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A rabbit model for evaluation of surgical anesthesia and analgesia: characterization and validation with isoflurane anesthesia and fentanyl analgesia

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Abstract
Purpose. With a clamp test, quantitative estimation of the level of surgical anesthesia/analgesia is not easy. We have developed a rabbit pain model allowing for quantitative evaluation of the level of surgical anesthesia/analgesia using both electrical and mechanical stimuli as simulated surgical stimuli. We evaluated whether this model allows for accurate tracing of dynamically changing levels of surgical anesthesia/analgesia induced by isoflurane and fentanyl.
Methods. Eight rabbits tracheotomized and vascularly cannulated under 3% isoflurane anesthesia were placed on a sling that allowed for free movement of the head and extremities. After the isoflurane concentration was reduced stepwise to 1.5% and then to 0%, four graded doses of fentanyl (5, 10, 20, and 40 μg·kg^{-1}) were injected intravenously at intervals of 120 min. At each dose, anesthetic/analgesic end-point variables were determined, including the number of animals behaviorally unresponsive to clamping the forepaw (nonresponders) and the threshold voltage of subcutaneous electrical stimulation (2, 5, and 50 Hz) required to evoke the head lift (HLT: pain detection/arousal threshold: sedative/hypnotic index) and the escape movement (EMT: pain tolerance threshold: analgesic index).
Results. With increasing doses of isoflurane and fentayl, HLTs and EMTs, especially those at 5 Hz, increased dose-dependently and proportionately to increases in the number of nonresponders to clamping the forepaw, a standard indicator of the anesthetic/analgesic level.
Conclusion. Using the HLT and EMT, especially at 5 Hz, combined with a clamp test, this rabbit model allows for repeated, quantitative, and distinctive evaluation of the dynamically changing levels of both sedative/hypnotic and analgesic components of surgical anesthesia/analgesia.

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Key words Animal pain model · Clamp test · Electrical stimulation · Fentanyl · Rabbit

Introduction

Traditionally, assessment of the depth or level of anesthesia or analgesia deep enough to allow surgical procedures (i.e., surgical anesthesia/analgesia) has mainly relied upon observation of movement responses to extremely painful stimuli, such as surgical skin incision in humans and clamping the tail or paw in animals [1–3]. With the use of such a crude method, however, the adequacy of surgical anesthesia/analgesia cannot be sensitively evaluated, especially in a small number of subjects, since a movement response to a given noxious stimulus has been defined only in a quantal manner (i.e., all or none, present or absent). Although "the percent probability of no movement response" to a given noxious stimulus, which is determined only after analyzing pooled quantal data with logistic regression analysis, may be used to estimate the level of surgical anesthesia/analgesia, it requires multiple-set experiments involving a substantial number of subjects [2–6]. In addition, in rats and mice commonly used for pain research, physiological variables such as the electrocardiogram (ECG), heart rate (HR), arterial blood pressure, respiratory rate (RR), arterial blood gases, and alveolar gas concentration cannot be easily monitored, and they may change dynamically due to both changing anesthetic doses and changing perioperative stimuli.

We, therefore, have developed a more sensitive and clinically relevant middle-sized animal (rabbit) model for investigation of surgical anesthesia/analgesia using both mechanical clamping and electrical stimuli as simulated surgical stimuli [7,8]. This animal

model allows for repeated, quantitative assessments of the level of surgical anesthesia/analgesia, and also allows for monitoring of all the clinically relevant physiological variables. In the previous studies, we characterized and validated this animal model using stepwise steady-state levels of anesthesia/analgesia induced by remifentanil infusion at constant rates and isoflurane inhalation at constant concentrations [7,8]. However, the performance of this animal model during continuously changing levels of surgical anesthesia/analgesia has not yet been evaluated. This study was conducted for further validation and characterization of this animal pain model, to assess whether this model allows for close tracking of dynamically changing levels of surgical anesthesia/analgesia induced by multiple-dose bolus injections of fentanyl, and if it does, which frequency of electrical stimulation allows for the most relevant estimation of the level of surgical anesthesia/analgesia.

Methods

Animals and preparations

The study protocol was reviewed and approved by the institution's animal research committee. Eight male New Zealand White rabbits weighing approximately 3 kg were studied. Anesthesia was induced with 5% isoflurane in oxygen delivered via a nose cone. During subsequent surgical preparations, anesthesia was maintained with 3% isoflurane in oxygen. After tracheotomy, a 3.5-mm pediatric tracheal tube was placed and secured with sutures to prevent air leak. The animal breathed spontaneously from an anesthesia breathing circuit incorporating a circle carbon dioxide absorption system. The marginal ear vein was cannulated with a 24 gauge plastic catheter for drug and fluid administration. Lactated Ringer's solution was infused at $8\,ml \cdot kg^{-1} \cdot h^{-1}$ for fluid maintenance. The central ear artery was cannulated with a 20 or 22 gauge plastic catheter for continuous blood pressure monitoring and blood sampling. The arterial blood pressure and the heart rate by percutaneous leads were continuously displayed on a multichannel polygraph recorder (Hewlett Packard 78304A and 78172A, Waltham, MA, USA). Body temperature was monitored with a rectal probe and maintained between 38° and 39°C with a heating lamp. A 16 French balloon catheter was inserted into the urinary bladder to monitor urine output.

The rabbit was then gently placed in the normal physiological position on a rubber sling that allowed the animal to move the head and all extremities freely (Fig. 1). A pair of subcutaneous platinum needle electrodes (Grass Type 2, Grass Medical Instruments, Quincy, MA, USA) was placed in the plantar aspect of a forepaw 10 mm apart and 3 mm deep into the skin. These electrodes were tightly secured with tape and connected to Grass S48 stimulator for electrical stimulation.

Anesthetic/analgesic variables

The forepaw clamp test was applied to evaluate the adequacy of surgical anesthesia/analgesia. A neoprene-covered 16-cm hemostat was applied across a forepaw and moved back and forth until the animal moved [1] or 10 s had passed [6]. An eventual motor response was

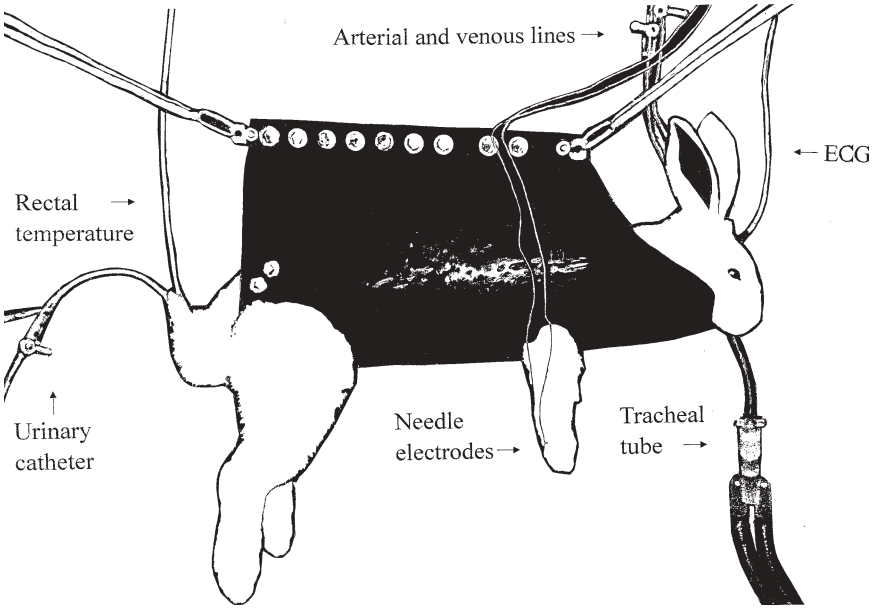


Fig. 1. Experimental settings including a rubber sling that allows for free movement of the head and all extremities

classified as a positive movement if the animal showed a gross purposeful movement, such as clawing or running with the extremities [1]. Coughing, swallowing, chewing, or movement solely of the clamped-side extremity unaccompanied by the movement of another part of the body was not considered a positive response [1,9]. At each measurement time point, the actual number of animals that did not show behavioral reactions in response to clamping the forepaw (the number of nonresponders) was determined as a standard indicator of the level of surgical anesthesia/analgesia [7], analogously to statistically determined "%probability of no response" [2–6].

Subcutaneous electrical stimulation was applied to another forepaw, and the threshold voltages required to evoke two consecutive end-point movement responses, the head lift response (HLT) and the escape movement response (EMT), were determined. Graded-voltage electric currents (square wave, 1 ms duration) at 3 different frequencies (2, 5, and 50 Hz, in that order) were applied subcutaneously. The intensity of the stimulus at each frequency was increased gradually from 0 to 15 V over 30 s until two consecutive movement responses were evoked. If these responses were not evoked, the voltage was gradually increased up to 150 V (an upper limit cutoff voltage to avoid tissue damage) over 30 s until two consecutive responses were elicited or the cutoff voltage was reached.

The HLT was defined as sudden lifting of the head accompanied by wide opening of the eyes (the arousal response: sedative-hypnotic index) [7]. The escape movement was running or jumping (analgesic index) [7]. A movement solely of the stimulated-side forepaw during electrical stimulation was not considered a positive response; simultaneous movement of another part of the body was required [3]. The thresholds of the HLT and EMT were determined with 2, 5, and 50 Hz stimuli, respectively.

Physiological variables

The systolic, diastolic, and mean arterial pressures (SAP, DAP, MAP), ECG, and HR were continuously monitored on a polygraph. RR and arterial blood gases were measured repeatedly. Rectal temperature and urine output were monitored throughout the experiment.

Dose-response studies with isoflurane and fentanyl

After completion of all preparations, the inspiratory concentration of isoflurane was reduced stepwise from 3% to 1.5%, and then to 0%. For each concentration, a steady-state dose was maintained for at least 30 min, and then anesthetic/analgesic and physiological variables were measured. After complete recovery from isoflurane anesthesia, four graded doses (5, 10, 20, and 40 $\mu g \cdot kg^{-1}$) of fentanyl were intravenously injected en bolus in this order at 120-min intervals. Naloxone 0.1 $mg \cdot kg^{-1}$ was injected 120 min after the last fentanyl injection.

Physiological variables, including SAP, DAP, MAP, HR, RR, and blood gases, and anesthetic/analgesic variables, including the number of nonresponders and HLTs as well as EMTs at 2, 5, and 50 Hz, were measured repeatedly at the following time points: at steady-state isoflurane concentrations of 3% and 1.5%; immediately before the first fentanyl injection; at least 30 min after discontinuation of isoflurane (baseline: isoflurane 0%, 0 min); 5, 15, 30, 60, 90, and 120 min following each fentanyl injection (5–480 min); and 15 min after naloxone injection (495 min).

Statistical analysis

The data are reported as means ± SEM. Changes in physiological and anesthetic/analgesic variables were analyzed with repeated-measures analysis of variance (ANOVA) followed by Fisher's protected least significant difference test. The number of nonresponders to clamping the paw at each time point was compared with that at 0 min by the chi-square test. To determine which frequency of electrical stimulation allows for the most relevant estimation of the level of surgical anesthesia/analgesia, the correlation between the number of nonresponders, a standard indicator of the anesthetic/analgesic level, and each of HLTs and EMTs was analyzed, and correlation coefficients were compared among electrical stimuli of different frequencies. Because the number of nonresponders could only be determined in the group as a whole, the mean HLT and EMT in the group as a whole at each of the 28 time measurement points, and not the HLTs and EMTs for individual subjects, were compared with the number of nonresponders, determined at the corresponding time point. The data are presented as X-Y scatter plots with either mean of the electrical stimulation thresholds on the Y-axis and the number of nonresponders on the X-axis. Linear regression lines were also calculated and plotted. Similar analytical methods using linear regression have been used to validate a newly developed pain assessment method by comparing it with a traditional method [7,10]. $P < 0.05$ was considered to indicate statistical significance.

Results

Changes in physiological variables

SAP, DAP, and MAP decreased dose-dependently with isoflurane and transiently increased rather than

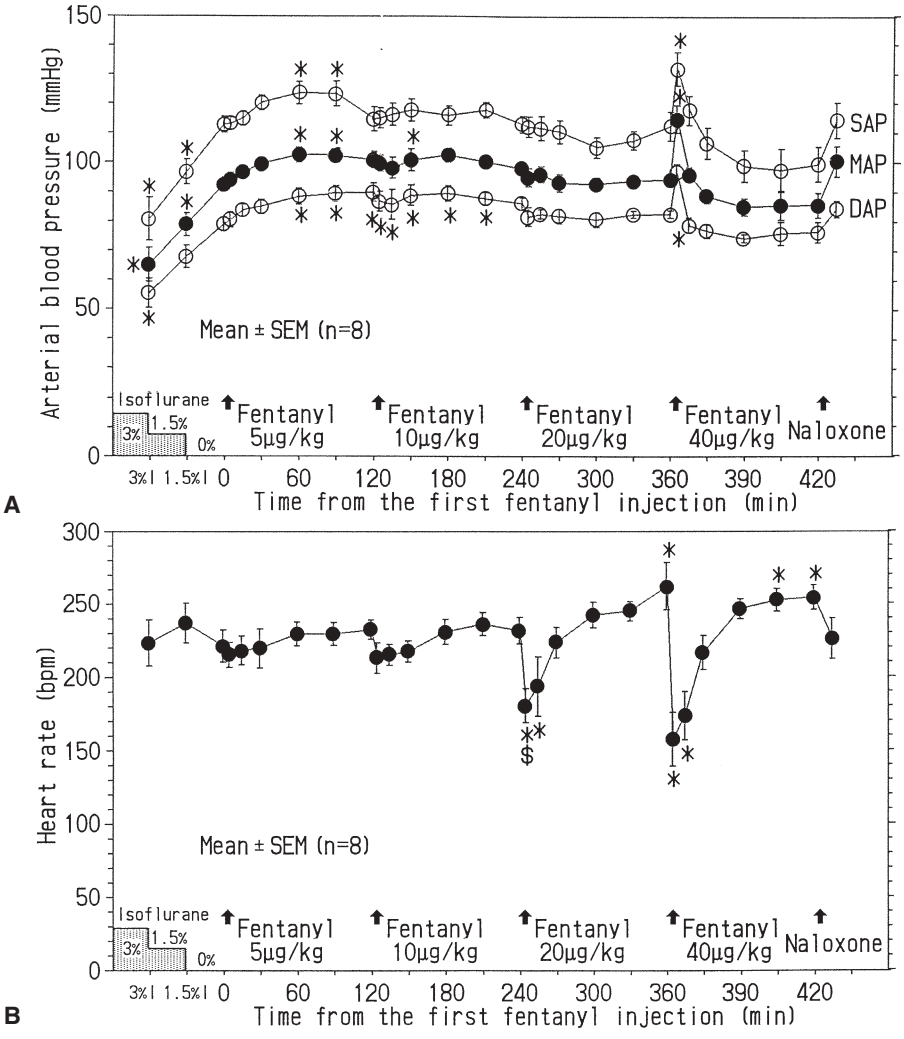


Fig. 2. Changes in systolic, diastolic, and mean arterial blood pressures (*SAP, DAP, MAP*) (**A**) and heart rate (**B**) during inhalation of isoflurane and following intravenous injections of fentanyl as well as naloxone. Data are means ± SEM. *Significantly different from the baseline value at 0 min. $Significantly lower than the lowest value following the previous fentanyl injection

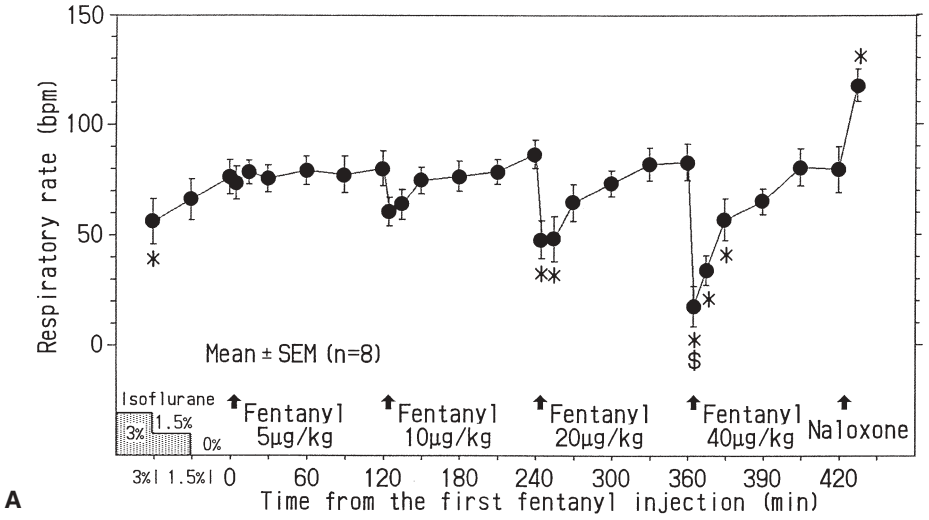
decreased with fentanyl (Fig. 2). Conversely, HR remained unchanged with isoflurane, whereas it decreased transiently and dose-dependently following graded-dose injections of fentanyl (Fig. 2). RR decreased dose-dependently with isoflurane, and it also decreased transiently and dose-dependently following graded-dose injections of fentanyl (Fig. 3). Pa_{CO_2} increased dose-dependently with both isoflurane and fentanyl (Fig. 3). All of these variables returned to baseline levels after naloxone injection (Figs. 2 and 3).

Changes in anesthetic/analgesic variables

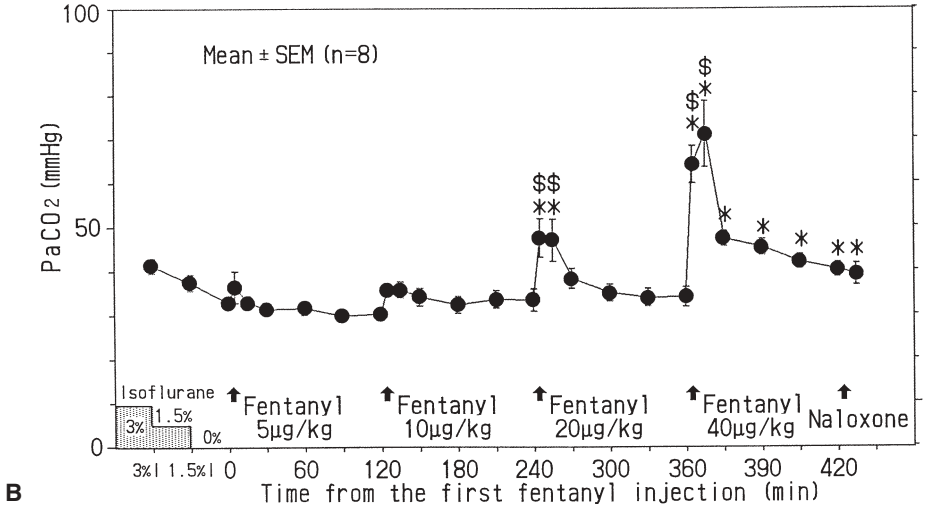
The number of nonresponders to clamping the forepaw increased dose-dependently with isoflurane, and it showed dose-dependent peaky increases following graded-dose injections of fentanyl (Fig. 4). After naloxone injection, it returned to the baseline value (0/8).

HLTs and EMTs at 2, 5, and 50 Hz increased dose-dependently with isoflurane, and these variables also showed peaky increases following graded bolus injections of fentanyl. After naloxone injection, all of these variables returned to baseline levels (Fig. 5). Throughout the experiment period, the HLTs and EMTs, especially the EMTs at 5 and 50 Hz, changed in direct proportion to the number of nonresponders to clamping the paw (compare Fig. 5 with Fig. 4). After injection of fentanyl, HLTs and EMTs, especially EMTs at 5 and 50 Hz, changed in direct proportion to changing Pa_{CO_2} and in reverse proportion to changing RR and HR (compare Fig. 5 with Figs. 2 and 3).

The increases in HLT and EMT with isoflurane and fentanyl were much smaller with a 50-Hz stimulus than a 5-Hz stimulus, and significant increases in EMT with fentanyl at lower doses (5 and 10 $\mu g \cdot kg^{-1}$) were observed with a 5-Hz stimulus, but not with a 50-Hz stimulus (Fig. 5B and C). Conversely, the increases in HLT and EMT with isoflurane and fentayl were much greater with a 2-Hz stimulus than a 5-Hz stimulus, and the dose-dependent increases in EMT with fentanyl at higher



A



B

Fig. 3. Changes in respiratory rate (**A**) and Pa_{CO_2} (**B**) during inhalation of isoflurane and following intravenous injections of fentanyl as well as naloxone. Data are means ± SEM. *Significantly different from the baseline value at 0 min. $Significantly lower than the lowest value following the previous fentanyl injection (**A**) or significantly higher than the peak value following the previous fentanyl injection (**B**)

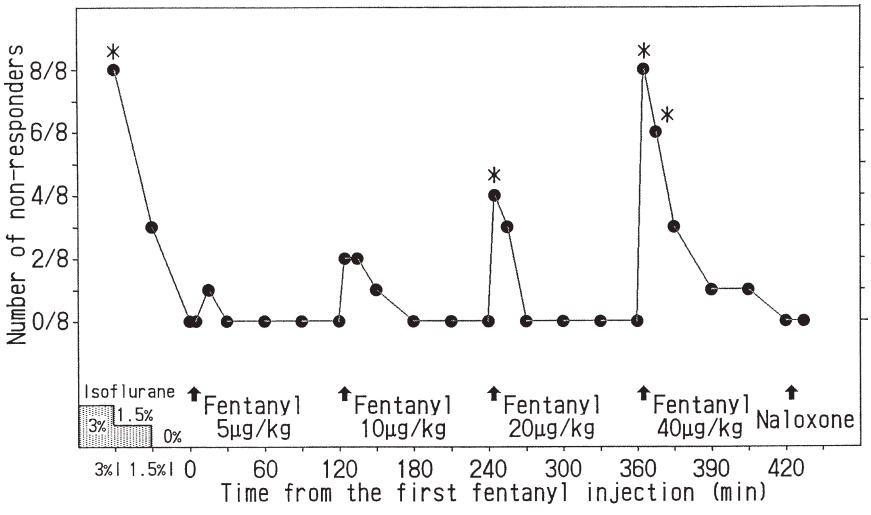


Fig. 4. Change in the number of nonresponders during inhalation of isoflurane and following intravenous injections of fentanyl as well as naloxone. The number of nonresponders = the number of animals that did not show behavioral reactions in response to clamping the forepaw. *$P < 0.05$ vs. the baseline value at 0 min

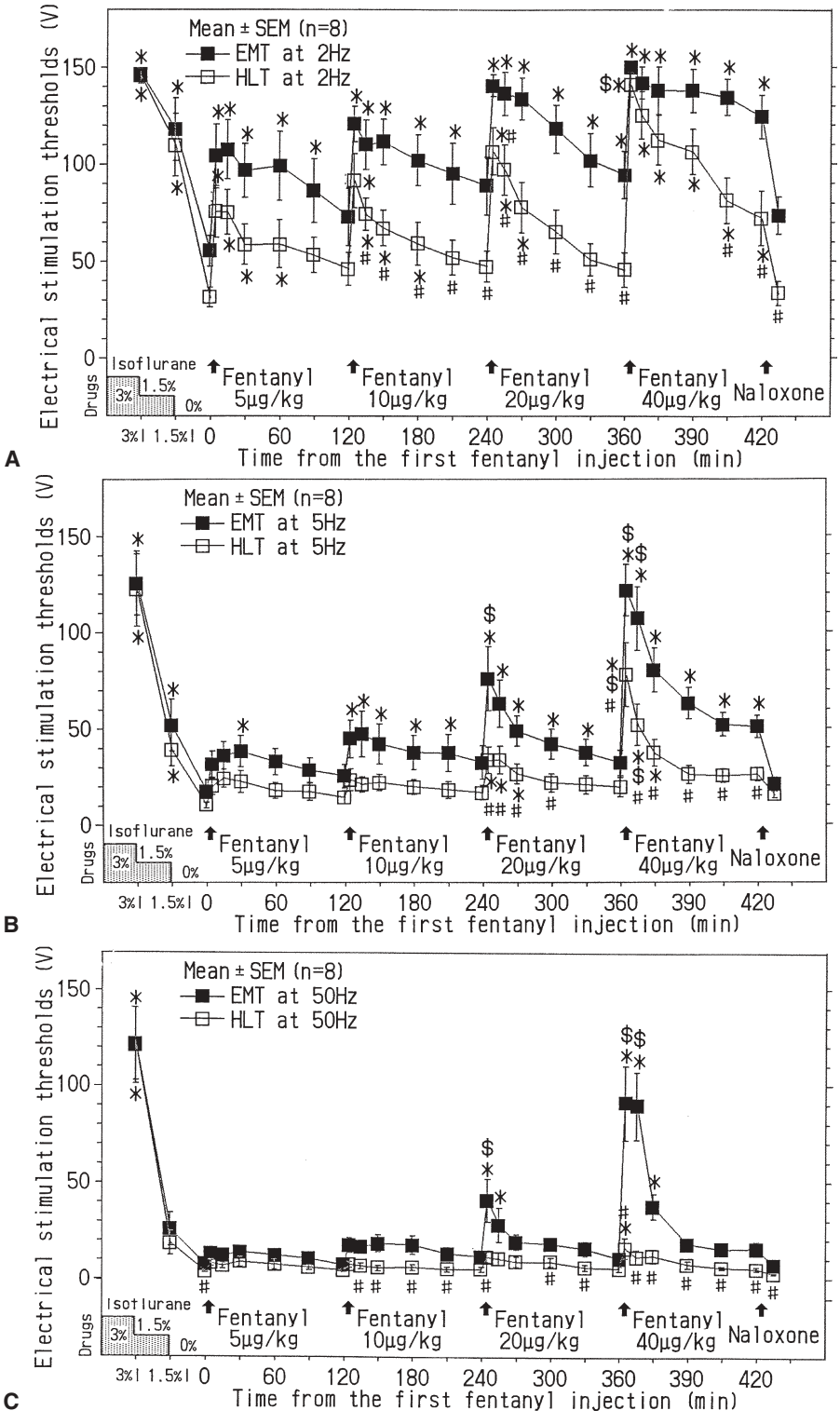


Fig. 5. Changes in electrical stimulation thresholds: HLTs and EMTs at 2 Hz (**A**), 5 Hz (**B**), and 50 Hz (**C**) during inhalation of isoflurane and following intravenous injections of fentanyl as well as naloxone. *HLT*, Electrical stimulation threshold of the head lift response; *EMT*, electrical stimulation threshold of the escape movement response. Data are means ± SEM. *Significantly different from the baseline value at 0 min. $Significantly higher than the peak value following the previous fentanyl injections (indicating a dose-dependent increase in an electrical stimulation threshold). #Significantly lower than the corresponding EMT at a corresponding time point

doses (20 and 40 μg·kg^{-1}) were observed with a 5-Hz stimulus, but not with a 2-Hz stimulus (Fig. 5A and B), since in many animals the EMT at 2 Hz reached the cutoff line after injection of higher doses of fentanyl; with a 2-Hz stimulus, more than 150 V might be required to elicit the maximal response (Fig. 5A). As a result, significant and dose-dependent increases in EMT with graded-dose injections of fentanyl could be more clearly demonstrated with a 5-Hz stimulus than with 2-Hz and 50-Hz stimuli (Fig. 5).

The HLTs did not significantly differ from corresponding EMTs during isoflurane inhalation, whereas

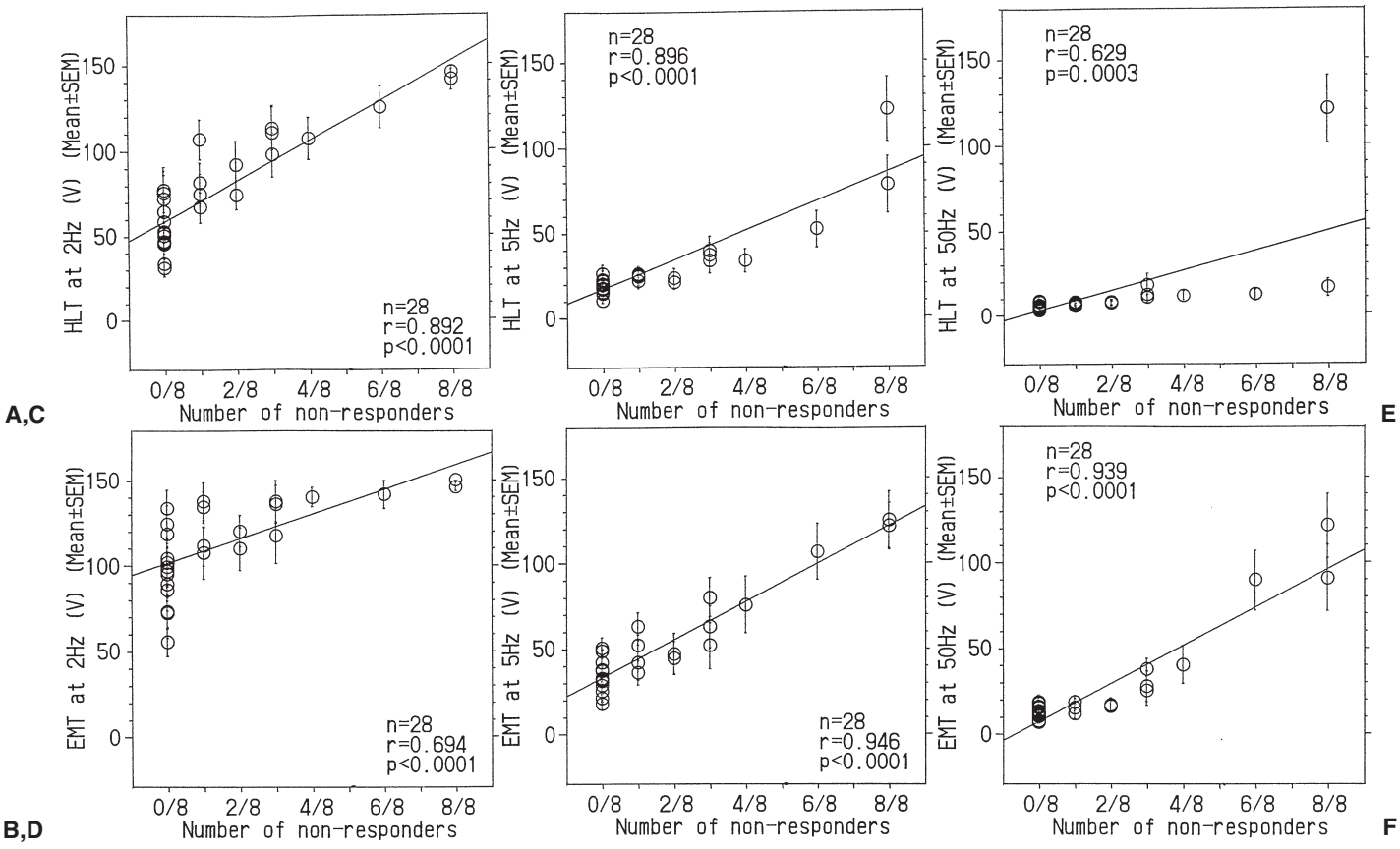


Fig. 6. Relations of the means(±SEM) of the HLT at 2 Hz (**A**), EMT at 2 Hz (**B**), HLT at 5 Hz (**C**), EMT at 5 Hz (**D**), HLT at 50 Hz (**E**), and EMT at 50 Hz (**F**) at measurement time points (n = 28) to the number of nonresponders at corresponding time points. *HLT*, Electrical stimulation threshold of the head lift response; *EMT*, electrical stimulation threshold of the escape movement response. The number of nonresponders = the number of animals that did not respond behaviorally to clamping the forepaw. Because the number of nonresponders could only be determined in the group as a whole, the means of HLTs and EMTs in the group as a whole at 28 measurement time points were plotted against the number of nonresponders, determined at the corresponding time points. Correlation coefficients were calculated, and linear regression lines were calculated and plotted

the HLTs remained significantly lower than the corresponding EMTs after fentanyl injections (Fig. 5).

Relations between anesthetic/analgesic variables

Each of the means of the HLT and EMT at 2, 5, and 50 Hz correlated significantly with the number of nonresponders to clamping the paw (Fig. 6). The means of the HLT and EMT at 5 Hz correlated more closely with the number of nonresponders than those at 2 and 50 Hz (Fig. 6), indicating that the means of the HLT and EMT at 5 Hz changed more proportionately to the changing number of nonresponders than those at 2 and 50 Hz (compare Figs. 4 and 5).

Discussion

In our acute pain rabbit model, both mechanical and electrical stimuli were used as simulated surgical stimuli, and anesthetic/analgesic and physiological variables were simultaneously monitored during dynamically changing levels of isoflurane anesthesia and fentanyl analgesia. By comparing various electrical stimulation thresholds with the number of nonresponders as a standard indicator of the level of surgical anesthesia/analgesia [7], we assessed in this study whether these electrical stimulation thresholds allow for precise tracing of the continuously and dynamically changing levels of surgical anesthesia/analgesia, and if they do, which threshold could be used as the most relevant indicator of the level of surgical anesthesia/analgesia.

In this test of surgical anesthesia/analgesia, we used clamping the rabbit forepaw instead of clamping the tail, commonly used in other species as a "supramaximal stimulus" to simulate surgical stimuli [1,2,11], since our preliminary study suggested that the rabbit tail was vulnerable to tissue damage after re-

peated clamping. In addition, one report suggested that clamping the tail was not a supramaximal stimulus and was a less intense stimulus than clamping the paw (dew claw) in pigs [9].

In tests of surgical anesthesia/analgesia, not only mechanical stimuli but also electrical stimuli have been used as simulated surgical stimuli [11]. As electrical stimuli, most investigators have used fixed-voltage, high-frequency (50 Hz or more) stimuli and observed the presence or absence of specific end-point responses [1,3,11–13]. However, because the level of anesthesia/analgesia can be measured not only in terms of the presence or absence of a response to a given noxious stimulus, but also in terms of the threshold intensity of a noxious stimulus required to elicit an end-point response [14], we applied variable graded-voltage stimulation and determined the thresholds of two distinctive movement responses, HLT and EMT, during a gradual constant-rate increase in stimulus intensity. Regression analysis of our data indicated that all the HLTs and EMTs changed, with statistically significant correlation, in parallel with the changing number of nonresponders, a standard indicator of the level of surgical anesthesia/analgesia. Therefore, it appeared that all these electrical stimulation thresholds could be used as quantitative measures of the anesthetic/analgesic level.

As the intensity of stimulation was gradually increased, the HLT and EMT were elicited in this order. Behaviorally, the HLT corresponds to the stimulus intensity at which the stimulus becomes sufficiently painful to trigger an arousal response (pain detection/arousal threshold: sedative/hypnotic index), and the EMT corresponds to the stimulus intensity at which the pain is perceived as intolerable (pain tolerance threshold: analgesic index) [7]. With isoflurane, the HLT was evoked only slightly earlier than or almost at the same time as the EMT during a gradual increase in voltage; the HLT was almost indistinguishable from the EMT, and isoflurane caused dose-related parallel increases in the EMT and HLT. With fentanyl, the HLT was elicited much earlier than the EMT during a gradual increase in voltage; fentanyl increased the EMT preferentially to the HLT, and the HLT remained significantly lower than the EMT after fentanyl injections. The distinctive dissociation between the EMT (analgesic index) and HLT (sedative/hypnotic index) with fentanyl, in contrast to the close similarity between these indices with isoflurane, may reflect the predominantly analgesic property of fentanyl, in contrast to the sedative/hypnotic/anesthetic property of isoflurane. In our previous, current, and unpublished studies, anesthetics such as isoflurane, propofol, and thiopental resulted in parallel increases in the EMT (analgesic index) and HLT (sedative/hypnotic index), whereas analgesics such as fentanyl, remifentanil, and adenosine preferentially increased the EMT [7,8,15]. This was presumably because inhaled or intravenous anesthetics suppress pain perception by producing unconsciousness, whereas opioids and other analgesics predominantly produce analgesia without producing unconsciousness [16]. This model thus seems to allow for differentiation of the sedative/hypnotic and analgesic components of drug actions.

In the previous study, we characterized and validated this animal model using constant-rate remifentanil infusion and constant-concentration isoflurane inhalation to achieve steady-state levels of anesthesia/analgesia [7]. In this study, however, we evaluated whether electrical stimulation thresholds can closely follow continuously and dynamically changing levels of anesthesia/analgesia produced by multiple bolus injections of fentanyl. In such situations, measurement of plasma concentration of fentanyl appeared to be of little significance, since the plasma concentration and the central nervous system (CNS) tissue concentration will never equilibrate in a short observation period [16]. In this study, HLTs and EMTs, especially EMTs at 5 and 50 Hz, changed in direct proportion to changing Pa_{CO_2} and in reverse proportion to changing RR and HR, suggesting that these electrical stimulation thresholds closely tracked the CNS effects of fentanyl that should be directly related to its tissue concentration.

In tests of surgical anesthesia using electrical stimuli, most investigators have used electrical stimuli at high frequency (50 Hz or more) as simulated surgical stimuli [1,3,11,13]. However, several clinical studies demonstrated that the segmental analgesia induced by epidural fentanyl and hypesthesia induced by systemic lidocaine were more sensitively detected with electrical stimulation at 5 Hz than at higher frequencies [17,18]. As the stimulation frequency of the electric current (square wave, 1 ms duration) is increased from 2 to 50 Hz, the current flowing in the tissue per second will increase accordingly, and conversely, the pain-threshold voltage will decrease [19]. Actually, the increases in the HLT and EMT with isoflurane and fentanyl were much smaller with a 50-Hz than with a 5-Hz stimulus, and conversely, much greater with a 2-Hz than a 5-Hz stimulus. As a result, a significant analgesic effect of fentanyl at its lower doses could be detected with the EMT at 5 Hz, but not with that at 50 Hz. Conversely, the dose-dependent increase in the analgesic effect of fentanyl at its higher doses could be detected with the EMT at 5 Hz, but not with that at 2 Hz. EMTs at 2 and 50 Hz might have more closely followed the analgesic effect of fentanyl if the cutoff voltage for the 2-Hz stimulus had been set at a higher level than 150 V, or the pulse duration of a square-wave current for 2-Hz and 50-Hz stimuli had been set at longer and shorter levels than 1 ms, respectively, to decrease and increase the pain-threshold voltage, respectively. In the settings of the present study,

the significant and dose-dependent analgesic effects of fentanyl over its wide dose range could be most clearly demonstrated with the EMT at 5 Hz. Furthermore, the EMT and HLT at 5 Hz correlated most closely with the number of nonresponders, a standard indicator of the anesthetic/analgesic level. These results indicate that electrical stimulation at 5 Hz, combined with clamping the forepaw, was the most relevant stimulus for assessment of the level of surgical anesthesia/analgesia. We could thus closely track continuously and dynamically changing levels of surgical anesthesia/analgesia even in a single subject by following changes in HLTs and EMTs, especially those at 5 Hz, in combination with the changing response to mechanical clamping.

As an acute pain model for research on surgical anesthesia/analgesia, a mid-sized animal, the rabbit, seems to have some advantages over smaller rodents such as rats and mice, which are commonly used for pain research. A rabbit is very easy to treat because of its extremely placid temperament. The ear vein and artery can be easily cannulated with a simple puncture technique [7,8,20], and various behavioral and physiological variables can be readily monitored, similarly to clinical anesthetic practice. In fact, in the current study we could distinctively demonstrate cardiovascular and respiratory depressant effects of isoflurane and fentanyl; isoflurane decreased AP, but not HR, dose-dependently. In contrast, fentanyl decreased HR, but not AP, dose-dependently; fentanyl decreased RR and increased Pa_{CO_2} in a dose-dependent manner. After injection of the highest dose of fentanyl, Pa_{CO_2} might reach a level high enough to augment its anesthetic/analgesic effect, since very high Pa_{CO_2} (>70 mmHg) produces a CO_2 narcosis syndrome of drowsiness [21]. Data with isoflurane, fentanyl, remifentanil, thiopental, ketamine, adenosine, and lidocaine in our previous, current, and unpublished studies suggest that effective dose ranges of various drugs in rabbits are quite similar to those in humans [7,8,15], whereas drug requirements (per kilogram) are generally much greater in smaller rodents than in humans [22,23]. Results obtained from a rabbit model thus may be more clinically relevant.

In conclusion, we have developed an animal model of surgical anesthesia/analgesia using both mechanical and electrical stimuli as simulated surgical stimuli. By combining electrical stimuli, especially at 5 Hz, with a clamp test, this model allowed us to closely track continuously and dynamically changing levels of surgical anesthesia/analgesia even in a single subject. In addition, our model enabled differentiation of two different endpoints of anesthesia/analgesia, namely, the pain detection/arousal threshold (sedative/hypnotic index) and the pain tolerance threshold (analgesic index). Furthermore, a variety of physiological variables could be readily monitored. This novel animal model with multimodal tests and monitoring, which closely mimic clinical anesthesia and surgical stimuli, may improve our understanding of surgical anesthesia/analgesia and thus facilitate laboratory investigations and development of new anesthetic/analgesic agents and monitoring and treatment modalities.

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