

Original Article

Comparison of Spinal Anesthetic Effect between Emulsified Isoflurane and Emulsified Nonimmobilizer F6

Jiao Guo^{1,2*}, Cheng Zhou^{1*}, Peng Liang², Xiao-jia Wang^{1,2}, Yi Zhao^{1,2}, and Jin Liu^{1,2}

ABSTRACT

Background: Our previous study found that emulsified isoflurane (EI) produced subarachnoid anesthesia in dogs. The spinal effect of isoflurane might account for its immobility action. 1, 2-dichlorohexafluorocyclobutane (F6) is a nonimmobilizer that is incapable of causing immobility, it is therefore interesting to know whether there are different spinal anesthetic actions between emulsified F6 and isoflurane and their underlying mechanisms.

Methods: EI and emulsified F6 were intrathecally injected into rats, and motor and sensory functions were evaluated. Sodium channel currents were recorded from spinal neurons. The effect of EI and emulsified F6 on sodium channel was examined.

Results: EI produced subarachnoid anesthesia (median effective concentration [EC₅₀] at 3.65%). Duration of actions of 8% EI was similar to 1% lidocaine. Emulsified F6 did not produce spinal anesthesia at 2% (5 folds of its predicted EC₅₀). Meanwhile, EI inhibited sodium channel currents with median inhibitory concentration (IC₅₀) at 0.81 ± 0.09 mmol/L and hyperpolarized voltage-dependent inactivation of sodium channel (from -57.5 ± 2.4 to -66.3 ± 1.8 mV, $P < 0.01$). Emulsified F6 slightly inhibited sodium channel currents and no effect was found to the channel gating.

Conclusions: Neither spinal anesthetic action nor effect to sodium channel was observed for nonimmobilizer F6, while EI inhibited spinal neurons sodium channels at clinically relevant concentrations and produced spinal anesthesia.

From the ¹Laboratory of Anesthesia & Critical Care Medicine, ²Department of Anesthesiology, Translational Neuroscience Center, West China Hospital, Sichuan University, Chengdu, China.

*Jiao Guo and Cheng Zhou contributed equally to this study.

Correspondence to Dr. Cheng Zhou at zhouc@163.com and Dr. Jin Liu at scujinliu@foxmail.com.

Citation: Jiao Guo, Cheng Zhou, Peng Liang, Xiao-jia Wang, Yi Zhao, Jin Liu. Comparison of spinal anesthetic effect between emulsified isoflurane and emulsified nonimmobilizer F6. *J Anesth Perioper Med* 2015; 2: 1-7.

Although volatile anesthetics have been used for more than 160 years, the exact mechanisms by which volatile anesthetics produce anesthesia are not fully known (1, 2). Previous studies have pointed out that spinal cord might be the most critical target for volatile anesthetics to produce immobility (3- 5), however, how volatile anesthetics produce immobility is unclear. Zhang et al. (6) found that intrathecal infusion of veratridine (sodium channel activator) significantly increased minimum alveolar concentration (MAC) of isoflurane, which revealed the important role of spinal sodi-

um channels on immobility of volatile anesthetics.

Recent studies found that volatile anesthetics could produce regional anesthesia (7- 11) and intrathecal injection of emulsified isoflurane (EI) (12) and sevoflurane (13) could also produce typical subarachnoid anesthesia in Beagle dogs. With intrathecal injection, the spinal effect of volatile anesthetics is similar to their effect of immobility with systemic inhalation because nociceptive reflex was both inhibited by these actions (12, 13). In addition, the subarachnoid anesthetic action of EI was comparable to the spinal effect of tradi-

tional local anesthetics such as lidocaine (12). Sodium channel is the main target for traditional local anesthetics and our previous study found that spinal sodium channel was inhibited by EI at clinically relevant concentrations, which indicated that spinal sodium channel might be the target for EI to produce subarachnoid anesthesia (12).

Nonimmobilizer compounds such as 1, 2-dichlorohexafluorocyclobutane (F6) could induce amnesia like isoflurane but are not able to produce immobility (14). Underlying molecular targets of general anesthetics are determined by comparison between volatile anesthetics and F6 such as GABA-type A (GABAA) and acetylcholine receptors (15-17). The most significant disadvantage of F6 is its possibility to induce seizure with systemic inhalation, which limits the use of the nonimmobilizer *in vivo* (14). The rat regional anesthesia model used in the present study could overcome this limitation. We hypothesized that emulsified F6 could not produce subarachnoid anesthesia as EI did because of its inability to induce immobility. This result would demonstrate the direct relationship between immobility and spinal effects of volatile anesthetics. In addition, we further examined the effect of emulsified F6 on sodium channels from acute isolated spinal neurons.

METHODS

With the approval of the Institutional Animal Experimental Ethic Committee of Sichuan University (Chengdu, Sichuan, China), adult (200-300 g) and natal (7-12 days) Sprague-Dawley rats were used in experiments *in vivo* and *in vitro*, respectively. All the animals were housed in standard conditions.

EI, containing pure liquid isoflurane and 30% Intralipid, was prepared by our laboratory (18). Briefly, 8% (v/v) EI 100 ml contained pure liquid isoflurane 8 ml. Isoflurane was supplied by the Abbott Pharmaceutical Co. Ltd. (Shanghai, China). 30% (w/v) Intralipid was from the Sino-Swed Pharmaceutical Corp. Ltd. (Wuxi, Jiangsu, China). F6 was purchased from Johnson Matthey Co. (Tianjin, China). Lidocaine was purchased from the Fortune Zhaohui Pharmaceutical Co. Ltd. (Shanghai, China).

Subarachnoid Anesthetic Effects of EI and Emulsified F6 in Rats

The subarachnoid catheter (PE-10, Scientific Commodities INC. Lake Havasu City, AZ, USA) was inserted between L5-L6 segments and about 2 cm advanced in the cephalic direction to place the distal end of the catheter at about lumbar intumescencia. The injection volume was 150 μ l/kg. Two days before formal experiment, 1% (w/v) lidocaine was injected to confirm the place of the catheter.

Spinal anesthetic median effective concentration (EC_{50}) of EI was measured. The rats with both successful motor and sensory blockades were defined as spinal anesthesia. Motor function was evaluated according to the score scale as 0-normal posture; 1-intact dorsiflexion of foot with impaired ability to splay toes when elevated by the tail; 2-toes and foot plantar flexed with no splaying ability; 3-loss of dorsiflexion, flexion of toes, and impairment of gait but could try to withdraw when noxious stimulus applied; 4-complete paralysis of hind limbs (19). Motor function blockade at score 4 was regarded as successful motor blockade, and no aversive response to tail-clamping stimulus (alligator clip, 10 A, type 85, length 2-1/8 inches, Newark Electronics, Dublin, CA, USA) was considered as successful sensory blockade. Rats were randomly divided into 7 groups (N=8 for each concentration group) receiving EI at concentrations of 6.4% (0.53 mol/L), 5.1% (0.42 mol/L), 4.1% (0.34 mol/L), 3.3% (0.27 mol/L), 2.6% (0.24 mol/L), 2.1% (0.17 mol/L), and 1.7% (0.14 mol/L), respectively.

Our previous studies demonstrated that 8% (v/v) EI was similar to 1% (w/v) lidocaine in regional anesthesia (7, 8). In the present study, 8% (v/v) EI, 2% (v/v) emulsified F6, 1% (w/v) lidocaine and 30% (w/v) Intralipid were intrathecally administered in rats (N=10 for each group). Onset time and duration of motor and sensory blockades were observed. Duration of motor blockade was the time from complete blockade (score 4) to recovery (under score 1). Sensory function was evaluated by tail-flick test (Tail-Flick Unit 7360; Ugo Basile, Comerio, Italy) and duration of sensory blockade was the time from complete blockade to baseline latency.

Preparation of Acute Isolated Spinal Neurons

The isolation of spinal neurons was described as our previous study (12) and rats (7-12 days) were used. Spinal cord at L3-L5 was quickly removed and placed in iced artificial cerebrospinal fluid (ASCF) containing (in mmol/L) 117 NaCl, 26 NaHCO₃, 5 KCl, 1 NaH₂PO₄, 2.5 CaCl₂, 3.4 MgCl₂, 10 glucose, pH at 7.30, saturated with 95% O₂/5% CO₂. Then the spinal cord was cut into pieces about 1 mm³ and placed into a digestion solution containing (in mmol/L) 117 NaCl, 26 NaHCO₃, 5 KCl, 1 NaH₂PO₄, 10 glucose, pH at 7.30, containing 1 mg/ml collagenase I, at 37 centidegree for about 70 minutes.

Electrophysiology Recording

Spinal neurons with diameter at 15-30 μm were selected for recording. After spinal neurons settled to the bottom of 35 mm Petri dish, whole-cell patch clamp technique was applied to examine the inhibition by EI and emulsified F6 to peak sodium currents and their effects on activation and inactivation of channel gating. Voltage-gated sodium channel currents were measured with holding potential at -90 mV and depolarized from -60 to 40 mV (12). Inactivation of sodium channel gating was recorded by pre-pulse potential from -100 to 0 mV at holding potential of -90 mV and tested at -10 mV. The neurons were bathed with extracellular solution containing (in mmol/L) 95 NaCl, 26 NaHCO₃, 5.6 KCl, 1 NaH₂PO₄, 0.1 CaCl₂, 5 MgCl₂, 20 TEA, 11 glucose, pH at 7.40. Electrode resistance was 3-5 MΩ. The pipette electrode solution contained (in mmol/L) 140 CsCl, 5 EGTA, 1 MgCl₂, 10 HEPES, 3 MgATP, pH at 7.30. Currents were sampled at 10 kHz and filtered at 1-3 kHz by Axon 200B amplifier, digitized using a Digi-data 1440A interface, and analyzed by pClamp 10.0 (Axon/Molecular Devices, Sunnyvale, CA, USA). Isoflurane and F6 were diluted from 500 mmol/L emulsion with extracellular solution and applied locally by gravity (at the speed of 100-150 μl/minute through a perfusion pipette with diameter of 0.2 mm, positioned 30-50 μm away from the patched neurons). Data analysis and curve fitting were utilized by the Clampfit 10.0 (Axon/Molecular Devices, Sunnyvale, CA, USA), Origin 8.0 (OriginLab Corp., Northampton, MA, USA) and Excel 2007 (Microsoft).

Statistical Analysis

The data were analyzed by SPSS 16.0 (SPSS Inc., Chicago, IL, USA). Probit analysis was applied to determine EC₅₀ of spinal anesthesia of EI. One-way analysis of variance (ANOVA) with S-N-K post hoc test was applied to compare the onset time and the duration of motor and sensory blockade among 1% lidocaine, 8% EI, 2% emulsified F6 and 30% Intralipid. Data were expressed as mean ± SD.

Concentration-dependent effect of EI and emulsified F6 on voltage-gated sodium channel currents was calculated by least squares fitting of data to the Hill equation as: $Y = 1 / (1 + 10^{((\log IC_{50} - X) \times h)})$, in which X is concentration of isoflurane or F6, Y is the inhibition effect, IC₅₀ is median inhibitory concentration, and h is Hill slope. Activation curves of sodium channel were fitted to the Boltzmann equation as the form: $G/G_{max} = 1 / (1 + e^{(V_{1/2} - V)/k})$, in which G_{max} is maximum conductance, G/G_{max} is normalized channel conductance, V_{1/2} is voltage activation of half-maximum, and k is slope factor. Channel conductance was calculated as: $G_{Na} = I_{Na} / (V_t - V_r)$, where I_{Na} is peak current, V_t is test potential, and V_r is sodium channel reversal potential. Inactivation curves of sodium channels were also fitted to the Boltzmann equation. Data were expressed as mean ± SD. Statistical significance was assessed by paired or unpaired t test (20). In all cases, P < 0.05 was considered as statistically significant.

RESULTS

With the subarachnoid anesthesia animal model, emulsified nonimmobilizer F6 did not produce any spinal anesthesia as EI did. No obvious effect on sodium channel was observed for F6 while EI inhibited spinal cord sodium channel currents at relevant concentrations and significantly hyperpolarized V_{1/2} of voltage-dependent inactivation.

Subarachnoid Anesthetic Effects of EI and Emulsified F6 in Rats

EI produced subarachnoid anesthesia which completely blocked sensory and motor functions of rats without obvious sedative effect, with EC₅₀ at 3.65% (0.29 mol/L, Figure 1). The

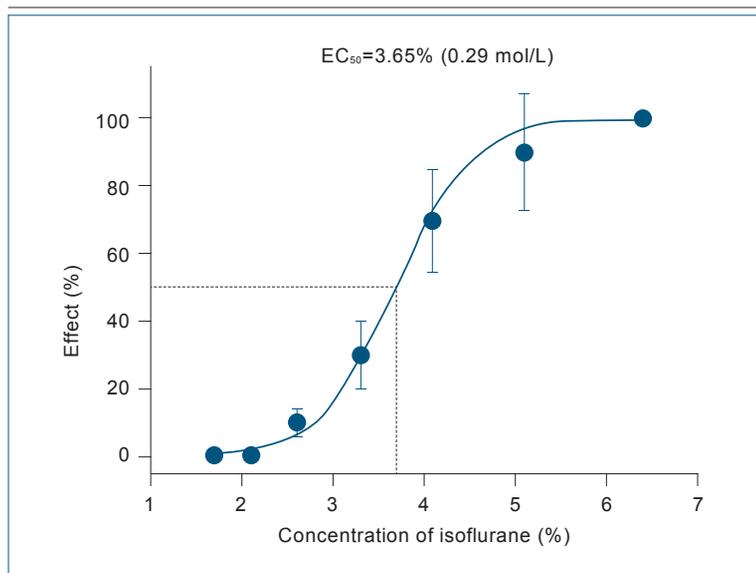


Figure 1. EC₅₀ of Subarachnoid Anesthesia of EI in Rats.

EC₅₀ of EI was determined by intrathecal injection at volume of 150 µl/kg. Seven different concentrations (N=8) of EI were applied intrathecally to rats and successful subarachnoid anesthesia was identified as loss of ability to support the body on hind limbs without reflex to tail-clamping stimulus. Subarachnoid anesthetic EC₅₀ of EI was calculated as 3.65% (0.29 mol/L).

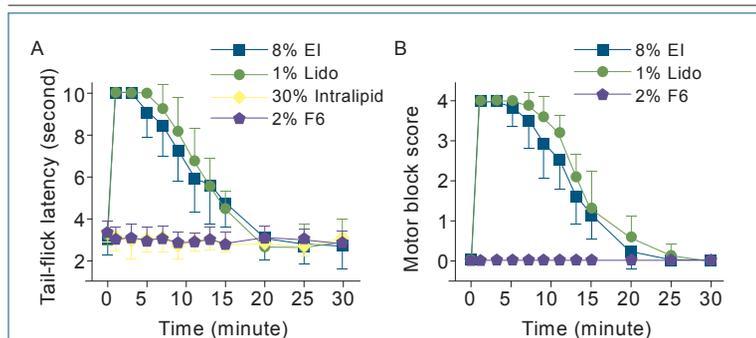


Figure 2. Spinal Anesthetic Actions of 8% EI, 2% Emulsified F6 and 1% Lidocaine.

All emulsified anesthetics and lidocaine were intrathecally injected into L5-L6 segments of rats at the volume of 150 µl/kg. Nonimmobilizer F6 at concentration of 2% (141 mmol/L) did not produce spinal effects on motor and sensory function. Most of the rats received emulsified F6 at concentration of 4% died. EI at 8% and lidocaine at 1% produced subarachnoid anesthetic effect immediately after intrathecal injection and their spinal anesthetic efficacy and durations were similar for both motor and sensory blockade. 30% Intralipid did not show any effect on motor and sensory function.

similar to 1% (w/v) lidocaine (N=10) both in maximum efficacy and duration (Figure 2). Both 8% EI and 1% lidocaine produced spinal anesthesia immediately after injection. Duration of motor function blockade was 16.3 ± 2.4 and 18.7 ± 2.1 minutes ($P=0.732$) for EI and lidocaine, respectively. Duration of sensory function blockade was 15.6 ± 2.3 and 16.9 ± 1.9 minutes ($P=0.518$) for EI and lidocaine, respectively. No spinal anesthetic effect was found by 30% Intralipid (Figure 2).

By previous studies, the predicted EC₅₀ of isoflurane and F6 in vitro were about 0.35 mmol/L (20) and 0.02 mmol/L (15), respectively, which indicated that F6 might be 15 folds more potent than isoflurane in local areas or tissues. In the present study, emulsified F6 (N=10) did not produce any spinal effect at concentration of 2% (141 mmol/L). Because EC₅₀ of EI in subarachnoid anesthesia was 290 mmol/L, thus, F6 at 141 mmol/L was about 7 folds to its predicted EC₅₀ (about 20 mmol/L). At the same time, EI at concentration of 2% (164 mmol/L) produced successful subarachnoid anesthesia in 2 out of 8 rats and analgesic action was observed in all the 8 rats. Most of the rats that received emulsified F6 at concentration of 4% died (data not shown).

Effects of EI and Emulsified F6 on Sodium Channels of Acute Isolated Spinal Neurons

Peak sodium channel current in spinal neurons was significantly inhibited by EI (Figure 3, Figure 4) in a concentration- dependent manner with IC₅₀ at 0.81 (0.09) mmol/L, and emulsified F6 inhibited peak sodium channel current merely by $20.5 \pm 4.2\%$ at concentration of 0.08 mmol/L (4 folds as predicted EC₅₀). At a concentration of 4 folds of predicted EC₅₀, the maximum inhibition of EI was $79.4 \pm 6.8\%$. The IC₅₀ of EI on spinal sodium channel determined in the present study was about 2 folds of its predicted MAC in vitro (20), which was similar to the IC₅₀ of isoflurane on sodium channels reported by previous studies (20- 27). At predicted MAC, 0.35 mmol/L isoflurane inhibited sodium channel current by $28.7 \pm 7.8\%$ and 0.02 mmol/L F6 inhibited sodium channel current only by $10.3 \pm 2.6\%$ (Figure 4, N=5-6).

For sodium channel activation, both EI and

potency of EI in subarachnoid anesthesia was similar to its potency in other regional anesthesia (7, 8) including IV regional anesthesia (8). The spinal effect of 8% (v/v) EI (N=10) was

emulsified F6 did not significantly shift $V_{1/2}$ of channel maximum activation (Figure 5, N=5-6). EI at concentration of 0.72 mmol/L (2 folds of predicted MAC) shifted $V_{1/2}$ of maximum activation from -12.4 ± 3.1 to -8.6 ± 2.4 mV ($P=0.067$). Emulsified F6 at concentration of 0.04 mmol/L (2 folds of predicted MAC) shifted $V_{1/2}$ of maximum activation from -12.4 ± 3.1 to -11.6 ± 2.9 mV ($P>0.5$). For sodium channel voltage-dependent inactivation (Figure 6, N=5-6), EI at concentration of 0.79 mmol/L (about 2 folds of predicted MAC) significantly hyperpolarized $V_{1/2}$ of maximum inactivation from -57.5 ± 2.4 to -66.3 ± 1.8 mV ($P<0.01$). And emulsified F6 at concentration of 0.038 mmol/L (about 2 folds of predicted MAC) did not significantly affect $V_{1/2}$ of maximum inactivation from -57.5 ± 2.4 to -58.4 ± 2.7 mV ($P>0.5$). EI was more effective on inactivation of sodium channel than activation.

DISCUSSION

The present study found that intrathecal injection of EI produced typical subarachnoid anesthetic actions in rats, which impaired motor and sensory functions. The effect of EI at concentration of 8% was similar to the effect of 1% lidocaine both in maximum efficacy and duration, which indicated that the regional effect of EI might share some common mechanisms with lidocaine. Traditional local anesthetics such as lidocaine produce nerve blockade mainly by inhibition to sodium channels. Increasing evidence demonstrated that volatile anesthetics could inhibit voltage-gated sodium channels at clinically relevant concentrations (20-27). Thus, we further evaluated the effect of EI on sodium channels of spinal neurons and found that EI inhibited sodium channel currents and hyperpolarized voltage-dependent inactivation of the channels. These results provide the direct evidence that EI produces regional anesthetic effect at least partially by its inhibition to sodium channels.

Zhang et al. (6) found that intrathecal infusion of veratridine (sodium channel activator) significantly increased systemic MAC of isoflurane, indicating that spinal sodium channel is a critical target for immobility of isoflurane. Our current results support their study. For the origi-

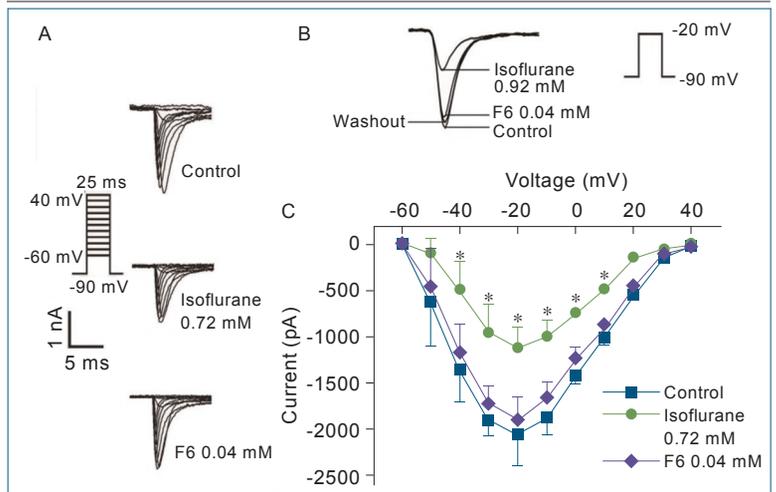


Figure 3. Effects of EI and Emulsified F6 on Sodium Channel Currents in Spinal Neurons of Rats.

A. At 2 folds of their predicted EC_{50} respectively, EI significantly inhibited sodium channel currents while emulsified F6 did not; B. EI and emulsified F6 at concentrations of 0.92 and 0.04 mmol/L (about 2 folds of predicted MAC) were applied to spinal neurons, and EI inhibited sodium channel currents significantly while emulsified F6 produced little effects; C. The current-voltage curves of sodium channels under conditions of control, application of EI and emulsified F6. * $P<0.05$ vs. control and F6.

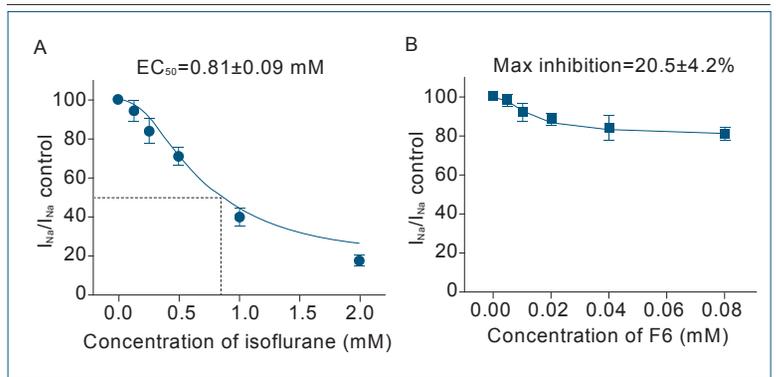


Figure 4. The Effect-Concentration Relationship of EI and Emulsified F6 on Sodium Channel Current in Spinal Neurons.

Predicted MAC of isoflurane in vitro was about 0.35 mmol/L (20) while predicted MAC of F6 in vitro was about 0.02 mmol/L (15). EI significantly inhibited sodium current in a concentration-dependent manner with EC_{50} at 0.81 ± 0.09 mmol/L, and emulsified F6 inhibited peak sodium channel current merely $20.5 \pm 4.2\%$ at concentration of 0.08 mmol/L.

nal design of the present study, we hypothesized that intrathecal injection of veratridine might also reverse the spinal effect of EI. However, all the rats received 4% EI in addition of 0.625 μ mol/L veratridine died. Veratridine activates sodium channel by keeping the channels in activated state. Therefore, the veratridine could inhibit

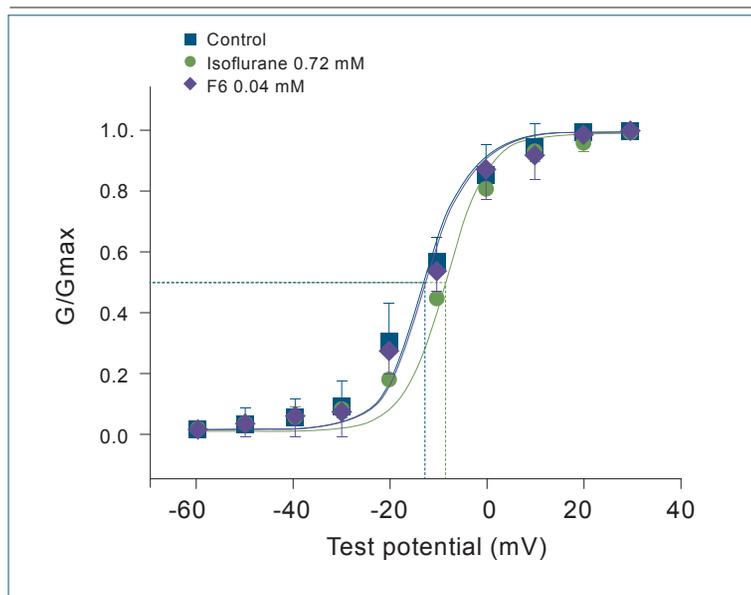


Figure 5. Effect of EI and Emulsified F6 on Voltage Activation of Voltage-Gated Sodium Channel in Spinal Neurons.

The concentrations of EI and F6 were used at their 2 folds of predicted MAC in vitro. Normalized sodium channel conductance was fitted to the Boltzmann equation to yield voltage of 50% maximal activation ($V_{1/2}$ of activation). Emulsified F6 did not affect activation curve of voltage-dependent sodium channel ($P>0.5$). EI shifted $V_{1/2}$ of maximum activation from -12.4 ± 3.1 to -8.6 ± 2.4 mV, without statistical significance ($P=0.067$).

further transmission of action potentials. At the same time, isoflurane could further hyperpolarize sodium channel inactivation. Thus, veratridine and isoflurane might produce synergism in toxicity. Further study could be designed to investigate how isoflurane and veratridine caused death in a synergistic manner when intrathecally administered.

The nonimmobilizer F6 produces amnesia with systemic inhalation but no effect on immobility like isoflurane (14). EI could induce spinal anesthesia effect; however, it is unclear whether the regional spinal effect of EI and its effect of immobility share similar mechanism. In the present study, we demonstrated that emulsified F6 produced little spinal effect at even high concentrations. The concentration of 2% (141 mmol/L) for F6 used in the present study was equal to about 25% (1,953 mmol/L) of isoflurane because the predicted in vitro potency of F6 is at least 15-fold higher than isoflurane (15). When emulsified F6 at 4% were injected, it induced injury and death. The fact that F6 did not produce spinal effect as isoflurane did indicated the direct relevance between spinal effect of isoflurane and its effect of immobility.

Since it is found from cut-off effect, nonimmobilizer F6 has been used as tool drug to explore anesthetic mechanism for a long time (14). It is a common way to determine anesthetic targets by comparing the effects of F6 and other potent inhaled anesthetics (14-17). The target that is sensitive to both F6 and isoflurane likely contributes to the effect of amnesia and the target that is sensitive to isoflurane and insensitive to F6 is likely the target for immobility. Until now, it is difficult to link its effect on molecular sites to behavior of F6. We investigated the regional spinal anesthetic effect of emulsified F6 and EI. Emulsified F6 did not produce any spinal effect like EI did. This might be an important evidence to demonstrate that regional spinal effect of isoflurane and its effect of immobility share the same mechanisms. Intrathecal injection could exclude the effect of brain, and spinal anesthetic model of EI could, thus, be a perfect approach to determine mechanisms of immobility of volatile anesthetics. In addition, like previous studies about F6 and isoflurane, we found that EI inhibited spinal sodium channels while emulsified F6

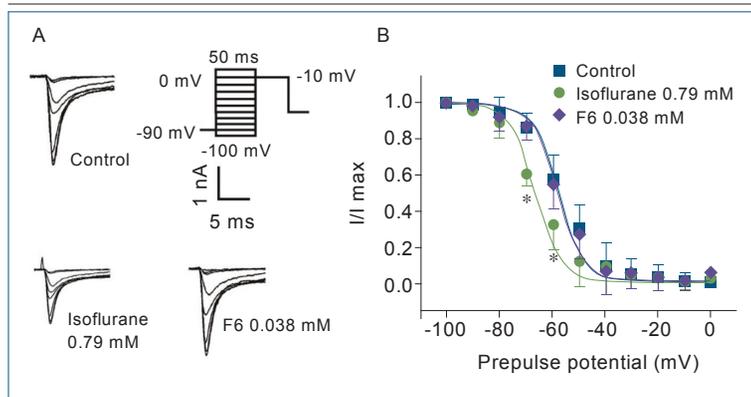


Figure 6. Effects of EI and Emulsified F6 on Inactivation of Sodium Channel in Isolated Spinal Neurons.

Inactivation of voltage-dependent sodium channel was recorded by pre-pulse potential from -100 to 0 mV at holding potential of -90 mV and tested at -10 mV. A. Current traces of voltage-dependent inactivation of sodium channels and the recording protocol. EI and emulsified F6 at concentrations of their 2 folds of predicted MAC were tested. B. The effects of EI and emulsified F6 on voltage-dependent inactivation of sodium channels. Normalized sodium channel inactivation was fitted to the Boltzmann equation to yield voltage of $V_{1/2}$ of maximal inactivation ($V_{1/2}$ of inactivation). EI at 0.79 mmol/L significantly hyperpolarized $V_{1/2}$ of inactivation from -57.5 ± 2.4 to -66.3 ± 1.8 mV ($P<0.01$). Emulsified F6 at 0.038 mmol/L did not affect $V_{1/2}$ of inactivation from -57.5 ± 2.4 to -58.4 ± 2.7 mV ($P>0.5$). * $P<0.05$ vs. control and F6.

did not. The target that is sensitive to isoflurane but not F6 is the candidate molecular target for immobility of general anesthetics (14). Our current results, thus, indicated that spinal sodium channel could account for effect of immobility for isoflurane and its spinal anesthetic effect, but not F6.

EC₅₀ of EI to produce spinal anesthetic effect in vivo is 290 mmol/L while its EC₅₀ to inhibit spinal sodium channel in vitro is 0.81 mmol/L. After intrathecally injected, EI would be significantly diluted by cerebrospinal fluid and the exact concentrations of isoflurane on spinal cord neurons were unclear. The most direct evidence to ensure the relationship between spinal effect of isoflurane and its effect of immobility is to compare the concentrations of isoflurane in spinal cord when these effects achieved. The limitation of the present study is that we could not know the real concentration of isoflurane in spinal cord because of the technical obstacle. In addition, for further study, it would be even more interesting to compare the effects of EI on spinal motor and/or sensory neurons respectively, by electrophysio-

logical recording on spinal cord slice.

Besides voltage-gated sodium channel, many other neuronal transmitters receptors might also be involved in spinal effects of volatile anesthetics, such as γ -aminobutyric acid (28), N-methyl-D-aspartic acid (29-31), opium (32), and acetylcholine and adrenergic (33) receptors. Thus, spinal effect of isoflurane might be a comprehensive outcome resulting from its modulations on multiple targets.

In summary, in the present study, EI could produce subarachnoid anesthetic effect in rats and inhibit spinal sodium channel current in relevant concentrations. At the same time, nonimmobilizer F6 could not produce any spinal effect even at very high concentrations and it also was insensitive to spinal sodium channels. These results supported that sodium channel plays an important role in spinal anesthetic effect of isoflurane as well as its effect of immobility.

This work was supported by the grant 2014M552361 (to Dr. Cheng Zhou) from the National Postdoctoral Foundation of China and the grant 81401139 (to Dr. Cheng Zhou) from the National Natural Science Foundation of China. The authors declare no other competing interests.

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