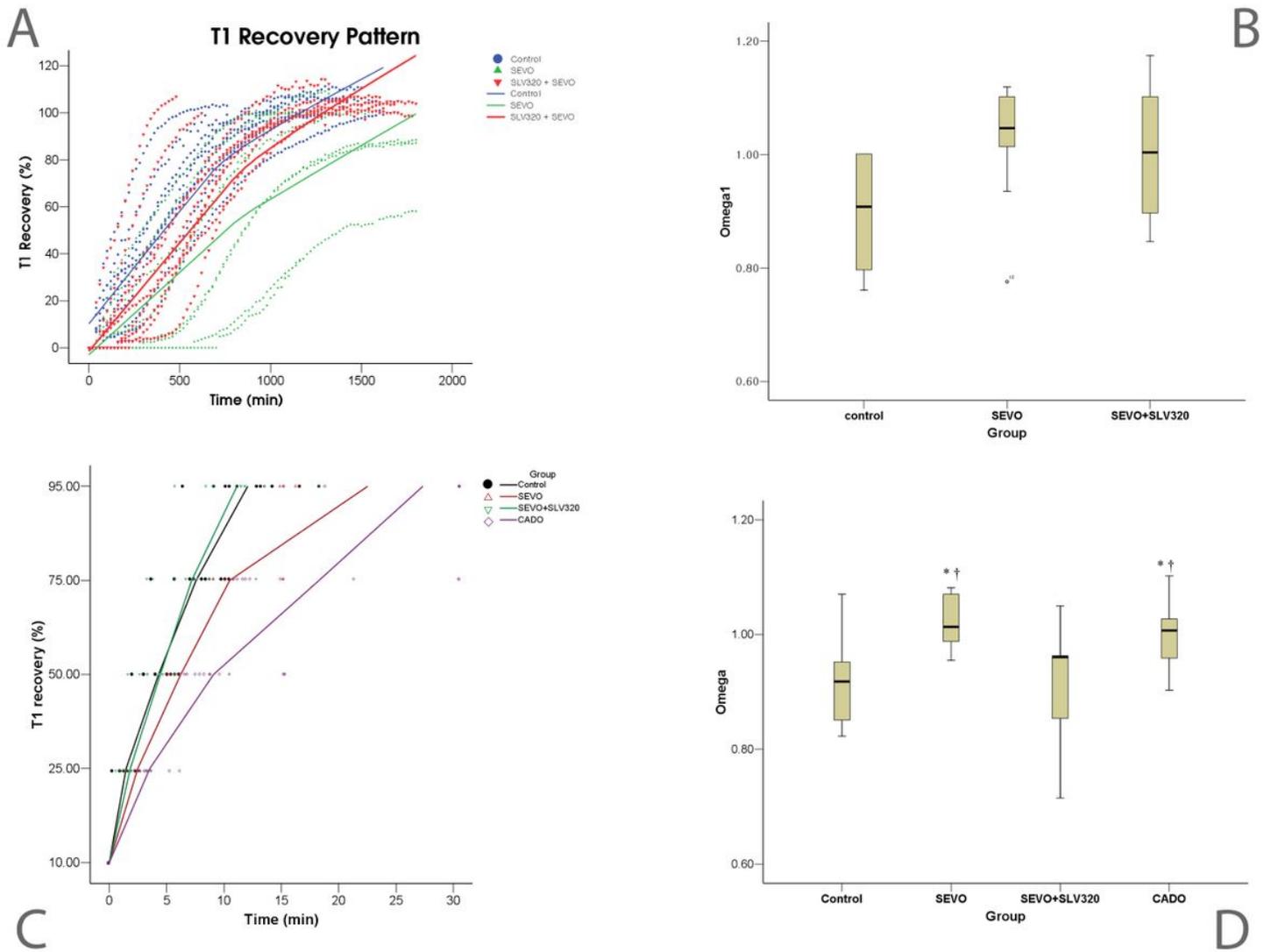


Figure 1

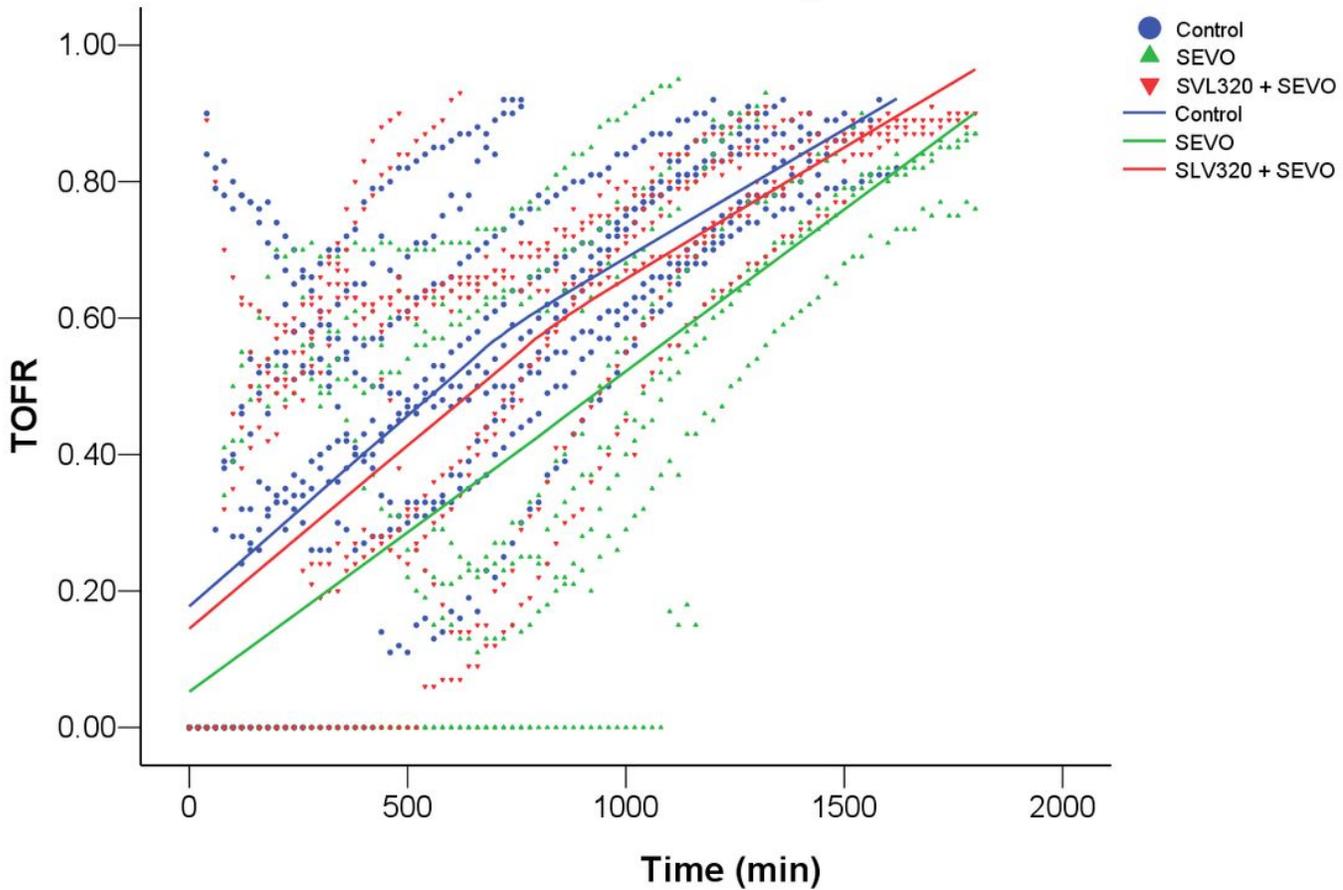
Study protocol. 10 min of reaction time was allowed after study drugs were added. Abbreviations: CADO, 2-chloroadenosine; SEVO, sevoflurane; SD, Sprague-Dawley rats; TOF, train-of four stimulation.



**Figure 2**

Scatter gram and regression of T1 twitch tension recovery. A) The zero point was set as the time of sugammadex administration. C) The zero point was set as the time when the recovery of the T1 twitch tension was 10%. Comparisons of omegas of T1 recovery are shown at B) and D). We set the equation for T1 recovery as  $y = 50 + 50\sin(\Omega x + b)$ , where  $y$  is the % recovery of T1 twitch tension and  $x$  is time. \*  $P < 0.050$  compared with the control. †  $P < 0.050$  compared with SEVO + SLV320. Abbreviations: CADO, 2-chloroadenosine; SEVO, sevoflurane; TOF, train-of-four stimulation; T1, the first twitch tension of TOF; TOFR, TOF ratio.

# TOFR Recovery Pattern



**Figure 3**

Comparison of TOFR recovery. There were no significant differences among the groups. Data of the CADO group are not shown because the TOFRs of this group did not reach 0.9 within 30 min. Abbreviations: SEVO, sevoflurane; TOF, train-of-four stimulation; T1, the first twitch tension of TOF; TOFR, TOF ratio.

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