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Supplementary Table 1 Organ distribution of SA-Ve modified with various amount of PEG-DSPE at 24 h. (Injected dose; 15mg/kg b.wt.)

Organs	SA-Ve	PEG(0.3)-[SA-Ve]	PEG(0.6)-[SA-Ve]	PEG(1.4)-[SA-Ve]	PEG(2.6)-[SA-Ve]
			% injected dose per organ ± SEM		
Blood	0.81 ± 0.14	0.47 ± 0.51	0.50 ± 0.05	0.55 ± 0.07	0.99 ± 0.26
Liver	20.05 ± 4.71	15.52 ± 1.98	14.13 ± 1.21	6.86 ± 1.15	7.52 ± 0.93
Bone marrow	23.05 ± 3.08	19.75 ± 1.71	30.35 ± 3.43	25.05 ± 1.48	22.42 ± 3.76
Spleen	2.90 ± 1.37	1.59 ± 0.51	1.44 ± 0.19	1.75 ± 0.23	3.48 ± 0.47
Skin	0.98 ± 0.41	0.38 ± 0.06	0.66 ± 0.11	0.53 ± 0.06	0.56 ± 0.04
Kidney	2.68 ± 0.40	2.35 ± 0.09	1.79 ± 0.25	1.58 ± 0.26	1.98 ± 0.39
Muscle	0.92 ± 0.20	0.74 ± 0.15	0.96 ± 0.33	0.76 ± 0.36	1.63 ± 0.75
Lung	0.05 ± 0.00	0.04 ± 0.01	0.08 ± 0.03	0.11 ± 0.05	0.12 ± 0.06
Heart	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00
Brain	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Testis	0.01 ± 0.00	0.02 ± 0.00	0.02 ± 0.00	0.02 ± 0.00	0.05 ± 0.00
Bowel	N.D.	N.D.	N.D.	N.D.	N.D.
Urine	25.73 ± 1.68	29.36 ± 2.73	24.51 ± 1.64	20.39 ± 2.79	21.16 ± 0.94
Feces	7.23 ± 6.50	2.82 ± 2.76	0.39 ± 0.23	9.07 ± 4.79	14.99 ± 1.34
Recovery	84.42 ± 8.88	73.02 ± 4.61	74.80 ± 1.48	66.69 ± 1.55	74.86 ± 3.67
			% injected dose per gram ± SEM		
Blood	0.01 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.01 ± 0.00
Liver	0.29 ± 0.05	0.21 ± 0.05	0.16 ± 0.01	0.06 ± 0.01	0.08 ± 0.01
Bone marrow	0.18 ± 0.05	0.16 ± 0.00	0.21 ± 0.02	0.18 ± 0.02	0.17 ± 0.02
Spleen	3.07 ± 0.62	2.17 ± 0.55	1.68 ± 0.04	1.97 ± 0.27	4.61 ± 0.92
Skin	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Kidney	0.19 ± 0.04	0.15 ± 0.01	0.10 ± 0.02	0.08 ± 0.00	0.10 ± 0.02
Muscle	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Lung	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.01
Heart	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Brain	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Testis	0.01 ± 0.00	0.01 ± 0.00	0.00 ± 0.00	0.01 ± 0.00	0.01 ± 0.00
Bowel	N.D.	N.D.	N.D.	N.D.	N.D.

Supplementary Table 2 Organ distribution of reference vesicles at 24 h. (Injected dose; 15mg/kg b.wt.)

Organs	Ve	PEG(2.6)-Ve	PG-Ve
	% injected dose per organ ± SEM		
Blood	16.97 ± 6.01	62.67 ± 3.43	18.70 ± 1.24
Liver	33.19 ± 9.12	9.17 ± 1.21	22.33 ± 0.76
Bone marrow	2.34 ± 0.39	5.49 ± 0.31	5.36 ± 0.65
Spleen	13.65 ± 4.27	7.62 ± 0.89	4.81 ± 0.46
Skin	1.08 ± 0.13	2.23 ± 0.25	1.36 ± 0.13
Kidney	2.04 ± 0.06	1.38 ± 0.15	2.50 ± 0.25
Muscle	1.22 ± 0.13	6.70 ± 2.50	3.13 ± 0.92
Lung	0.20 ± 0.07	0.93 ± 0.12	0.16 ± 0.02
Heart	0.06 ± 0.02	0.12 ± 0.01	0.06 ± 0.00
Brain	0.01 ± 0.01	0.06 ± 0.01	0.01 ± 0.00
Testis	0.05 ± 0.02	0.33 ± 0.09	0.10 ± 0.01
Bowel	9.55 ± 1.86	6.71 ± 0.44	8.86 ± 1.59
Urine	19.54 ± 1.95	5.24 ± 0.40	25.01 ± 5.48
Feces	1.62 ± 1.14	0.32 ± 0.29	0.05 ± 0.04
Recovery	94.53 ± 2.79	108.98 ± 5.48	92.45 ± 5.51
	% injected dose per gram ± SEM		
Blood	0.13 ± 0.05	0.46 ± 0.02	0.12 ± 0.01
Liver	0.51 ± 0.14	0.14 ± 0.01	0.26 ± 0.02
Bone marrow	0.02 ± 0.00	0.05 ± 0.00	0.04 ± 0.01
Spleen	17.27 ± 2.42	7.63 ± 0.28	3.90 ± 0.38
Skin	0.00 ± 0.00	0.01 ± 0.00	0.01 ± 0.00
Kidney	0.14 ± 0.01	0.10 ± 0.01	0.15 ± 0.01
Muscle	0.00 ± 0.00	0.01 ± 0.00	0.00 ± 0.00
Lung	0.02 ± 0.00	0.11 ± 0.01	0.02 ± 0.00
Heart	0.02 ± 0.00	0.03 ± 0.00	0.01 ± 0.00
Brain	0.00 ± 0.00	0.01 ± 0.00	0.00 ± 0.00
Testis	0.02 ± 0.00	0.09 ± 0.03	0.03 ± 0.01
Bowel	0.03 ± 0.01	0.02 ± 0.00	0.02 ± 0.00

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Effects of magnesium sulfate on neuromuscular function and spontaneous breathing during sevoflurane and spinal anesthesia

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Abstract
The purpose of the present study was to determine the effects of magnesium sulfate ($MgSO_4$) on the neuromuscular function and spontaneous breathing of patients under sevoflurane and spinal anesthesia. Twenty-two patients with a history of arrhythmia undergoing elective knee surgery were randomly assigned to two groups: group M (n = 11), administered with $MgSO_4$ 40 mg·kg^{-1}, and group S (n = 11), administered with saline. A combination of spinal anesthesia with 2% sevoflurane inhalation was applied to all patients under spontaneous breathing. Tidal volume (VT), respiratory rate (RR) and end-tidal carbon dioxide (ET_{CO_2}) were measured before the $MgSO_4$ or saline injection and measurements were repeated at 5, 15, 30, and 45 min after the injection. Neuromuscular function was continuously monitored with an acceleromyograph to record the acceleration of the adductor pollicis by stimulating the ulnar nerve at a frequency of 0.1 Hz. The VT, RR, and ET_{CO_2} showed little change in either group, and there was no significant difference between, the groups. The single-twitch response showed significant differences between the two groups (P = 0.0006). The present study indicated that the $MgSO_4$ had a minimal effect on spontaneous breathing in patients undergoing sevoflurane and spinal anaesthesia, but that it attenuated the safety margin of neuromuscular function.

Key words Magnesium sulfate · Neuromuscular function · Respiratory function · Sevoflurane

Magnesium sulfate ($MgSO_4$) has been used as an antiarrhythmic agent during non-cardiac anesthesia as well as cardiac anesthesia [1,2]. Although the prophylactic use of $MgSO_4$ for arrhythmia has been controversial [3], Terzi et al. [2] showed that the prophylactic use of $MgSO_4$ reduced the incidence of atrial arrhythmias in thoracic surgery.

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On the other hand, $MgSO_4$ has been shown to potentiate the action of muscle relaxants and is reported to cause muscle weakness associated with respiratory insufficiency when administered at a comparatively high dose [4,5]. The mechanism of the muscle relaxant effect of $MgSO_4$ is shown as a result of competition with calcium ion (Ca^{2+}) for membrane channels and the inhibition of acetylcholine (ACh) release from the neuromuscular junction [6]. During inhalational and spinal anesthesia, these effects of $MgSO_4$ in patients with arrhythmia may be enhanced by interaction with volatile anaesthetics, and calcium channel blockers, and by advanced age [7–9]. However, it is still unknown how $MgSO_4$ per se affects neuromuscular function and spontaneous breathing under anesthesia. The purposes of the present study were to determine the effect of $MgSO_4$ in attenuating neuromuscular function and spontaneous breathing during sevoflurane and spinal anesthesia.

After obtaining institutional ethics committee approval from the Keio University School of Medicine, and obtaining informed consent from the patients, 22 patients undergoing elective knee surgery were enrolled in this double-blinded, randomized, placebo-controlled, prospective study. The patients fulfilled the following criteria: (1) American society of Anesthiologists (ASA) physical status II or III and (2) a history of arrhythmias. Patients who had any neurological abnormalities were excluded from the study.

All patients were premedicated with hydroxyzine 25–50 mg and atropine sulfate 0.5 mg intramuscularly 1 h before induction. The patients received spinal anesthesia with 0.5% isobaric bupivacaine through the L3/4 interspace. Fifteen minutes after spinal anesthesia was induced, and at the end of the operation, the blocking height was confirmed by the pin-prick method below Th10. Anesthesia was induced with propofol 2–2.5 mg·kg^{-1} intravenously, for the placement of a laryngeal mask airway, and maintained with the inhalation of oxygen-air (fraction of inspired oxygen) ([$F_{I_{O_2}}$], 0.4) and

2% sevoflurane under spontaneous breathing. A standard optimal circle system was used with high fresh gas flow ($6 l \cdot min^{-1}$) during anesthesia.

Thirty minutes after induction, baseline respiratory variables, including tidal volume (V_T), respiratory rate (RR), and end-tidal carbon dioxide (ET_{CO_2}), were measured by using an expiration gas analyzer (Datex; Capnomac Ultima-SVi, Helsinki, Finland). Mean indirect arterial pressure (MAP) and heart rate (HR) were also measured. The eligible patients were randomly assigned to two groups: group M received $40 mg \cdot kg^{-1}$ $MgSO_4$ injection, considered as the initial dose for the treatment of arrhythmias [10], and group S received an equal volume of saline. An independent investigator, who was not involved in the data collection, prepared the solution in advance. The measurements were repeated at 5, 15, 30, and 45 min after injection.

Neuromuscular function was evaluated by an acceleromyograph (TOF-Guard; Organon Teknika, Turnhout, Belgium) to record the acceleration of the adductor pollicis by stimulating the ulnar nerve. After induction, the stimulating mode was initially set as the autonomic stimulating mode (TOF I mode) to stimulate supramaximally, and reset as single-twitch mode with a frequency of 0.1 Hz. The control single-twitch height was recorded before sevoflurane anesthesia, and the single-twitch height was monitored continuously during the study. We measured TOF values at the end of study if the single-twitch height was reduced by more than 30%. Data were recorded and analyzed using Card Reader Ver.1.1 software (Organon Teknika). Palmar skin and body temperature were monitored and kept above 34°C and 36.5°C, respectively. Supplemental oxygen ($6 l \cdot min^{-1}$) was administered via a face mask during 6 h after the operation.

All data values are expressed as means (SD) [ranges] unless otherwise described. Before we started the present study, in 8 other patients administered with $MgSO_4$, we determined the number of subjects needed to achieve 90% power to detect a difference of 20% in single-twitch height, with $\alpha = 0.05$. Based on a power calculation, it was shown 22 subjects were needed. The patients' characteristics were analyzed by using Student's t-test and the χ^2 test for differences between the groups. The measurement variables were analyzed by two-way repeated-measures analysis of variance between the groups, followed by Student's t-test with Bonferroni correction at each time point. The Mann-Whitney U-test was used to compare differences in the minimum single-twitch height. A P value of <0.05 was considered as statistically significant.

Patient characteristics were not significantly different between the two groups (Table 1). Three of the 11 $MgSO_4$ patients had been medicated with nifedipine to treat hypertension. No patient was receiving steroid therapy or aminoglycoside antibiotics.

Hemodynamic and respiratory variables in the two groups showed similar changes and did not change significantly throughout the study period (Table 2). The single-twitch responses in group M were significantly depressed throughout the study period ($P = 0.0006$) (Fig. 1). The minimum twitch-height responses were significantly lower in group M than in group S (76.2% (18.3%) [40%–90]% versus 95.8% (3.7%) [90%–100]%, respectively; $P = 0.002$), and the duration from $MgSO_4$ or saline injection to that point was 246.3 (65.2) [200–360] s in group M. Three patients in group M (aged 71, 76, and 76 years) showed over 30% reductions in twitch responses (51%, 70%, and 67% of control, respectively). The TOF ratio (T4/T1) of these three patients was not decreased at the end of the study (110%, 102%, and 108%, respectively).

While three patients in group S showed arrhythmias (one, premature ventricular contractions and the others, supraventricular premature contractions) during anesthesia, no patient in group M had any arrhythmias ($P = 0.06$; χ^2 test). In group M, no patient was observed to have hypoventilation, desaturation (defined as

Table 1. Patients' characteristics

	Group S ($n = 11$)	Group M ($n = 11$)
Age (years)	66 (9.3) [55–75]	73 (4.6) [66–81]
Height (cm)	151.5 (9.4) [139–171]	149.2 (7.8) [141–162]
Weight (kg)	57.8 (9.0) [45–73]	53.8 (7.0) [44–62]
Sex (M/F)	3/8	3/8
ASA physical status (II/III)	8/3	8/3
Type of arrhythmia		
Supraventricular	9	7
Ventricular	2	4
Treatment with calcium channel blocker	2	3

Values are means (SD) [ranges] or numbers of patients. There were no significant differences between the groups

Table 2. Respiratory and hemodynamic changes after injection

	Group	Baseline	Time after injection (min)			
			5	15	30	45
Heart rate (min^{-1})	S	75.8 (9.8) [57–90]	75.3 (9.9) [56–92]	73.6 (10.2) [55–90]	73.6 (9.9) [54–85]	73.9 (9.1) [54–84]
	M	67.2 (10.1) [54–84]	67.5 (13.2) [49–94]	65.6 (10.0) [53–80]	66.3 (11.1) [50–78]	67.3 (13.3) [50–81]
Mean arterial pressure (mmHg)	S	66.5 (8.0) [54.3–78]	65.4 (7.3) [53–78]	63.6 (7.0) [55.3–77.3]	63.8 (8.1) [54.7–77]	67.3 (11.1) [53.7–93]
	M	70.8 (7.7) [62–83]	64.5 (6.8) [56.3–79]	68.2 (5.1) [64–79]	66.4 (7.4) [63–81]	71.8 (4.0) [65.3–78.3]
Tidal volume ($ml \cdot kg^{-1}$)	S	4.8 (0.8) [3.7–6.3]	4.8 (0.8) [3.7–6.4]	4.8 (0.9) [3.7–6.2]	4.7 (0.7) [3.8–6.0]	4.7 (0.8) [3.6–6.0]
	M	4.9 (0.8) [4.0–6.1]	4.9 (0.8) [4.0–6.3]	4.9 (0.9) [3.7–6.7]	4.8 (0.8) [3.8–6.4]	4.8 (0.7) [4.0–6.2]
End-tidal CO_2 (mmHg)	S	43.1 (3.6) [37–48]	42.6 (3.2) [37–46]	43.0 (3.6) [37–48]	42.6 (3.3) [37–46]	42.0 (3.8) [38–48]
	M	40.6 (3.8) [36–47]	40.4 (3.7) [36–47]	40.4 (3.9) [35–47]	39.7 (3.9) [33–47]	39.1 (4.0) [31–45]
Respiratory rate (min^{-1})	S	17.9 (4.2) [10–26]	18.1 (4.1) [11–26]	18.2 (3.9) [11–26]	18.1 (4.1) [10–25]	18.6 (4.0) [11–26]
	M	17.6 (5.7) [11–31]	18.0 (5.4) [11–29]	18.0 (5.8) [11–32]	18.6 (6.3) [11–35]	19.8 (6.3) [11–36]

Values are means (SD) [ranges]. There were no significant differences between the groups

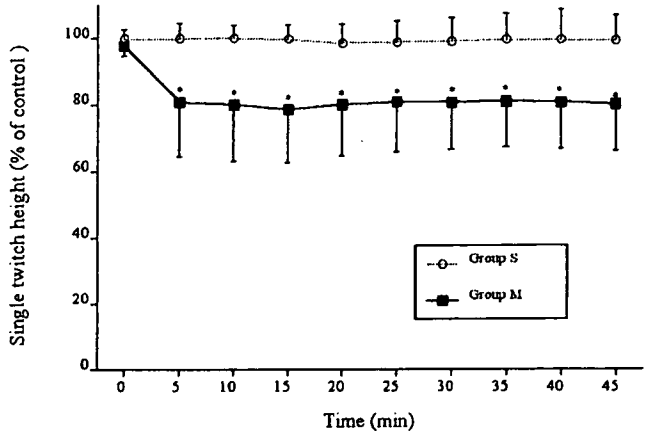

Fig. 1. The single-twitch responses in *group M* (treated with $MgSO_4$ 40 $mg \cdot kg^{-1}$) were significantly depressed compared with those *group S* (treated with saline) until 45 min after the injection ($P = 0.0006$ two-way repeated measures analysis of variance). $^*P < 0.002$ vs group S (Student's *t*-test with Bonferroni correction)

peripheral oxygen saturation [Sp_{O_2}] < 95% under supplemental oxygen therapy), or delayed awakening.

Although $MgSO_4$ use has been controversial as a prophylactic anti-arrhythmic agent, it has been used as such an agent during non-cardiac anesthesia. We therefore studied patients with a history of arrhythmia who were likely to be administered with $MgSO_4$ during non-cardiac anesthesia and who may have been more sensitive to the neuromuscular inhibition of $MgSO_4$ as a result of aging and administration of a calcium channel blocker. The present study indicated that the administration of $MgSO_4$ at a moderate dose (40 $mg \cdot kg^{-1}$) in patients with a history of arrhythmia seemed to have little effect on spontaneous breathing and did not cause hypoventilation during sevoflurane and spinal anesthesia. However, the single-twitch responses had diminished by about 20% at the end of the study. These findings suggest that $MgSO_4$ should be used cautiously in patients with a history of arrhythmia during sevoflurane and spinal anesthesia.

The major factors that enhanced the effect of $MgSO_4$ on the neuromuscular system in present study might be considered as an interaction with sevoflurane, the spinal anesthesia, the age of the patients, and the use of calcium channel blockers [7–9]. First, although sevoflurane brought about little change of the twitch responses in the control group, sevoflurane has been shown to potentiate the effect of neuromuscular blocking agents [7]. Sevoflurane may act synergistically with $MgSO_4$ on the neuromuscular junction, but the present study did not confirm this effect. On the other hand, $MgSO_4$ administration had little effect on spontaneous breathing. Inhalational anesthetics depress the function of the parasternal intercostal muscles and cause diaphragmatic

function to be dominant under spontaneous respiration [11,12]. Furthermore, the diaphragma is more resistant to neuromuscular blocking agents than peripheral muscle [13]. Therefore, there was some possibility that such a sparing effect of respiratory muscles could have occurred in our patients treated with $MgSO_4$.

Secondly, spinal blockade may modify the functional state of the neuromuscular junctions through the depression of afferent impulses. The blockade of afferent impulses into the central nervous system is reported to have lessened the dose of sedatives required [14]. Central neural influences on neuromuscular transmission may explain the frequent failure of evoked electromyographic responses, but no investigation has been performed to clarify the alterations of neuromuscular function during spinal anesthesia. The possibility of an effect of spinal blockade, however, could not be excluded in our study.

Thirdly, with aging, there may be overactivity in response to magnesium at the neuromuscular junction, because the acetylcholine (ACh) content at the neuromuscular junction is reduced with aging [9]. In the present study, three patients aged over 70 years showed a marked reduction of single-twitch height. Because the TOF ratio in these three patients did not decrease, it seems that $MgSO_4$ did not inhibit the release of ACh from the neuromuscular junction with the moderate dose administered. However, high-dose administration of $MgSO_4$ during anesthesia should be used cautiously in older patients.

Finally, patients with arrhythmias may have other cardiovascular complications, e.g., hypertension and ischemic heart disease, and these patients may have been medicated with a calcium channel blocker, such as nifedipine, which has been shown to enhance neuromuscular blockade [8]. In our patients, 3 of the 11 treated with $MgSO_4$ had been medicated with nifedipine to treat hypertension. The reduction of single-twitch height in these patients was similar to that in the other patients, but further study is needed to evaluate the possible interaction of calcium channel blockers with $MgSO_4$.

In regard to the limitations of our study, we observed the responses of single-twitch height for only 45 min after $MgSO_4$ injection, because $MgSO_4$ ($40\,mg \cdot kg^{-1}$) had been reported to prolong the duration of recovery to 75% of the twitch height of vecuronium ($0.1\,mg \cdot kg^{-1}$) for approximately 30 min [15]. However, it should be noted that the responses of single-twitch height were depressed about by 20% at 45 min after the $MgSO_4$ injection. Such prolonged effects of $MgSO_4$ on the neuromuscular system suggest that we should keep monitoring the single-twitch for a longer time period. We did not measure obvious indicators in our subjects, such as the plasma magnesium concentration.

In summary, the current preliminary study indicated that $MgSO_4$ injection at the initial dose used for the treatment of arrhythmias caused a substantial, but not clinically apparent, risk of limiting spontaneous breathing in patients with a history of arrhythmia under sevoflurane and spinal anaesthesia. However, we investigated only V_T, RR, and ET_{CO_2}, and did not determine other optimal variables, such as maximum inspiratory pressure. A further study would be needed to evaluate in detail the effects of $MgSO_4$ on the respiratory system during anesthesia.

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Impact of Sonoclot hemostasis analysis after cardiopulmonary bypass on postoperative hemorrhage in cardiac surgery

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Abstract
Purpose. The Sonoclot Analyzer provides a functional test of whole blood coagulation by measuring the viscous property of the blood sample. In this study, we used a modified Sonoclot assay, using cuvettes with a glass bead activator containing heparinase, and compared the Sonoclot data before and after cardiopulmonary bypass (CPB) to assess the usefulness in predicting postoperative hemorrhage.
Methods. In 41 cardiac surgery patients, Sonoclot data were obtained immediately after heparin administration (pre-bypass) and just before protamine administration (post-bypass). Excessive bleeding was defined as chest tube drainage greater than $2 ml \cdot kg^{-1} \cdot h^{-1}$ in 1 h during the first 4 h after surgery.
Results. There were no significant differences in Sonoclot values before and after CPB in patients with acceptable bleeding (n = 29). In patients with excessive bleeding (n = 12), Sonoclot variables reflecting fibrin formation (activated clotting time [ACT], rate of fibrin formation [clot rate], and peak clot signal) were preserved after CPB; however, the variables reflecting platelet-fibrin interaction (time to peak, peak angle, and clot retraction rate) were significantly different from their respective pre-bypass values. Sonoclot analysis showed impairment of clot maturation after CPB in patients with excessive postoperative bleeding.
Conclusion. Our results suggest that abnormal postoperative hemorrhage can be predicted by Sonoclot analysis with a new glass bead-activated heparinase test performed after CPB.

Key words Sonoclot · Cardiac surgery · Postoperative hemorrhage

Introduction

Postoperative bleeding is a major cause of morbidity and mortality after cardiac surgery [1,2]. Because the etiology of postoperative hemorrhage is multifactorial, it is often difficult to predict bleeding risks with conventional tests, including activated clotting time (ACT), prothrombin time (PT), activated partial thromboplastin time (aPTT), platelet count, fibrinogen concentration, and platelet function tests. Each of these tests reflects an isolated portion of the hemostatic sequence, and therefore the overall complex interactions of coagulation defects may not be reliably evaluated.

The Sonoclot Coagulation and Platelet Function Analyzer (Sienco, Morrison, CO, USA) traces the transition of whole blood, from fluid to viscous clot, with a high-frequency (400-Hz) vibrating probe. Both clotting and late fibrinolytic state can be assessed with Sonoclot tracings, and defects of plasma factors, fibrinogen, and platelets may be detectable [3–5]. In this study, we report our initial experience of a modified Sonoclot assay using a cuvette containing a glass bead activator and heparinase. The use of glass beads improves the sensitivity of Sonoclot to platelet dysfunction, and heparinase allows testing to be performed during cardiopulmonary bypass (CPB). The aim of this study was to characterize the Sonoclot variables that are associated with increased postoperative bleeding after CPB. We hypothesized that hemostatic defects might be characterized by platelet-mediated mechanisms, and that the major proportion of cardiac surgical patients would develop characteristic Sonoclot variables during CPB.

Patients and methods

After Institutional Review Board (IRB) approval, informed written consent was obtained from 41 consecutive patients (24 men and 17 women) undergoing elective cardiac surgery using CPB. No patients had liver dysfunction, thrombocytopenia, or coagulopathy. Patients were excluded if they had received anticoagulant (warfarin or heparin) or antiplatelet (aspirin or ticlopidine) medications 7 days before surgery.

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Anesthesia care and perfusion management were according to our routine protocol. Briefly, general anesthesia was maintained with fentanyl, midazolam, vecuronium, and sevoflurane. CPB was managed with a membrane oxygenator at a flow rate of between 2.2 and 2.6 l·min^{-1}·m^{-2}, and hypothermia during CPB was in the range of 28°C–32°C. Anticoagulation was maintained with unfractionated heparin (300 U·kg^{-1}) for CPB. The celite ACT was measured every 30 min and if the ACT was less than 480 s, additional heparin (100 U·kg^{-1}) was administered during CPB. No antifibrinolytic drug was used during surgery. A Cell Saver (Haemonetics, Braintree, MA, USA) was used in all patients. After termination of CPB, protamine (1 mg per 100 U of heparin) was given to all patients.

Whole blood samples were obtained from the brachial artery immediately after full heparin (300 U·kg^{-1} i.v.) administration (pre-bypass) and on termination of CPB just before protamine administration (post-bypass). Sonoclot coagulation analysis was performed by placing a small amount of whole blood (0.4 ml) into a cuvette consisting of a glass-bead activator with heparinase and a stir bar in which a vertically vibrating probe is suspended [3]. In contrast to the conventional celite or kaolin activators, the glass-bead activator initiates less contact activation, and therefore the onset of clot formation (ACT result) is more sensitive to heparin. With the use of heparinase, the glass-bead activation test may provide greater sensitivity to factor deficiencies in clot formation and clot retraction (platelet function). A comparative set of Sonoclot tracings from the preliminary experiments is shown in Fig. 1.

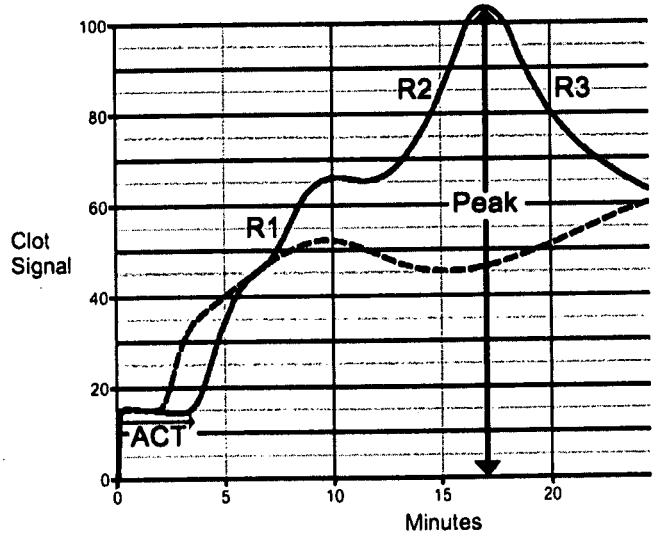

Fig. 1. Production of normal Sonoclot analysis (*solid line*) and abnormal Sonoclot analysis (*dotted line*). *ACT*, activated clotting time; *R1*, the primary slope; *R2*, the secondary slope; *R3*, the third downward slope; *peak*, peak impedance

The changes in mechanical impedance exerted on the probe by the changing viscoelastic properties of the forming clot are measured on a recorder. As fibrin strands form, impedance rises at various rates until the peak impedance is achieved. As shown in Fig. 1, the ACT is the onset time of the beginning of fibrin formation and the clot rate is the primary slope (R1) that reflects further fibrin formation from fibrinogen. This reaction is affected by both the quality of thrombin and the quantity of fibrinogen. After primary fibrin formation, an inflection point is often seen where the platelets start contracting the fibrin strands. The secondary slope (R2) reflects further fibrin formation and platelet-fibrin interaction that represents the completion of clot formation. The peak impedance (peak clot signal) reflects the completion of fibrin formation and represents the fibrinogen concentration. The time to peak is the value for the speed of clot formation, which depends not only on early fibrin formation but also on the dynamic combination of fibrin formation and early clot retraction. A downward slope after the peak signal (R3) represents clot retraction and is produced as platelets induce contraction of the completed clot. The peak angle is the angle between R2 and R3, and the clot retraction rate is the R3. The number of available platelets, as well as platelet function, determines the time to peak, peak angle, and clot retraction rate. Further decrease in impedance is caused by clot lysis [3,4]. Sonoclot data were analyzed by a single person who was blinded to the patients' demographics.

A separate blood sample was placed into an ethylene diamine tetraacetic acid (EDTA) tube, and platelet count and fibrinogen level were obtained.

The patient care team was not informed of the results of the Sonoclot measurements. Hemostatic product transfusion was guided by the conventional laboratory tests; fresh frozen plasma for PT more than 15 s and/or aPTT of more than 45 s, and platelet concentrates for platelet counts of less than $100 \times 10^3 \cdot mm^{-3}$.

We reviewed the Sonoclot and coagulation data of the patients based on the occurrence of excessive bleeding after surgery. Excessive bleeding was defined as chest tube drainage greater than 2 ml·kg^{-1}·h^{-1} in 1 h during the first 4 h after surgery [6]. Results are expressed as means ± SD. Statistical analysis was performed using two-way repeated analysis of variance, Student's *t*-test, or the X^2 test. A P value of less than 0.05 was considered significant.

Results

Table 1 shows the demographics, transfusion requirements, and blood loss of the study patients with excessive bleeding ($n = 12$) and those with acceptable bleeding

Table 1. Patient characteristics

	Acceptable bleeding ($n = 29$)	Excessive bleeding ($n = 12$)
Age (years)	50 ± 16	66 ± 8*
Sex (male/female)	16/13	8/4
Height	163 ± 9	161 ± 6
Weight	60 ± 11	56 ± 8
Operation performed		
Coronery artery bypass surgery	9	3
Aortic valve replacement	5	2
Mitral valve surgery	2	5
Atrial septal defect closure	13	0
Myxoma extraction	0	2
Aortic clamp time (min)	77 ± 38	129 ± 39*
Duration of CPB (min)	136 ± 37	188 ± 48*
MAP administration after CPB	4/29 (14%)	8/12 (67%)*
FFP administration after CPB	5/29 (17%)	8/12 (67%)*
Plt administration after CPB	1/29 (3%)	5/12 (42%)*
Postoperative blood loss during the first 4 h after surgery (ml)	97 ± 59	350 ± 134*

* Value significantly different from acceptable bleeding; $P < 0.05$
Values are means ± SD
CPB, cardiopulmonary bypass

Table 2. Coagulation data before and after CPB

	Acceptable bleeding ($n = 29$)		Excessive bleeding ($n = 12$)	
Variable	After heparin	Before protamine	After heparin	Before protamine
Platelet number ($10^3 \cdot mm^{-3}$)	221 ± 75	149 ± 57*	216 ± 69	90 ± 28*:**
Fibrinogen concentration ($mg \cdot dl^{-1}$)	268 ± 71	160 ± 37*	284 ± 49	154 ± 40*
ACT (s)	255 ± 48	241 ± 52	250 ± 58	246 ± 64
Clot rate ($signal \cdot min^{-1}$)	16.9 ± 7.3	15.7 ± 6.1	17.1 ± 5.2	16.3 ± 4.3
Peak clot signal	94 ± 12	94 ± 8	99 ± 13	93 ± 13
Time to peak (min)	12.1 ± 3.4	13.4 ± 3.7	12.9 ± 3.6	17.3 ± 7.2*:**
Peak angle (degrees)	83 ± 29	85 ± 24	86 ± 22	109 ± 29*:**
Clot retraction rate ($signal \cdot min^{-1}$)	3.0 ± 1.6	3.0 ± 1.4	2.7 ± 1.2	1.5 ± 1.1*:**

* Value before protamine significantly different from after heparin within group; $P < 0.05$;
** Value before protamine significantly different between acceptable and excessive bleeding; $P < 0.05$
Values are means ± SD
ACT, activated clotting time

($n = 29$). There were no significant differences between the two groups in terms of sex distribution, height, or weight. Patients with excessive hemorrhage were older than the nonbleeders. Aortic clamp and CPB times were significantly longer in patients who experienced excessive bleeding.

Coagulation data are shown in Table 2. Before CPB, platelet counts, fibrinogen levels, and Sonoclot values were similar in the groups with and without excessive bleeding. After CPB, platelet counts and fibrinogen concentrations significantly decreased from baseline in both groups, although post-bypass platelet counts were significantly less in bleeders than in nonbleeders (90 ± 28 and 149 ± 57 × $10^3 \cdot mm^{-3}$, respectively). There were no significant differences in Sonoclot values before and after CPB in nonbleeding patients. In the group with excessive bleeding, Sonoclot variables reflecting primary fibrin formation (ACT, clot rate, and peak clot signal) were preserved after CPB; however, the later variables, reflecting platelet-fibrin interaction (time to peak [12.9 ± 3.6 vs 17.3 ± 7.2 min], peak angle [86 ± 22° vs 109 ± 29°], and clot retraction rate [2.7 ± 1.2 vs 1.5 ± 1.1 $signals \cdot min^{-1}$]) were significantly different from their respective pre-bypass values.

The number of hemostatic product transfusions was naturally higher in patients who bled excessively, but none of the study patients required surgical re-exploration of the chest for postoperative hemorrhage.

Discussion

In the present study, we observed that bleeding diathesis was associated with reduced platelet-fibrin

interaction that was evident on the Sonoclot tracings. Of note, initial Sonoclot variables reflecting primary fibrin formation were preserved even in patients who experienced excessive postoperative hemorrhage after CPB. Therefore, obtaining late Sonoclot variables that reliably reflect platelet-fibrin interaction is important for predicting bleeding tendency. The glass-bead activator preferentially activates platelets, and improves the sensitivity of Sonoclot to platelet dysfunction. The addition of heparinase to the glass beads cuvette circumvents the susceptibility of the clot signal to heparin anticoagulation. In our study, we used heparinase to assess coagulation function during rewarming (35.5°C) on CPB, and the total time required for obtaining pertinent Sonoclot variables was in the range of 14 to 27 min, which is reasonable considering the typical laboratory turnaround time of 45–60 min for coagulation assays. If Sonoclot variables are used to obtain pertinent variables reflecting platelet-fibrin interaction (prolonged time to peak, blunt peak angle, and reduced clot retraction rate) prior to protamine administration, they may allow us to determine the risk of bleeding and prepare for allogeneic transfusion if necessary.

A bleeding tendency after CPB reflects multiple coagulation defects, including platelet defects in quantity and/or quality, reduced coagulation factors, inadequate reversal of heparinization, and increased fibrinolysis [7–11]. Nevertheless, a number of investigators have attributed post-CPB bleeding to platelet defects [8–10]. Sonoclot variables have been shown to reflect platelet count as well as platelet function [3,4]. Our finding of decreased platelet-fibrin associated with bleeding is in agreement with such views. Shore-Lesserson et al. [12] reported that the intraoperative guidance of hemostatic transfusion with a Thrombelastograph (TEG; Haemoscope, Niles, IL, USA) could reduce bleeding after surgery. One useful TEG variable is the maximum amplitude, which reflects the interaction of fibrin with platelets [13]. Therefore, reduced platelet-fibrin interaction seems to be a critical indicator of platelet abnormality after CPB.

In our study, platelet concentrates were transfused to maintain the platelet counts at more than $100 \times 10^3 \cdot mm^{-3}$. However, 12 of the 41 study patients met the criteria of excessive bleeding after surgery. Possible factors that affect the efficacy of transfused platelets include the occupation of glycoprotein IIb/IIIa receptors by fibrin degradation products [14], and plasmin-mediated fibrinolysis and platelet activation [15]. The above receptors function as a major fibrinogen binding site, and therefore the platelet-fibrin interaction is reduced by their inhibition [16]. Because we did not implement routine antifibrinolytic therapy (e.g., aminocaproic acid or aprotinin), fibrinolysis might have contributed to the postoperative bleeding diathesis.

Conventional clotting tests such as PT and aPTT only reflect early clot formation, and therefore the kinetics and quality of clot formation are not reflected. Sonoclot coagulation analysis enables bedside determination of clot strength and function in a timely fashion. Several investigators have reported that the Sonoclot Analyzer is useful in predicting postoperative coagulation defects after CPB [17–19]. Tuman et al. [17] reported that Sonoclot analysis can be a reliable predictor of abnormal clinical hemostasis after CPB. Stern et al. [18] assessed platelet function by the Sonoclot parameter of the clot retraction rate in cardiac surgery patients taking nonsteroidal anti-inflammatory drugs. Miyashita and Kuro [19] reported that time to peak for the Sonoclot signature can predict approximate platelet function in cardiac surgery. The use of point-of-care coagulation assays has been consistently associated with reduced blood transfusion and postoperative bleeding after CPB [12,20].

In conclusion, Sonoclot analysis with the glass-bead activated heparinase test indicated the impairment of clot stability after CPB in patients who developed excessive postoperative bleeding. Prolonged CPB was associated with poor hemostasis performance and resultant postoperative hemorrhage. Our retrospective analyses provided us with preliminary data to support the usefulness of Sonoclot in guiding hemostatic therapy, and an additional study is ongoing to evaluate this monitor and antifibrinolytic therapy in a prospective manner.

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INHIBITION OF NEUTROPHIL ELASTASE ATTENUATES GUT MUCOSAL INJURY EVOKED BY ACUTE ALVEOLAR HYPOXIA IN RABBITS

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ABSTRACT—The aim of the present study was to examine whether neutrophil and its elastase activity played consequential roles in the progression of gut barrier dysfunction during acute alveolar hypoxia by using a specific neutrophil elastase inhibitor, sivelestat. With our institutional approval, 20 male rabbits (weight, 2.0–2.5 kg) were randomly allocated into two groups: control (n = 11) or sivelestat group (n = 9; bolus, 10 mg/kg, followed by 10 mg/kg per hour). At 4 h of alveolar hypoxia exposure (fraction of inspired oxygen, 0.10) under mechanical ventilation, the white blood cell counts and their function to produce oxygen radicals were measured. Intestinal permeability and myeloperoxidase activity were also assessed concurrently with the examination of histological changes of gut mucosa. The examination of sham animals (n = 4) exposed to normoxia was performed under the same study protocol. The circulating leukocyte counts and the neutrophil chemiluminescence were not different between the groups, whereas the neutrophil elastase activity was significantly increased in the control but not in the sivelestat and sham groups. Permeability, leukocyte accumulation, and myeloperoxidase activity of ileal wall in the control group were significantly elevated, accompanied by apparent destruction of gut mucosa compared with the sivelestat group ($P < 0.05$). Despite no significant differences in systemic inflammatory responses, the neutrophil elastase activity is a key element in the progression of functional and structural injury of gut mucosa during acute alveolar hypoxia.

KEYWORDS—Bacterial translocation, leukocyte, permeability, sivelestat, high-mobility group box 1

INTRODUCTION

Critically ill patients, who are at high risk of developing serious conditions such as septic shock or myocardial infarction, are frequently susceptible to acute hypoxic episodes because of their complicating lung injury or other conditions (1–3). Although alveolar hypoxia modulates almost all organ function, previous studies have mainly focused on the lungs. For example, decreased alveolar oxygenation induced lung inflammation (i.e., the recruitment of macrophage, the enhancement of inflammatory mediators, and the increase of albumin leakage in lungs) (4). Another study demonstrated that alveolar hypoxia increased leukocyte adhesion in the microcirculation through the involvement of adhesion molecules, resulting in vascular leak syndrome (5, 6). As a promising approach, the inhibition of neutrophil elastase with a specific neutrophil elastase inhibitor, sivelestat, (ONO-5046; *N*-[2-{4-(2,2-dimethylpropionyloxy) phenylsulphonyl-amino} benzoyl] amino acetic acid; Ono pharmaceuticals, Osaka, Japan) has been shown to provide therapeutic effects on such lung injury in laboratory investigations (7, 8). However, the roles of this mediator in the pathophysiology of extrapulmonary organs have not been fully investigated, particularly under noninflammatory insults such as acute hypoxia.

Gut, the most fragile organ to hypoxia because of its anatomical characteristics (9), has become a therapeutic target in critically ill patients (10). Thus, a loss of gut barrier function causes the translocation of microorganisms and/or toxins and, subsequently, the development of multiple organ failure (10). Indeed, we previously demonstrated that acute hypoxia deteriorated the gut mucosal barrier function and structure, resulting in the translocation of endotoxin (11, 12). However, substantial mechanisms of hypoxia-induced gut mucosal injury remain to be fully clarified. We therefore designed the present study to examine, by using sivelestat, whether the inhibition of neutrophil elastase activity minimized the elevation of gut mucosal permeability and structural alterations through the neutrophil-dependent mechanisms during acute hypoxia. Simultaneously, we examined the contribution of plasma high-mobility group box 1 (HMGB1), originally identified as a DNA-binding protein and a consequential mediator of lung injury (13), to the development of gut barrier dysfunction due to moderate level of hypoxia.

MATERIALS AND METHODS

This protocol was approved by the Keio University Council on Animal Care in accordance with the guidelines of the National Institutes of Health.

Preparation surgery

Twenty healthy rabbits (Japanese white rabbits [male]; SEASCO, Saitama, Japan), weighing 2.0 to 2.6 kg (average, 2.3 kg) and fasted for 24 h, were used. With sevoflurane 3% to 4% inhalation in oxygen (flow rate, 3–4 L/min) *via* facemask, the rabbits underwent tracheostomy and i.v. line access on the marginal ear vein. The animals were then mechanically ventilated to maintain normocapnia (fraction of inspired oxygen [F_{IO_2}], 0.21; inspiratory pressure, 12–15 cm H_2O; breath rate, 10–15 breaths/min) using an intensive care unit–type ventilator (Newport E100; Newport Medical Instruments, Costa Mesa, Calif). The right carotid artery was cannulated to monitor the mean arterial pressure (MAP) and to obtain blood samples. After a

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midline abdominal incision, a perivascular probe (Transit Time Ultrasound flowmeter, model T206; Transonic Systems, Inc., Ithaca, NY) was attached around the descending aorta for the measurement of abdominal aortic flow. After the closure of laparotomy incision, the administration of inhalational anesthesia was discontinued; then, a continuous 1-mL/h infusion of sedatives consisting of buprenorphine (dose, 0.1 mg/mL), midazolam (dose, 2 mg/mL), and pancuronium (dose, 0.05 mg/mL) was performed throughout the study period to suppress the vigorous, spontaneous inspiratory efforts during hypoxia described in the study protocol. Rectal temperature was monitored and maintained at approximately 37°C. The animals were observed for 1 h before baseline measurements were made.

Study protocol

After 45-min equilibration period, the baseline measurements described in the Specific Measurements section were performed (baseline). Thereafter, 20 rabbits were randomly assigned to two groups by means of computer-generated random numbers; then, all animals were exposed to acute hypoxia (Fio_2, 0.10) for 4 h. After the baseline study, 11 rabbits (control group) received isotonic sodium chloride infusion, whereas nine rabbits received i.v. injection of sivelestat (sivelestat group; dose, 10 mg/kg), followed by continuous infusion at a rate of 10 mg/kg per hour throughout the study period. By mixing air with 100% nitrogen through the oxygen blender of the ventilator, the Fio_2 was reduced to hypoxia (Fio_2, 0.10), under monitoring using an anesthetic gas analyzer (Ohmeda 5250RGM; BOC Health Care, Louisville, Colo). Our pilot study demonstrated that the moderate level of acute hypoxia (Fio_2, 0.10) for 4 h caused a moderate level of gut mucosal alterations (i.e., significant elevation of mucosal permeability and apparent structural changes of gut mucosa). Therefore, in the current study, we applied this condition to examine the roles of neutrophil and HMGB1 in the development of gut mucosal injury. The measurements were then repeated after 2 and 4 h. After the experiments were executed, the rabbits were killed by means of i.v. pentobarbital overdose. In addition, to determine the time course effects in our experimental setting, we studied four sham animals that received the same catheterization without acute hypoxia under the same study protocol.

Specific measurements

Arterial pH, $Paco_2$, Pao_2, and lactate concentration were determined by using a blood gas analyzer (Chiron 860 series; Chiron Diagnosis Corporation, East Walpole, Mass). The white blood cell counts in whole blood were determined (Celltac MEK-5153; Nihon Kohden Corporation). Simultaneously, blood sample was stained with Giemsa stain to determine the changes on peripheral neutrophils count.

Neutrophil priming was assayed by means of the release of reactive oxygen species from *in vitro* stimulated neutrophils with luminol-dependent neutrophil chemiluminescence (luminescence reader BLR-201, Aloka, Tokyo, Japan) (14). The reaction mixture consisted of 1.7 mL of diluted whole blood and 0.2 mL of a 1-mmol/L luminol solution. After preincubation at 37°C, 5 mg/mL opsonized zymosan (dose, 0.1 mL) was added to the mixture; then, the peak chemiluminescence value during the 20-min assay time was used as a neutrophil-priming indicator. The neutrophil elastase activity in plasma was determined using the synthetic substrate *N*-methoxysuccinyl-Ala-Ala-Pro-Val *p*-nitroaniline, which is highly specific for neutrophil elastase by using the method described previously (15). Briefly, the samples were incubated in 0.1 mol/L Tris-HCl buffer (pH value, 8.0) containing 0.5 mol/L NaCl and 1 mmol/L substrate for 24 h at 37°C. After incubation, the *p*-nitroaniline release was measured spectrophotometrically at 405 nm and was considered as neutrophil elastase activity.

Plasma HMGB1 concentration was measured with a new, highly sensitive, and specific enzyme-linked immunosorbent assay (Shino-Test Corporation, Tokyo, Japan), which could be applied to rabbits and other species (16). Briefly, polystyrene microtiter plates were coated with monoclonal anticalf HMGB1 antibody. The remaining binding sites in the wells were blocked by bovine serum albumin. After washing, the calibrator and the samples were added to the wells. After another washing, antihuman HMGB1 peroxidase-conjugated monoclonal antibody (a synthetic peptide was used as immunogen) was added to each well. After another washing step, the luminescence reagent was added to each well. The luminescence was measured using a microplate luminescence reader. Measurements of all parameters were performed in duplicate, and mean values were used for analysis.

Mucosal permeability and myeloperoxidase activity in ileal wall

Using fluorescein isothiocyanate–conjugated Dextran with a molecular weight of 4,000 d (FD4), we determined the alterations of gut permeability as previously described (17). At 4 h, the abdomen was opened for the preparation of an *in situ* loop of gut. Briefly, double ligatures at both ends were made on the 10-cm length of terminal ileum. Through a cannula placed into this segment of terminal ileum, FD4 (weight, 50 mg) was injected. After 30 min, blood samples from both the portal vein and the carotid artery were taken and centrifuged; subsequently, plasma FD4 concentrations were measured using fluorescence spectrometry (spectrofluorophotometer model RF-1500; Shimadzu Corporation, Kyoto, Japan). The results were corrected for the plasma protein contents measured by using the Lowry method.

As an index of leukocyte sequestration, the myeloperoxidase (MPO) activity of ileal wall was assayed, as reported previously, with minor modification (18). Briefly, a small portion of the terminal ileum was frozen in liquid nitrogen and stored at a temperature of −80°C for subsequent assay. The samples were disrupted by homogenization at a temperature of 4°C and were placed into 0.5% hexyldecyltrimethyl ammonium bromide in 50 mmol/L potassium phosphate solution (pH value, 6.0; 1 mL per 100 mg ileum). The tissue was sonicated on ice and underwent three freeze/thaw cycles (liquid nitrogen bath and 37°C water bath). The solution was centrifuged at 18,000*g* for 20 min at 4°C. Aliquots (volume, 0.04 mL) of supernatant were added to 0.96 mL of assay buffer (concentration, 0.17 mg/mL) and measured after 5 min of incubation by means of spectrophotometry at 492 nm (spectrofluorophotometer model RF-1500; Shimadzu Corporation). The MPO activity was expressed in units per milligram of protein.

Histological analyses

At the completion of experiments, the microstructure of terminal ileum was examined. Ileal samples were fixed for microscopic examination by using luminal perfusion and were processed as described by Deitch et al. (19). After processing, semithin (thickness, 2–4 μm) sections were cut by means of a diamond knife and were stained with hematoxylin and eosin. Histological sections were randomly coded and evaluated in a blinded manner at ×100 magnification using light microscopy. With a modification of the grading system of Chiu et al. (20), the degree of mucosal damage was graded in a blinded fashion by an independent observer on a scale of 0 (normal) to 4 (severe cell disruption). Twenty-five random fields from each tissue were examined in a blinded fashion by an independent observer under light microscopy.

Statistical analysis

Data are expressed as mean ± SD unless otherwise specified. One-way analysis of variance was applied to examine the differences, and the means of all groups were compared using Student-Newman-Keuls test for multiple comparisons. The histological scoring data were analyzed by using chi-square test. Differences were considered statistically significant if $P < 0.05$.

RESULTS

Table 1 shows the changes on systemic circulatory and oxygenation variables during acute hypoxia. As a matter of course, the Pao_2 and the arterial O_2 content were significantly depressed at 2- and 4-h study periods to a similar extent in

TABLE 1. **Changes on systemic circulatory and oxygenation variables during acute hypoxia**

	Group	Baseline	2 h	4 h
Pao_2 (mmHg)	Control	71 ± 10	28 ± 3**	31 ± 3**
	Sivelestat	70 ± 10	34 ± 5**	30 ± 4**
Arterial O_2 content (mL O_2/L)	Control	14.8 ± 1.6	7.8 ± 0.8**	7.4 ± 1.2**
	Sivelestat	14.7 ± 1.1	8.7 ± 1.3**	7.4 ± 1.2**
Arterial pH	Control	7.39 ± 0.06	7.26 ± 0.08	7.18 ± 0.09*
	Sivelestat	7.37 ± 0.06	7.29 ± 0.13	7.21 ± 0.14
Arterial lactate (mmol/L)	Control	3.0 ± 0.8	10.8 ± 4.0**	13.1 ± 3.5**
	Sivelestat	2.6 ± 0.9	10.1 ± 5.1**	13.8 ± 5.7**
MAP	Control	74 ± 6	79 ± 15	68 ± 14
	Sivelestat	86 ± 6	78 ± 9	77 ± 15
Heart rate (beats/min)	Control	267 ± 27	249 ± 19	235 ± 54
	Sivelestat	257 ± 46	247 ± 19	250 ± 42
Abdominal aortic flow (mL/min)	Control	281 ± 65	243 ± 49	252 ± 32
	Sivelestat	259 ± 16	250 ± 44	220 ± 35

All values are expressed as mean ± SD.
*$P < 0.05$;
**$P < 0.01$ *versus* baseline.

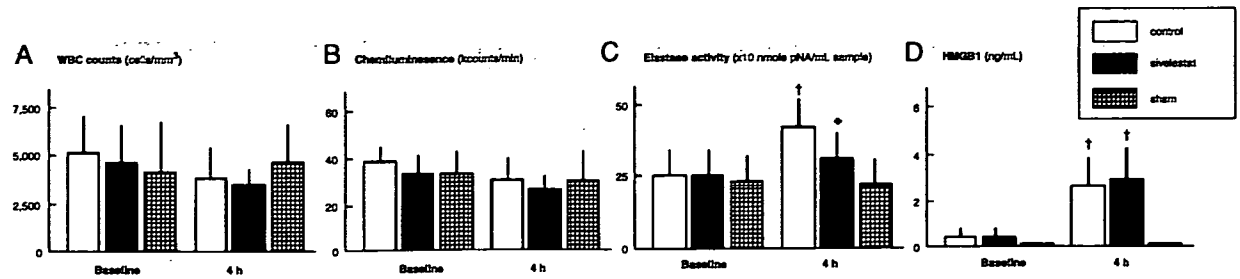

FIG. 1. **Changes on circulating leukocyte counts, chemiluminescence, neutrophil elastase activity, and HMGB1 activity during acute hypoxia.** Data are expressed as mean ± SD. $^{*}P < 0.05$ *versus* control group; $^{\dagger}P < 0.05$ *versus* baseline of each group.

both study groups. Arterial pH was also depressed at 4 h, whereas arterial lactate showed a marked elevation at 2- and 4-h study periods. On the contrary, systemic hemodynamics, such as MAP and heart rate, were not significantly different between both study groups throughout the study periods, whereas these parameters showed a slight reduction at 4 h *versus* the baseline. Simultaneously, the abdominal aortic blood flow, possibly reflecting cardiac output, remained constant during acute hypoxia.

Figure 1 illustrates the changes on circulating white blood cell counts, chemiluminescence, neutrophil elastase activity, and HMGB1 activity at baseline and at 4 h of acute hypoxia. The number of circulating leukocytes and their chemiluminescence were not changed significantly at baseline and at 4 h of acute hypoxia in all study groups. The number of neutrophils showed results similar to those of leukocytes (data not shown). The plasma neutrophil elastase activity in the control group increased significantly at 4 h compared with the baseline period and the sivelestat group, whereas the plasma neutrophil elastase activity in both the sivelestat and the sham groups remained constant throughout the study periods. The plasma HMGB1 activity, which was undetectable in most animals at baseline, showed a marked elevation at 4 h of acute hypoxia in both study groups *versus* the sham. Figure 2 shows the changes on plasma FD4 concentration and ileal MPO activity. The plasma FD4 concentration, indicating mucosal permeability, was markedly increased in the control group compared with that of the sivelestat group. Simultaneously, the ileal MPO activity level in the control group was significantly higher compared with that in the sivelestat groups ($P < 0.05$), indicating that the number of accumulated leukocytes in the ileal wall during hypoxia was significantly reduced with sivelestat infusion.

Figure 3 illustrates, using light microscopy, the representative pictures of the villi of distal ileum in the control and the sivelestat groups. The villi of the animals subjected to hypoxia in the control group showed definitive evidence of mucosal injury, such as disruption of microvilli, lifting of the epithelium from the basal lamina with submucosal edema, and accumulation of leukocytes (A), whereas those in sivelestat group showed near-normal picture (B). Apparently, the leukocyte accumulation in the ileal mucosa was augmented in the control *versus* the sivelestat group. Simultaneously, the data of injury scores using Chui's modified scoring system demonstrated that the mucosal injury of distal ileum observed in the control group was significantly attenuated in the sivelestat group (Fig. 4) ($P < 0.01$). Thus, sivelestat infusion in this experimental model minimized the acute hypoxia-induced alterations of both structure and barrier function against macromolecules in ileal mucosa.

DISCUSSION

The present study demonstrates that the exposure of moderate hypoxia (FIO_2, 0.1) for short-term period induces both functional and structural alterations of gut, accompanied by a marked accumulation of leukocytes in the mucosa. This study also showed that the inhibition of neutrophil elastase activity with a specific elastase inhibitor ameliorated such gut mucosal dysfunction evoked by acute hypoxia. Inasmuch as the changes of hemodynamics and neutrophil-related parameters detected at systemic level were paralleled in both

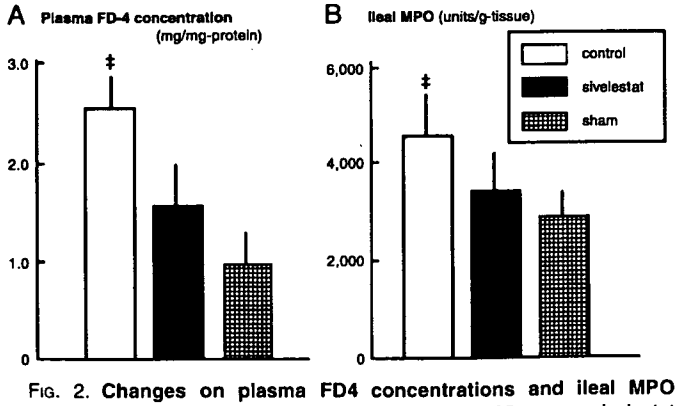

FIG. 2. **Changes on plasma FD4 concentrations and ileal MPO activity.** Data are expressed as mean ± SD. $^{*}P < 0.05$ *versus* sivelestat group.

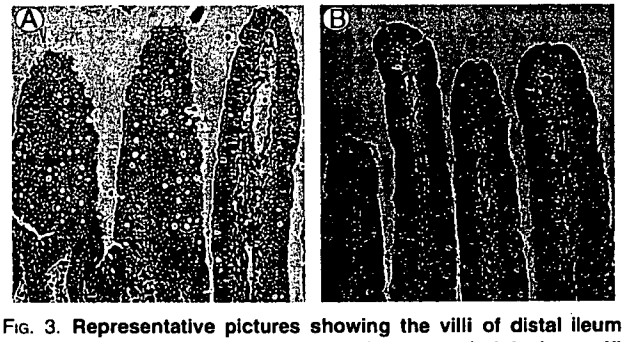

FIG. 3. **Representative pictures showing the villi of distal ileum of rabbits, as captured by means of light microscopy (original magnification, ×100).** A, Representative villi of distal ileum from a rabbit in control group. Note the degenerated epithelial cells of villi and apparent leukocyte accumulation in ileal mucosa. B, Representative villi of distal ileum from a rabbit in sivelestat group. Note the normal-looking structure of ileal mucosa.

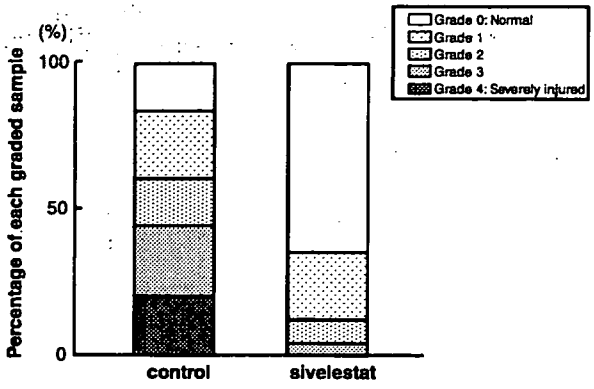

FIG. 4. **Severity of mucosal injury during 4 h of acute hypoxia.** The degree of mucosal injury was graded on a scale of 0 (normal) to 4 (severe cell disruption). Data are expressed as the percentage of 25 fields of each animal. Chi-square test showed significant difference between the control and the sivelestat groups.

hypoxia study groups, the activity of neutrophil elastase could be a key element to induce the significant accumulation of neutrophils and the alterations of gut mucosa. Previous study showed that neutrophil elastase directly degraded the components of basement membrane to allow for neutrophil migration into the interstitial space (21). Hence, the augmented activity of neutrophil elastase caused by hypoxia contributes to facilitate the migration of neutrophils into gut mucosa *via* chemotactic factor production as observed in lungs (22, 23), thereby allowing gut mucosal dysfunction.

In clinical situations potentially complicated by hypoxia, the severity of diseases was reported to correlate well with the increased number of primed and/or activated leukocytes in blood (24). In addition, the neutrophils obtained from rats exposed to hypoxia (F_{IO_2}, 0.09) for 12 h enhanced the responses against stimulation, indicating that hypoxic exposure could prime the circulating neutrophils (25). Therefore, it is rational to hypothesize that the inhibition of neutrophil elastase suppressed the moderate level of hypoxia-induced gut mucosal injury. Although acute hypoxia in the present study did not modulate neutrophil priming *per se* as assessed by chemiluminescence, the enhanced discharges of neutrophil elastase in the control group compared with those of the sham indicated that neutrophil degradation rather than production of reactive oxygen species might play more consequential roles in the development of hypoxia-induced gut injury. In addition, neutrophil accumulation into gut mucosa evoked by hypoxia was minimized by the inhibition of neutrophil elastase activity with sivelestat. This finding suggests that activated neutrophils exposed to acute hypoxia are adhered in the intramucosal microcirculation, thereafter migrating into intramucosal tissues of gut through the involvement of neutrophil elastase activity. Laboratory reports indicated that the inhibition of neutrophil elastase with sivelestat attenuated the endotoxin-induced alterations of pulmonary microcirculation, including the number of adhered leukocytes and tissue edema (26), whereas it remains unclear whether the substantial mechanisms of neutrophil elastase inhibition with sivelestat to lung injury are applied to the other organs. However, the present study demonstrated that neutrophil elastase *per se* played a consequential role for the development of noninflammatory insult-elicited gut injury.

Another important finding of the present study was to show that HMGB1, usually detectable 12 to 16 h after endotoxin infusion (27), was discharged and quantified significantly for 4 h of alveolar hypoxia. Although HMGB1 has been appreciated as a consequential mediator of mortality and acute lung injury (13), the role for the development of gut mucosal dysfunction has not been previously examined. The present study showed that 4-h hypoxic insult was long enough to discharge HMGB1 from macrophages or other cells, such as neutrophils. A different model to evoke ventilator-induced lung injury recently demonstrated that injurious mechanical ventilation expressed the marked elevation of HMGB1 in bronchoalveolar lavage fluid only for 4 h and showed a decrease in tumor necrosis factor (TNF) α concentration in bronchoalveolar lavage fluid by treatment with anti-HMGB1 antibody (28). Collectively, noninflammatory insults, such as hypoxia or mechanical stretch, may evoke HMGB1 discharges through different mechanisms from lethal endotoxemia. It should also be noted that plasma HMGB1 levels in both study groups significantly increased and remained unchanged with or without the inhibition of neutrophil elastase activity. Therefore, HMGB1 was unlikely to be involved directly in this model of gut mucosal dysfunction during acute hypoxia, although we did not assess the changes on TNF-α and interleukin 1, both of which were considered as the upstream regulators of HMGB1 release. Further investigation using HMGB1 antagonist is warranted to explore the involvement of this mediator for hypoxia-induced gut injury.

There are several limitations to interpret the data in this study. First, inasmuch as the results of this study were obtained during 4 h of acute hypoxia, the very short term or the long-term effects, which could be more clinically relevant, remain to be fully determined. Indeed, the length of exposure to hypoxia could be a key factor to reflect the responses. For example, *in vivo* 3-min exposure of rats to severe hypoxia induced a reversible increase of leukocyte adhesion in the mesenteric venules, whereas 30-min exposure to hypoxia provided leukocyte activation (29, 30). On the other hand, the present study showed that the gut mucosal barrier function deteriorated through the involvement of proinflammatory cells and mediators at least within 4 h of moderate hypoxia. Second, neutrophil priming was not significantly augmented in this experimental setting, whereas other reports indicated that circulating neutrophils were primed even under noninflammatory insults (31). Although the reason for such discrepancy remains unclear, the difference on study model or design might be involved. Third, inasmuch as enzyme-linked immunoassay kits for many kinds of cytokines were not commercially available for rabbits, we were unable to examine these markers, which seem to be of interest to elucidate the underlying mechanisms of hypoxia-induced mucosal injury in combination with the changes on HMGB1. Previous studies indicated that hypoxia induced the production of proinflammatory cytokines, such as TNF-α and IL-1β, from macrophages and endothelial cells (32, 33). However, hypoxia of

these studies using *ex vivo* experimental settings were too severe to mimic clinical studies. Further study is needed to explore the roles of proinflammatory cytokines in acute hypoxia–induced tissue injury. Finally, the clinical effectiveness of neutrophil elastase inhibitor in heterogenous patients with lung injury still remains controversial (34), although neutrophil elastase has been recognized as a key element in the development of acute lung injury in multiple animal models (35). Accordingly, the therapeutic effects of neutrophil elastase inhibitor to protect the barrier function of gut must be examined in clinical situations.

In conclusion, moderate hypoxia induces both functional and structural alterations of gut mucosa through the involvement of neutrophil infiltration in the intestinal wall and the activation of HMGB1, and that the inhibition of neutrophil elastase activity with a specific inhibitor ameliorates such modulation of gut barrier dysfunction during acute hypoxia.

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総　説

新しい非脱分極性神経筋遮断薬 ロクロニウム(Org 9426)の紹介

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1. 本邦と欧米における神経筋遮断薬の現況

本邦では1988年のベクロニウム販売開始以来新しい神経筋遮断薬が導入されておらず，最近の神経筋遮断薬の市場ではベクロニウムが90%以上を占めているとのことである．

一方，欧米では1994年よりロクロニウムの臨床使用が開始され，これまでに1億例以上の使用実績がある．さらにatracurium，cisatracurium，mivacuriumなどベンジルイソキノリン系神経筋遮断薬も使用可能であり，非脱分極性神経筋遮断薬の選択肢が多い点が本邦と異なる点である．加えて1999年には，より作用発現が早いrapacuroniumの使用が開始されたが，気管支攣縮のため2001年に自主回収となっている．

本邦でも1994年以降，ロクロニウムの臨床使用が待たれていたが[1-3]，2003年に第III相臨床治験が再実施され，現在承認申請中である．

ところで，神経筋遮断薬の理想として以下のような特徴が挙げられている[4]．

① 非蓄積性，非脱分極性であること．
② 作用発現および回復が速やかであること．
③ 適当な拮抗薬投与により作用が確実に消失すること．
④ 心血管系およびその他の有意な副作用をもたないこと．

2番目に挙げられている作用発現および回復が速やかな優れた神経筋遮断薬が必要とされる理由は以下のような点である．

① 安全な気道確保とdifficult airwayへの対策[5-8]．
② full stomach対策としてrapid sequence intubationへの対応（後述）．
③ 残存筋弛緩による問題点への対策[9,10]．

本稿ではロクロニウムの特徴を，当施設を含めて実施した臨床治験の結果を加えて紹介し，ロクロニウムが理想の筋弛緩薬にどの程度該当するか，について解説する[11-14]．

2. 神経筋遮断薬に関する基本用語の解説

ロクロニウムの特徴を解説する前に，神経筋遮断薬に関する基本事項を整理しておく．

キーワード：神経筋遮断薬，臭化ロクロニウム，Org 9426

Review
Introduction of New Non-depolarizing Neuromuscular Blocking Agent, Rocuronium (Org 9426)
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❶ 力価（potency）

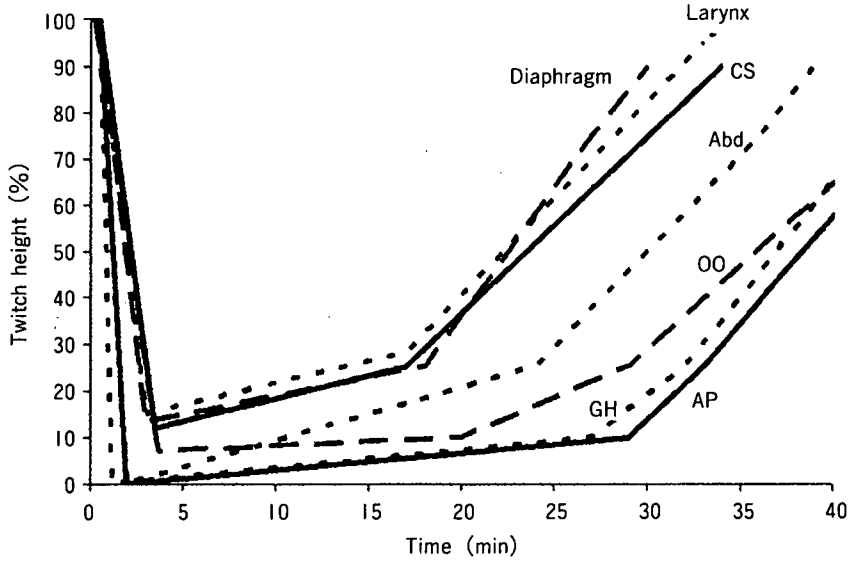
神経筋遮断薬の dose-response は投与量と単収縮高の抑制によって表現され，S 字曲線となる．一般的な筋弛緩モニターでは 4 連刺激が行われることがほとんどで，初回刺激に対する反応（T_1）を単収縮高とする場合が多い．一般的に T_1 を 95% 抑制する量，すなわち ED_{95} を力価の指標とする．当然のことながら ED_{95} が低い神経筋遮断薬ほど力価は高い．気管挿管の際の標準的な投与量としては ED_{95} の 2 倍量を選択することが多い．

❷ 作用発現時間（onset）

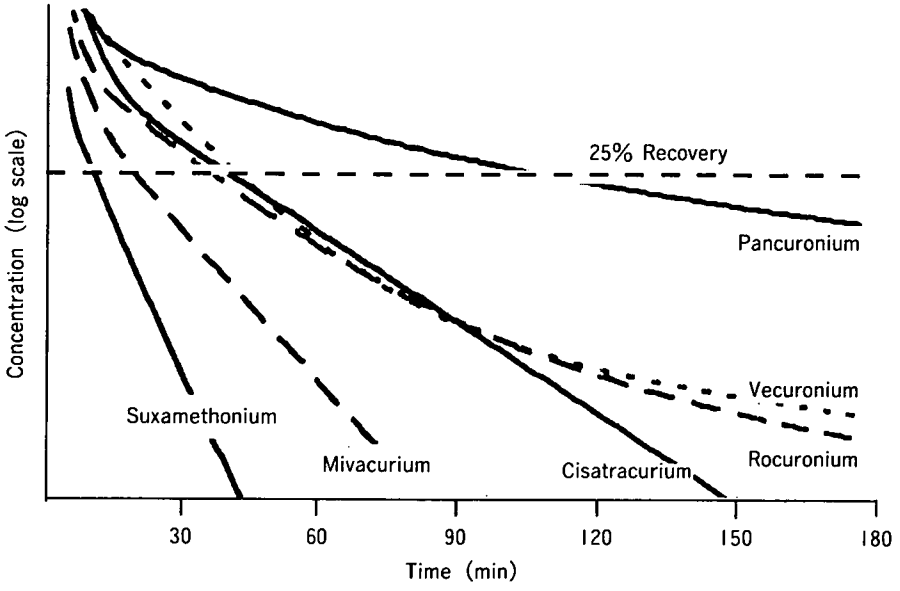
神経筋遮断薬投与完了から最大遮断（T_{max}）までの時間．一般的に力価が低い神経筋遮断薬ほど作用発現が早い傾向が認められる[15]．この理由としては，分子量が同程度であれば，力価が低い神経筋遮断薬ほど投与量が多く，投与される神経筋遮断薬の分子数が増える．結果として筋弛緩薬分子の血中から間質液への移行，神経筋接合部への到達が速やかになるため，と説明されている．通常，筋弛緩のモニタリングは母指内転筋（AP）で行われるため，作用発現および後述する作用持続は AP における神経筋遮断薬の効果に基づいて算出されている．しかし，臨床的には喉頭筋における作用発現，持続が重要である．喉頭筋における神経筋遮断薬の効果は AP よりも作用発現，持続ともに短いが，ED_{50} および ED_{95} が大きく，AP と同程度の筋弛緩効果を得るためには高用量を必要とする[16]．図❶にロクロニウムの標準投与量である 0.6 mg/kg を投与した場合の横隔膜，喉頭筋，皺眉筋（CS），腹筋（Abd），眼輪筋（OO），上気道開存に重要なオトガイ舌骨筋（GH）および AP における神経筋遮断作用のシミュレーションを示す[17]．

❸ 作用持続時間（duration）

神経筋遮断薬投与完了から T_1 がコントロールの 25% あるいは 95% まで回復するまでの時間．25% 回復までの時間（$Duration_{25}$）が臨床的作



図❶ ロクロニウム 0.6 mg/kg による神経筋遮断作用の筋肉別のシミュレーション[17]．
縦軸は対照に対する単収縮高（T_1）の割合（%），横軸は時間経過を示す．Diaphragm：横隔膜，Larynx：喉頭筋，CS：皺眉筋，Abd：腹筋，OO：眼輪筋，GH：オトガイ舌骨筋，AP：母指内転筋．



図❷ 神経筋遮断薬の神経筋接合部における濃度のシミュレーション[17]． 各神経筋遮断薬を ED_{95} の2倍量投与した場合のシミュレーションであり，点線で示した25%回復時点での濃度が一致するように各濃度曲線を移動してある．

用持続時間の指標として一般的に用いられており，本稿でもこちらを用いる．各神経筋遮断薬の作用持続時間は主として血中からの神経筋遮断薬の消失する速度に依存する．スキサメトニウムやmivacuriumは血中のコリンエステラーゼによって代謝され，血中濃度が速やかに低下するため作用持続が短い．一方，ロクロニウム，ベクロニウムの場合，組織における再分布，肝臓における取り込みによって濃度が低下し，中間時間作用性となる（図❷）．

❹ 回復時間（recovery index）

T_1 が25%から75%まで回復するのに必要な時間．

❺ 臨床的持続時間（clinical duration）

神経筋遮断薬投与完了から4連刺激比（TOFR）が0.7または0.9まで回復するのに必要な時間．最近は筋弛緩からの回復の指標としてTOFR 0.9が推奨されており，clinical durationについても0.9を用いる場合が多い．

3. ロクロニウムの特徴

今回紹介するロクロニウムはアミノステロイド系非脱分極性神経筋遮断薬のうち，中間作用時間型に属している．すなわちベクロニウムと同じカテゴリーに属しており，本稿でも本邦で現在最も用いられているベクロニウムとの比較を中心として記述を進めていくこととする．構造上はベクロニウム，パンクロニウムに認められるステロイド構造のA-リングにアセチルコリン様の構造（Me-N^+-C-C-OAc）を欠いている点と4級アミンの側鎖がメチル基からアリル基に置換されている点が特徴である（図❸）．これらの構造上の特徴によりロクロニウムはパンクロニウム，ベクロニウムと比較して力価が低い．ロクロニウムの開発番号はOrg 9426であり，rocuroniumという一般名はrapid onsetのrとoに由来するとのことである．ちなみに欧州での商品名はEsmeron，米国での